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Improving Algae Growth Kinetics in Suspension Bioreactors for the Production of Recombinant Proteins

by

Brendan Robert Clark

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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Abstract

Millions of individuals rely on recombinant proteins such as essential biopharmaceuticals. Recently, genetically engineered microalgae have been identified as a potentially inexpensive and fast growing host organism for recombinant protein production. Using *Chlamydomonas reinhardtii*, a species of unicellular green microalgae, the goal was to improve algal cell growth kinetics, genetically engineer the cells and develop a bioprocess to analyze recombinant protein production.

C. reinhardtii growth kinetics were improved under mixotrophic growth conditions using acetate in small scale 10 mL cultures. This process was scaled-up to 500 mL spinner flask suspension bioreactors and through the use of a fed-batch acetate feeding strategy, cell growth rates and maximum cell concentrations were improved. A genetic construct was designed, manufactured, isolated and used to genetically transform *C. reinhardtii*. A bioprocess was then developed to isolate and analyze protein production rates from these cells. Results indicated product concentrations of 8.44 mg/L of culture.

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Chapter 1: Introduction

1.1 Introduction

Millions of people rely on recombinant proteins. Examples of commonly used recombinant proteins include livestock vaccines, antibodies for research, and clinically prescribed pharmaceuticals such as insulin. A recombinant protein is generated in a host cell type (e.g. bacteria, yeast) that has been genetically modified to produce that specific protein. To generate commercially relevant quantities of recombinant proteins, genetically modified host cells are grown in large bioreactors. Bioreactors are scalable vessels in which a controlled environment can be provided that is capable of supporting the culture of the host cells which, in turn, produce the recombinant protein of interest. Protein collected from the culture is put through a multistage separation process in order to isolate it from other soluble proteins, and purified to a concentration which may be used for the intended application. Commercially available recombinant proteins are traditionally produced via the manipulation of bacteria, yeast or mammalian cells. For these cell types, production systems have been optimized to generate recombinant proteins for as little as $50 per gram [1]. Recently, it has been suggested that algae may be superior host organisms for recombinant protein production [2], [3]. In comparison to traditional host organisms, algae represent an inexpensive, scalable and safe alternative for recombinant protein production [4]. Previous reports have indicated that an algal-based protein production system could reduce production costs to $0.05 per gram, a thousand-fold decrease compared to currently optimized systems [1]. This potential for a significant decrease in the production costs could translate into easier access to relevant recombinant proteins for a number of industries, including the chemical, nutritional, agricultural and biopharmaceutical industries. It is therefore important to explore the possibility of generating algae-derived recombinant proteins in bioreactors.

1.2 A Comparison of Host Organisms

Recombinant proteins have been produced from a wide range of host organisms. In industry, bacteria, yeast and mammalian cells represent the established host systems. Transgenic

animals have also been investigated as a host organism, as have plants and microalgae more recently. The advantages and disadvantages of these host organisms will be discussed below.

1.2.1 Bacteria

The bacterium *Escherichia coli* has been a useful tool in the biotechnology industry for decades and represents one of the most widely used hosts for the production of heterologous proteins [5]. Although *E. coli* can produce recombinant proteins quickly and at a relatively low cost, this organism is best suited for the production of small and structurally simple proteins [6]. Typically, bacteria struggle to produce proteins that are rich in disulphide bridges, cannot carry out post-translational modifications such as glycosylation, and are inefficient at protein folding and secretion [5]. Measures can be taken to properly modify the proteins produced by *E. coli*, such as *in vitro* denaturation and refolding, expression of protein fragments and the addition of fusion partners, among others [7], [8]. Whereas these techniques can aid in proper protein production of more complex proteins, they represent a significant cost and time investment. Even with these advancements, many *E. coli* heterologous proteins fail to perform properly; for example, antibodies often fail to recognize mammalian proteins [9]. Although suitable for myriad purposes in the biotechnology sector, *E. coli* is not the host organism of choice for many complex recombinant proteins utilized by the chemical and medical industries.

1.2.2 Yeast

Yeasts are single-celled eukaryotic organisms that are capable of post-translational protein modifications that cannot be performed in *E. coli*. Many recombinant proteins that require glycosylation or protein folding can be produced in yeast, whereas this is typically not possible in bacterial systems. *Saccharomyces cerevisiae* is the most common yeast strain utilized for protein production, and has several advantages over bacterial hosts [10]. *S. cerevisiae* has a long history in industrial fermentation applications; it can secrete heterologous proteins outside of the cell, and it carries out glycosylation of proteins. However, the glycosylation performed by *S. cerevisiae* is often unacceptable for the production of more complex mammalian proteins.

Yeasts have a tendency to over-glycosylate N-linked sites, leading to a reduction in activity and receptor recognition [6]. For this reason, yeast cannot reproduce the necessary function and activity of higher eukaryotic proteins.

1.2.3 Mammalian Cells

Mammalian cells are most commonly used for the production of recombinant proteins which require complex mammalian post-translational modifications. Chinese Hamster Ovary (CHO) cells are considered the "workhorse" of the biotechnology industry and are the primary system for the production of higher eukaryotic proteins such as monoclonal antibodies. Mammalian host systems are particularly useful because the proteins produced are properly folded and glycosylated, eliminating the need for costly in vitro protein modifications [6]. Furthermore, the productivity of mammalian cells is typically high: protein titers of monoclonal antibodies have reached up to 26 g/L in industry [11]. Despite these benefits, mammalian systems do have some major drawbacks. Mammalian cells are very expensive to maintain, slow growing, often have poor secretion and can be contaminated by viruses [6], [12].

1.2.4 Transgenic Animals

Transgenic animals such as mice, goats, cows, pigs, rabbits and sheep have been developed as recombinant protein production systems [6]. Transgenic expression is high, and production volumes are unparalleled. A dairy animal can produce 1 to 14 g of heterologous protein in one litre of milk every day for 305 days out of the year [13]. Several disadvantages are encountered with animal systems, including the length of time necessary to assess protein levels, in addition to ethical issues involving the use of animals.

1.2.5 Transgenic Plants

For recombinant protein production, the use of transgenic plants, in comparison to live animals or animal cell cultures, represents a cost effective, safe and simple system. In fact, plant expression systems are even thought to be better than microbial systems in terms of cost,

ability to produce complex proteins, storage and distribution [6]. Furthermore, unlike the systems described above, plants require only simple forms of sustenance to proliferate. This simple and cost-effective alternative protein production system has only one major downfall: production time. Similar to transgenic animals, it may take months before protein production levels can be assessed in transgenic plants.

1.2.6 Transgenic Microalgae

Microalgae have recently been attracting increasing attention as an alternative host organism for the production of high-value recombinant proteins [3], [12], [14–18]. Transgenic microalgae possess many of the same benefits as plants and photosynthesize in a similar manner, but can direct energy to cellular growth and reproduction, resulting in total soluble protein levels ranging from 30%-50% of dry weight biomass [14]. Unlike plants, the time required to transform, culture, harvest and assess protein production levels from microalgae is on the scale of days or weeks, rather than months or years [12]. Therefore, it is possible that microalgae offer all the benefits of plants coupled with the high productivity and low production time of microbial host organisms [4], [14].

The green eukaryotic unicellular microalgae *Chlamydomonas reinhardtii* may represent the future of the biotechnology industry. This species of microalgae is inexpensive to culture, a generally regarded as safe (GRAS) organism, easily scalable, fast growing, CO_2-fixing, easily transformed and capable of producing high quality eukaryotic proteins [4]. The fact that *C. reinhardtii* is a GRAS organism may allow for oral administration of recombinant vaccines or pharmaceuticals, eliminating the need for costly downstream purification processes [19]. Furthermore, its nuclear, chloroplastic and mitochondrial genomes have been sequenced, and a number of genetic tools have been previously developed [14]. It also has the unique ability, like some other species of microalgae, to be cultured heterotrophically (in the absence of light), which may help to promote high cell concentrations without light limitations. [20].

All host systems have unique advantages and disadvantages (see Table 1-1). The potential of microalgae, as discussed above, make it a fascinating subject for recombinant biotechnology research. The biggest driving factor is the prediction that recombinant proteins produced by genetically engineered microalgae may be up to several orders of magnitude more cost efficient than the current industrial biotechnologies [1], [21]. The vast majority of industrially produced biopharmaceuticals are manufactured in traditional host organisms. A pie chart comparing these percentages is presented in Figure 1-1. Though the traditional host systems are well established, they are typically very expensive.

The potential cost reductions stemming from the use of a microalgae host system would ultimately result in increased worldwide access to necessary recombinant proteins such as vaccines and biopharmaceuticals. It is for these reasons that unicellular green microalgae were the focus host organism for this project.

There is currently a lack of research in the field of microalgae biotechnology. There is a need to improve and ultimately optimize cell growth kinetics, established algal bioprocesses must be developed, and the protein production rates from genetically engineered algae must be analyzed and optimized. This thesis will investigate some of these key questions to help fill some of the knowledge gaps that are currently present in the literature.

Table 1-1: A comparison of host organisms (data for table from [12])

Features	Bacteria	Yeast	Mammalian Cells	Transgenic Animals	Transgenic Plants	Transgenic Microalgae
Production time	Days	Weeks	Months	Months	Months	Days
Production cost ($USD)	Thousands	Thousands	Tens of Thousands	Tens of Thousands	Hundreds	< $100
Scale up cost ($USD)	Thousands	Thousands	Thousands	Thousands	Hundreds	< $100
Cost and type of storage	< $100: -20°C	< $100: -20°C	Hundreds: Liquid N_2	Hundreds: Liquid N_2	< $100: Room Temp.	< $100: Room Temp.
Protein glycosylation	Absent	Incorrect	Correct	Correct	Correct	Correct
Protein yield (mg/L)	10-100	100-10,000	100-10,000	100-10,000	100-10,000	Unknown

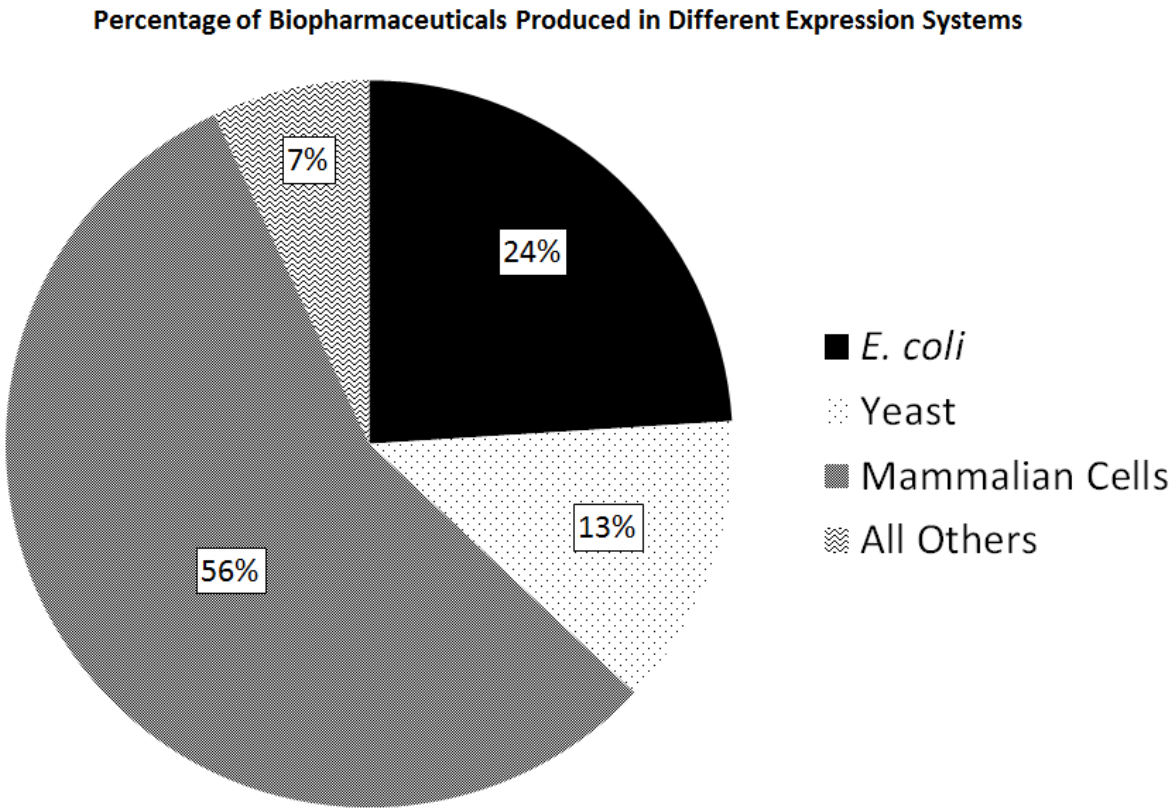

Figure 1-1: A comparison of the percentage of all biopharmaceuticals approved by the United States Food and Drug Administration and European Medicines Agency produced from various expression systems (data for figure from [22]). A majority of biopharmaceuticals are produced in mammalian cells, while only 7% of all biopharmaceuticals are produced from transgenic plants, algae, animals and all other hosts combined. Although there is great potential for transgenic plants and microalgae as host organisms, the current low dependence on these organisms is primarily due to a lack of established and approved bioprocesses [22].

1.3 Genetic Tools and Techniques

Chlamydomonas reinhardtii is a species of unicellular algae which has been particularly well researched; the genome of *C. reinhardtii* has been fully sequenced, it has been genetically engineered to produce recombinant proteins and growth kinetics have been recorded and improved through various means [23], [24]. Although a complete set of genetic tools is still being developed for *C. reinhardtii*, this organism has demonstrated significant promise as a platform for recombinant protein production. All three genomes (mitochondrial, chloroplastic and nuclear) of *C. reinhardtii* have been transformed with each genome showing distinct transcriptional, translational, and post-translational properties [3]. Additionally, each genome has been fully sequenced which provides a strong foundation for future genetic transformation research in *C. reinhardtii* [25–27]. Since each genome has unique properties, it may be desirable to target a recombinant protein to a particular genome or region of a cell. This type of approach has been successfully applied to the plant *Arabidopsis thaliana* [28], although the targeting of recombinant proteins to cellular organelles in algae is an ongoing area of research.

1.3.1 Algal Transformation Methods

The three genomes of algae can be reliably transformed through homologous recombination with exogenous DNA [3]. Several transformation technologies of varying efficiencies have been developed to facilitate homologous recombination in algae. Robust *in vivo* recombinant protein reporters, such as Green Fluorescent Protein (GFP) and luciferases, are commonly used to detect successful algal transformations [29], [30]. Figure 1-2 shows a typical *in vivo* assay using a GFP reporter.

The simplest and most readily available transformation method involves the use of glass beads, polyethylene glycol and DNA [24]. Unfortunately, this method is typically undesirable because the transformation obtained is transient; in other words, the gene of interest is not incorporated into the genome and cannot be passed from generation to generation.

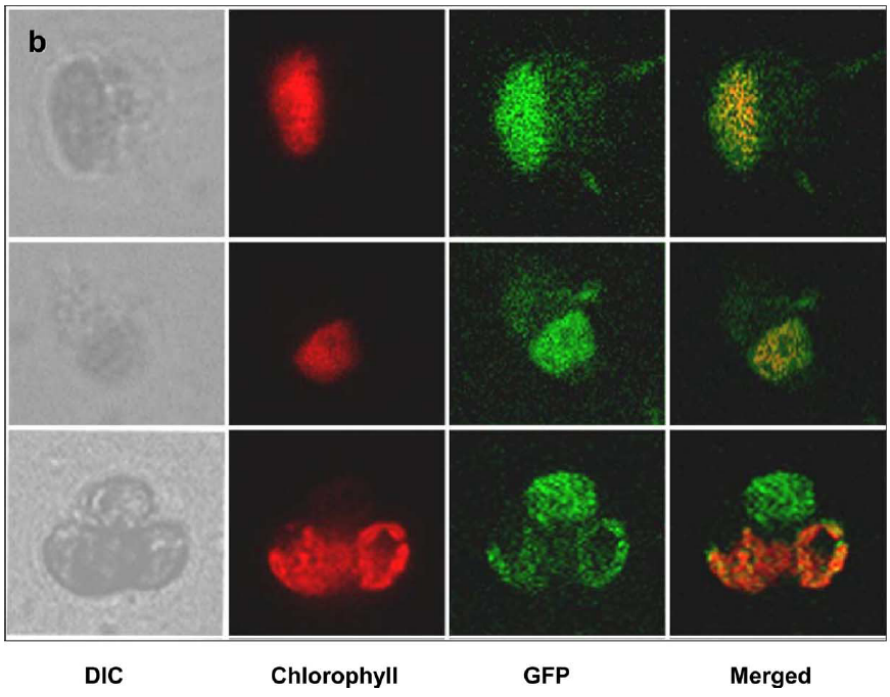

Figure 1-2: The detection of Green Fluorescent Protein (GFP) in *Chlamydomonas reinhardtii* cells using confocal microscopy (image reproduced with permission from [31]). The red chlorophyll images show autofluorescence characteristic of algal chloroplasts, while the green GFP images indicate areas of the cell which express GFP. The merged image superimposes the chlorophyll and GFP panels. Copyright information for this figure is provided in Appendix C.

Stable transformation techniques, while typically more complicated than transient techniques, are much more desirable because the gene of interest is passed down through generations. One of the simplest stable transformation techniques involves the use of *Agrobacterium tumefaciens* [31]. *A. tumefaciens* are gram-negative pathogenic bacteria found in soil, which infect a wide range of plants with crown gall disease through the transfer of an infectious plasmid [31]. This plasmid incorporates into the host's genome, effectively transforming the DNA of the plant cell. The beneficial traits inherent within the *A. tumefaciens* may be exploited to preferentially transform other organisms, including microalgae.

Another widely used stable transformation method utilizes a large voltage gradient which briefly opens some of the protein channels found on the cell membrane of the organism. This method, called electroporation, typically involves the use of a 5.0 kV voltage gradient applied for approximately 0.05 seconds [32]. Prepared plasmids containing a gene of interest enter the cell during the application of the voltage gradient. Both transient [32] and stable [33] nuclear transformations have been successfully completed in algae using electroporation.

Many other algal transformation methods exist, including particle gun bombardment [34][35], protoplasting [36] and the use of silicon carbide whiskers [37]. Particle gun bombardment is of particular note due to the high efficiency of this stable transformation technique. However, the high costs of the required materials for this method prevent it from being more popular in modern laboratories.

1.3.2 Targeting to the Chloroplastic Genome

The production of genetically engineered proteins in the chloroplast is of particular interest for a number of reasons. Transgenic proteins often accumulate to much higher levels in the chloroplast as compared to expression in the nuclear genome, primarily because the chloroplastic genome lacks gene silencing mechanisms found frequently in the nuclear genome [38]. Furthermore, proteins produced in the chloroplast are not glycosylated [39], which may

be essential for the production of certain recombinant proteins such as antibodies or vaccines which must maintain a specific, non-glycosylated organization in order to be functional [40].

1.3.3 Screening

Following genetic transformation, the genetically engineered cells which expressed the gene of interest must be separated or "screened" from other cells which did not take up the exogenous DNA or did not properly express the gene of interest. In *C. reinhardtii*, this is accomplished using a hygromycin B selection system [41]. The antibiotic hygromycin B, at a concentration of 20 μg/mL, will kill *C. reinhardtii* cells by inhibiting protein synthesis. However, the cells which take up and express the gene of interest convey resistance to hygromycin B and survive. In this way, genetically engineered cells can be selected from non-genetically engineered cells.

1.3.4 Gene Promoters

To create a gene of interest which will be successfully expressed in an organism such as a unicellular alga, genetic engineers must ensure that the gene of interest is properly constructed. To properly regulate the expression of the protein of interest from the gene of interest, gene promoters must be utilized in conjunction with appropriate codon optimization techniques, as described above [42]. Some promoters, called constitutive promoters, cause continual protein expression in all cells; in other words, they cause the gene to always be turned "on". This causes the protein of interest to be constantly expressed by the host organism.

The opposite of a constitutive promoter is called an inducible promoter. Inducible promoters vary from constitutive promoters in that the activity of the promoter is regulated by a stimulus. In this case, the gene of interest can be upregulated or downregulated at the will of the genetic engineer. This may be desirable for stepwise process design. For example, it may be desirable to culture cells in optimal conditions for cell production, and then transfer the culture to conditions ideal for protein production. Hence, a constitutive promoter may be undesirable, as

energy that would otherwise be used for cell growth would be used to produce recombinant proteins in non-optimal protein production conditions. Depending upon the scenario, an inducible promoter may provide the genetic engineer with better control over the system.

In *C. reinhardtii*, the HSP70A-RBCS2 fusion promoter is the most commonly used genetic promoter [43]. It is an inducible promoter which acts by either a temperature shock or a light shock stimulus, or a combination of these two stimuli. The heat shock portion of the promoter is stimulated via an incubation of the algae culture at 40°C for 30 minutes. The light shock portion is best stimulated by culturing the algae in the dark, then subjecting the cells to a light flux of 40 $\mu E \bullet m^{-2} \bullet s^{-1}$ for one hour [43]. This promoter system is used by genetic engineers to control the production of recombinant proteins from *C. reinhardtii*, and represents the system that was used for this project. Other genetic promoters have been developed for *C. reinhardtii*, but have not matched the productivity or robustness of the HSP70A-RBCS2 promoter system.

1.3.5 Codon Optimization

As with many other protein expression systems, the use of codon optimization in algae plays a major role in the transcription and translation of a recombinant gene. A codon is an RNA triplet of nucleotides; for example, GCU. One codon is specific to one amino acid, although the opposite is not necessarily true. The codon GCU codes for the amino acid alanine, although alanine is also coded by the codons GCC, GCA and GCG. Since the last nucleotide in the triplet is irrelevant in this case, this nucleotide is defined as being in the "wobble position" [3]. Genes within the chloroplastic genome prefer an A or U to be in the wobble position while genes in the nuclear genome prefer a G or C [44]. The use of codon optimization strategies is often essential for the expression of a recombinant gene. Using GFP it has been shown that codon optimization could improve transgenic protein accumulation up to 5-fold in the nuclear genome and up to 80-fold in the chloroplastic genome [30].

1.3.6 Alternative Factors Affecting Protein Expression

The transformation approach, promoters and codon usage are the three most important factors which affect protein expression [45]. Several other factors which may impact the protein expression levels in recombinant algae include protein localization, mRNA stability, the effect of introns, enhancers and intron-mediated sequences, transcription factor binding sites, mRNA translation initiation, trait stacking, transgene stabilization, riboswitches and inhibition of post-transcriptional gene silencing [45]. These other factors will not be described in detail, but must remain a concern for genetic engineers.

1.4 *Chlamydomonas reinhardtii* Growth Strategies

Chlamydomonas reinhardtii has the ability to grow and proliferate in both the presence and absence of light. In the light, algae grow phototrophically, while in the dark, the cells grow heterotrophically with the addition of a carbon source. It is possible for *C. reinhardtii* to grow both phototrophically and heterotrophically at the same time in a metabolic process known scientifically as mixotrophic growth. A visual comparison of these three growth methods is presented in Figure 1-3, and Table 1-2 outlines the key differences between the methods.

1.4.1 Phototrophic Growth

In the presence of light, algae grow phototrophically. Under phototrophic growth regimes, the cells take in carbon dioxide and, using light as an energy source, produce carbon-based products which are then broken down for energy [46]. Two major factors which influence the phototrophic growth of algae include carbon dioxide concentration as well as light intensity.

Figure 1-4 displays the major phototrophic pathways active in *C. reinhardtii* cells during phototrophic metabolism. As shown by the thickness of the arrows, the majority of external carbon is used within the chloroplast for the Calvin cycle [47]. The Calvin cycle uses light energy to make organic compounds which are then transferred to the mitochondria and broken down through the Krebs cycle.

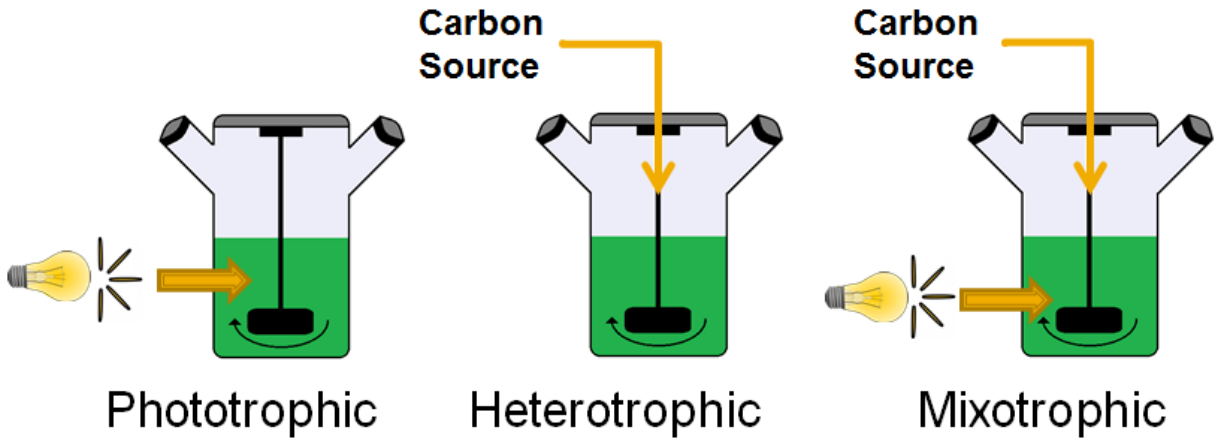

Figure 1-3: A comparison between phototrophic, heterotrophic and mixotrophic growth schemes for *C. reinhardtii*. Phototrophic algae cultures are grown in the presence of light and undergo photosynthesis. Heterotrophic cultures are grown in the absence of light and have an external carbon source added which is consumed by the cells for energy. If the cells are grown in the presence of light but also have an external carbon source added, this method is called mixotrophic growth. Copyright information for this figure is provided in Appendix C.

Table 1-2: A comparison of the three major algal growth methodologies (data for table from [50])

Growth Method	Energy Source	Carbon Source	Light Requirements
Phototrophic	Light	Inorganic	Obligatory
Heterotrophic	Organic	Organic	No requirements
Mixotrophic	Light and Organic	Inorganic and Organic	Not obligatory

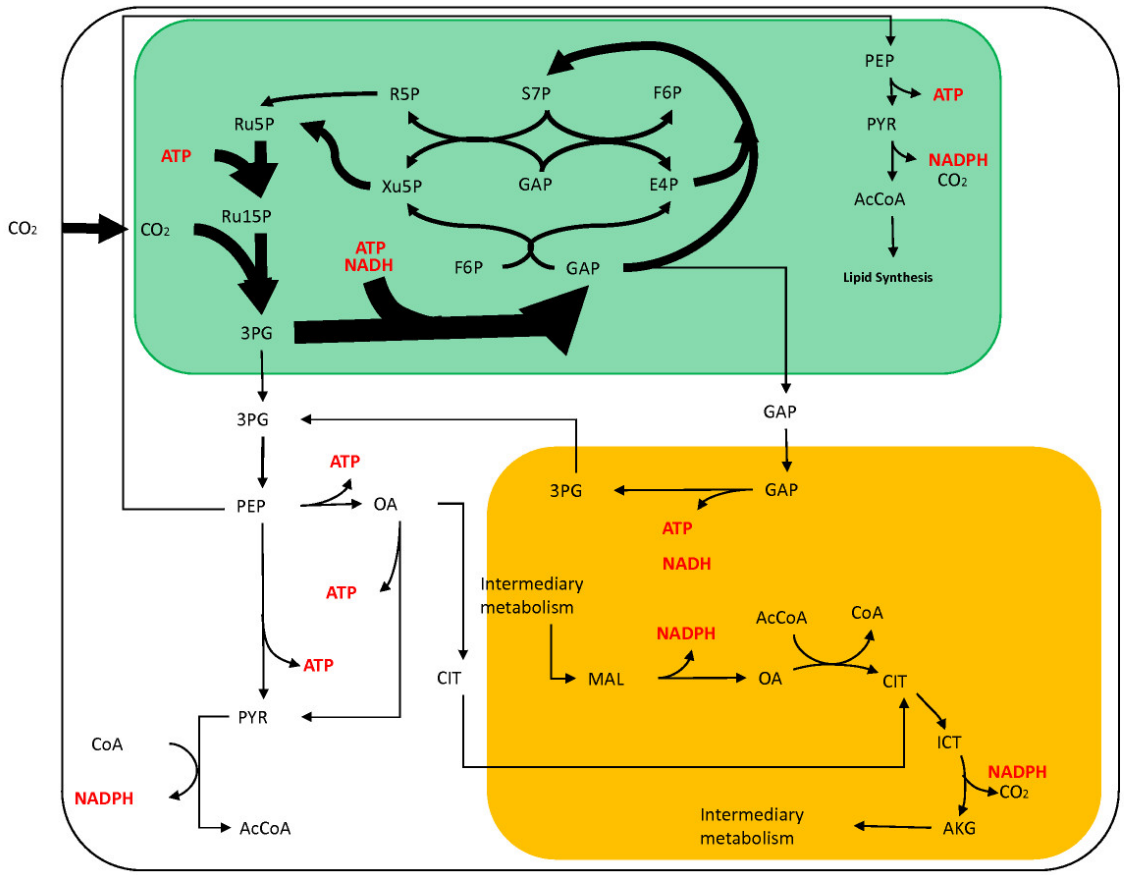

Figure 1-4: An outline of the major metabolic pathways of *C. reinhardtii* under phototrophic growth (figure reproduced with permission from [47]). The green compartment represents the chloroplast, while the orange compartment represents the mitochondria. The thickness of the arrows has been normalized to the total carbon dioxide uptake of 100 moles. The molecules in red represent direct (ATP) and indirect (NADH and NADPH) energy sources. Copyright information for this figure is provided in Appendix C.

1.4.2 Heterotrophic Growth

In the absence of light, certain species of algae are able to grow and proliferate with the addition of an external carbon source; this process is known as heterotrophic growth. *C. reinhardtii* has been found previously to metabolize acetate as a sole carbon source in the dark [48]. Other substances such as glucose have been investigated for use as a heterotrophic metabolite for *C. reinhardtii*, but the only substance found to be consumable by this species was acetate [49]. The major pathways responsible for heterotrophic growth in *C. reinhardtii* are presented in Figure 1-5. The majority of external acetate is used to produce energy via the glyoxylate cycle, a variation of the Krebs cycle [47]. One noteworthy point is that energy in the form of ATP is required to convert acetate into a useful form which can be consumed by the cells. Hence, cultures undergoing heterotrophic growth must have a supply of energy available to convert the acetate into a consumable product.

The ability of *Chlamydomonas reinhardtii* to grow in the absence of light in heterotrophic conditions is a trait which has been investigated previously, although not exhaustively. Previous studies have investigated using sodium acetate as the sole acetate source; however, substrate inhibition was determined to be a major restrictive factor [20], [51]. To combat this, fed-batch systems were developed with recycle streams [52]. Using these recycle streams, it was determined that a high sodium concentration was causing the inhibition observed. The use of sodium acetate as a major feeding substrate was the cause of the inhibition [52]. However, in other microbial cell systems, relatively low acetate concentrations have also been shown to cause growth inhibition, perhaps due to the accumulation of NADH [53]. Through various optimization methods, a heterotrophic cell concentration of 1.2e7 cells/mL was achieved using sodium acetate [54]. It was later suggested that the acetate source be altered to one that does not contain sodium; for example, ammonium acetate or acetic acid [54], although no studies have ever been reported on the use of alternative acetate sources.

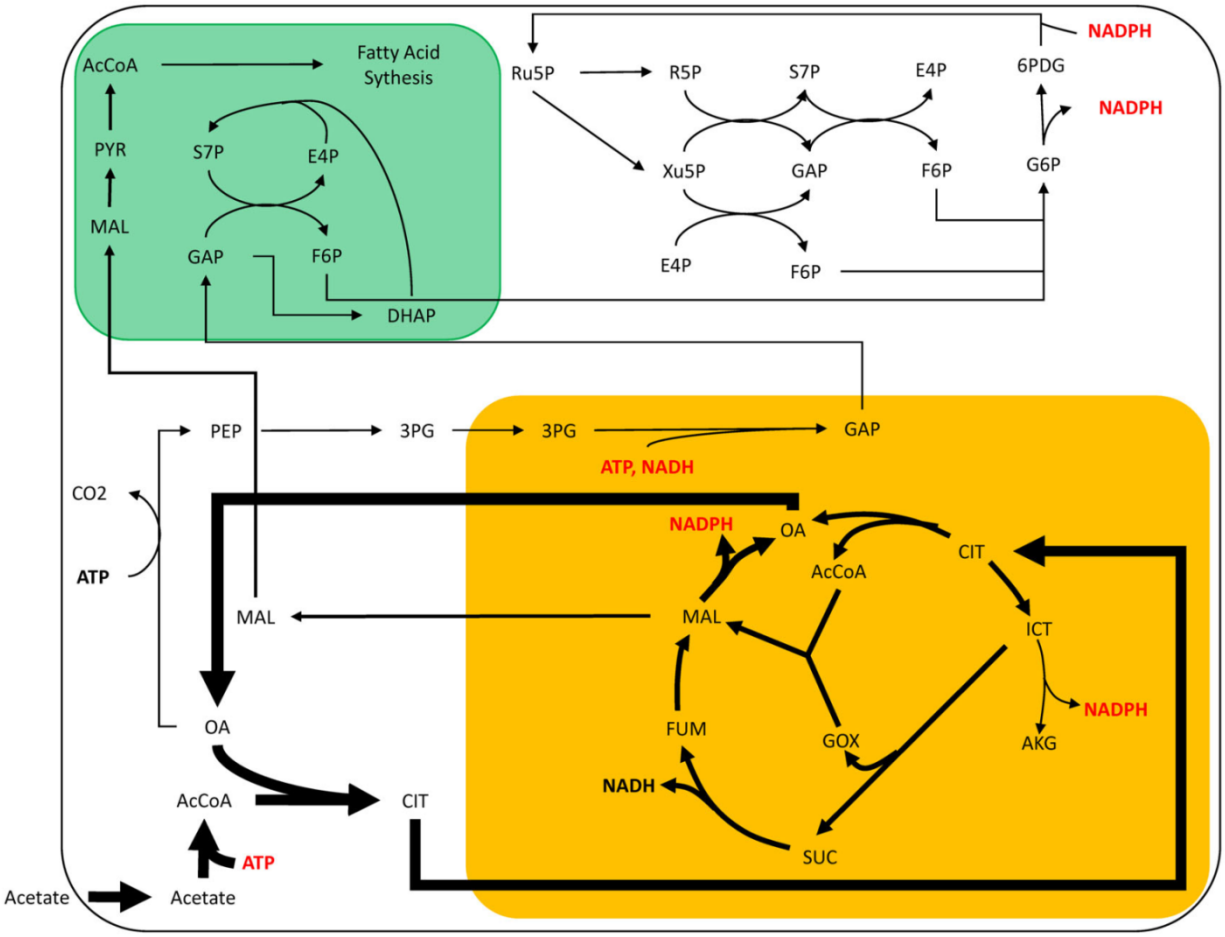

Figure 1-5: An outline of the major metabolic pathways used by *C. reinhardtii* for heterotrophic growth (figure reproduced with permission from [47]). The green compartment represents the chloroplast, while the orange compartment represents the mitochondria. The arrow thickness has been normalized to the total acetate uptake of 100 moles. The molecules in red represent direct (ATP) and indirect (NADH and NADPH) energy sources. Copyright information for this figure is provided in Appendix C.

1.4.3 Mixotrophic Growth

In the presence of both light and an external acetate carbon source, *C. reinhardtii* can be cultured through a process known as mixotrophic growth. Under these growth conditions, algae utilize both of the pathways presented in Figure 1-4 and Figure 1-5 [47]. In a comparison study investigating the growth of *C. reinhardtii* using phototrophic, heterotrophic and mixotrophic growth regimes, the highest *C. reinhardtii* cell densities and recombinant protein production rates were observed under mixotrophic growth conditions [55], [14]. Further, the addition of acetate to culture medium has been shown previously to cause *C. reinhardtii* cultures to become less susceptible to photo-inhibition compared to phototrophic cultures [56]. As such, for the production of high-value products such as recombinant proteins, mixotrophic growth conditions may be the ideal growth methodology.

1.5 Large Scale Growth Strategies of *Chlamydomonas reinhardtii*

Although there are many methods to produce algae on a large scale [57], the choice, design and operation of one particular system depends upon the product of interest. There has recently been an increased interest in research focusing on renewable biological fuel sources (biofuels) to partially replace the current dependence on non-renewable fossil fuels as a major energy source [58]. In this case, the oil bodies found within the algae cells are the products of interest. Large, open algae ponds which utilize the Sun's light energy can be used to maximize the total number of cells [59]. Numerous circulating "raceway" style ponds can be placed over several acres of land. For the production of algal biofuels these systems are suitable [59]. However, if the product of interest is a medically relevant protein for use in humans, this method of culture is unacceptable.

Due to issues with contamination, recombinant proteins utilized for medical purposes must be produced in closed bioreactor systems. For recombinant protein production in algae, one major assumption is that an increase in the number of cells will result in an increased amount of protein. Therefore, recombinant algal culture systems often try to maximize the total number

of cells [60]. Depending upon the size and purpose of the system, algae may be grown phototrophically in photobioreactors or heterotrophically in fermentors.

1.5.1 Photobioreactors

A photobioreactor is defined as a closed vessel which supports phototrophic growth using light energy supplied via electric lights, sunlight or any other light source [61]. Photobioreactors are designed to promote photosynthesis in algae; as such, a system providing optimal access to light, CO_2 and additional nutrients is essential for maximum efficiency. Several configurations have been used, including tubular, cylindrical, conical and flat-sided vessels [62]. The design of any photobioreactor system, regardless of configuration, must consider how to provide light, how to circulate the algae in culture, which materials to use for the bioreactor construction, how to provide CO_2, how to remove O_2, how to maintain sterility and how to control pH and temperature [57].

1.5.2 Fermentors

In the presence of acetate, *Chlamydomonas reinhardtii* can be grown heterotrophically without a light supply in vessels called fermentors [63]. Instead of the focus being on photosynthesis, algal fermentors are designed to promote cellular respiration of the algae cells. Unlike photobioreactors where the limiting growth factor is often the access to light, algal fermentors are limited by the supply of oxygen [61]. Therefore, the major design considerations of a fermentor include access to O_2, access to acetate (or alternative carbon source), a method to circulate the culture, and ways to maintain sterility and control pH and temperature [61].

1.5.3 Photobioreactors versus Fermentors

The choice of whether to use a photobioreactor or a fermentor for use in the recombinant protein production in algae is dictated by a number of constraints. Typically, fermentors are large, complex and heavily controlled vessels which may range anywhere from a size of 1 litre to more than 500,000 litres [61]. On the other hand, photobioreactors are commonly small,

simple vessels which rarely exceed more than a few hundred litres in size [57]. The main drawback of a photobioreactor system is that the larger the required vessel, the more difficult and expensive it becomes to supply light [62]. As such, photobioreactors are ideal for bench-top laboratory experiments using transgenic algae due to the relative simplicity of the system, while fermentors are more relevant for the industrial production of recombinant proteins. A detailed comparison between the use of photobioreactors and fermentors is shown in Table 1-3. Laboratory scale photobioreactor vessels were used in this project as they are commonly used for algal growth, are easily sterilized, can be used for mixotrophic growth experiments, and can easily be converted for use in heterotrophic growth experiments using aluminum foil to block external light sources.

Table 1-3: Characteristics of photobioreactors and fermentors for algal growth (data for table from [61])

Feature	Photobioreactor	Fermentor
Energy source	Light	Organic carbon
Relative cell density	Low	High
Limiting factor for growth	Light	Oxygen
Vessel geometry	Dependent on light penetration	Independent of energy source
Sterility	Usually sanitized	Can be completely sterilized
Relative construction costs	High per-unit volume	Low per-unit volume
Relative operating costs	High per-kg biomass	Low per-kg biomass
Applicability to algae	Photosynthetic algae	Heterotrophic algae

1.6 Project Goal

C. reinhardtii is an organism capable of being cultured using several different growth strategies in a wide variety of culture vessels. Furthermore, it is able to produce a number of products, and can also be genetically manipulated to produce recombinant products. While many reports indicate the production of reporter proteins in microalgae, the production of more industrially useful complex mammalian proteins such as biopharmaceuticals has not been well investigated. Although it has been projected that microalgae such as *C. reinhardtii* may be able to produce high value recombinant proteins at a low cost, this is a field of ongoing research with few reports. Ergo, the goal of this project was to investigate the general feasibility of an algae-derived recombinant protein production system.

1.7 Hypothesis

It was hypothesized that:

1) *Chlamydomonas reinhardtii* algae may be genetically engineered to produce complex mammalian recombinant proteins at a level detectable using traditional protein analysis techniques;

2) A particular set of environmental parameters exist such that total algal protein production may be improved over a base control under mixotrophic growth in suspension bioreactor systems.

1.8 Specific Aims

The three specific aims for this project were:

1. *Characterizing and improving the mixotrophic growth kinetics of Chlamydomonas reinhardtii*;
2. *The manufacture of a genetic construct and the production of a recombinant Chlamydomonas reinhardtii strain capable of producing a mammalian-derived recombinant protein;*
3. *Analysis of recombinant protein production from 500 mL transgenic cultures.*

This thesis is divided into three major results chapters, with one chapter written for each of the specific aims above. Chapter 2 outlines the improvements made to the growth of *C. reinhardtii* using supplemental acetate in suspension bioreactors. Chapter 3 describes the production of a genetic construct and its transformation into *C. reinhardtii*. Chapter 4 investigates the development of a bioprocess to analyze algal recombinant protein production rates using the transgenic algae strain produced from Chapter 3 and the cell culture methods developed in Chapter 2. Finally, Chapter 5 offers a summary, areas of future work and overall conclusions.

Chapter 2: Improving Mixotrophic Algae Growth Kinetics in Suspension Bioreactors using Supplemental Acetate

2.1 Introduction

For decades, microalgae have been investigated by researchers, governments, and entrepreneurs as a source of food, biodiesel, wastewater treatment, carbon capture, and, in genetically engineered microalgae strains, recombinant proteins [64]. One species of particular interest is *Chlamydomonas reinhardtii*, a unicellular green microalga. One major dilemma restricting the widespread use of microalgae stems from low biomass cultures. This greatly affects the overall cost of developing microalgae product systems. The growth of *Chlamydomonas reinhardtii* commonly occurs in photobioreactor (PBR) systems, following photoautotrophic growth processes. There are significant engineering challenges in the design of PBRs, specifically to produce cost-efficient systems that also allow for high cell concentrations. For microalgae that are able to utilize and consume exogenous carbon sources, mixotrophic or heterotrophic cultures may provide a cost effective alternative to photoautotrophic systems [65]. It has been hypothesized that these culture techniques may be able to achieve higher cell densities and cell productivities relative to photoautotrophic cultures. Heterotrophic and mixotrophic culture media are very similar in composition to media used for traditional photoautotrophic growth, with the sole exception being the addition of a consumable organic carbon source [66]. Under mixotrophic growth, microalgae assimilate both CO_2 for photosynthetic metabolism, as well as organic carbon for growth under respiratory metabolism. In some large scale open pond systems, organic carbon such as acetate or glucose is added continuously in small quantities [65]. Heterotrophic growth removes the requirement for light, eliminating the issue of light limitation and penetration.

2.2 Materials and Methods

2.2.1 Organism

Wild type *Chlamydomonas reinhardtii* 137c was obtained from Invitrogen (Catalog #A14258). Cells were maintained at -80°C for up to one year prior to resuscitation, as per the supplier's

guidelines. Following the supplied manual, frozen cells were resuscitated by removing vials from the freezer and immediately placing them in a 35°C water bath for 1-2 minutes until the cells were completely thawed [67].

2.2.2 Medium

Tris-Acetate-Phosphate (TAP) medium was used as a control, since it is commonly used for mixotrophic and heterotrophic growth of *C. reinhardtii*. The TAP medium consisted of 100 mL per L of Beijerinck's Solution, consisting of (per litre) 8 g NH_4Cl (Sigma A-9434), 1 g $CaCl_2 \bullet 2H_2O$ (Sigma C-7902) and 2 g $MgSO_4 \bullet 7H_2O$ (Sigma M-1880); 8.5 mL per L of Phosphate Buffer Solution, consisting of (per litre) 11.62 g Na_2HPO_4 (Sigma S-3264) and 7.26 g KH_2PO_4 (Sigma P-0662); 10 mL per L of Tris Acetate Solution, consisting of (per litre) 242 g Trisma Base (Sigma T-1503) and 100 mL glacial acetic acid (EM Science B10001-78); 1 mL per L of Hutner's Trace Element Solution, consisting of (per litre) 50 g $Na_2EDTA \bullet 2H_2O$ (Sigma E-5134), 22 g $ZnSO_4 \bullet 7H_2O$ (Sigma Z-0251), 11.4 g H_3BO_3 (Sigma B-6768), 5.1 g $MnCl_2 \bullet 4H_2O$ (Sigma M-3634), 5 g $FeSO_4 \bullet 7H_2O$ (Sigma F-8633), 1.6 g $CoCl_2 \bullet 6H_2O$ (Sigma C-8661), 1.16 g $CuSO_4 \bullet 5H_2O$ (Sigma C-8027) and 1.1 g $(NH_4)_6Mo_7O_{24} \bullet 4H_2O$ (Sigma M-1019). pH was adjusted to 7.0 with HCl (EM Science HX0603-53) using a VWR SB20 pH Meter.

Note that the acetate concentration in the control TAP medium was 17 mM. Various acetate salts or buffered acetic acid were added to TAP medium for experimental conditions to raise the acetate concentration. Acetate salts were added to TAP medium prior to sterilization. Acetic acid was diluted with ddH_2O to a 2 M solution and was buffered with Trisma Base to a pH of 7.0 prior to being added to TAP medium. The acetic acid buffered with Trisma Base to pH=7.0 will henceforth be known as the Tris-acetate solution.

All media were sterilized by autoclaving at 121°C for 20 minutes.

2.2.3 Cell Culture and Cell Counting

All photoautotrophic and mixotrophic cultures were grown under a light flux of 50 $\mu E \bullet m^{-2} \bullet s^{-1}$. Light intensity was measured with a light meter (VWR 62344-944) placed at the edge of the culture vessel. Heterotrophic culture vessels were blocked from all sources of light using an aluminum foil covering. A 50,000 cell/mL starting inoculum was used to begin every culture, taken from a stock culture growing under the exponential growth phase. Cultures were grown in ambient air conditions, and temperature was maintained at 23°C using a BOD incubator (VWR 10753-894). All procedures relating to cell culture were performed using sterile technique under aseptic conditions.

All cell concentrations were observed using cell counting via an Olympus CKX41 light microscope and a hemocytometer. It was found that under mixotrophic and heterotrophic conditions, an OD_{750} measurement was not a reliable method to infer cell concentration.

2.2.4 Small Scale Cultures

10 mL small scale cultures were performed in 25 mL Erlenmeyer shake flasks. Cells were agitated at 120 rpm on a gyratory shaker. All culture conditions were performed in triplicate.

2.2.5 Large Scale Cultures

500 mL large scale cultures were performed in 500 mL working volume Wheaton Celstir suspension culture flasks (Wheaton 356882). Agitation was provided via a digitally controlled 4-way multi position stir plate (VWR 12621-022). Acetate concentrations in the medium were determined using an acetate colourimetric assay kit (Sigma-Aldrich MAK-086). All culture conditions were performed in triplicate.

2.2.6 Osmolality Measurements

Osmolality measurements were obtained using an Osmette A Osmometer (Precision Instruments Inc).

2.2.7 Statistical Analysis

Statistical analysis of data was performed using the Real Statistics Data Analysis Tool add-on in Microsoft Excel. A two-factor mixed-design ANOVA followed by a post-hoc Tukey Honest Significant Difference (HSD) test was used to analyze the experimental data, and p-values less than 0.05 were considered statistically significant.

2.3 Results

The identification of optimal cell growth parameters aids the efficiency of algal product production. By maximizing cell concentrations, it is assumed that the product concentration is also maximized. Small scale 10 mL cultures were used to characterize cell growth kinetics and identify potential areas for improvement. The use of small scale cultures allowed for easy observation of several experimental conditions at once. Following initial growth characterization and improvement, the process was scaled up into larger 500 mL suspension bioreactor vessels.

2.3.1 Small Scale Culture

Initial studies were performed using sodium acetate as an external carbon source. Using TAP medium as a control, experimental cases were undertaken by adding sodium acetate to the control medium. Additional sodium acetate concentrations ranging from 10 mM to 60 mM were investigated, in 10 mM increments. Figure 2-1 outlines the results from this study. Additional data for the remaining sodium acetate concentrations can be found in Appendix B.

Following the protocol for the sodium acetate experiments with 10 mM concentration increments, the effect of adding potassium acetate was tested. As outlined in Figure 2-2, the best potassium acetate concentration was observed at 40 mM. Additional data for all potassium acetate concentrations is presented in Appendix B. Below a concentration of 40 mM, the addition of potassium acetate did not have a statistically significant effect to the

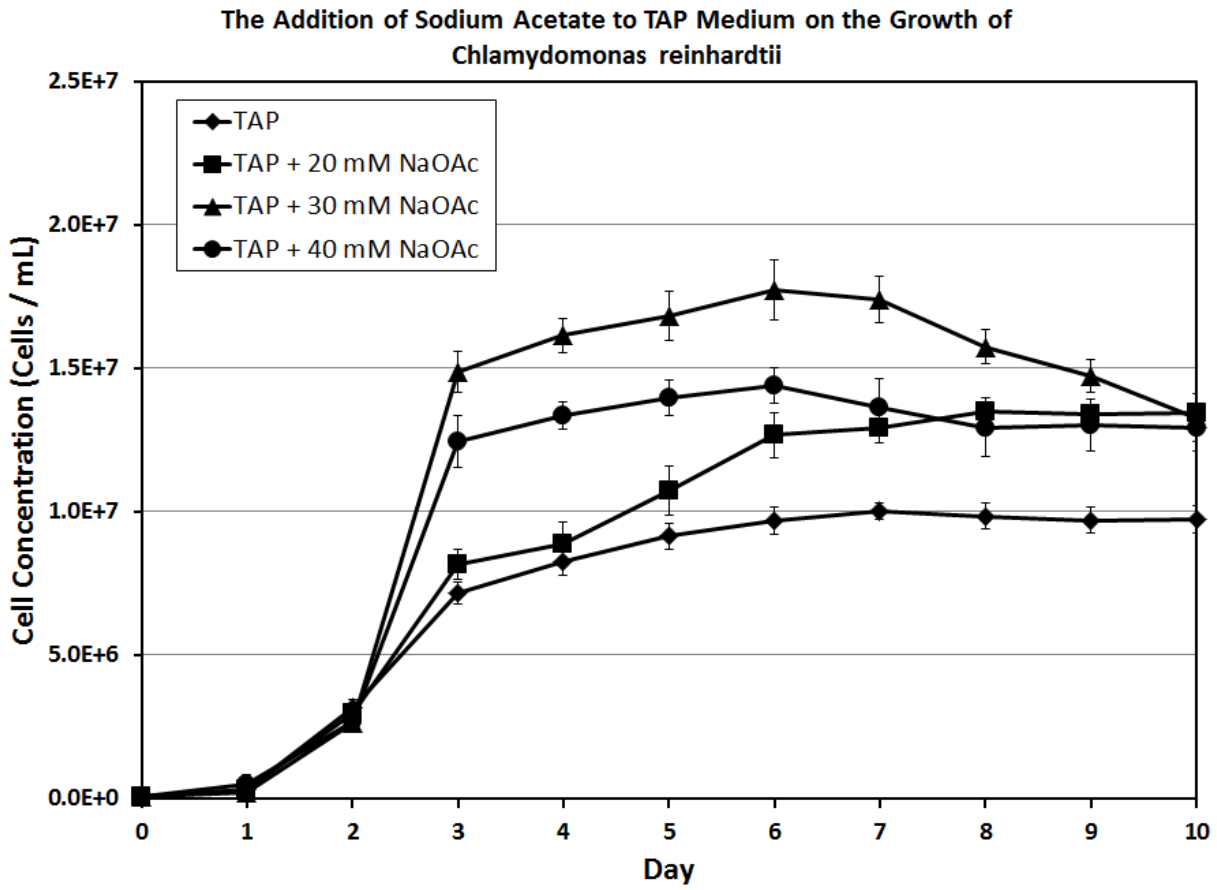

Figure 2-1: The effect of sodium acetate (NaOAc) when added to TAP medium on the growth of *C. reinhardtii* under photoautotrophic culture conditions. As the sodium acetate concentration was increased, both the maximum cell concentration attained and the cell growth rates measured during the exponential phase increased. This was observed up to a sodium acetate concentration of 30 mM, where a peak was observed. When added in concentrations above 30 mM, cell growth was reduced relative to the peak at 30 mM. At 30 mM NaOAc, the maximum cell concentration observed was 1.77e7 cells/mL, with a cell growth rate of 1.71 ± 0.07 day^{-1}. All data is statistically significant with a p-value < 0.01 with exception to the comparison of TAP + 20 mM NaOAc and TAP + 40 mM NaOAc, where the data was not found to be statistically significant. All cases were performed in triplicate (n=3), and error bars represent the standard error of the mean. Supplemental data is presented in Figure B-1.

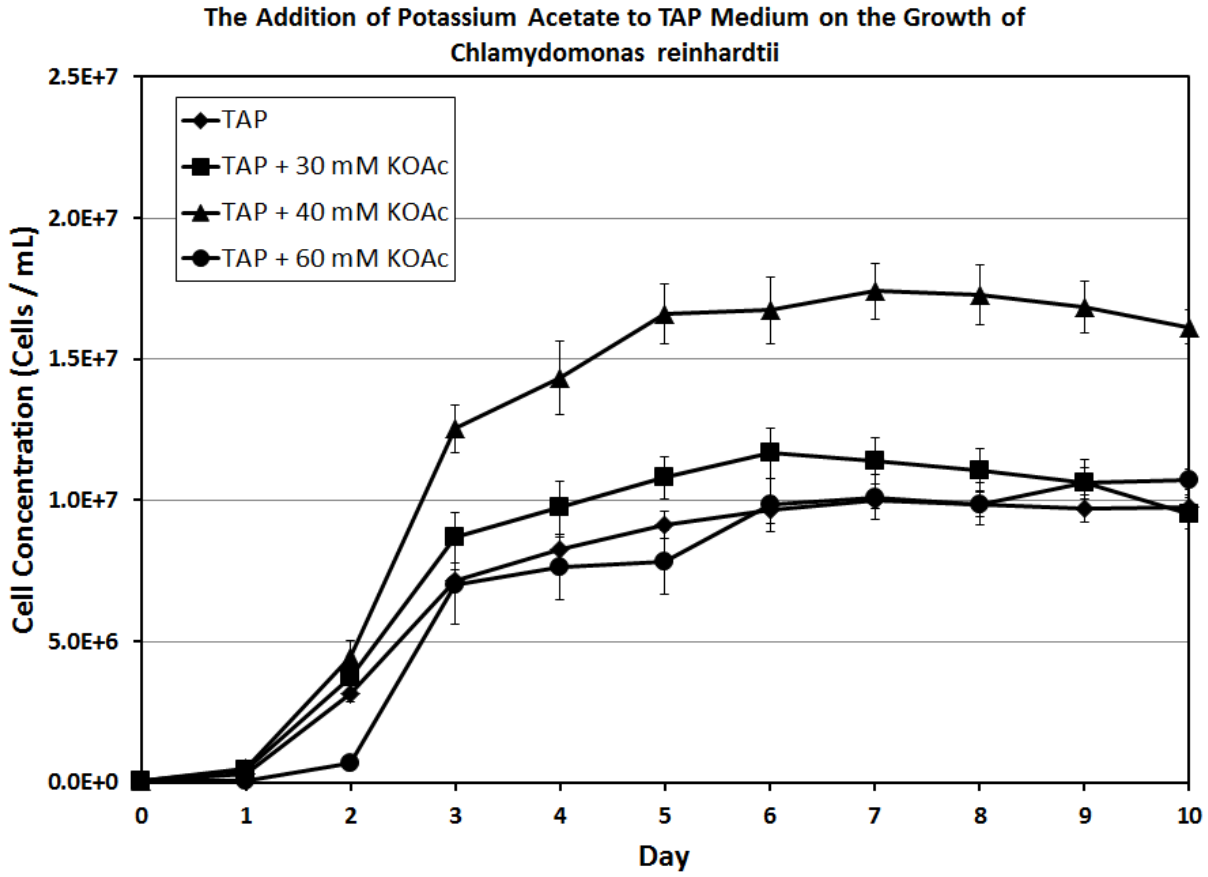

Figure 2-2: Potassium acetate (KOAc) and its effect on the growth of C. reinhardtii when added to TAP medium. A peak in cell concentration and cell growth rates was observed at a potassium acetate concentration of 40 mM. At this concentration, the maximum cell concentration observed was 1.74e7 cells/mL with a cell growth rate of 1.62 ± 0.09 day^{-1}. Compared to any other data set, the TAP + 40 mM KOAc case is statistically significant with $p<0.001$. All other data set comparisons were not found to be statistically significant. All cases were performed in triplicate (n=3), and error bars represent the standard error of the mean. Supplemental data is presented in Figure B-2.

growth of *C. reinhardtii*. Above 40 mM, cell growth kinetics lowered to near the level of the control TAP medium condition.

An attempt was made to remove the potentially negative influence of the accumulation of positive cations, and the effect of adding Tris-acetate to TAP was investigated, as outlined in Figure 2-3. Additional data for all Tris-acetate concentrations tested can be found in Appendix B. It was found that a Tris-acetate concentration of 40 mM added to TAP medium provided the highest maximum cell concentration and cell growth rates. A summary of the results obtained from the various acetate sources is prepared in Table 2-1.

Two other acetate salts were also investigated: ammonium acetate and lithium acetate. It was found that any concentration of these salts added to TAP caused decreased growth relative to the control.

It should be noted that the osmolality of the medium increases with the addition of external substances. To observe the effect of osmolality on cell growth, sucrose, a neutral substance not metabolized or used in any way by *C. reinhardtii*, was added in various concentrations to TAP medium to mimic the osmolality increase of the acetate compounds. TAP medium osmolalities were increased by up to 120 mOsm/L using sucrose, and this increase in the osmolality of the medium was found to have no impact on cell growth (data not shown). 120 mOsm/L corresponds to the osmolality increase of 60 mM of a two ion compound such as sodium acetate.

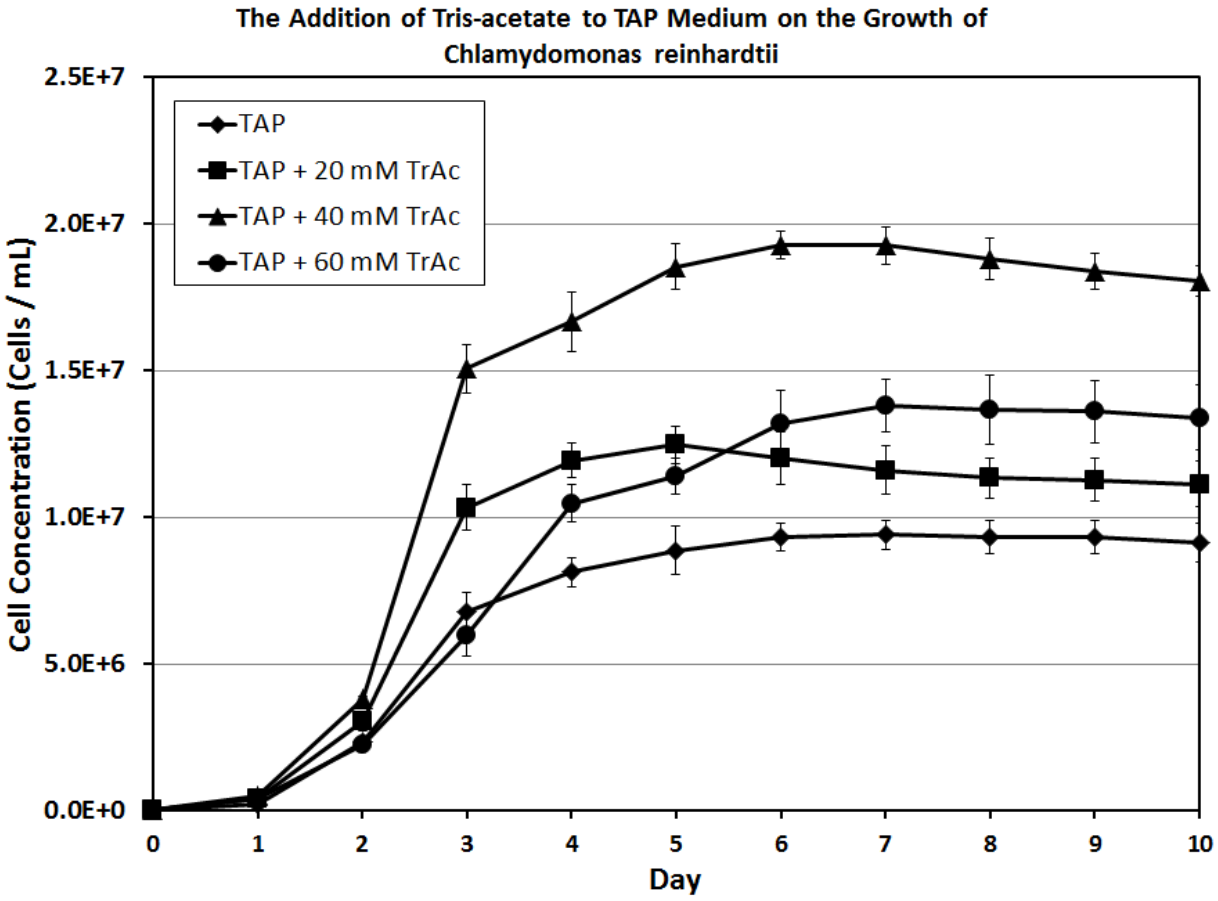

Figure 2-3: The effect of adding additional Tris-acetate (TrAc) to TAP medium on the growth of *C. reinhardtii*. A Tris-acetate concentration of 40 mM added to TAP medium was found to maximize both cell concentration and cell growth rates. Relative to all other acetate sources used, a Tris-acetate concentration of 40 mM provided the highest cell concentration and cell growth rates observed: 1.93e7 cells/mL and 1.72 ± 0.07 day^{-1}, respectively. All data set comparisons were found to be statistically significant with $p<0.05$, with exception to the comparison between TAP + 20 mM TrAc and TAP + 60 mM TrAc. All cases were performed in triplicate (n=3), and error bars represent the standard error of the mean. Supplemental data is presented in Figure B-3.

Table 2-1: The maximum cell concentrations and net specific growth rates observed from the various sources of acetate added to TAP medium

	Best tested concentration added to TAP	Maximum Cell Concentration (cells/mL)	Net Specific Growth Rate (day^{-1})
TAP	-	1.01e7	1.34 ± 0.04
TAP + NaOAc	30	1.77e7	1.71 ± 0.07
TAP + KOAc	40	1.74e7	1.62 ± 0.09
TAP + TrAc	40	1.93e7	1.72 ± 0.07

The final small scale experiment undertaken was designed to observe the impact of light during the stationary growth phase. For this experiment, the cultures were subjected to light for the first three days of the culture period resulting in mixotrophic growth. After the third day, cultures were removed from the light and placed into darkness for heterotrophic growth. This study was undertaken using a Tris-acetate carbon source added to TAP medium as it provided the highest growth of the acetate sources investigated. The results of this study are shown in Figure 2-4. Following the initial exponential growth phase from days 1 to 3, a secondary lag phase was observed when the cultures were transferred to heterotrophic growth. After the secondary lag phase, a secondary growth phase was observed for the experimental cultures. Using a medium consisting of 40 mM Tris-acetate added to TAP medium, a cell growth peak was observed at day 6 at 1.87e7 cells/mL.

Additional studies were performed to investigate the effect of the initial mixotrophic growth period. Initial culture time in the light was varied between 0, 1, 2, 3 and 4 days (data not shown). It was found that a 3 day initial mixotrophic period provided the optimal transition from mixotrophic to heterotrophic growth.

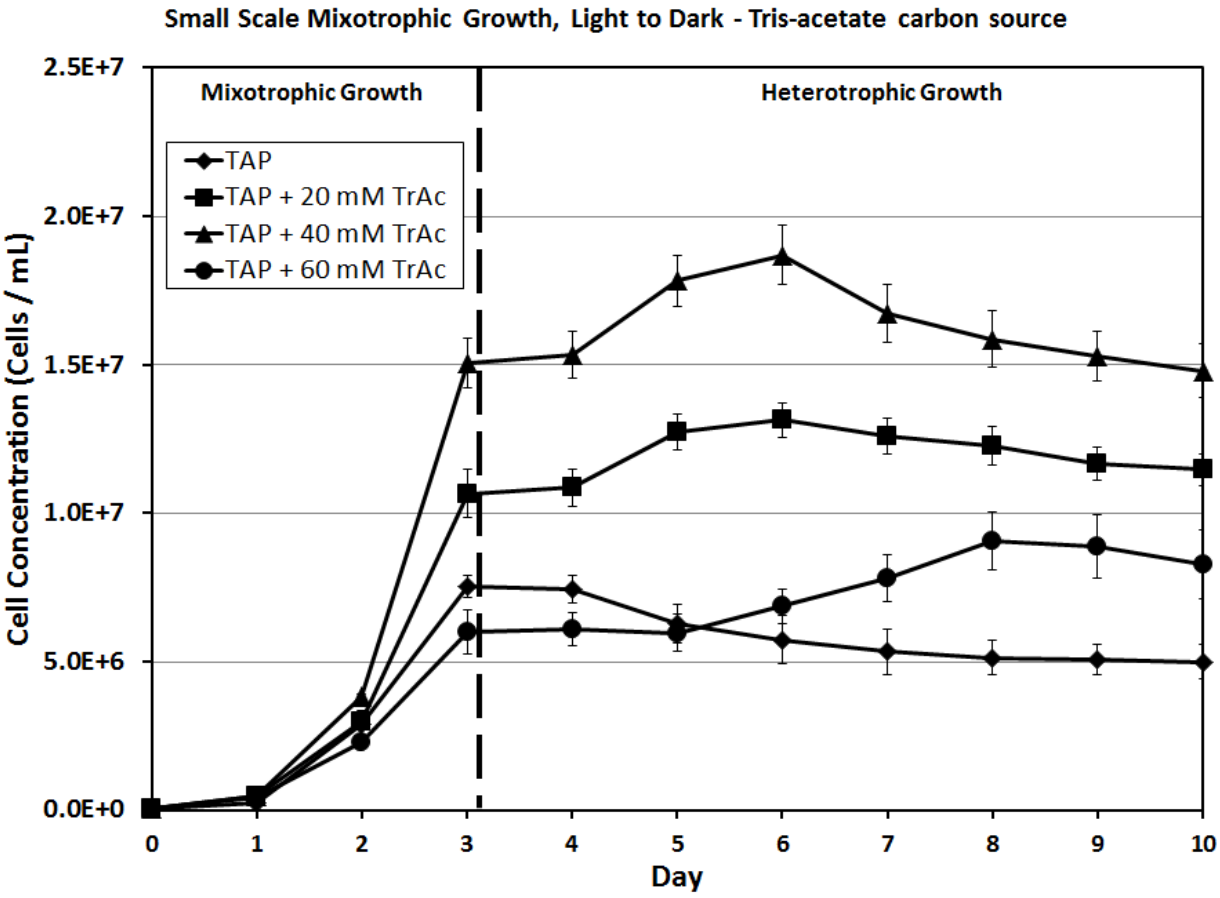

Figure 2-4: The effect of transitioning from mixotrophic to heterotrophic culture conditions on the growth of *C. reinhardtii* with a Tris-acetate (TrAc) carbon source. For the first three days of the culture period, the culture was grown mixotrophically under the presence of light. After the third day, the lights were removed and the cultures were subjected to heterotrophic growth for the remainder of the culture period. Using TAP + 40 mM Tris-acetate, a maximum cell concentration of 1.87e7 cells/mL was observed on day 6. All comparisons between experimental conditions were found to be statistically significant with $p<0.05$, with exception to the comparison between TAP and TAP + 60 mM TrAc. All cases were performed in triplicate (n=3), and error bars represent the standard error of the mean.

2.3.2 Large Scale Culture

Initial experiments performed using 500 mL spinner flasks were designed to investigate the impact of agitation rate and the addition of Tris-acetate to TAP medium. A factorial experiment was designed using two agitation rates and two different media. A moderate agitation rate of 200 RPM and a high agitation rate of 400 RPM were analyzed. An agitation rate of 400 RPM represents the maximum rate at which the impellers of these spinner flasks could rotate, given the limitations of the stir plate and the vessels themselves. Using these agitation rates, it was assumed that all cultures were well mixed. Using the results from the small scale cultures, two different media were used: a control consisting of TAP medium, and an experimental case consisting of TAP medium + 40 mM Tris-acetate. The medium consisting of TAP + 40 mM Tris-acetate represents the medium which produced the highest maximum cell concentration and net specific growth rate in the small scale experiments. Figure 2-5 outlines the results from this experiment.

Increasing the agitation rate from 200 RPM to 400 RPM caused the cell growth curve to shift towards the left. Hence, increasing the agitation rate from 200 RPM to 400 RPM increased the net specific growth rate. The addition of 40 mM Tris-acetate to TAP medium caused an upward shift in the cell growth curve, resulting in a higher maximum cell concentration. Of these four scenarios, the best case was observed using a high agitation rate of 400 RPM, with the improved medium consisting of TAP + 40 mM Tris-acetate.

Following the characterization of the growth of *C. reinhardtii* in 500 mL spinner flask culture vessels, experiments moved on to observe how acetate concentrations changed throughout the culture period. For these experiments, 1 mL aliquots of culture medium were taken every day and stored for analysis. Acetate concentrations were analyzed for the initial starting medium, as well as on culture days 3, 5, 7 and 9. Figure 2-6 shows the results for a control and experimental culture grown entirely under mixotrophic conditions.

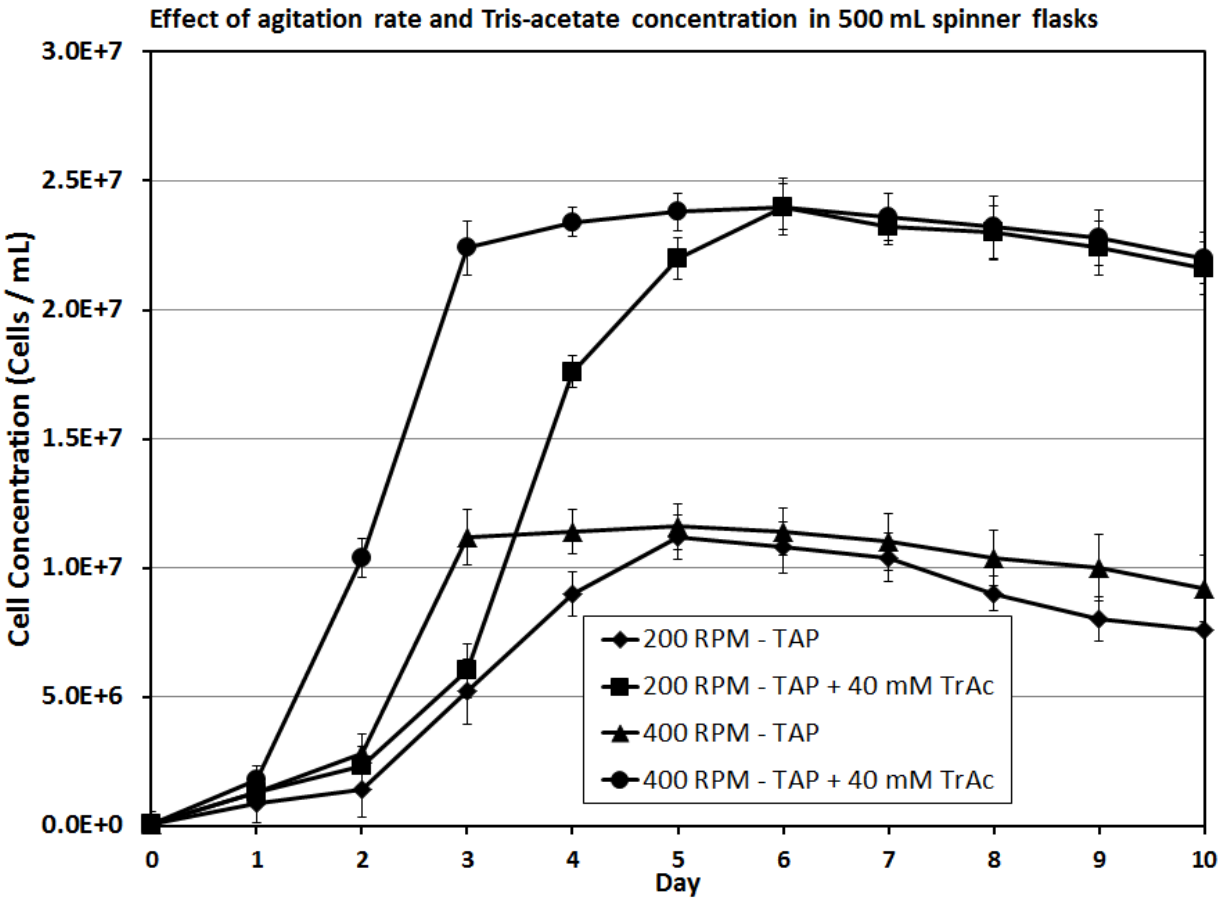

Figure 2-5: A factorial experiment under mixotrophic metabolism to analyze the effect of agitation rate and medium on the growth of *C. reinhardtii*. The two agitation rates chosen were 200 RPM and 400 RPM, while the two media selected were TAP medium and TAP + 40 mM Tris-acetate (TrAc). All data comparisons were found to be statistically significant with $p<0.05$. All cases were performed in triplicate (n=3), and error bars represent the standard error of the mean.

Due to the promising results from Figure 2-4 where small scale cultures were transferred from mixotrophic growth conditions to heterotrophic growth conditions on Day 3, the experiment performed in Figure 2-6 was repeated using this growth method alteration protocol, and is shown as Figure 2-7.

Figure 2-6 and Figure 2-7 show that the available acetate was consumed partway through the culture period, and at the same time a cell concentration peak was observed due to the full consumption of the carbon source. As shown in Figure 2-3, adding more Tris-acetate to the starting inoculum medium was not the solution to this problem; inhibition was observed at Tris-acetate concentrations above 40 mM when added to TAP medium. Instead, a fed-batch feeding strategy was used to overcome these limitations. In this fed-batch strategy, Tris-acetate was added to the culture medium to increase the acetate concentration by 40 mM. This helped to return the acetate concentration back to the initial levels while avoiding the issue of inhibition. Figure 2-8 outlines the results from a wholly mixotrophic, fed-batch culture system. In this experiment, 40 mM Tris-acetate was added to the culture in a fed-batch fashion on Day 5 to increase the culture acetate concentration, resulting in prolonged mixotrophic growth. Under these conditions, the cell concentration was maintained above 2.5e7 cells/mL until the culture period ended at Day 10.

The experiment in Figure 2-8 was repeated using the mixotrophic-heterotrophic culture strategy, and the results can be seen in Figure 2-9. Similar to the wholly mixotrophic case, a fed-batch addition of 40 mM Tris-acetate rejuvenated the culture acetate concentration, resulting in a high cell concentration that was maintained until the culture period ended on day 10.

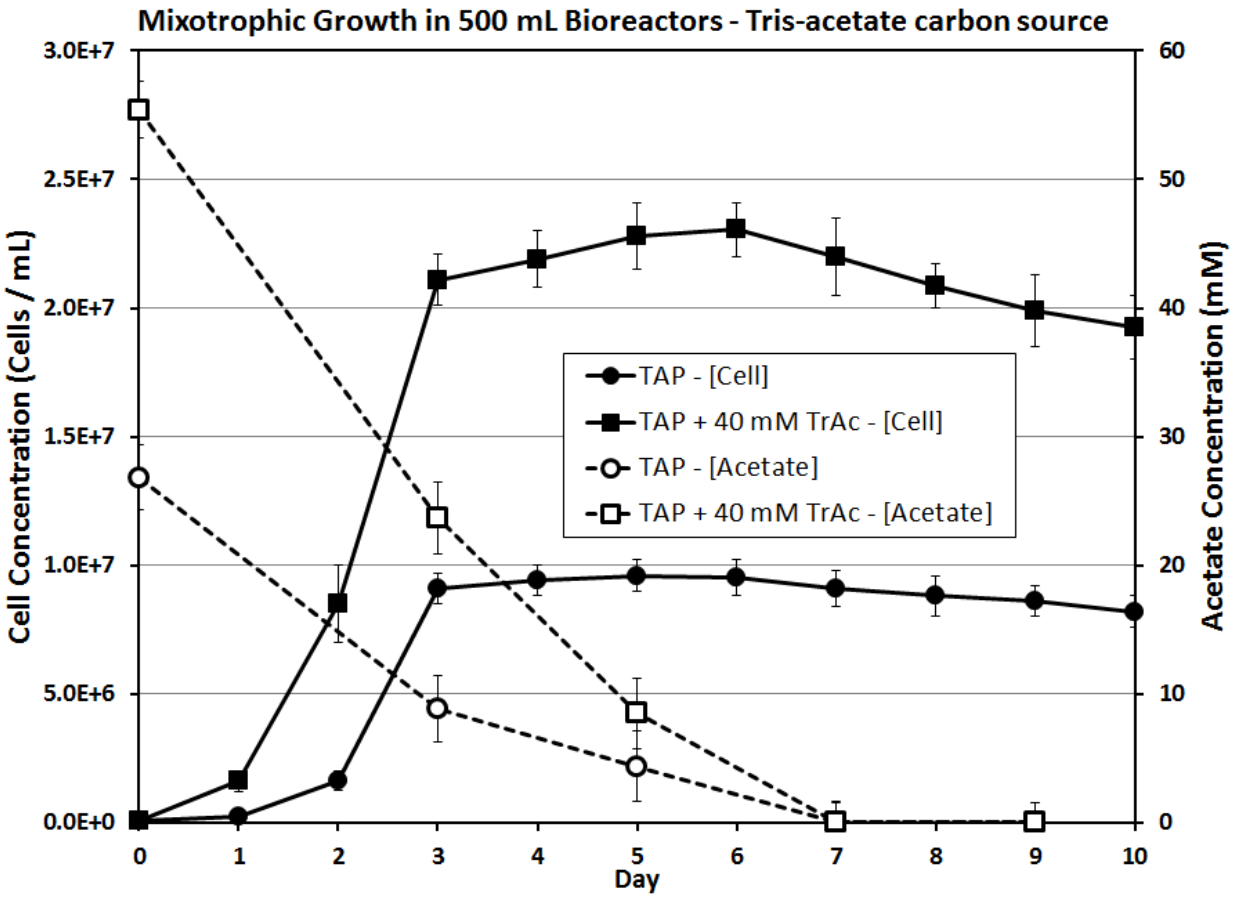

Figure 2-6: A mixotrophic growth curve of *C. reinhardtii* showing the decline of acetate concentration using TAP medium and TAP + 40 mM Tris-acetate (TrAc). Cell concentrations are depicted as solid black lines and correspond to the primary y-axis, while acetate concentrations are depicted as dashed lines and use the secondary y-axis. For both the control and experimental cases, the majority of the acetate was consumed during the exponential growth phase. The remainder of the acetate was consumed by day 7, resulting in a slight peak in cell concentration for both cases. Following the peaks, the cell concentrations slowly declined until the end of the culture period. All relevant data comparisons were determined to be statistically significant with $p<0.001$. All cases were performed in triplicate ($n=3$), and error bars represent the standard error of the mean.

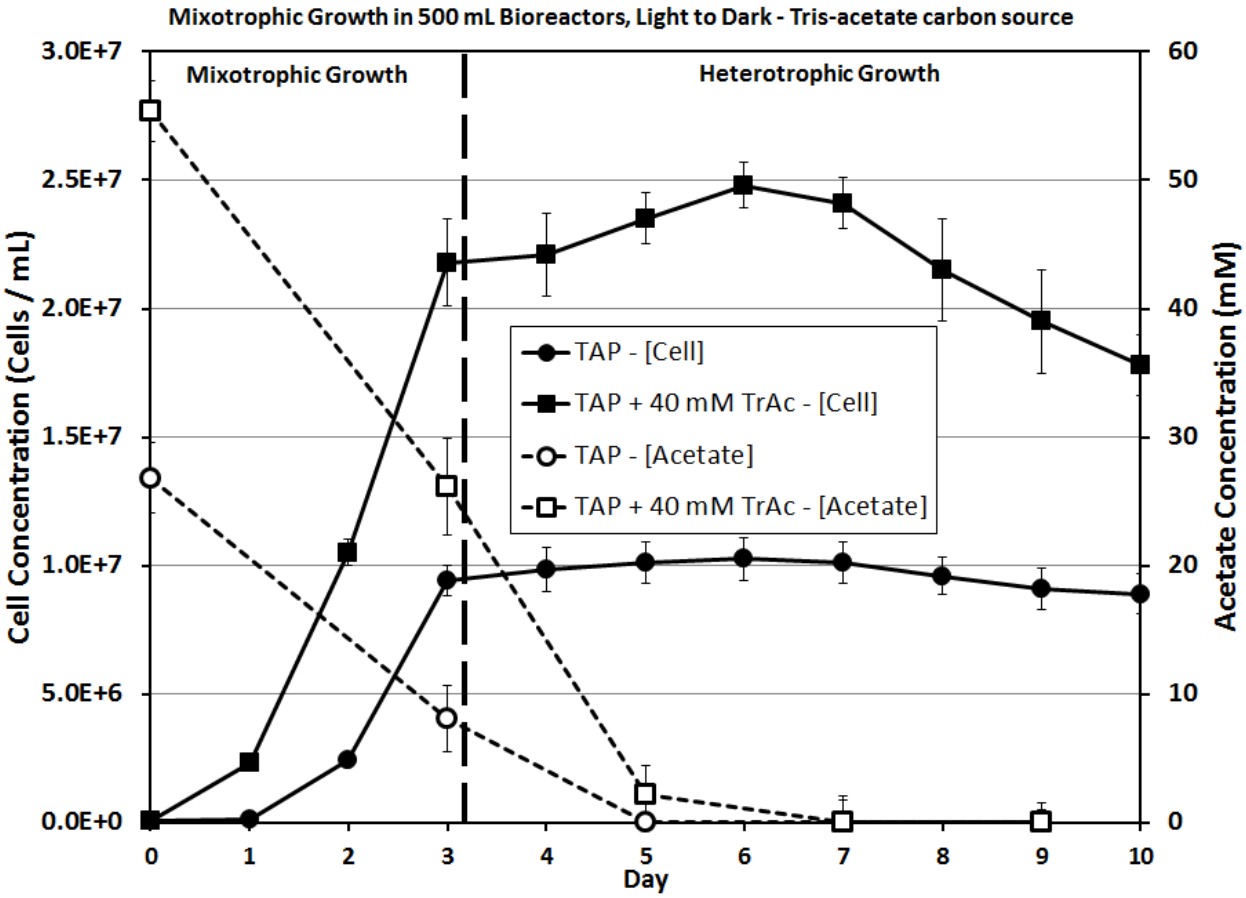

Figure 2-7: Investigation of Tris-acetate (TrAc) concentration under the mixotrophic-heterotrophic growth method alteration protocol. For the first three days the cultures were subjected to light and grew mixotrophically. On day 3, the light was removed and cultures grew heterotrophically until the end of the culture period. The majority of the acetate was consumed during the mixotrophic growth phase, with the remainder nearly consumed by day 5 for both the experimental and control cases. All relevant data comparisons were determined to be statistically significant with $p<0.001$. All cases were performed in triplicate ($n=3$), and error bars represent the standard error of the mean.

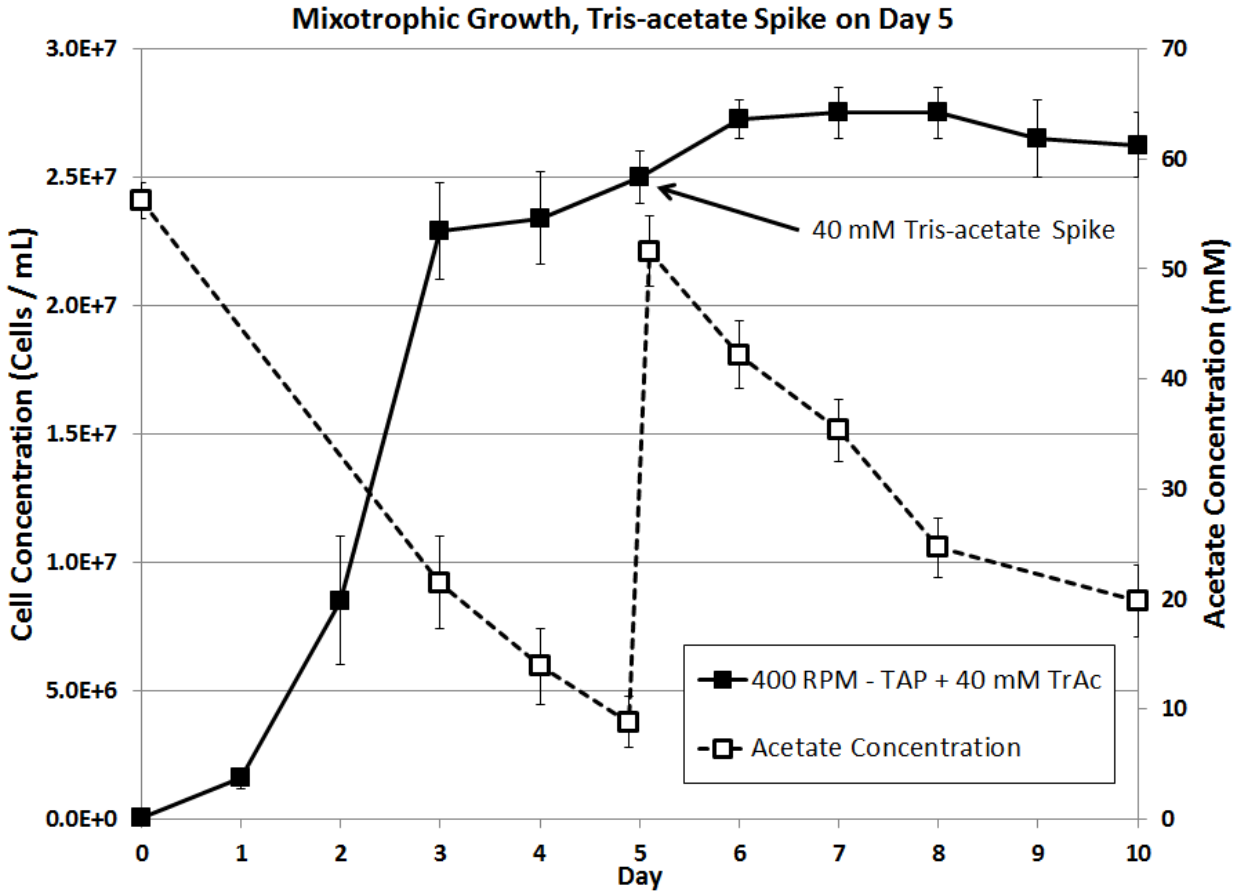

Figure 2-8: The effect of a fed-batch addition of 40 mM Tris-acetate (TrAc) on culture Day 5 under mixotrophic growth. Through the addition of the Tris-acetate on Day 5, acetate concentrations were restored to allow for the continuation of mixotrophic growth. Cell concentrations responded by increasing at Day 6 and maintaining until the end of the culture period. The acetate concentration at the end of the culture period declined to approximately 20 mM. This study was performed in triplicate (n=3), and error bars represent the standard error of the mean.

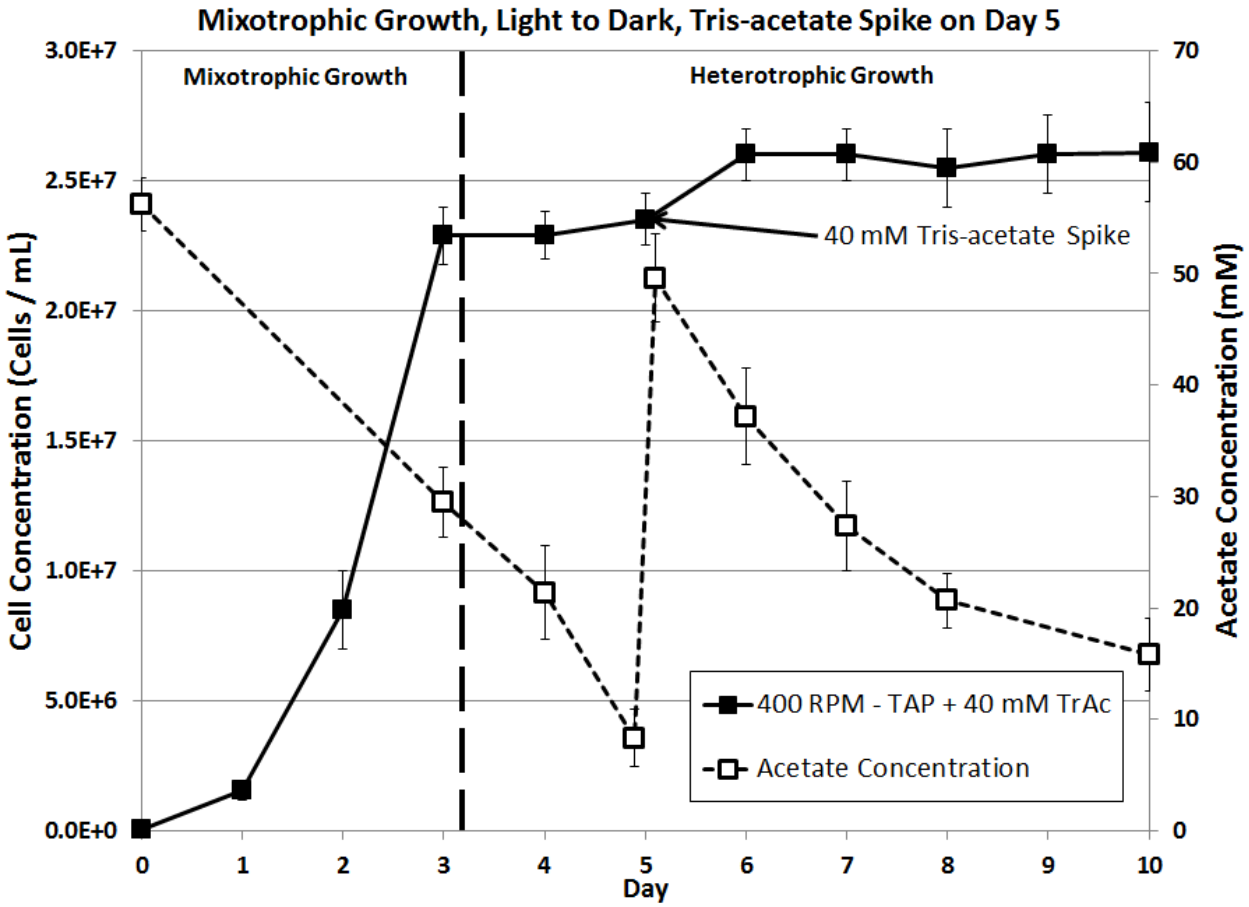

Figure 2-9: The effect of a fed-batch addition of 40 mM Tris-acetate (TrAc) on culture Day 5 following the mixotrophic-heterotrophic growth strategy. The majority of the acetate was consumed during the mixotrophic growth phase between days 0 and 3, while a sharp decline in acetate concentration was observed following transition to heterotrophic metabolism between days 3 and 5. Following the fed-batch addition of 40 mM Tris-acetate, heterotrophic growth continued for one additional day, and cell concentrations remained constant for the remainder of the culture period. This study was performed in triplicate (n=3), and error bars represent the standard error of the mean.

2.4 Discussion

2.4.1 Small Scale Culture

Under the proper concentration, the addition of acetate to TAP medium clearly had a positive impact on the growth of *C. reinhardtii*. The addition of sodium acetate to culture medium has been investigated extensively in the past [20], [51], [52], [54]. It was shown previously that although sodium acetate aids cell growth, the accumulation of the Na^+ cation inhibits cell growth [52]. In small scale 10 mL cultures, inhibition was clearly observed, as shown in Figure 2-1, under sodium acetate concentrations above 30 mM. A similar result was observed for the addition of potassium acetate in Figure 2-2; however inhibition was observed above 40 mM. The addition of Li^+ or NH_4^+ ions via lithium acetate or ammonium acetate, respectively, caused significant inhibition. Relative to basic TAP medium, the addition of either of these acetate salts in the culture medium caused significant detrimental effects to the growth of *C. reinhardtii*.

Previous studies have employed the use of a hollow-fiber recycle stream to prevent accumulation of the Na^+ cation in the culture medium [52]. An alternative and simple solution to this problem was presented in Figure 2-3, where Tris-acetate was added to the culture medium. The use of Tris-acetate means that potentially inhibitory cations cannot accumulate in the culture medium; however, there was also inhibition observed at concentrations above 40 mM due to the accumulation of acetate itself. This is hypothesized to be due to feedback inhibition of acetate within the glyoxylate cycle. Thus, it is thought that there was cation inhibition and acetate inhibition occurring with the addition of sodium acetate or potassium acetate. It was observed that the addition of 40 mM Tris-acetate to TAP medium caused the greatest improvement to net specific growth rate and maximum cell concentration, relative to basic TAP medium. The impact of the addition of these various acetate salts or Tris-acetate on the cell growth kinetics of *C. reinhardtii* has not been previously reported.

Figure 2-4 outlines a unique strategy to allow for heterotrophic growth in *C. reinhardtii*. Following a three day mixotrophic growth period, cultures were subjected to darkness. Once transferred to the heterotrophic phase on day 3, a one day lag phase was observed. This was

likely due to the shock of the removal of the light, and the transfer to fully heterotrophic metabolism. Following the secondary lag phase, cell growth continued to a peak at day 6 with a substantial final cell concentration of 1.87e7 cells/mL. Removing the three day mixotrophic growth period and instead following a wholly heterotrophic growth regime resulted in very limited cell growth. It is hypothesized that the initial three day mixotrophic growth period is required to prepare the cells for the heterotrophic growth phase. This is because the glyoxylate cycle, the cycle responsible for heterotrophic metabolism, can only metabolise acetate by first expending some energy in the form of ATP. Without sufficient energy reserves, the acetate cannot be metabolized. This novel method of promoting algal heterotrophic growth has not been investigated previously.

2.4.2 Large Scale Culture

The experiments performed in 10 mL small scale cultures showed promising improvements to the cell growth kinetics of *C. reinhardtii*. The high-throughput approach used for the small scale cultures is not possible in larger vessels. Thus, the best results from the small scale cultures were scaled up and analyzed in large scale 500 mL spinner flasks.

It was assumed that the data found in the small scale cultures would be relevant and reconcilable in a larger system, despite changes in the vessel type and culturing method. Although the vessels would ideally be similar and directly scalable via predetermined quantities, the two vessel types used in these experiments were the only ones available for use by our laboratory. It should also be noted that there are several other large scale culture vessels that may have been more ideal for the culture of *C. reinhardtii*, including but not limited to: thin plate photobioreactors, computer-controlled chemostat photobioreactors, or thin-tube continuous bioreactor systems. The use of such systems may be investigated in the future as they may more properly control culture conditions. For the experiments presented, the results indicate that, generally speaking, the trends found from the small scale culture experiments translated to the large scale cultures.

Initial studies with the 500 mL vessels found that higher agitation rates led to shifts in the growth curve to the left, while the addition of 40 mM Tris-acetate to TAP medium caused an increase to the net specific growth rate and the maximum cell concentration.

Rigorous agitation of the culture results in a more well-mixed culture. This aids in the homogeneity of the culture as a whole, and also may increase the amount of time, on average, that a single cell would have access to the light source (for cultures with an external light source). Agitation also helps overcome mass transfer limitations. Two major limiting factors in this system include cellular access to oxygen and carbon dioxide. Oxygen is required for heterotrophic metabolism while carbon dioxide is required for phototrophic metabolism. Under mixotrophic metabolism, access to both oxygen and carbon dioxide is essential for *C. reinhardtii*. Increasing the agitation rate helps overcome the mass transfer limitations within this large scale culture system. As the results indicated, cell growth kinetics improved under increasing agitation rates. This investigation was limited due to the equipment restriction of a maximum agitation rate of 400 RPM. There are likely still mass transfer limitations presents in this system which may possibly be overcome by further increasing agitation rates in a more rigorous culture vessel system, or perhaps by direct injection of necessary gases.

Although it may be beneficial to increase the agitation rates on these cultures, the shear generated by the impellers must remain a concern. *C. reinhardtii*, relative to other organisms such as mammalian cells, have a cell wall as well as a cell membrane, which helps protect the cells from being damaged by high shear stress. Higher agitation rates above 400 RPM may increase the shear stress to a point where the cells lyse and the culture dies. This must remain a concern for future studies.

Ample concentrations of nitrogen, phosphorus and potassium are also very important factors in the growth and proliferation of algae. The impact of these elements was not analyzed in this project; rather, it was assumed that the TAP medium provided the necessary quantities of

these nutrients to the cultures. If nitrogen, phosphorus or potassium were identified as lacking, this would be a limiting factor for algal growth and would need to be addressed.

Access to light is also a limiting factor for cultures grown phototrophically and mixotrophically. As cell density increases in a photobioreactor, light penetration into the culture becomes more of a concern. This limitation can be circumvented by using alternative forms of photobioreactor vessels, increasing access to light, or by maintaining lower cell concentrations perhaps in a continuous cell culture process. Issues due light limitations were not assumed to be present for this project, but would be an essential consideration for larger scale operations or larger culture vessels.

Under 400 RPM and TAP medium + 40 mM Tris-acetate, the cell growth curve was further analyzed via an acetate concentration assay. The results of this study, outlined in Figure 2-6, indicate the rate at which acetate was being consumed in the culture. By culture day 7, the acetate was entirely consumed by both the experimental culture and the control culture. As a result of the acetate consumption, the culture transferred from mixotrophic growth to phototrophic growth only. This impacted the cell growth curve, resulting in cell death from day 6 until the end of the culture period. A similar result was observed under the mixotrophic-heterotrophic growth method shown in Figure 2-7.

It was clear that feeding of acetate was necessary to maintain a supply of acetate past day 5. A fed-batch feeding regime of Tris-acetate was suggested, with a one-time feed on day 5. Unlike the acetate salts, the Tris-acetate can be added in a fed-batch manner without accumulation of inhibitory cations. The fed-batch acetate feeding regime was a success, as shown in Figure 2-8. A similar growth curve was observed utilizing the mixotrophic-heterotrophic growth strategy, as outlined in Figure 2-9.

A computer-controlled chemostat photobioreactor system would be of particular use to aid in these findings. A chemostat photobioreactor system could be automatically programmed to

continuously feed Tris-acetate into the system to maintain an appropriate acetate concentration. This would eliminate the concern over diminishing acetate concentrations, and would maintain culture conditions at the ideal levels. Such a culture system was not available for this project, but may be used in the future as a more rigorous cell culture system.

Research on the optimization of algal cell growth kinetics under mixotrophic growth has not been well investigated. *C. reinhardtii* cell concentrations of up to 1.2e7 cells/mL have been reported through the addition of sodium acetate [54], but the addition of potassium acetate, lithium acetate, ammonium acetate or buffered acetic acid to algal cell culture medium for the purpose of improving cell growth kinetics has not been previously investigated.

The work presented in this chapter represents novel advancements in the culturing of *C. reinhardtii*. First, the analysis of the growth kinetics of *C. reinhardtii* under mixotrophic growth conditions was performed using a variety of forms of supplemental acetate. Second, the development of the mixotrophic-heterotrophic growth regime was used to offer an alternative method for culturing cells heterotrophically. Finally, a fed-batch culture system was developed which can maintain *C. reinhardtii* cell concentrations above 2.5e7 cells/mL.

2.5 Conclusions

The addition of acetate to *Chlamydomonas reinhardtii* culture medium can drastically improve cell growth kinetics and maximum cell concentrations relative to control cases. In this study, the effect of adding sodium acetate, potassium acetate, lithium acetate, ammonium acetate or Tris-buffered acetic acid to TAP medium on the growth of *C. reinhardtii* was investigated. In 10 mL cultures, the addition of 40 mM Tris-acetate to TAP medium was shown to improve the net specific growth rate and maximum cell concentration by 28% and 91%, respectively, relative to TAP medium alone. In 500 mL spinner flasks, the addition of 40 mM Tris-acetate to TAP medium resulted in nearly a 2.5x increase in the maximum cell concentration over regular TAP medium in mixotrophic conditions. A mixotrophic-heterotrophic culture regime was also investigated, and showed promising results reaching a similar maximum cell concentration as

cultures grown mixotrophically for the entire culture period. Overall, the findings presented in this chapter could help improve *C. reinhardtii* cell growth kinetics for maximum cell growth through a very simple alteration to the culture medium. This could have a major impact in the fields of algal biofuels, agriculture and in the production of algal-derived recombinant proteins.

2.6 Future Work

Under mixotrophic growth conditions, *C. reinhardtii* requires access to both CO_2 for photosynthesis and O_2 for acetate consumption. The system described above was limited by having ambient air as a gas source. It was discovered that mass transfer limitations are present in this system. It is clear that these are major limiting factors and would need to be addressed in future studies. Feeding in O_2 and/or CO_2 to the culture medium under the proper concentrations could greatly impact mixotrophic cell growth. Increasing the agitation rate may also have a beneficial impact on the cell growth kinetics. The effects of temperature and light intensity may also play a significant role, and could be the focus of future studies.

The culture vessels used in the experiments presented above were the only readily available and cost effective vessels for the production of *C. reinhardtii* in our laboratory. In the future, more specialized culture systems such as computer-controlled chemostat photobioreactors would provide continuous monitoring and adjustment of key culture parameters. Such systems can be designed to measure and control temperature, pH, acetate concentration, light flux and gas concentrations in a continuous manner. With appropriate control systems in place, the culture vessels used for these experiments could be modified to control these parameters for further laboratory scale experiments.

Chapter 3: Production of a Genetic Construct and its Transformation into *Chlamydomonas reinhardtii*

3.1 Introduction

This project was aimed at producing recombinant proteins in genetically engineered algae. The genetic manipulation of these organisms is not a trivial task, and requires significant work proceeding from a conceptual idea for a product to actually producing that product in the genetically engineered host. This chapter begins with an introduction to the genetic engineering methodology. While this general method is well established for experienced genetic engineers, the novelty in this chapter comes from the unique construct that was developed and its transformation into a *C. reinhardtii* host.

The emerging field of genetic engineering has allowed for dramatic advances to the fields of medicine, manufacturing, research and agriculture, among others. In short, the goal of genetic engineering is to design and utilize a genetic construct to produce a desirable product through a favourable change to a host organism's genome [68]. The key to achieving this goal is the proper design and manufacture of a DNA construct. If a protein is the final product, the genetic construct must be verified, transformed into a host organism and the recombinant proteins verified. A simplified diagram outlining the design process is outlined in Figure 3-1.

3.1.1 Conceptual Design

The first and most important step in the conceptualization phase is to determine a host organism which will produce the protein of interest. Once a host is selected, the desired traits of the genetic construct must be chosen. With an end goal or product in mind, genetic engineers can conceptually design a construct and piece together important parts of the genetic code. The design typically involves the selection and positioning of promoters, screening techniques, restriction sites, the protein of interest and other valuable traits within the construct.

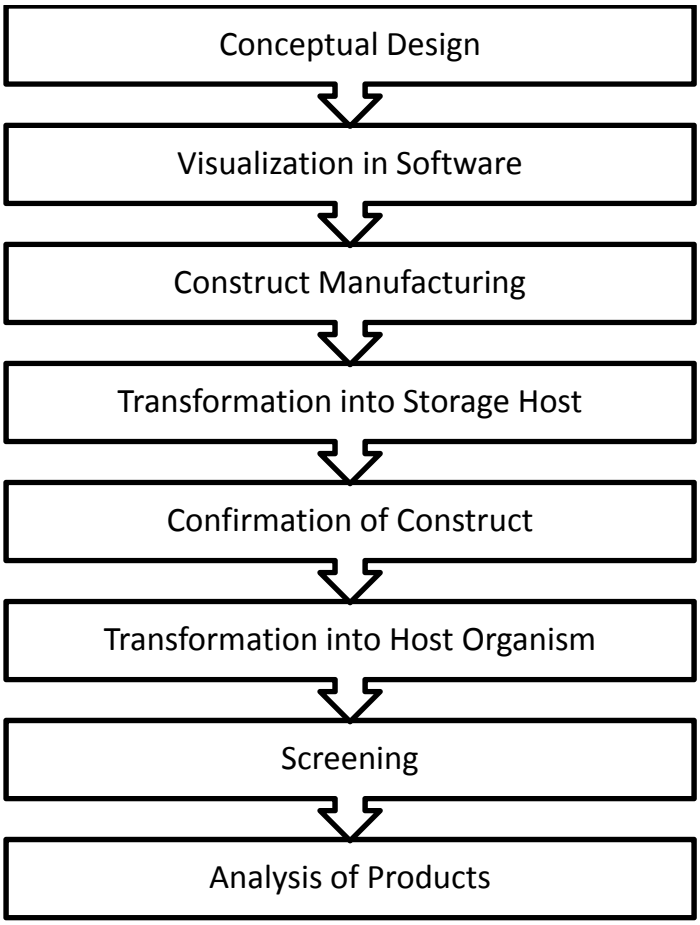

Figure 3-1: A simplified flow chart outlining the key steps to construct design.

In many cases, the goal of genetic engineering is to produce a valuable or useful protein. It is often desirable to link proteins together into what is called a fusion protein. Consisting of two or more proteins conjoined into one protein of interest, fusion proteins are used to link several desired traits, components or products.

3.1.2 Visualization in Software

Using software designed for genetic engineering purposes, the exact genetic sequence of an entire construct can be visualized. One example of genetic visualization software is Gene Construction Kit (Textco BioSoftware Inc.). Using existing information from a gene library, DNA sequences for a particular gene can be determined and inputted into the software. By cutting and pasting genes together, a genetic engineer can design an entire genetic construct optimized for a particular goal. Inputting this information into software provides key information into restriction enzyme sites, DNA sequences and the corresponding amino acid sequences. Although still a conceptual design at this point, the exact sequence of a construct of interest can be analyzed and altered as necessary.

3.1.3 Construct Manufacturing

Once a final product construct is determined, it must be physically manufactured. Many popular genes used in the past exist in what is known as a gene library. Using this library, genetic engineers can use genes made by other researchers. If a gene does not exist in a gene library, it must be manufactured through artificial gene synthesis using existing microbiology techniques such as polymerase chain reaction. Gene pieces can be cut and pasted together using restriction enzyme and ligation techniques. In this way, a gene of interest can be inserted into a vector previously optimized for a particular host organism. Once manufactured, DNA sequences are commonly separated or analyzed using agarose gel electrophoresis. As an alternative to the manual approach, industrial companies such as GenScript will manufacture custom genetic constructs on demand.

3.1.4 Transformation into Storage Host

For long term storage of a genetic construct, the most common host is the bacterium *Escherichia coli*. The *E. coli* represent a simple and cost effective means of mass construct multiplication and storage. A genetic construct of interest can be genetically transformed into *E. coli*, recombinant cells selected using antibiotics, and DNA analyzed for confirmation of vector insertion using agarose gel electrophoresis. If correct, the *E. coli* are then stored in glycerol at -80°C. When a high copy number of the construct of interest is desired, the genetically engineered *E. coli* are resuscitated, cultured and the DNA construct is isolated using plasmid prep techniques.

3.1.5 Confirmation of Construct

A technique known as DNA sequencing is used to confirm that a gene of interest is indeed the product first conceptualized. Using modern machinery and techniques, the exact genetic sequence of a DNA construct can be determined using a DNA sequencer. This device analyzes the DNA and determines the genetic sequence represented by guanine (G), cytosine (C), adenine (A) and thymine (T). Once the results of the sequencing are complete, the DNA sequence can be compared to the conceptual design outlined using the genetic software. If the sequence is confirmed, the construct of interest may then be used to transform a host organism.

3.1.6 Transformation into Host Organism

Using the storage host to increase copy numbers, the genetic construct may be isolated in a high concentration and used to genetically transform the selected host organism. The transformation technique selected will vary depending upon the organism. The transformation procedure allows the exogenous construct DNA to interact with the genomic DNA of the host, with the end goal being the insertion of the construct of interest into the host's genome via homologous recombination.

3.1.7 Screening

Only a small fraction of cells are properly transformed with the construct of interest. The vast majority do not express the gene of interest and are of no use to genetic engineers. One commonly used technique to separate recombinant cells from non-recombinant cells involves the use of antibiotics. When added to the culture medium in the proper concentration, the antibiotics cause cell death to the majority of non-recombinant cells. However, the small fraction of cells which express the gene of interest will convey resistance to the antibiotics and survive. When cultured on solid media, the surviving cells proliferate to produce cell colonies. These colonies can then be individually removed and transferred to liquid media for further scale-up.

Even though the gene of interest may be inserted into the genome of the host organism, positional effects within the host may limit recombinant protein production. Furthermore, occasional mutations may give a non-recombinant cell resistance to an antibiotic, causing their proliferation. Hence, many transformants are selected for further analysis.

3.1.8 Analysis of Products

The final step in the process is to analyze the ability of the host organism to produce the product protein of interest. Further analysis can determine whether the proteins are properly folded and biologically active. For more complex products, proper post-translational modifications must be made to the protein by the host to elicit the desired activity.

In some cases, a protein is not the main product. Instead, some genetic engineering processes preferentially alter an organism's genome to meet a particular goal. This could be as simple as providing antibacterial resistance to a host strain. In this case, the product is the cell strain itself and proper analysis comes from observation aimed at ensuring that the culture meets the desired goal.

3.1.9 Summary

Genetic engineering seeks to alter the genetic code of a host organism to manufacture a useful product. Although a broad field, this process generally follows a specified set of steps, regardless of the final host organism chosen. The conceptual design of the construct is the main foundation upon which all further work is based. For this reason, the underlying construct design is of great concern. All steps beyond conceptual design, including manufacturing of the construct, host transformation and product analysis typically all follow established techniques. For this project, the focus was on the design of a single genetic construct. Owing to the flexibility of the genetic engineering process, this construct could be re-designed, altered and re-manufactured at any time to produce alternative products.

3.2 Methods

The methods described below represent the process and techniques used to genetically engineer *C. reinhardtii*, starting from the conceptual design of the construct to the physical transformation of the cells. These methods are not novel; on the contrary, they are techniques commonly used by microbiologists and genetic engineers. Rather, what is unique is the makeup of the construct itself, specifically the gene of interest. The gene of interest will be discussed further in the results section.

3.2.1 Basic Plasmid

An “empty” plasmid was obtained from Invitrogen using the commercially available GeneArt *Chlamydomonas* Engineering Kit (Invitrogen #A14258). This plasmid, known as the pChlamy_1 vector and shown in Figure 3-2, consisted of 4,283 base pairs and had several important and useful sites.

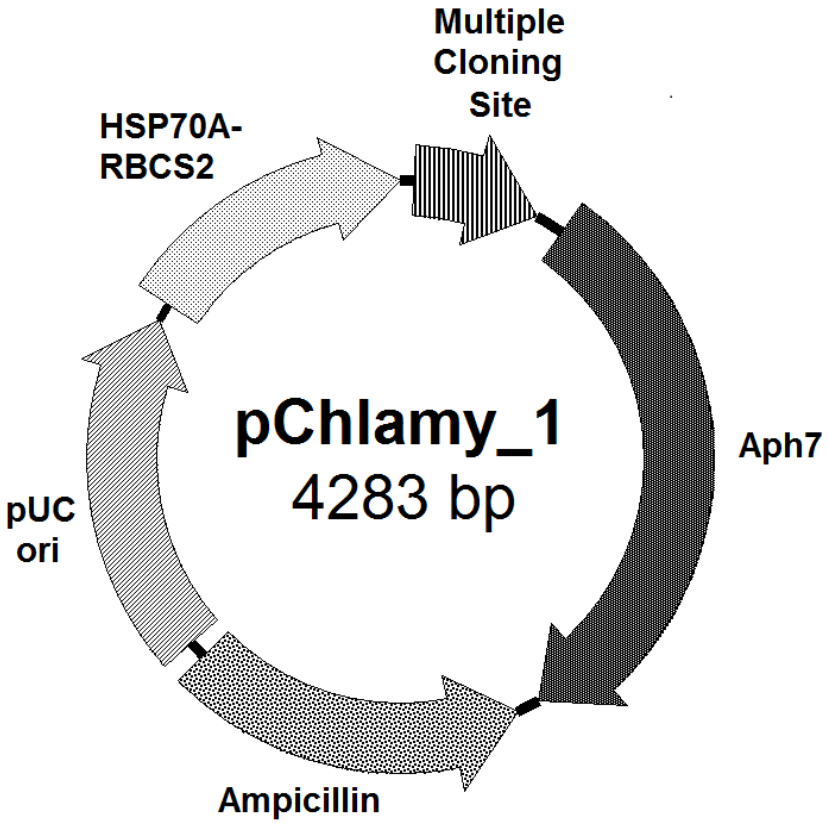

Figure 3-2: The pChlamy_1 vector. Notable sites include: the HAP70A-RBCS2 site, which represents the fusion heat shock and light shock genetic promoter; the Multiple Cloning Site, into which the gene of interest is inserted; the Aph7 site, which corresponds to algal hygromycin B resistance and is used for algal genetic selection; the Ampicillin site, which represents a bacterial genetic selection tool using ampicillin; and finally the pUC ori site, representing the origin of replication site.

The pChlamy_1 vector was used as a tool into which any gene of interest may be inserted. The pChlamy_1 vector can be thought of as the skeleton of the genetic construct, while the gene of interest represented the working gene responsible for producing the protein of interest. The vector was theoretically optimized for use in *C. reinhardtii*, using the appropriate promoters and selection genes. A genetic construct was designed, produced, cloned into the pChlamy_1 vector and was used for transformation of wild type *C. reinhardtii*.

3.2.2 Construct Design

Theoretical construct design began using a computer program called Gene Construction Kit (GCK; v4.0.2, Textco BioSoftware Inc.). This program gives a visual representation of the genetic sequence, genes and corresponding amino acid residues produced. Using the genetic sequence of the pChlamy_1 plasmid provided by Invitrogen [69], the sequence was inserted into GCK for visualization and digital manipulation.

Using GCK, a visual representation of a genetic construct may be designed from DNA sequences. Several existing DNA sequences can be merged together to form fusion proteins. From the DNA sequences, the corresponding amino acid sequences can be determined and analyzed. GCK was used an important tool for construct design, restriction enzyme site identification and finally for inferring DNA and protein sizes.

3.2.3 Construct Development

DNA primer sequences were designed using GCK and were manufactured by the University of Calgary Oligonucleotide Synthesis Laboratory. Overlapping primers were designed to extend and produce a desired genetic construct. Successive Polymerase Chain Reactions (PCRs) were used to produce and extend the gene of interest, while also greatly increasing the copy number.

A single 20 µL PCR reaction consisted of:

- 5.0 µL 5x Phusion PCR buffer (New England Biolabs B-0518S)
- 2.5 µL 2.5 mM deoxynucleoside triphosphates (dNTPs)
- 2.5 µL 2.5 mM forward primer
- 2.5 µL 2.5 mM reverse primer
- 1.0 µL template DNA
- 0.25 µL Phusion DNA polymerase (New England Biolabs F-530L)
- 11.25 µL PCR quality ddH_2O (Thermo Fisher 10977015)

The PCR reagents were mixed in PCR tubes and placed in a Bio-Rad MyCycler Thermal Cycler. The temperature profile used to amplify the DNA is shown in Figure 3-3.

3.2.4 Insertion of the Gene of Interest into the Host Vector

The amplified gene of interest must be inserted into the host vector before it can become a useful tool. The host vector, the pChlamy_1 vector, is a circular DNA vector. To insert the gene of interest, first the pChlamy_1 vector was linearly cut at the vector's multiple cloning site using the restriction enzyme KpnI. A 20 µL restriction enzyme digestion reaction consisted of:

- 2 µL 10x FastDigest Buffer (Thermo Fisher Scientific B64)
- 10 µL circular pChlamy_1 plasmid DNA
- 1 µL FastDigest KpnI Restriction Enzyme (Fermentas FD0524)
- 7 µL ddH_2O

The digestion solution was mixed thoroughly in a 1.5 mL tube and incubated at 37°C for one hour. Following digestion and linearization of the pChlamy_1 vector, the gene of interest could be inserted into the multiple cloning site. The gene of interest was designed to have terminal DNA sequences with "sticky ends" which correspond to the sticky ends produced by the KpnI restriction enzyme. Hence, the sticky ends attach to the gene of interest. A ligation reaction was performed to re-circularize the DNA into a circular vector consisting of the pChlamy_1 vector with the gene of interest insert.

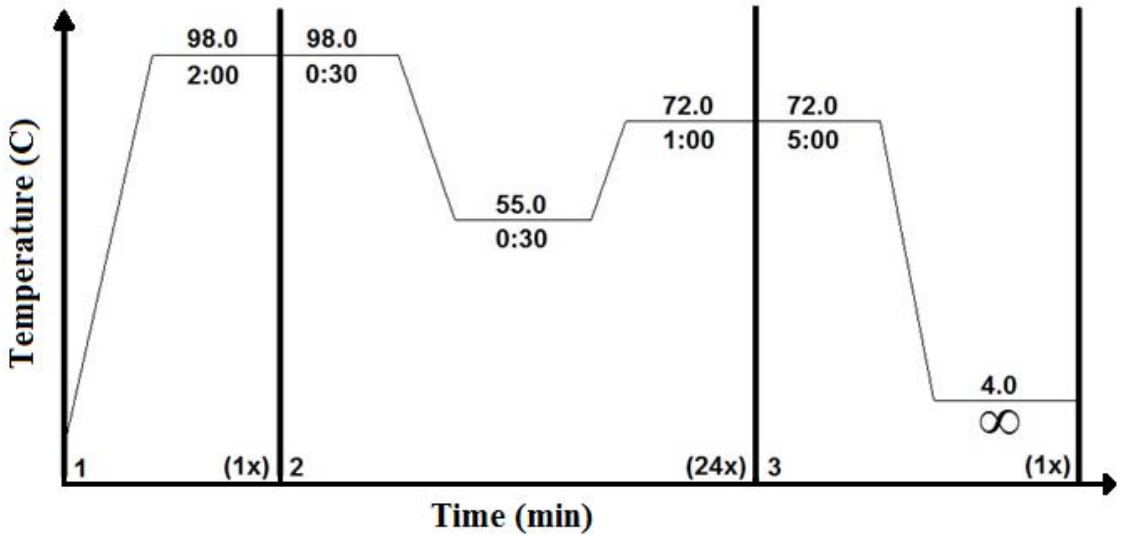

Figure 3-3: The temperature profile curve used by the PCR thermal cycler. The numbers above the thin line represent the temperature in °C, while the numbers below the line represent the time in hours and minutes. The temperature profile is divided into 3 sections, separated by the thick vertical lines. The first section is repeated once, the second 24 times, and the third and final section ends by maintaining the PCR mixture at 4°C for an indefinite period of time.

The 20 µL ligation reaction consisted of:

- 10 µL 2x Ligase Buffer (New England Biolabs B-2200)
- 4 µL of the linearized pChlamy_1 vector
- 5 µL of the insertion gene of interest
- 1 µL T4 DNA Ligase (New England Biolabs M-0202)

The ligation reaction was incubated at room temperature for 10 minutes and used for *E. coli* transformation.

3.2.5 Construct Multiplication, Analysis, Purification and Long Term Storage

E. coli was used as a host for vector multiplication and long term storage. Using the ligation reaction product, chemically competent TOP10 *E. coli* cells (chemically competent TOP10 *E. coli* produced in the Alcantara laboratory) were transformed by gently mixing 40 µL of competent cells with 5 µL of plasmid DNA. The *E. coli* were placed on ice for 30 minutes, after which a 37°C heat shock was applied for 30 seconds to seal the cell membrane. The *E. coli* were then cultured in 600 µL of Luria Broth (LB, Sigma L-3022) at 37°C for one hour in a VWR 1550 incubator. Following the incubation, cells were plated onto solid selection media consisting of LB + agar + 100 µg/mL Ampicillin (VWR J637; Sigma A-0166) in petri dishes (VWR 25384-208). Solid plates were incubated overnight at 37°C in a VWR 1550 incubator. 10 individual cell colonies were picked using a pipette tip and cultured overnight in separate 15 mL tubes containing 1 mL of LB + 100 µg/mL Ampicillin.

Plasmid DNA was purified using the E.Z.N.A. Plasmid Mini Kit (Omega Bio-Tek D6942-02). Plasmid DNA was digested using KpnI and analyzed using agarose gel electrophoresis.

An agarose gel was made by first microwaving a solution of 50 mL 1x TAE buffer and 0.5 g of agarose in a 500 mL Pyrex flask to dissolve the agarose in the TAE buffer. Upon cooling to approximately 40°C, 5 µL of ethidium bromide was added and mixed thoroughly. The warm gel solution was cast into the electrophoresis apparatus and a 20 well comb was placed in the

solution. Following hardening of the gel, 18 µL of the purified plasmid DNA was mixed with 2 µL of 10x FastDigest Green Dye Buffer (Thermo Fisher Scientific B72) and pipetted into an individual well in the electrophoresis gel. A DNA ladder marker prepared by the Alcantara laboratory was added for approximate DNA size comparison.

1 Litre of 50x TAE Buffer Solution (diluted to 1x using ddH_2O prior to use) consisted of:

- 242 g Tris
- 100 mL 0.5 M EDTA, pH 8.0 (Sigma E-5134)
- 57.1 mL glacial acetic acid
- Dissolved in ddH_2O

The gel was subjected to a constant voltage of 100 V for 30 minutes to separate the DNA according to size. A BioKan Scientific Tek Star ultraviolet transilluminator was used to observe the DNA. The size of the DNA band was compared to the theoretical size of the pChlamy_1 vector plus insert, and if the size appeared correct, the DNA plasmid was stored at 4°C and the corresponding *E. coli* strain was stocked using glycerol for long term storage at -80°C.

3.2.6 Construct Sequencing

Construct sequencing was performed by the University of Calgary Oligonucleotide Synthesis Laboratory.

3.2.7 Genetic Transformation of *C. reinhardtii*

Many methods exist to genetically transform *C. reinhardtii*; however, following the GeneArt *Chlamydomonas* Engineering Kit [67], electroporation was selected as the transformation method. Electroporation is commonly used for nuclear transformation of *C. reinhardtii*; chloroplastic transformation is not possible using the electroporation method [67]. The genetic construct was linearized using the SspI restriction enzyme (Thermo Scientific FD-0774) and was prepared for genetic transformation.

Wild type *Chlamydomonas reinhardtii* 137c were cultured and the electroporation protocol was followed using a Bio-Rad Gene-Pulser Electroporator and the following electroporation parameters:

- A voltage of 600 V
- A capacitance of 50 μF
- An infinite resistance

Following electroporation, the cells were left to recover from the physical shock of the electroporation for 24 hours. After the recovery period, the cells were plated onto solid TAP + agar + 20 μg/mL Hygromycin B (Invitrogen 10687-010) plates and incubated at 23°C and a light flux of 50 μE/(m^2s). Individual colonies were observed on the plate after approximately two weeks. Several colonies were selected regardless of shape, colour or size.

A control group consisting of wild type *C. reinhardtii* was electroporated without the addition of exogenous DNA. These cells were plated on solid selection media and colonies were selected in a similar manner to the experimental group.

3.2.8 Scale-up and Storage of Recombinant *C. reinhardtii*

Individual colonies were removed from the selection medium using a pipette tip and transferred into separate wells of a 48 well plate containing 1 mL of TAP medium. After approximately one week, the 1 mL cultures were used as the inoculum in 25 mL shake flasks containing 9 mL of TAP medium, for a total culture volume of 10 mL. Once cultured into the exponential growth phase, the 10 mL culture could then be used as a stock for long term liquid nitrogen storage. Existing *C. reinhardtii* cryopreservation techniques were followed [70].

3.3 Results

3.3.1 Conceptual Construct Design

Using existing genetic sequences as a guide, a genetic construct was designed to produce a fusion protein with the following characteristics:

- A histidine tag for nickel column chromatography purification
- A 10x Elastin-Like Polypeptide (ELP) sequence for temperature-dependent protein separation
- An intein cleavage site for pH-dependent protein cleavage
- A TEV cleavage site
- Proinsulin, consisting of A, B and C chain polypeptides
- A KDEL targeting site for protein targeting to the endoplasmic reticulum

The components of the genetic construct are summarized in Figure 3-4, while Figure 3-5 shows a cartoon layout of the protein product. The genetic sequence itself with the corresponding amino acid sequence is presented in Figure 3-6. Each piece of the fusion protein will be described in detail below.

The main product from the fusion protein is the biopharmaceutical precursor to insulin, proinsulin. Proinsulin must undergo several post-translational modifications to become biologically active in the form of insulin. This protein hormone was chosen as a model protein for several reasons: a) it is relatively small; b) it is a complex mammalian protein which contains complex disulphide bridges. This would challenge the algae cells to perform the proper post-translational modifications and folding necessary to produce an active protein; c) it is a well-known biopharmaceutical that would be familiar to most audiences; and d) there is existing research on alternative methods of recombinant proinsulin production, allowing for comparisons to be made to existing technologies. Theoretically speaking, any protein could have been chosen as a model protein; the DNA sequence could simply be redesigned for another biopharmaceutical or product. However, for the reasons discussed, proinsulin was chosen.

Histidine Tag	Elastin-Like Polypeptide Chain	Intein Cleavage Site	TEV Cleavage Site	Proinsulin	KDEL Sequence

Figure 3-4: A summary of the components of the fusion protein of interest.

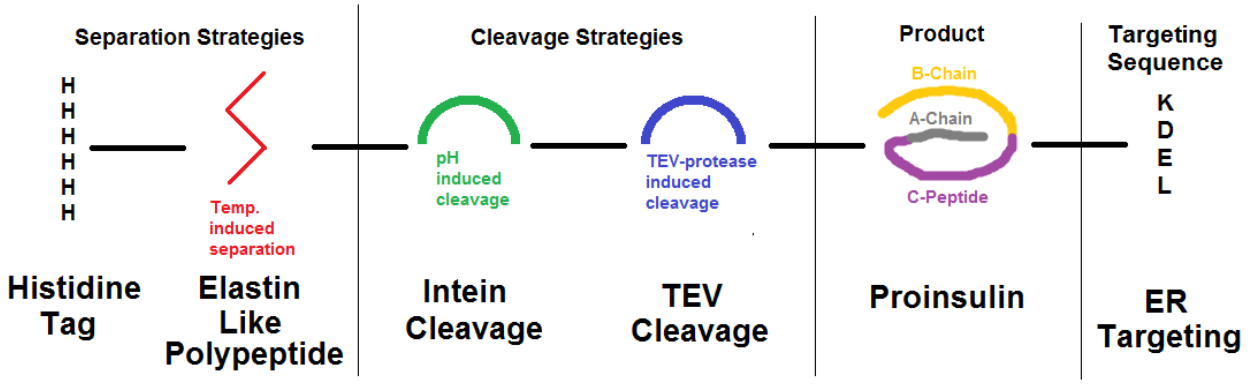

Figure 3-5: A cartoon graphic of the individual components of the fusion protein of interest.

Figure 3-6: The genetic sequence of the designed construct with the corresponding amino acid sequence and individual sites. Each arrow corresponds to a section of the fusion protein. Starting at the top, the fusion protein sections proceed as follows: methionine initiation, histidine tag, Elastin-Like Polypeptide chain, intein cleavage site, TEV cleavage site, proinsulin A chain, proinsulin C peptide, proinsulin B chain, KDEL endoplasmic reticulum targeting chain.

To separate the main product from all other proteins in solution, the construct was designed with both histidine tag purification and ELP purification techniques. The ELP represents a relatively fast and inexpensive, yet more difficult separation strategy. The histidine tag purification is a simple and established technique, but is more costly and time consuming relative to ELP separation. Although ideally ELP separation would be used, the construct was designed with both strategies.

A histidine tag consists of a sequence of at least 6 histidine amino acids in a row. This construct was designed to have a sequence of 8 consecutive histidine residues. The histidine tag allows for nickel-column chromatography separation of the fusion protein from other soluble proteins. This separation technique was chosen and utilized, and the procedure is described in Chapter 4.

The Elastin-Like Polypeptide (ELP) chain is a novel protein separation technique which has a thermally reversible phase transition trait [71], [72]. By simply changing the temperature of the protein solution, the ELP chains in the fusion protein aggregate and the protein will precipitate out of the solution. The temperature at which this phase transition occurs depends upon the ELP sequence. The ELP chain consists of repeating subunits of the amino acids VPGXG, where X is any amino acid, and typically range from 10 to 100s of consecutive repeating subunits. The gene of interest contained 10 consecutive subunits of VPGXG. The ELP technique was attempted in this project, but it resulted in low separation efficiencies. Rather than optimizing the ELP separation protocol, the histidine tag separation process used and the time was instead invested in the development of the bioprocess. The development and use of the ELP separation strategy remains as important future work in this project.

Two protein cleavage strategies, an intein cleavage site and a TEV cleavage site, were designed into the fusion protein of interest. The TEV cleavage site represents an established yet expensive methodology, while the intein cleavage site represents a newer, more inexpensive alternative. Ideally the intein cleavage site would be used for cost effectiveness reasons, but if intein cleavage fails the established TEV cleavage strategy acts as a backup. Due to the low final

protein concentrations obtained from the genetically engineered cells (see Chapter 4), protein cleavage was not investigated in this project. Upon improvement of the protein production rates, protein cleavage would be necessary to obtain an active product needed for a functional protein assay.

The intein cleavage site was included as an option for protein splicing. At a particular pH and temperature, the intein causes protein cleavage [73]. This offers an inexpensive method to cleave proteins, separating a product protein (ex. a biopharmaceutical) from the remainder of the fusion.

Similar to an intein cleavage site, a TEV cleavage site is also used for protein splicing. The TEV cleavage site is spliced by TEV protease. This protein cleavage methodology is a commonly used biotechnology tool for fusion proteins [74]. Compared to intein cleavage, TEV cleavage is more expensive primarily because it requires the enzyme TEV protease.

The final piece of the fusion protein is the messenger peptide KDEL attached to the C-terminus. This KDEL sequence directs the protein to the endoplasmic reticulum, preventing secretion. It has been shown for other host systems that the KDEL sequence led to increased stability and accumulation of recombinant proteins [75].

If the weights of all the amino acids in this fusion protein are added together, a total weight of 31.0 kDa is expected. Figure 3-6 outlines the exact theoretical sequence of the amino acids, and GCK provided an indication of the expected weight of this protein.

3.3.2 Insertion into pChlamy_1 and Construct Confirmation

Following PCR amplification of the gene of interest, the gene was inserted into the pChlamy_1 vector and this product was named the pBC_1 vector. The pBC_1 vector was then transformed into *E. coli*. The expected size of the pBC_1 vector was 5,177 base pairs, as it contained the

pChlamy_1 vector (4,283 base pairs) with the insert gene (894 base pairs). A visualization of the complete theoretical pBC_1 vector is shown in Figure 3-7.

The ligation and transformation process was confirmed with agarose gel electrophoresis, as seen in Figure 3-8. With confirmation of the approximate size of the pBC_1 construct, the vector was sent to the University of Calgary Oligonucleotide Synthesis Laboratory for genetic sequencing. This sequencing confirmed the pBC_1 genetic construct as consisting of the pChlamy_1 vector containing the appropriate insertion gene of interest. The gene of interest was also confirmed to be in the proper orientation capable of producing the desired fusion protein.

3.3.3 Genetic Transformation of *C. reinhardtii*

Following the protocol provided by the GeneArt *Chlamydomonas* Engineering Kit [67], the wild type *C. reinhardtii* were transformed with the desired genetic construct. Transgenic *C. reinhardtii* cultures were cryopreserved in liquid nitrogen for long term storage. As needed, transgenic *C. reinhardtii* were resuscitated from liquid nitrogen and used for protein production experiments described in Chapter 4.

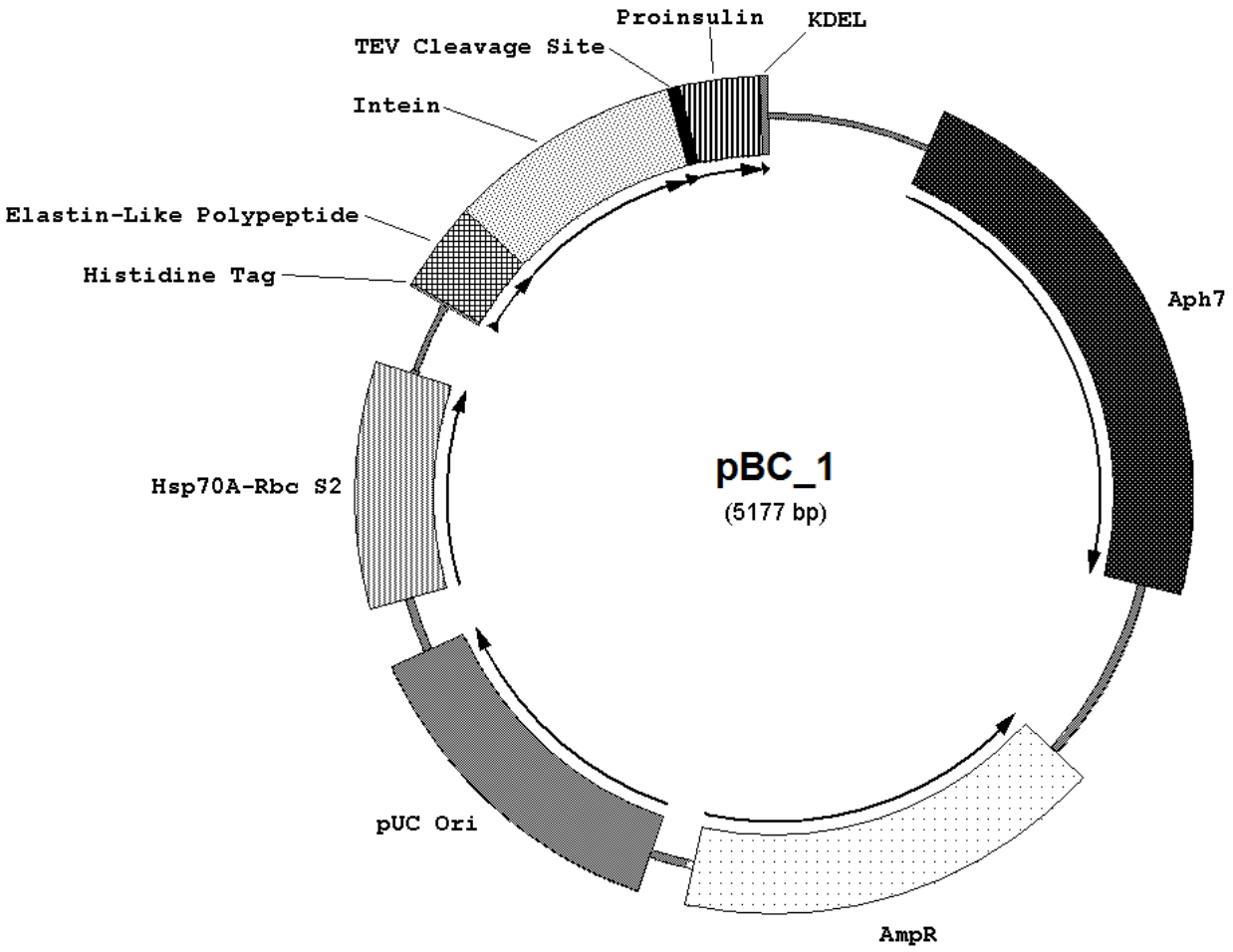

Figure 3-7: The pBC_1 construct as designed on GCK. The total size is 5,177 base pairs. The pUC Ori site is the origin of replication; the HSP70A-RBCS2 site is the fusion heat/light shock promoter; the histidine tag marks the beginning of the fusion protein, followed by the ELP sequence, the intein, the TEV cleavage site, the proinsulin site and finally the KDEL targeting peptide; The Aph7 site corresponds to algal hygromycin B resistance; and finally the AmpR site represents the gene for bacterial ampicillin resistance.

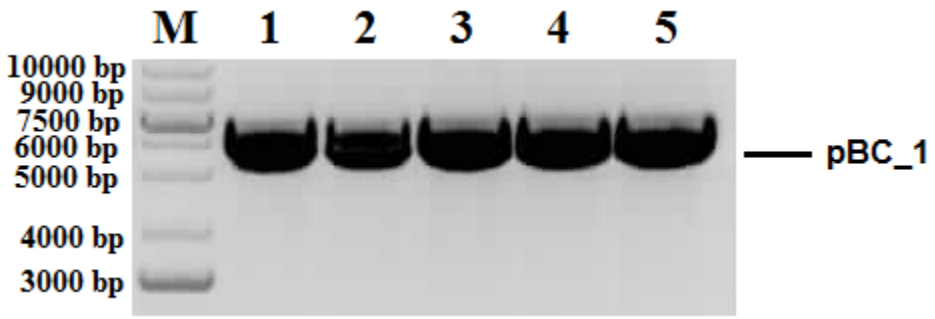

Figure 3-8: An agarose gel outlining the approximate size of pBC_1 construct. The marker (M) gives a guide of the approximate size of the experimental DNA. Base pair (bp) size is indicated to the left of the marker. Columns 1-5 indicate 5 different DNA constructs expected to contain the construct of interest. The 5 constructs were all shown to be near the expected approximate size of 5,177 base pairs.

3.4 Discussion

The production of a usable algal genetic construct was by no means a simple endeavour. The work presented in this chapter alone took over two and a half years to successfully develop. Three different transformation methods were attempted before the eventual purchase of the GeneArt *Chlamydomonas* Engineering Kit. The first several methods used for algal transformation failed for various reasons. The most challenging aspect was the maintenance of a stable transformation which could pass the transgenic traits through successive generations. Transformation efficiencies were either very low or were transient in nature instead of stable. The purchase of the GeneArt *Chlamydomonas* Engineering Kit enabled the production of stable transformations at relatively high transformation efficiencies.

Apart from the transformation process, the genetic construct used for the transformation procedure was challenging to clone and isolate. The complexity and length of the fusion genes meant that multiple rounds of polymerase chain reactions, confirmations and isolation steps were required. Furthermore, a multitude of side constructs were produced and rejected before the final product construct outlined in this thesis was produced. These side constructs had varying combinations of products, separation techniques and cleavage strategies.

The product that came of this work, however, is a novel construct unlike anything ever produced in the past. The unique nature of the construct, with its various separation and cleavage techniques, makes it a very useful tool for the analysis of algal recombinant protein production and the isolation of the product itself. Genetic constructs developed in the past lack the unique separation and cleavage strategies present in this new construct. Furthermore, this construct is optimized through strategies such as codon optimization for use in an algae host organism. Despite the advantageous qualities of this genetic construct, it is by no means the final product; it was developed in a modular fashion such that individual components can readily be exchanged, depending on the desired application or final product.

One of the greatest challenges in this thesis was the selection of a recombinant product. Although many biopharmaceuticals and other recombinant proteins were considered, proinsulin was chosen as the model product protein. It is important to note that any biopharmaceutical or protein product could have taken the place of proinsulin; it was chosen simply as a model protein. Selection of a relatively complex mammalian protein was one of the most important factors. Alternative host systems such as bacteria and yeast often do not provide the proper post-translational modifications to properly fold complex mammalian proteins. Instead, CHO cells are often used for this purpose at an increased cost. Algae have been previously shown to be capable of properly folding complex mammalian proteins, and therefore may represent a cost-efficient alternative to CHO cells. Proinsulin contains several complex disulphide bridges and was thought to challenge the algae cells to properly produce recombinant proteins. Although a functional protein assay was not performed in this project, it is a key piece of work that can be investigated in the future.

With regard to the product of the genetic construct itself, there are myriad combinations of potential fusion proteins that could have been designed and used. The construct designed for this project had several advantageous components. First, two different separation strategies were designed into the construct: a histidine tag and an ELP sequence. The histidine tag provided an established separation technology that could readily be used to separate the fusion protein from other soluble proteins. The ELP sequence was added as a novel alternative to the histidine tag.

Similar to the pair of separation strategies, a pair of protein cleavage strategies was also designed into the construct. The TEV cleavage site, similar to the histidine tag, represents a commonly used and proven methodology in the biotechnology industry. TEV cleavage is commonly used as a genetic engineering tool, because it allows for selective cutting of fusion proteins through the addition of TEV protease. A novel alternative to TEV cleavage, the intein biotechnology, was also designed into the construct.

Ideally, the ELP separation technique would be used alongside the intein cleavage strategy to purify an active product protein. These two biotechnologies do not require expensive reagents; rather, only minor alterations to temperature and pH. In terms of cost effectiveness, these two biotechnologies could greatly impact the final cost to produce the protein of interest as protein separation and cleavage are often complex and expensive procedures.

Several alternative constructs were designed, cloned and stored in addition to this construct. In these various constructs, traits were added, removed or modified to meet specific goals. However, the protein analysis described in Chapter 4 was performed using the construct developed and described.

3.5 Conclusions

Genetically engineering the DNA construct used for this project was not a trivial task and took over two years to develop. A gene of interest coding for a fusion protein consisting of a proinsulin product and several separation and cleavage strategies was designed and produced. Following insertion of this gene of interest into the algal pChlamy_1 vector to produce the pBC_1 vector, genetic transformation was performed on wild type *C. reinhardtii* using electroporation. Genetically engineered *C. reinhardtii* cultures were stored in liquid nitrogen and used for the experiments in Chapter 4. The construct developed in this chapter represents a novel tool unlike anything ever produced. The unique properties of the construct were meant to aid in the efficiency and cost effectiveness of the algal recombinant protein production system.

3.6 Future Work

As new research discoveries are made, more efficient promoters, separation techniques, cleavage strategies and targeting peptides may be desirable. For this construct in particular, future work will investigate the use of the ELP separation strategy, as well as protein cleavage using the TEV and intein cleavage sites. Although these strategies have all been investigated

previously, their use in recombinant algae has not been reported and would be of interest for future research.

One of the key areas of future work for this project is the substitution of the model proinsulin protein with other high value biopharmaceuticals or product proteins. This algal system represents a platform technology into which it is thought any complex mammalian protein product could be inserted. Therefore, following optimization of the parameters of the construct above, it is likely that new constructs coding for other protein products could be developed and used for algal genetic engineering. The process for these subsequent designs would follow the general procedure described in this chapter. If algae are indeed found to be a cost-efficient alternative for recombinant protein production, the utilization of it for various products would be greatly desired and valuable.

Chapter 4: A Bioprocess for the Production and Analysis of Recombinant Proteins in *Chlamydomonas reinhardtii*

4.1 Introduction

With the genetic construct developed and transformed into *C. reinhardtii* (Chapter 3), and improved cell culture methods identified (Chapter 2), this project turned toward the development of more down-stream processing techniques, protein isolation and protein analysis strategies. Together, the cell culture and downstream processing represents the bioprocess as a whole.

The purpose of bioprocessing is to use a living cell or organism to produce useful products. Many bioprocesses begin with cell culture. The culture process increases the concentration of the desired product, and from here downstream processing is required to isolate the product. Most downstream processing involves the use of one or more of the following unit processes: centrifugation, chromatography, cooling, crystallization, dialysis, distillation, drying, evaporation, filtration, heating, homogenization, humidification, membrane separation, milling, mixing, precipitation, solid handling and solvent extraction [76]. A subset of these unit processes are selected depending upon the product, its solubility, the scale of the process and the value of the product [76].

The key unit processes used in this project include homogenization for cell disruption, centrifugation and chromatography. Following these processes, the concentrated protein product was analyzed. These key processes will be introduced below in more detail as they are essential to the algal bioprocess developed and described in this chapter.

4.1.1 Cell Disruption

Downstream processing of cell cultures typically begins with cell separation via filtration or centrifugation [76]. Although some genetic constructs allow for the secretion of a recombinant

protein [77], many host organisms retain the product within the cell. In the case that a product is retained intracellularly, cell lysis is required to gain access to the product. One widely used technique for cell disruption involves high pressure homogenization [76]. The shear forces generated in a homogenization device are sufficient to disrupt many cell types.

4.1.2 Centrifugation

Centrifugation uses centrifugal forces to separate material based on density. In a solution consisting of a lysed cell culture, microscopic insoluble material such as cell debris is mixed together with soluble components. Through the use of centrifugation, the relatively dense cell debris components are separated from the less-dense soluble components.

4.1.3 Chromatography

The principle of chromatography lies in differential migration, and a selective slowing of solute during passage through a packed column bed [76]. For separation of proteins containing histidine tags, a column packed with charged nickel ions is used [78]. The nickel selectively binds proteins containing histidine tags due to covalent bonding, while other proteins are not retained and are washed away as waste. In this way, a recombinant fusion protein can be separated from other soluble proteins. To elute the fusion protein from the column, a metal ion ligand such as imidazole is used [78]. When introduced, the imidazole competes with the histidine for the active sites of the nickel, releasing the histidine and eluting the product. The column can be recharged using wash buffers.

4.1.4 Protein Analysis

To analyze proteins, one common approach involves the use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [79]. This technique separates proteins of different sizes using an electric field. Boiling the proteins denatures them to a linear shape, leaving only the primary protein structure. In this way, the size of proteins can be compared. The addition of SDS causes the proteins to be covered in negative ions, which is required for

separation using an electric field [79]. A polyacrylamide gel provides the medium through which the proteins migrate and separate based upon size. Smaller proteins migrate faster through the gel, while larger proteins migrate more slowly. Once separated, the gels can be analyzed using Coomassie blue staining, which dyes the proteins making them visible to the naked eye when present in high concentrations.

Western blotting is a common technique used for precise detection of proteins [79]. Protein samples are separated using SDS-PAGE and transferred to a membrane for antibody staining. Typically, Western blots use a two-stage antibody reporter system. The primary antibody has an antigen binding site specific to the protein of interest. The secondary antibody has an antigen binding site specific to the primary antibody, and also contains a reporter system to allow for observation of bound antibody. When activated, the reporter system indicates the presence of the secondary antibody, which in turn implies detection of the protein of interest.

4.2 Methods

4.2.1 Process Overview

The process began by culturing cells in spinner flasks. These vessels allowed for algal culture volumes of up to 1 litre, from which an observable amount of recombinant protein was produced from genetically engineered algae. Following cell culture for 8 days, cultures were subjected to a 40°C heat shock to stimulate the genetic promoters. Cultures were then harvested via centrifugation and homogenized in a cell homogenizer. Two further rounds of centrifugation were required to separate insoluble proteins and cell debris from soluble proteins, including the soluble protein of interest. Soluble proteins were then passed through a histidine purification column, which separated most impurities from the recombinant product. This purified recombinant proinsulin was then analyzed using traditional protein analysis techniques such as Western blotting. A simplified process flow diagram is presented in Figure 4-1.

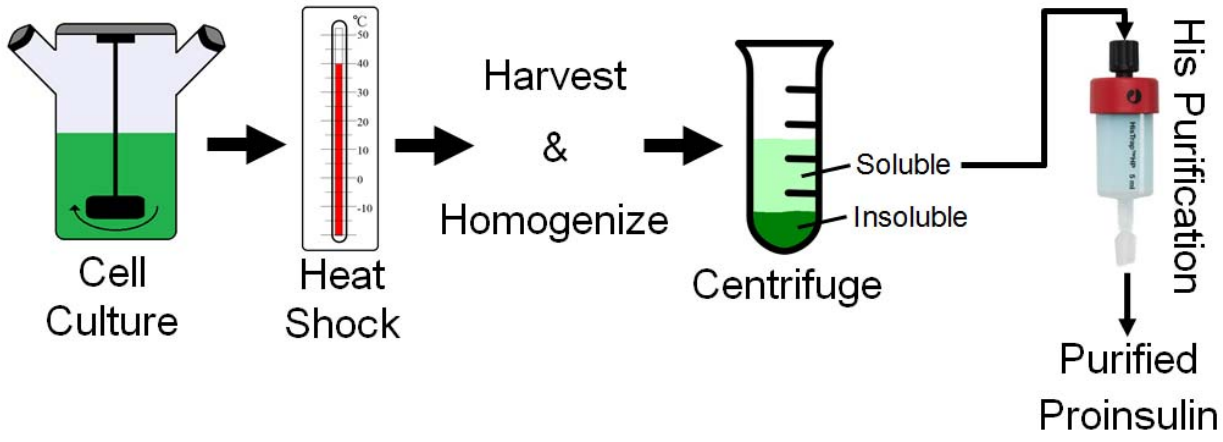

Figure 4-1: A simplified process block diagram outlining the major steps to obtain a purified amount of the recombinant proinsulin product.

4.2.2 Algae Strain

A genetically engineered strain of *Chlamydomonas reinhardtii* capable of producing recombinant proinsulin was used to analyze recombinant protein production rates. The details of this genetic construct and its transformation into *C. reinhardtii* can be found in Chapter 3. As a control, wild type *C. reinhardtii* 137c was also used (Invitrogen #A14258). Transgenic and wild type *C. reinhardtii* were found to have similar growth kinetics (data not shown).

4.2.3 Cell Culture

To obtain an amount of recombinant protein which could be observed using Western blot techniques, a total of one litre of genetically engineered *Chlamydomonas reinhardtii* was cultured in two separate 500 mL spinner flasks. The improved culture conditions found in Chapter 2 were used to grow these cells to the highest cell concentration possible. The starting medium was TAP + 40 mM acetic acid buffered to a pH of 7.0 with Tris (to produce 40 mM Tris-acetate). Following the best protocol developed from Chapter 2, the culture was grown mixotrophically for the entire culture period using a constant light flux of 50 $\mu E \bullet m^{-2} \bullet s^{-1}$, an agitation rate of 400 RPM and a temperature of 23°C. To prevent full consumption of the acetate, a one-time fed-batch addition of 40 mM Tris-acetate was supplied on culture day 5. The cell growth was suspended on day 8 when cell concentrations reached a maximum near 2.7e7 cells/mL. At this point, the two 500 mL cultures selected for promoter stimulation were subjected to a heat shock. Some control cultures were not subjected to promoter stimulation.

4.2.4 Promoter Stimulation

To initiate recombinant protein production, the recombinant *C. reinhardtii* was subjected to heat shock to stimulate the HSP70A heat shock promoter. Optimization of heat shock parameters were previously reported for *C. reinhardtii* [43], and were used for these experiments. Following culture growth at 23°C until the cell concentration reached a maximum, culture vessels were transferred into a heated incubator operating at 40°C. Agitation and light intensity parameters remained unchanged throughout the procedure.

Cultures remained in a 40°C environment for 30 minutes, after which they were returned to 23°C for 5 hours. After 5 hours had elapsed, the cultures were returned back to the 40°C chamber for another 30 minutes to initiate a secondary heat shock. It was found in the literature that performing two sequential 30 minute 40°C heat shocks stimulated the highest recombinant protein production of the conditions tested [43]. Following the secondary heat shock, cultures were returned to the 23°C environment for 90 minutes to allow for the accumulation of recombinant protein products. It should be noted that the HSP70A/RBCS2 fusion heat/light shock promoter is the only well developed and readily available promoter system for *C. reinhardtii* at this time.

4.2.5 Cell Harvesting and Homogenization

Upon completion of the heat shock promoter stimulation and accumulation of the product proteins, cultures were harvested by centrifugation in an Eppendorf 5810R at 10,000 RPM for 10 minutes. The supernatant was discarded, and the cell pellets were resuspended to a total volume of 50 mL using cell lysis buffer. The lysis buffer was designed for genetic constructs containing a 6x histidine purification tag, and consisted of: 50 mM NaH_2PO_4 (Sigma S-0876), 300 mM NaCl (Sigma S-9888) and 10 mM imidazole (Sigma I-2399) [78]. pH was adjusted to 8.0 using NaOH (Sigma S-0899). A thorough pipette resuspension of the cell pellet was performed prior to homogenization to ensure the cell suspension was well mixed.

The cell solution was homogenized using an EmulsiFlex-C3 homogenization device. Following preparation and cleaning of the device, the air pressure was set to 40 psi and the cell solution was passed through the device 3 times to ensure a well-homogenized product. Homogenization was confirmed via observation using an Olympus CKX41 light microscope to ensure near complete homogenization of whole cells.

4.2.6 Protein Isolation

One of the major challenges in this bioprocess was the separation of the recombinant protein of interest from all other soluble proteins. This task was accomplished using histidine tag purification in a nickel column. Prior to column purification, all insoluble proteins and cell debris had to be separated.

The homogenized mixture underwent two rounds of centrifugation to remove all insoluble material. First, the solution underwent a primary centrifugation at 10,000 RPM for 10 minutes to remove much of the cell debris present following homogenization. The supernatant from the initial centrifugation was then subjected to a second centrifugation at 50,000 RPM for 30 minutes in a Beckman Optima L-100K ultracentrifuge (Beckman SW41 Ti Swinging Bucket rotor) to ensure all insoluble proteins and cell debris were removed from the sample.

Following the secondary centrifugation step, the supernatant was ready for nickel column purification. A 1 mL HisTrap FF Nickel Column (GE Healthcare Life Sciences 17-5255-01) was used. A 15 mL syringe was used to flow solutions through the column at the recommended flow rate of approximately 1 mL/min. Three different buffers are required for the nickel column purification [78]:

1. Lysis Buffer
    - 50 mM NaH_2PO_4
    - 300 mM NaCl
    - 10 mM Imidazole
    - pH adjusted to 8.0 using NaOH
2. Wash Buffer
    - 50 mM NaH_2PO_4
    - 300 mM NaCl
    - 20 mM Imidazole
    - pH adjusted to 8.0 using NaOH

3. Elution Buffer
    - 50 mM NaH_2PO_4
    - 300 mM NaCl
    - 250 mM Imidazole
    - pH adjusted to 8.0 using NaOH

To prepare the nickel column, 10 mL of lysis buffer was flowed through. The sample, typically consisting of a volume of 30 to 40 mL, was flowed through the column, with the flow-through solution saved in a fresh 50 mL tube. Following addition of the sample, the column was washed with 10 mL of wash buffer. Next, 1 mL of elution buffer was flowed through the column and the column was left to incubate at room temperature for 15 minutes. During this incubation time, the imidazole competes for the nickel binding sites, eluting the histidine-tagged fusion protein. After incubation, a further 9 mL of elution buffer was flowed through the column for a total of 10 mL of eluted product. 1 mL fractions of product were collected and ordered from 1-10 based on the order in which they were flowed through the column. Typically, the first 1 mL elution contains the highest concentration of the product. Finally, 5 mL of lysis solution was flowed through the column for re-equilibration, and the column was stored long term at 4°C.

4.2.7 Protein Standard

A protein standard was needed to allow for quantification of recombinant protein amounts on the Western blot. This was done using an *E. coli* host. A genetic construct containing the same insert sequence used for the algae was inserted into an *E. coli* vector with the appropriate promoters. The *E. coli* were cultured and the proteins analyzed. Using a commercially available Bradford protein assay kit (Bio-Rad 5000201), the protein concentration was determined to be 504 µg/mL. Serial dilutions were performed to allow for 5 different recombinant protein weights ranging from approximately 5 ng to 100 ng to be added to the protein gel as a standard curve.

4.2.8 Protein Analysis – SDS-PAGE

Proteins were separated based on size using the commonly used Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) technique. 1.5 mm thick acrylamide SDS-PAGE gels were prepared by first setting the 10% acrylamide separating gel. Each 10 mL separating gel consisted of [80]:

- 4.0 mL ddH_2O
- 3.3 mL 30% acrylamide mix (Bio-Rad 161-0156)
- 2.5 mL 1.5 M Tris (pH 8.8)
- 0.1 mL 10% SDS (Sigma L-3771)
- 0.1 mL 10% ammonium persulfate (Bio-Rad 1610700)
- 0.004 mL tetramethylethylenediamine (Bio-Rad 1610800)

Following the setting of the separating gel, the 5% acrylamide stacking gel was set over top of the resolving gel. Each 5 mL stacking gel consisted of [80]:

- 3.4 mL H_2O
- 0.83 mL 30% acrylamide mix
- 0.63 mL 1.0 M Tris (pH 6.8)
- 0.05 mL 10% SDS
- 0.05 mL 10% ammonium persulfate
- 0.005 mL tetramethylethylenediamine

10 or 15 well combs were placed into the top of each stacking gel prior to setting, depending upon the number of samples and the application required.

Several buffers were prepared prior to using the gel. These buffers include 2x SDS-PAGE sample buffer, SDS running buffer and 10x transfer buffer. Their components are described below [81]:

2x SDS-PAGE Sample Buffer

- 100 mM Tris-Cl (pH 6.8)
- 200 mM dithiothreitol (added prior to use; Sigma D-0632)
- 4% w/v SDS
- 0.2% bromophenol blue (Sigma B-0126)
- 20% v/v glycerol (Sigma G-5516)
- Dissolved in ddH_2O

1 Litre of 5x SDS Running Buffer (diluted to 1x using ddH_2O prior to use)

- 15.1 g Tris
- 94.0 g glycine (Sigma G-7126)
- 50 mL 10% SDS
- Dissolved in ddH_2O

Protein samples were prepared by first mixing equal parts sample and 2x sample buffer. Samples were then placed in boiling water for 5 minutes to break all intermolecular bonds. Following cooling of the boiled samples, 15 µL of each sample was added to an individual well of the prepared gel. Blanks consisted of ddH_2O and 2x sample buffer, while a commercially available marker was used to infer sample sizes (PageRuler Prestain Protein Ladder; Thermo Scientific #26616).

Gels were prepared in the proper SDS-PAGE apparatuses, and SDS running buffer was added appropriately as both the anode and cathode buffer. The gels were then subjected to a constant voltage of 100 V for approximately 1.5 hours, or until the dye front emerged from the separating gel.

4.2.9 Protein Analysis - Coomassie Blue Gel Staining

Protein gels selected for Coomassie blue staining were separated from those selected for Western blot analysis. Coomassie Brilliant Blue R-250 dye binds to all proteins through ionic

bonding. This allows for a simple, visual observation of all proteins present on a protein gel and their relative amounts. Two solutions are required for Coomassie blue staining: the Coomassie blue staining solution, and the destain solution used to remove all unbound Coomassie dye. The makeup of these solutions is described below [81]:

1 Litre Coomassie Blue Staining Solution

- 2.5 g Coomassie Brilliant Blue R-250 (Sigma B-8647)
- 500 mL methanol (Fisher Scientific A-412)
- 100 mL glacial acetic acid
- 400 mL ddH_2O

1 Litre of Destain Solution

- 500 mL methanol
- 100 mL glacial acetic acid
- 400 mL ddH_2O

The protein gels selected for Coomassie staining were removed from the gel apparatuses and each gel was placed in a separate 100 mL dish. 20 mL of Coomassie blue staining solution was added to each dish, and the gel was incubated at room temperature on a rocking shaker for 15-20 minutes. The binding of the Coomassie dye was observed throughout the procedure, and the incubation was halted by removing the Coomassie blue staining solution from the dish when protein bands were clearly visible.

To remove unbound Coomassie dye from the gels, 20 mL of destaining solution was added to the dish, along with a folded sheet of paper towel. The paper towel absorbed the residual Coomassie dye, and was replaced periodically. After further incubation on a rocking shaker for 20-30 minutes, the gels were fully destained and ready for photography and analysis. A Bio-Rad Molecular Imager ChemiDoc XRS + Imaging System running the ImageLab Software (v4.1, Bio-Rad Laboratories) was used for imaging of the Coomassie gels and the Western blots.

4.2.10 Protein Analysis – Transfer to PVDF and Western Blotting

Gels not selected for Coomassie blue staining were used for Western blotting. Following separation of proteins via SDS-PAGE, gels were transferred onto polyvinylidene fluoride (PVDF) membrane (Bio-Rad 1620177). A gel sandwich was constructed using filter paper and the sandwich apparatus. Two buffers were required for protein transfer onto PVDF membrane; a 10x transfer buffer stock solution and a 1x transfer running buffer. The components of these two buffers are explained below [81]:

10x Transfer Buffer Stock Solution (1 litre)

- 58.1 g Tris
- 29.3 g glycine
- 37.5 mL 10% SDS
- Dissolved in ddH_2O

1x Transfer Running Buffer (1 litre)

- 100 mL 10x transfer buffer stock solution
- 200 mL methanol
- 700 mL ddH_2O

The sandwich apparatus was placed into the transfer apparatus along with an appropriate amount of the 1x transfer running buffer. The transfer was left overnight at 4°C at a constant voltage of 20 V.

Following a successful transfer of the proteins from the polyacrylamide gel to the PVDF membrane, the Western blotting procedure could commence. The PVDF membrane was first washed with a 20 mL solution of 5% skim milk powder (Difco BD-232100) in Tris-Buffered Saline (TBS) for 10 minutes on a rocking shaker. The TBS consisted of [81]:

1x Tris-Buffered Saline (1 litre)

- 8 g NaCl
- 0.2 g KCl (Sigma P-9541)
- 3 g Tris
- Dissolved in ddH_2O
- pH to 7.4 with HCl

Subjecting the PVDF membrane to the skim milk solution blocked all of the non-specific antibody binding sites. Next, 20 µL of primary antibody was added to the skim milk solution, and the membrane was left to incubate on a rocking shaker at room temperature for 2 hours. Two primary antibodies were tested; both were rabbit-derived polyclonal antibodies. One had a binding site specific to a histidine tag, while the other had a binding site specific to proinsulin. The primary antibodies were produced and collected by members of the Alcantara laboratory.

The PVDF membrane was then rinsed with TBS for 5 minutes to wash off all unbound primary antibody. The TBS was discarded and 20 mL of fresh 5% skim milk in TBS was added, followed by 20 µL of the secondary antibody. The secondary antibody was a commercially available goat anti-rabbit horseradish peroxidase (HRP) antibody (Bio-Rad 1706515). The secondary antibody was left to incubate at room temperature on a rocking incubator for 1 hour. A final 5 minute TBS wash step was performed to remove all unbound antibody. Following this wash step, the PVDF was ready for HRP development.

The HRP development solution consisted of 20 mL TBS, 5 mL 4CN solution (Bio-Rad 170-6534) and 19 µL of 35% H_2O_2 (Fisher Scientific 382171). The 4CN solution consisted of 120 mg of 4CN dissolved in 40 mL methanol. The HRP development solution was applied to the PVDF membrane, and left to incubate on the rocking shaker at room temperature for 5 minutes. Purple bands indicated a positive development. The PVDF membrane was dried overnight and photographed the next day using a Bio-Rad Molecular Imager ChemiDoc XRS + Imaging System running the ImageLab Software (v4.1, Bio-Rad Laboratories).

4.3 Results

The main results that come from this lengthy procedure are a Coomassie blue stained protein gel and a Western blot. These results provide vital information about the purification process as well as the concentration of the product protein.

The Coomassie blue stained protein gel is presented as Figure 4-2. This gel stains for all proteins present. Using this figure, it is clear that an abundance of unwanted soluble and insoluble proteins exist within the cell homogenate solution. This is expected as the *C. reinhardtii* cell culture, when homogenized, contains a multitude of various soluble and insoluble proteins. Following centrifugation to remove insoluble proteins, a wide variety of unwanted soluble proteins still exist in solution, as shown in column 'P' of Figure 4-2. The nickel column purification process separates out the majority of the unwanted soluble proteins, leaving primarily the proinsulin fusion protein in solution, as seen in column 'I'.

Two Western blots are presented in Figure 4-3 and Figure 4-4. The Western blot presented in Figure 4-3 used a rabbit-derived primary antibody specific to the histidine tag, while the Western blot presented in Figure 4-4 used a rabbit-derived primary antibody specific to proinsulin. Although both Western blots used identical protein amounts, the signal observed from the Western blot in Figure 4-3 was approximately 100 times greater than in Figure 4-4. As a result, Figure 4-3 was used to calculate the results of the protein analysis study.

Using Figure 4-3, it was determined that the experimental heat shock case produced a total of 31.7 ng of recombinant proinsulin from a 15 µL concentrated sample, which corresponds to a concentration of 8.44 mg/L produced by the entire one litre culture of recombinant *C. reinhardtii*.

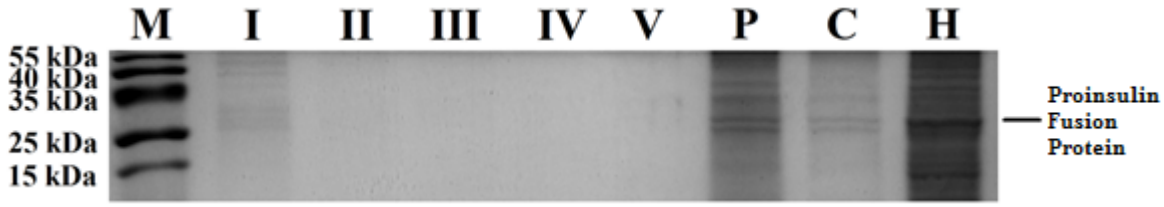

Figure 4-2: A stained Coomassie blue protein gel presenting the protein content of various sample fractions taken throughout the purification process. Starting on the left side of the blot, the protein marker denoted 'M' is used to estimate the approximate size of experimental proteins. The protein sizes of each marker band are presented directly to the left in kiloDaltons (kDa). Roman numerals 'I' through 'V' represent elution fractions 1 through 5, respectively. As expected, the greatest amount of product is observed in the very first elution fraction. 'P' represents a sample of the clarified supernatant solution prior to being flowed through the nickel purification column. 'C' represents a sample of the nickel column flow-through. 'H' represents a sample from the raw homogenate prior to any insoluble protein separation.

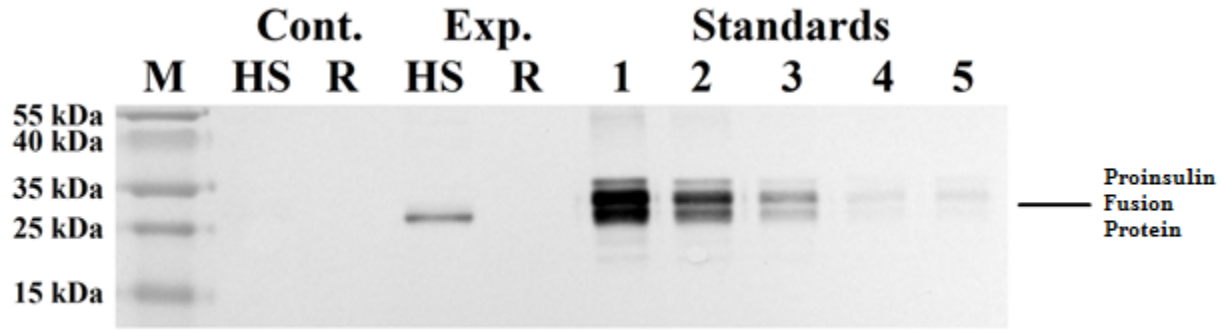

Figure 4-3: A Western blot outlining the production of recombinant proinsulin from genetically engineered *C. reinhardtii* using a rabbit-derived anti-histidine primary antibody. The left side of the blot denoted 'M' is the commercially available marker, which is used to estimate the approximate size of experimental proteins. The recombinant proinsulin fusion product, as described in Chapter 3, is approximately 31 kDa in size. The size of each marker band is denoted directly to the left, presented in kilodaltons. Two lanes are denoted 'Cont.' and refer to the control. This represents the recombinant proinsulin production from wild type *C. reinhardtii*. The two lanes denoted 'Exp.' refer to the experimental condition; the recombinant proinsulin production from genetically engineered *C. reinhardtii*. 'HS' refers to a culture which underwent the heat shock promoter stimulation, while 'R' refers to a culture which did not undergo heat shock stimulation. The standards are presented in the final 5 lanes. Standard lanes 1 through 5 contain, respectively, 94 ng, 47 ng, 23 ng, 12 ng and 6 ng of recombinant proinsulin. Using these standards, it was determined using the Bio-Rad ImageLab software that the experimental case which underwent the heat shock produced a band containing 31.7 ng of recombinant proinsulin. The experimental case which did not undergo the heat shock produced 1.6 ng of recombinant proinsulin. Both of the control cases did not produce an observable amount of recombinant proinsulin.

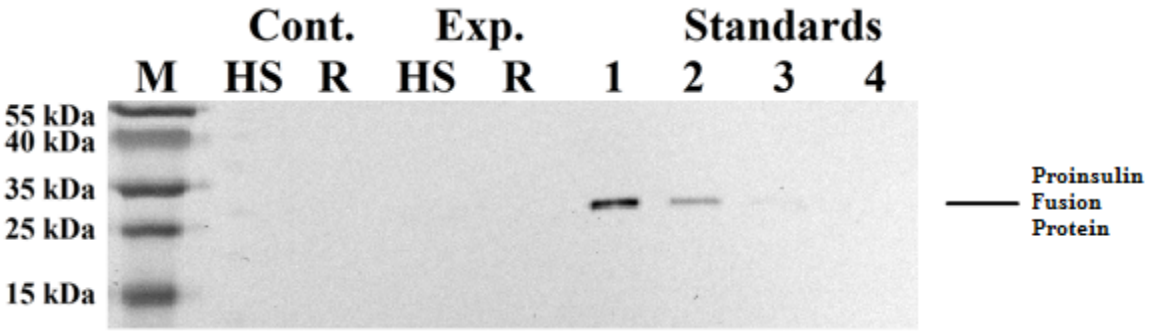

Figure 4-4: A Western blot outlining the production of recombinant proinsulin from genetically engineered *C. reinhardtii* using a rabbit-derived anti-proinsulin primary antibody. The left side of the blot denoted 'M' is the commercially available marker, which is used to estimate the approximate size of experimental proteins. The recombinant proinsulin fusion product, as described in Chapter 3, is approximately 31 kDa in size. The size of each marker band is denoted directly to the left, presented in kilodaltons. Two lanes are denoted 'Cont.' and refer to the control. This represents the recombinant proinsulin production from wild type *C. reinhardtii*. The two lanes denoted 'Exp.' refer to the experimental condition; the recombinant proinsulin production from genetically engineered *C. reinhardtii*. 'HS' refers to a culture which underwent the heat shock promoter stimulation, while 'R' refers to a culture which did not undergo heat shock stimulation. The standards are presented in the final 5 lanes. Standard lanes 1 through 4 contain, respectively, 94 ng, 47 ng, 23 ng and 12 ng. The signal observed in the control and experimental lanes was very low. The anti-histidine primary antibody produced signals approximately 100x greater.

4.4 Discussion

The entire procedure, starting from the inoculation of cultures to the development of the Western blot took approximately 2 weeks to perform. It was very important to improve cell growth kinetics and identify ideal growth conditions as cell concentration plays a major role in the total amount of recombinant protein produced. This was the purpose of the work presented in Chapter 2. Similarly, designing and creating a genetic construct for use in *C. reinhardtii* and the genetic transformation process, as described in Chapter 3, was a key precursor to the work presented in this chapter. Together, Chapter 2 and Chapter 3 come together and allow for the analysis of the process as a whole.

The development of this bioprocess was challenging and took over a year to learn and master. The number and complexity of methods involved in this bioprocess were substantial, from culturing the cells, to cell harvesting and homogenization, to protein isolation, to analysis and quantification of recombinant protein amounts. Identifying growth conditions for cell culture was achieved using the results from Chapter 2. The next challenge was to separate the cells from the media and resuspend the cells in a buffer suitable for homogenization. Given the relative size of algae cells (approximately 10 µm in diameter), centrifugation for 10 minutes at 10,000 RPM produced a tight pellet that allowed for the complete removal of all spent medium.

Cell culturing was performed using the standard suspension bioreactor systems discussed in Chapter 2. One assumption for this project was that greater cell numbers would produce more of the product. Although this assumption should hold true given the nature of the promoter system, it was not investigated. It should also be noted that alternative culture vessels may very well increase cell concentrations and therefore product concentrations. The culture vessels used for these experiments were readily available, and were also easily transferrable for the necessary heat shock application process. In the future, alternative algal photobioreactor systems could be investigated.

Prior to homogenization, the cell solution was very viscous due to high cell concentration. Following the standard cell homogenization protocol, the solution's viscosity became similar to that of water. This, along with an observation under a light microscope, gave indication that the cells in solution were lysed.

Following homogenization, a high concentration of cell debris was present inside the cell solution. A primary centrifuge step separated the large pieces of cell debris from the remainder of the solution. However, significant debris still remained. An ultracentrifuge separated the remaining debris from the soluble proteins within the lysis buffer. At this point, the green colour of the cell solution, due to the chloroplasts of the algae, was pelleted by ultracentrifugation leaving only a clear lysate supernatant.

This clarified supernatant could then be used in the nickel chromatography column without fear of clogging or fouling the packed bed. Following histidine tag separation, 10 separate 1 mL elution fractions were collected. As expected, the first fraction collected contained the highest concentration of the proinsulin fusion proteins, as shown in Figure 4-2. The chromatography purification appeared to remove the vast majority of unwanted proteins; however, Figure 4-2 shows that a significant portion of the product was lost during the purification steps. The raw homogenate appeared to show a strong indication of the protein product, but the chromatography elution contained considerably less amounts of product, relatively speaking.

Using the first chromatography elution fraction and the bacterial protein standards, it was determined via Western blotting that the genetically engineered algae produced recombinant proinsulin at a concentration of 8.44 mg/L. A comparison of this value to other proinsulin production systems is shown in Table 4-1.

Table 4-1: A comparison of proinsulin production systems [22]

Source	Culture Method	Typical Net Specific Growth Rate (1/h)	Product Concentration (mg/L)
E. coli (Cytoplasm)	Bioreactor Fed-Batch	0.08-0.12	434
E. coli (Secreted)	Shake Flask Batch	Not specified	9
S. cerevisiae (Secreted)	Shake Flask Batch	<0.33	75
P. pastoris (Secreted)	Bioreactor Fed-Batch	<0.03	3075
***C. reinhardtii* (Cytoplasm)**	**Bioreactor Fed-Batch**	**0.07**	**8.44**

There was a large discrepancy in signal produced from Western blots using the histidine-specific primary antibody and the proinsulin-specific primary antibody. This could be because the histidine-specific primary antibody is simply a better and more robust antibody. However, if conformational position is taken into account, it may be possible that the histidine-specific antibody was more effective because the histidine tag was present on the end of the fusion protein. This positional effect would result in more of the primary antibody binding to the protein of interest, resulting in a larger signal. The proinsulin-specific primary antibody was specific to a portion of the product that was more in the centre of the protein, relative to the histidine-specific antibody. This may have resulted in fewer antibodies binding and may explain the relatively low signal observed.

The functionality of the recombinant proinsulin produced by the genetically engineered algae is unknown, but is identified as an area of future research. The size of the protein corresponds to the theoretical size of the fusion protein designed during the genetic engineering stage of the project (31 kDa). Furthermore, proinsulin-specific primary antibodies were used to detect the presence of proinsulin (although at a low signal), indicating at least partial congruence with the proper amino acid sequence. The determination of whether the proper folding, glycosylation and post-translational modifications had occurred in the algae-derived recombinant proinsulin is currently unknown. Animal studies will be pursued in the future to analyze the activity of the recombinant proinsulin.

Investigating recombinant protein production rates in genetically altered algae is an upcoming area of global research, with little published data. Typically, recombinant protein concentrations are reported as being “low” relative to other traditional microbial host platforms [82]. In one study, the highest protein concentration rate observed in *C. reinhardtii* was 1.65 mg/L of culture [14]. Although this study did achieve a protein concentration of 8.44 mg/L of culture, this is still well below the product concentrations produced in the traditional host organisms. These low protein concentrations are a major hindrance to the commercial production of algae-derived recombinant proteins.

For cost-associated reasons, recombinant proinsulin is typically produced in bacterial or yeast host systems [22]. Mammalian cell host systems are too expensive to be used for this purpose, and this is often attributed to the high cost of the cell culture medium. Recombinant algae also represent a theoretically low-cost alternative for recombinant protein production. This comes primarily from the relative simplicity of algal cell culture. *C. reinhardtii* medium is a simple and well defined solution containing only water, salt, trace minerals and acetate. External ongoing costs to produce the recombinant product from algae include lighting and the cost of agitation. A summary of these costs is presented in Table 4-2.

The majority of the cost of culturing one litre of *C. reinhardtii* under the mixotrophic growth conditions described above comes from the cost of the medium and the cost of agitation. However, the approximate cost amounts to only $1.47 per litre for an 8 day culture period. Accounting only for the cost of cell culture results in a cost of $174 per gram of recombinant protein produced. Compared to bacterial and yeast host systems which typically run in the $50 - $100 per gram range [83], this cost is high. This is due primarily to the low product concentration observed in the algae culture. Note that this estimate does not include the costly processes of cell homogenization and histidine tag chromatography as the cost for these steps would be difficult to estimate on a laboratory scale.

It may be possible, depending on the application, that recombinant proteins could be produced in a less technologically advanced method. For example, if the application is to produce biopharmaceuticals for veterinary or agricultural needs, it may be possible that the culture need not be sterile. As such, larger and more cost efficient culturing techniques such as raceway ponds could potentially be employed.

Unlike bacterial and yeast systems, algae represent a safe host for recombinant protein production which can theoretically fold complex recombinant proteins into a proper and active configuration. There is a great potential for genetically engineered algae to be used commercially as a cost-effective source of recombinant proteins. Unfortunately, low protein

Table 4-2: An estimate of the approximate cost culturing 1 litre of *C. reinhardtii* for 8 days

Item	Approximate Cost
TAP Medium	$0.50 per litre (Based on the purchase of reagents for 1,000 litres)
Addition of 40 mM Tris-acetate	$0.23 per litre, per fed-batch addition (Two additions per 8 days)
Operation of a 2.7 W LED light array for 8 days, operating 24 hours per day	$0.03 for 8 days (Assuming a cost of $0.05/kWh [84])
Operation of a 50 W multi-position magnetic stir plate for 8 days, operating 24 hours per day	$0.48 for 8 days (Assuming a cost of $0.05/kWh [84])
TOTAL COST	**$1.47 per litre, 8 day culture period**

production rates are the current bottleneck in this system. Further research is needed to improve algal protein production rates.

4.5 Conclusions

Using a genetically engineered strain of *C. reinhardtii* capable of producing recombinant proinsulin, a process was developed to isolate, analyze and quantify recombinant proinsulin production rates. Ideal growth conditions identified in Chapter 2 were used to culture algae cells in spinner flask bioreactors. Upon reaching a peak cell concentration, cultures underwent a heat shock to activate the genetic promoters, stimulating the production of recombinant proinsulin. Several steps were then taken to isolate the recombinant proinsulin, beginning with homogenization of the cells. Soluble proteins were separated from insoluble proteins through several steps of centrifugation. Once a clear solution of soluble proteins was obtained, this solution underwent histidine tag chromatography separation in a nickel column. This process separated the majority of the unwanted soluble proteins from the product. Using SDS-PAGE and Western blotting techniques, protein amounts were analyzed and compared to a standard. It was determined through this process that the 1 litre of *C. reinhardtii* culture produced 8.44 milligrams of the recombinant proinsulin fusion protein, which is greater than previously reported product concentrations of 1.65 mg/L of *C. reinhardtii* culture. However, this value is significantly less than the protein concentrations produced by alternative host systems such as *P. pastoris*, which have reached as high as 3,075 mg/L. A cost analysis determined that culturing one litre of *C. reinhardtii* for 8 days costs approximately $1.47. Given the amount of protein produced, this resulted in an estimated cost of $174 per gram of recombinant protein. Although algae are very inexpensive to culture, the low protein production rates result in a very high cost per gram, relatively speaking. There is potential for algae to be used as a host organism for recombinant protein production; however, the main challenge will be increasing protein production rates to levels near or above traditional methods.

4.6 Future Work

Using algae as a host organism for recombinant protein production has been shown to be a promising alternative to traditional hosts. Unfortunately, this system is currently limited by low protein production rates. Several methods could potentially be used to increase the protein production concentrations.

The genetic construct itself and the transformation procedure represent major components in this challenge. The orientation of the genetic construct within the genome plays a major role in the expression of the genes. A more efficient transformation and screening procedure could be used to find an algae strain producing at an optimal level. Alterations could also be made to the genetic construct itself. For example, targeting the product protein to be secreted from the cell would eliminate cell processing steps such as cell homogenization. Doing so would also reduce the product losses experienced in these processing steps. As algal biotechnology advances, it is likely that new genetic promoters or screening methodologies could be developed which would aid product production.

Increasing cell concentrations to a maximum volume prior to protein production stimulation is another important factor to consider. As discussed in Chapter 2, utilizing various oxygen or carbon dioxide concentrations above atmospheric levels could have a positive impact on cell concentration, and hence protein concentration. Similarly, the use of a specialized bioreactor or chemostat could greatly improve protein production levels over those observed from this project.

One area of concern lies within the protein separation process itself. Small amounts of recombinant protein were lost during each purification step. Optimizing these steps, or alternatively the use of more efficient purification steps, could greatly impact the final protein concentration observed.

Future work will also investigate the use of alternative algae hosts other than *Chlamydomonas reinhardtii*. Advances in algal biotechnology are allowing for the genetic manipulation of other species of algae. Though this technology has not yet reached a level similar to *C. reinhardtii*, this may change in the future. The use of alternative algae species will remain an area of future research and investigation.

Chapter 5: Summary, Future Work and Conclusions

5.1 Summary

As described previously, the specific aims for this project were identified as follows:

1. *Characterizing and improving the mixotrophic growth kinetics of Chlamydomonas reinhardtii*;
2. *The manufacture of a genetic construct and the production of a recombinant Chlamydomonas reinhardtii strain capable of producing a mammalian-derived recombinant protein;*
3. *Analysis of recombinant protein production from 500 mL transgenic cultures.*

5.1.1 Characterizing and improving the mixotrophic growth kinetics of *C. reinhardtii*

Chapter 2 focused on the first specific aim; namely, characterizing and improving the mixotrophic growth kinetics of *C. reinhardtii*. The underlying hypothesis behind this work was that an increase in the maximum cell concentration would lead to an increase in total protein production in recombinant algae strains. Given the nature of the HSP70A/RBCS2 fusion promoter, an increase in the total number of cells capable of producing a recombinant protein would indeed lead to a greater amount of product produced. For these reasons, a key portion of this project was the characterization and improvement of *C. reinhardtii* cell culture concentrations.

Using methods previously described in the literature, baseline *C. reinhardtii* growth curves were established using small scale shake flask vessels and TAP medium. The selection of TAP medium was based primarily on previous research indicating that the supplementary acetate present in the medium improved cell growth relative to other media via mixotrophic growth. Following the research of other investigators, the addition of sodium acetate was found to have a profound benefit on the maximum cell concentration. This research was extended to the supplementary addition of other acetate salts, and eventually to the addition of Tris-acetate. In small scale cultures, the addition of 40 mM Tris-acetate to TAP medium resulted in a maximum

cell concentration of 1.93e7 cells/mL; a significant improvement over the 1.01e7 cells/mL cell concentration observed using regular TAP medium.

This process was then scaled up from a 10 mL volume to a 500 mL volume in spinner flask bioreactors. Initial bioreactor studies investigated the effects of agitation, as well as the impact of supplemental acetate. Similar to the small scale studies, a supplemental Tris-acetate concentration of 40 mM to TAP medium provided a significant benefit to maximum cell concentration. Furthermore, it was found that in these systems, the acetate was fully consumed by the algae by culture day 5. By adding 40 mM Tris-acetate in a fed-batch manner on day 5, cell concentrations were further improved and maintained. Using only TAP medium with no supplemental acetate, a cell concentration of 1.2e7 cells/mL was observed in 500 mL spinner flasks with an agitation rate of 400 RPM. However, with the addition of 40 mM Tris-acetate to the starting TAP medium, as well as a 40 mM Tris-acetate spike on day 5, cell concentrations as high as 2.6e7 cells/mL were observed.

This research into the use of supplementary acetate salts and buffered acetic acid would be of significant interest not only for genetic engineers working with transgenic *C. reinhardtii*, but also for researchers investigating biofuel production, cell farming or carbon capture, among other disciplines. The process of maximizing cell concentrations is highly beneficial to many investigators, as it improves the efficiency of a culture, regardless of the product.

5.1.2 The manufacture of a genetic construct and the production of a recombinant *Chlamydomonas reinhardtii* strain capable of producing a mammalian-derived recombinant protein

With *C. reinhardtii* cell growth kinetics well characterized, attention turned in Chapter 3 to the manufacture of a genetic construct for use in the genetic engineering of a wild type *C. reinhardtii* strain. This process began with the conceptual design of the construct itself. Several components were designed into the construct, including a histidine tag for protein separation purposes, as well as an alternative separation technique and two protein cleavage techniques.

The model product was selected to be proinsulin. Although any complex protein could have been selected for this purpose, proinsulin was chosen as it is a widely recognized, relatively small yet complex mammalian protein. As this project aimed to produce a platform technology, the modular characteristics of the genetic construct allow for individual genes to be easily inserted or removed. If a particular trait of the fusion protein is to be added or removed, this can be done with relative ease. Similarly, the product can be exchanged for any other protein, while the remaining construct manufacturing, transformation, cell culture and downstream processing would stay unchanged.

The gene of interest was manufactured as conceptually designed using existing microbiology techniques, and inserted into the pChlamy_1 host vector to create the pBC_1 vector. After being transformed into an *E. coli* host for long term storage, the sequence of the pBC_1 vector was confirmed using a commercial laboratory. The recombinant *E. coli* was used as a multiplication host to obtain a high copy number of the pBC_1 vector, which was then purified and used for the transformation of wild type *C. reinhardtii*. Using the electroporation transformation protocol, the *C. reinhardtii* were genetically engineered with the pBC_1 vector and placed under hygromycin selection. Several colonies observed on the solid TAP medium selection plates were selected and used as the inoculum for liquid cultures. Transgenic cultures were stored long term in liquid nitrogen.

5.1.3 Analysis of recombinant protein production from 500 mL transgenic cultures

Using the genetically engineered *C. reinhardtii* strain developed in Chapter 3, the work in Chapter 4 focused on the development of a bioprocess to analyze the protein production from the recombinant algae. Using the improved cell growth techniques from Chapter 2, the recombinant *C. reinhardtii* were cultured in 500 mL spinner flask bioreactors to a cell concentration of 2.7e7 cells/mL. Following cell harvesting via centrifugation, the cells were lysed using a cell homogenizer.

The lysed cell solution was separated by relative density using two rounds of centrifugation to separate all insoluble proteins from soluble proteins. Using a nickel chromatography column, the fusion protein of interest was separated from all other soluble proteins. The product was collected and analyzed using SDS-PAGE and Western blotting.

By comparing the algal protein amounts to those of a standard, it was determined that the recombinant *C. reinhardtii* produced the fusion protein of interest at a concentration of 8.44 mg/L of culture, at a cost of $174/g for cell culture only. Relative to the existing methods of proinsulin production, this protein concentration was determined to be quite low. Previous studies on recombinant protein concentration in *C. reinhardtii* yielded a product concentration of 1.65 mg/L of culture, which is well below the value observed with the methodology developed in this thesis.

The production rate of recombinant proteins from genetically engineered algae has not been well characterized in the literature. Therefore, this value will help other researchers understand future challenges. Although the protein concentration was determined to be quite low, as a first attempt this does show promise. A multitude of techniques and strategies could be employed in the future to help increase the recombinant protein production rate, making it more competitive with traditional methods.

5.2 Future Work

The potential for advancement in this research field is staggering. Modifications to culture techniques, media and growth conditions may have a significant impact on the growth kinetics of *C. reinhardtii*. The most reasonable next step from this project is to determine the effect of CO_2 and O_2 on mixotrophic cell growth kinetics. This work has already begun, and is presented in Appendix A. Preliminary results show very promising improvements to cell growth rates and maximum cell concentrations by simply increasing the ambient CO_2 concentration from 0.04% to 3%.

Advancements to the algal genetic construct could lead to significant changes in the downstream processing. The pBC_1 construct includes the new and innovative ELP separation technology, which could potentially reduce product losses observed in the presented bioprocess. Hence, future research will also look into the use of the ELP protein separation technique. Future research will also investigate further improvements to the promoter design and stimulation. It is the genetic construct, more specifically the genetic promoter, which ultimately drives the production of the product.

By improving recombinant protein production rates and decreasing costs, the field of genetically engineered algae could evolve into a new and cost effective alternative for recombinant products. Many different approaches could be taken to increase production rates, including having better recombinant algae screening techniques, more refined and efficient genetic promoters, increased cell concentrations and fewer losses in downstream processing.

Furthermore, the product itself must be identified to be biologically active. There is currently not enough research to conclude that algae can indeed produce complex mammalian proteins such as antibodies, although it is strongly believed to be possible. Ergo, the biological activity of the proinsulin will be assayed in the future using animal models to determine the functional biological activity. It is estimated that protein amounts on the order of grams will be required for a complete functional assay.

The final goal of this research is to establish a new and cost efficient recombinant protein production technology. If literature estimates are true, the use of algae-derived recombinant proteins could result in a cost reduction of up to 1000x compared to traditional techniques. This significant cost decrease would result in a greater availability of recombinant proteins such as biopharmaceuticals, vaccines and antibodies to the entire world. As a result, essential medicines would be more accessible to all.

5.3 Conclusions

Recombinant proteins are essential to modern medicine, industry, research and agriculture. Traditional host organisms such as bacteria, yeast and mammalian cells are currently used industrially for the production of recombinant proteins. In many cases, these systems are complex, expensive and slow. The use of algae as a host organism for recombinant protein production has recently been widely discussed in the literature as a potentially cost efficient, scalable, safe and fast-growing alternative to traditional host organisms.

Using the well characterized unicellular algae *Chlamydomonas reinhardtii*, this project set out to identify the feasibility of producing recombinant proteins from genetically engineered algae. The cell growth kinetics of *C. reinhardtii* was characterized and improved using mixotrophic growth techniques. Through the fed-batch addition of 40 mM Tris-acetate to TAP medium, cell concentrations of up to 2.7e7 cells/mL were observed. Next, a novel genetic construct was designed, manufactured, isolated and used to transform wild type *C. reinhardtii*. Finally, a bioprocess was developed to analyze the protein production from the genetically engineered algae. It was determined that the transgenic algae were capable of producing the recombinant fusion protein product at a concentration of 8.44 mg/L of cell culture. Ignoring downstream processing, this corresponded to a production cost of $174/g. Although the economic goal was not met, this bioprocess represents an improvement over previous *C. reinhardtii* recombinant protein production systems which had product concentrations of 1.65 mg/L of culture.

Ultimately this project aims to provide a cheaper, safer and more efficient source of recombinant proteins, including biopharmaceuticals such as proinsulin. If successful, the impact on the fields of medicine, agriculture, research and industry could be extensive. Although not cost-competitive in its current form, the field of recombinant protein production in algae hosts is a developing field of research. The experiments performed in this project will help future researchers better understand the challenges that lie ahead. It is my hope that researchers in the future will solve these problems and make recombinant proteins, such as essential medicines, more available to the worldwide population.

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Appendix A: Preliminary investigation of the effect of CO_2 concentration on the growth of *Chlamydomonas reinhardtii*

Ben Marsall-Moritz, an undergraduate research student, is credited with the experimental design, setup and all data observations present in Appendix A. It is separated from the body of the thesis because it represents preliminary data in an area previously identified as future work.

A.1 Introduction

In Chapter 2, the growth kinetics of *C. reinhardtii* were investigated and improved through the addition of acetate. The future work section in this chapter indicated that increasing CO_2 and/or O_2 concentrations above ambient conditions may greatly impact cell growth. Furthermore, it was discussed that various light fluxes above the standard 50 $\mu E \bullet m^{-2} \bullet s^{-1}$ could also have an impact on cell growth kinetics. In this appendix, preliminary studies were undertaken to determine the effect of CO_2 concentration and light flux on the growth of *C. reinhardtii*. The data presented here was gathered by experiments performed by Ben Marsall-Moritz, a 4^{th} year biomedical engineering undergraduate student. It represents a promising preliminary investigation into the effect of gas concentration and light flux.

A.2 Methods

Methods of cell culture are similar to those described in Chapter 2 for small scale cultures. In all cases, 10 mL of TAP medium supplemented with 40 mM acetic acid (buffered with Tris to pH 7.0 to produce Tris-acetate) was used as the culture medium, and wild type *Chlamydomonas reinhardtii* 137c was used as the cell source (Invitrogen A14258). Cultures were grown mixotrophically under the presence of acetate and light. All cultures were placed within a VWR Symphony air-jacketed CO_2 incubator (VWR Model 5.3A) to provide a precise CO_2 concentration control. All cultures were performed in quadruplicate, and statistical significance was determined as in Chapter 2.

Cell concentration was observed using standard techniques involving an Olympus CKX41 light microscope and a hemocytometer.

A.3 Results

Studies were performed to investigate the effect of CO_2 concentration on the growth of *Chlamydomonas reinhardtii*. All experiments described in Chapter 2 were undertaken in ambient air conditions (containing 0.04% CO_2). It was hypothesized that increasing the CO_2 concentration could benefit cultures growing mixotrophically. Hence, several CO_2 concentrations were selected to determine the impact on *C. reinhardtii*. As presented in Figure A-1, four separate CO_2 concentrations were selected: atmospheric (0.04%), 1.5%, 3.0% and 4.0%.

Various levels of light flux were also analyzed to determine their effect on *C. reinhardtii*. As shown in Figure A-2, four separate light fluxes were analyzed in a 0.04% CO_2 environment: 50 $\mu E \bullet m^{-2} \bullet s^{-1}$, 65 $\mu E \bullet m^{-2} \bullet s^{-1}$, 90 $\mu E \bullet m^{-2} \bullet s^{-1}$ and 135 $\mu E \bullet m^{-2} \bullet s^{-1}$. The high light fluxes of 90 and 135 $\mu E \bullet m^{-2} \bullet s^{-1}$ resulted in reduced cell growth relative to more moderate light fluxes of 50 and 65 $\mu E \bullet m^{-2} \bullet s^{-1}$.

The final preliminary growth experiment investigated the effect of light flux in a 3.0% CO_2 environment, presented as Figure A-3. Following the results from Figure A-1, 3.0% CO_2 was selected as the CO_2 concentration which resulted in the highest maximum cell concentration. Under a 3.0% CO_2 environment, cell concentrations were observed to be at a maximum under 50 and 65 $\mu E \bullet m^{-2} \bullet s^{-1}$.

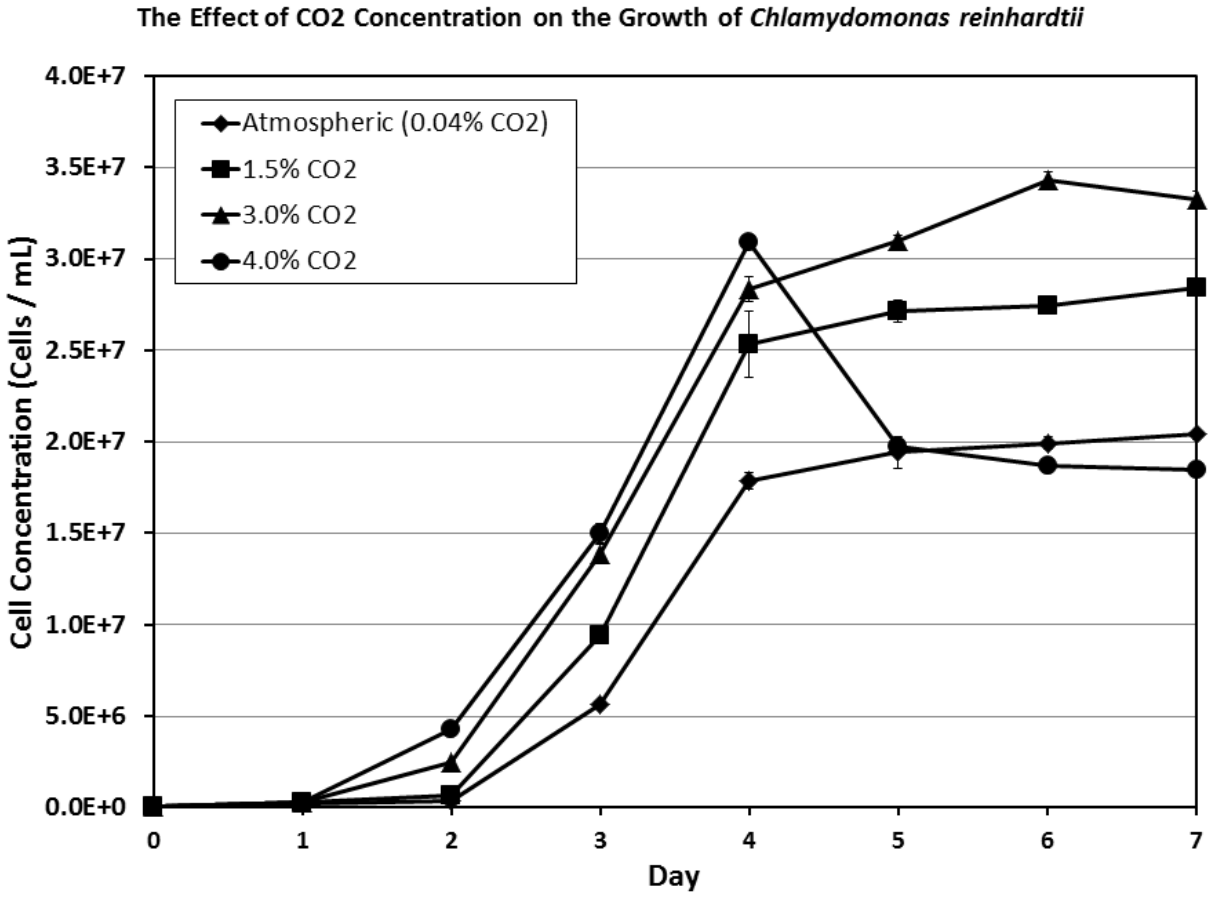

Figure A-1: The effect of CO_2 concentration on the growth of *C. reinhardtii*. 4 different CO_2 concentrations were selected: atmospheric (0.04%), 1.5%, 3.0% and 4.0%. Increasing CO_2 concentration above atmospheric concentrations appeared to benefit cell growth up to 3.0%. At 4.0% CO_2 concentrations, cell death was observed beyond a cell concentration peak at day 4. All comparisons between experimental conditions were found to be statistically significant with $p<0.05$. All cases were performed in quadruplicate (n=4), and error bars represent the standard error of the mean.

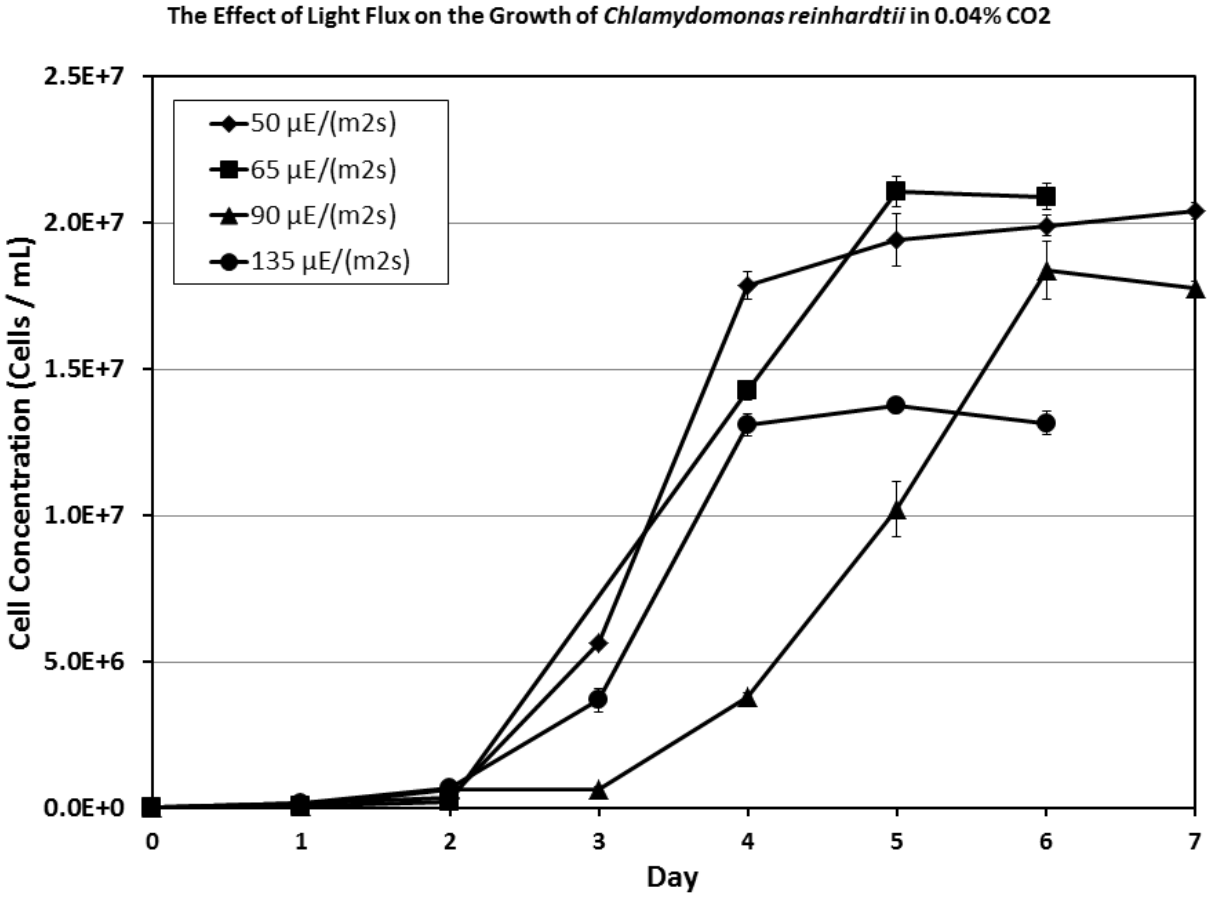

Figure A-2: The effect of light flux on the growth of *C. reinhardtii* in 0.04% CO_2 conditions. Four separate light fluxes were investigated; 50, 65, 90 and 135 $\mu E \bullet m^{-2} \bullet s^{-1}$. Cell concentrations were observed to be at a maximum under 65 $\mu E \bullet m^{-2} \bullet s^{-1}$. Light fluxes above this value resulting in a relative decrease in cell growth rates and maximum cell concentration. All comparisons between experimental conditions were found to be statistically significant with $p<0.05$. All cases were performed in quadruplicate (n=4), and error bars represent the standard error of the mean.

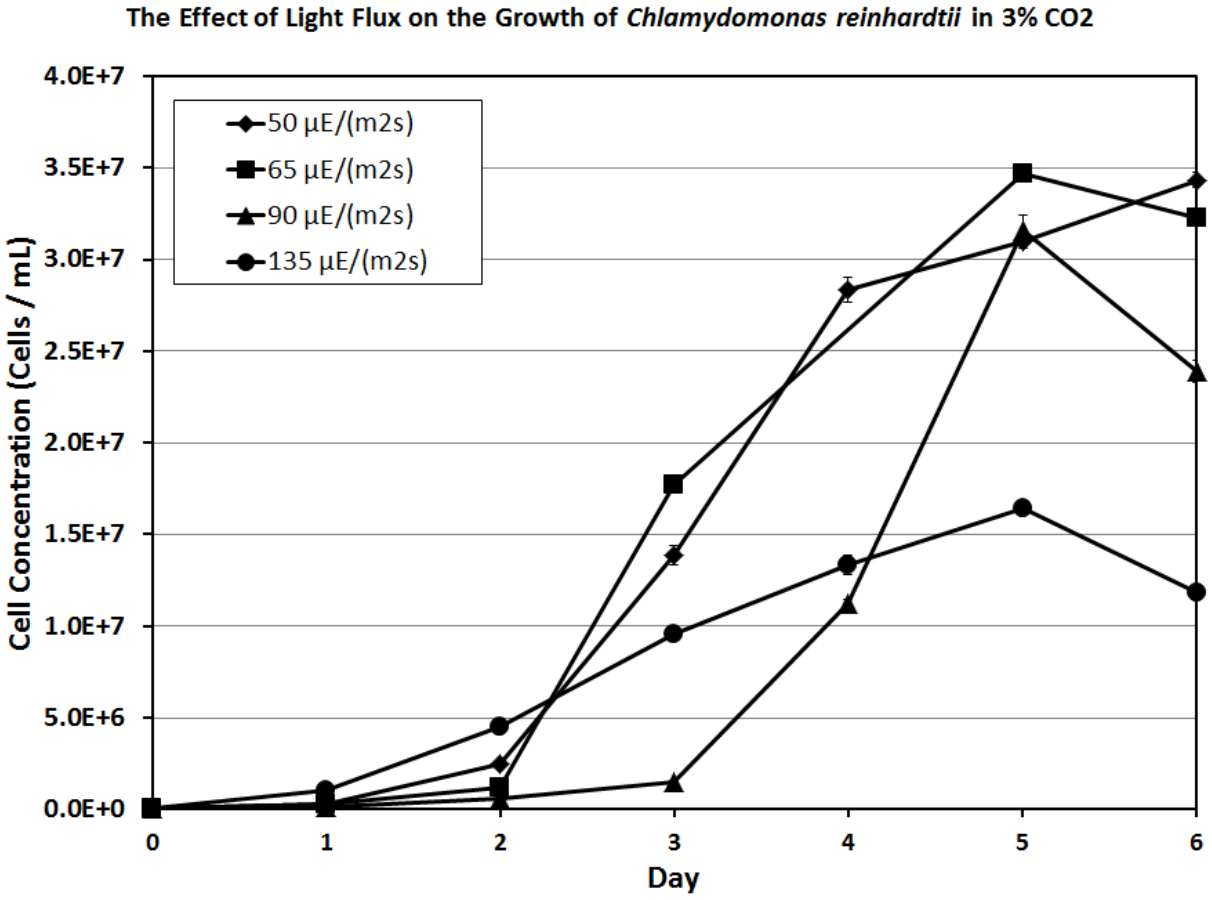

Figure A-3: The effect of light flux on the growth of *C. reinhardtii* in a 3% CO_2 environment. Four separate light fluxes were investigated; 50, 65, 90 and 135 $\mu E \bullet m^{-2} \bullet s^{-1}$. Moderate light fluxes of 50 and 65 $\mu E \bullet m^{-2} \bullet s^{-1}$ were found to have similar maximum cell concentrations, peaking at nearly 3.5e7 cells/mL. Higher light fluxes of 90 and 135 $\mu E \bullet m^{-2} \bullet s^{-1}$ caused a relative decrease in maximum cell concentration. All comparisons between experimental conditions were found to be statistically significant with $p<0.05$. All cases were performed in quadruplicate (n=4), and error bars represent the standard error of the mean.

A.4 Discussion

CO_2 concentration is likely to play a major role in the growth kinetics of *C. reinhardtii* cultured under phototrophic or mixotrophic conditions. CO_2 is a necessary molecule for photosynthesis, and it was hypothesized that increasing CO_2 concentrations could result in increased cell growth. Similarly, increasing the light flux was hypothesized to have an impact on photosynthesis and cell growth.

Under the addition of exogenous acetate, *C. reinhardtii* grow under mixotrophic conditions. It is also hypothesized that increased O_2 concentrations would have a beneficial impact on cell growth due to potentially increased acetate breakdown via cell respiration. Time and equipment restraints resulted in the investigation of CO_2 concentrations only, as well as the impact of light flux.

Under mixotrophic cell growth conditions, it was observed in Figure A-1 that increasing the CO_2 concentration to 3.0% provided a maximum benefit to cell growth kinetics and maximum cell concentration. At 4.0% CO_2, cell death was observed following day 4, which could potentially be attributed to a decreased culture pH. Under increasing CO_2 concentrations, more CO_2 is dissolved in the culture medium, resulting in increased concentrations of carbonic acid. This carbonic acid could, in turn, lower culture pH to undesirable levels causing cell death. Culture pH levels were not investigated and this remains an area of future research.

The effect of various light fluxes was also investigated in air in Figure A-2 and in 3.0% CO_2 in Figure A-3. For both of these cases, moderate light fluxes of 50 and 65 $\mu E \bullet m^{-2} \bullet s^{-1}$ were observed to produce the scell concentrations or cell death. This can likely be attributed to the process of photoinhibition. Under this phenomenon, the photosynthetic capacity of the alga is reduced due to light-induced damage to cellular components responsible for photosynthesis [85].

The highest cell concentration observed in small scale shake flask cultures in Chapter 2 was approximately 2.0e7 cells/mL. The preliminary results presented here have increased this cell concentration to nearly 3.5e7 cells/mL by simply increasing the CO_2 concentration from 0.04% to 3.0%. This adjustment in and of itself could significantly improve the protein production rates observed in Chapter 4. Further improvements and optimizations using a mixture of O_2 and CO_2 could result in even greater improvements. Slightly increasing the light flux from 50 to 65 $\mu E \bullet m^{-2} \bullet s^{-1}$ may also slightly improve cell growth kinetics, although further work will be necessary to investigate the impact. It is clear that CO_2 concentration plays a significant role in the mixotrophic culture of *C. reinhardtii*, and this will continue to be investigated.

A.5 Conclusions and Future Work

CO_2 concentration and light flux were found to play an important role in the mixotrophic growth kinetics of *C. reinhardtii*. Cultures in a 3.0% CO_2 environment were found to have a maximum cell concentration of nearly 3.5e7 cells/mL. Light fluxes above 50 $\mu E \bullet m^{-2} \bullet s^{-1}$ were not observed to produce a significant increase in cell growth kinetics in both a 0.04% CO_2 and 3.0% CO_2 environment. Higher light fluxes of 90 and 135 $\mu E \bullet m^{-2} \bullet s^{-1}$ were found cause a significant reduction in cell growth kinetics, likely due to the phenomenon of photoinhibition. The goal of this research was to improve the maximum cell concentration of *C. reinhardtii* cultures for the protein production rates in genetically engineered cell cultures. However, this data could also be useful for the production of increased biofuels, biomass or any other algae-derived product. Regardless of the application, these preliminary results show promising steps forward in mixotrophic cell growth kinetics, which will prompt further investigation.

Future work will investigate the effect of increasing CO_2 concentrations on culture pH, as pH can greatly impact the well-being of the culture. Furthermore, the impact of increasing O_2 concentrations will be investigated as it is hypothesized that this could impact heterotrophic and mixotrophic cell growth kinetics. Under mixotrophic conditions, the impact of increasing both O_2 and CO_2 concentrations will also be investigated. Finally, under improved or optimized conditions, the recombinant protein production rates will be analyzed.

Appendix B: Supplemental Data

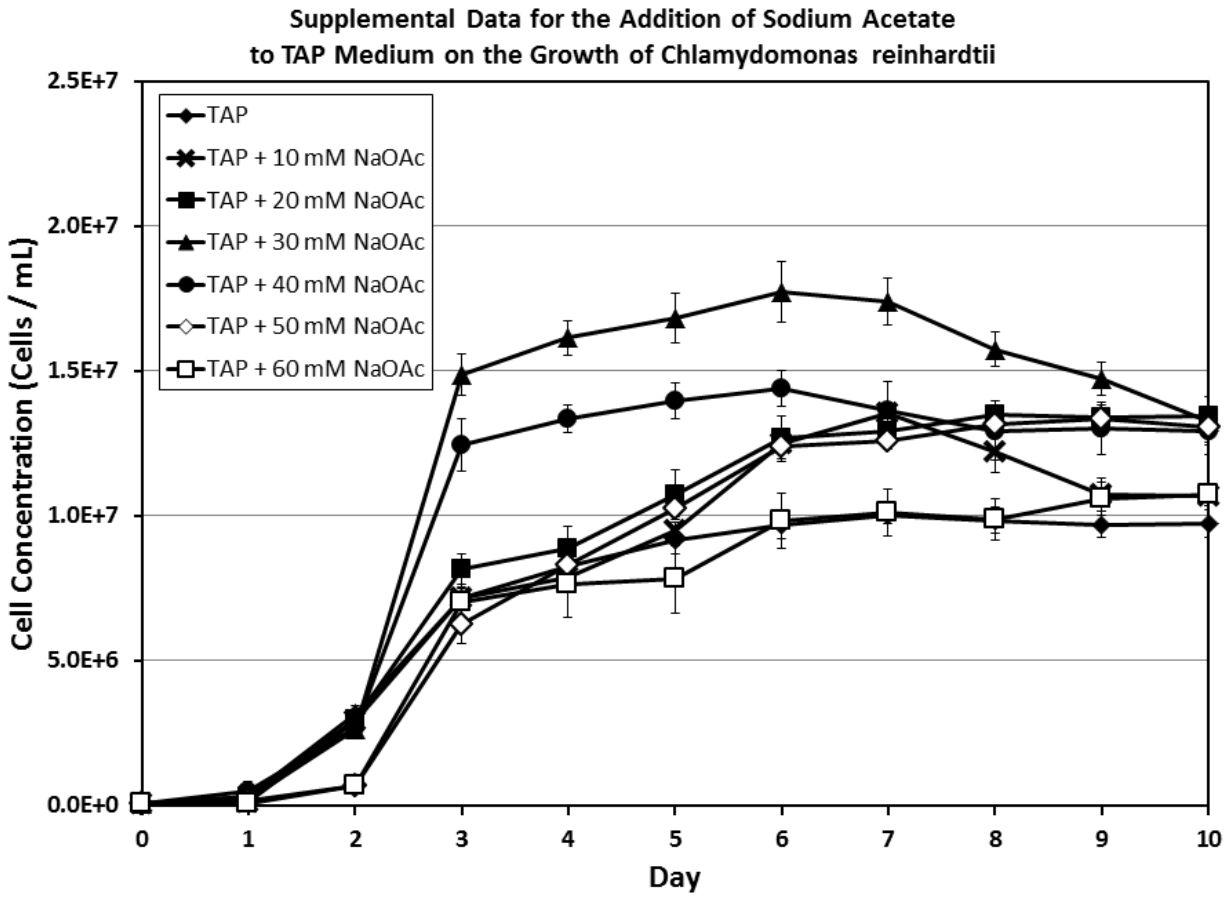

Figure B-1: Supplemental data for Figure 2-1 showing all the data obtained from studies on the growth of *C. reinhardtii* using sodium acetate (NaOAc). All cases were performed in triplicate (n=3), and error bars represent the standard error of the mean.

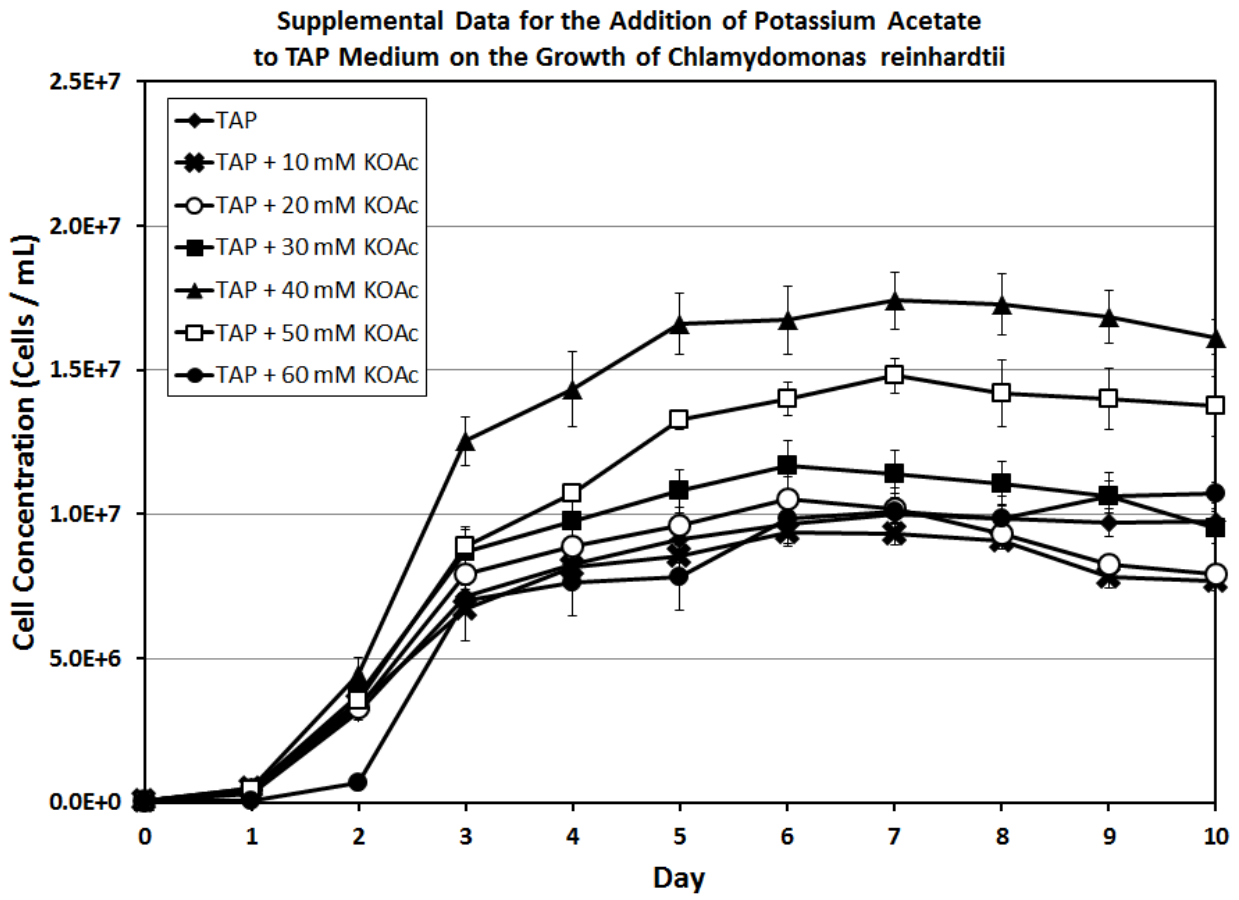

Figure B-2: Supplemental data for Figure 2-2 showing all the data obtained from studies on the growth of *C. reinhardtii* using potassium acetate (KOAc). All cases were performed in triplicate (n=3), and error bars represent the standard error of the mean.

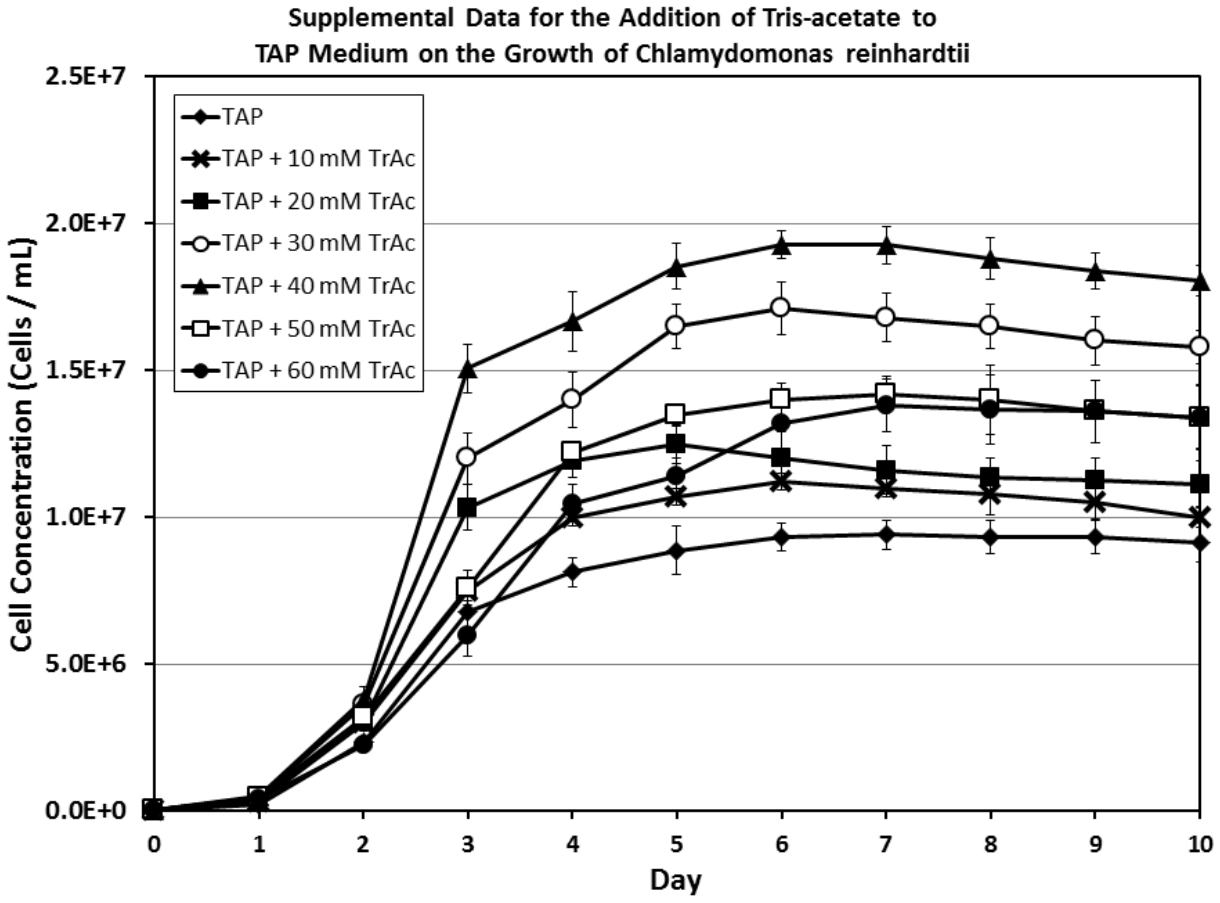

Figure B-3: Supplemental data for Figure 2-3 showing all the data obtained from studies on the growth of *C. reinhardtii* using Tris-acetate (TrAc). All cases were performed in triplicate (n=3), and error bars represent the standard error of the mean.

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