ABSTRACT

Carbon Metabolism in Lipid-Producing, Nitrogen-Starved, Chlamydomonas reinhardtii

Angeline Nguyen

Director: Dr. Sung Joon Kim, Ph.D

In recent years, studies have shown that lipid bodies produced by nitrogen-starved green algae such as *Chlamydomonas reinhardtii* may serve as viable alternatives of fossil fuels. While there is no doubt that these organisms are capable of producing energy-packed lipids, the exact pathway of how carbon is incorporated into the cells to make energy-storage molecules has yet to be determined. In order to determine this, the mutant strains sta6 and cw15 were grown using acetate as their main carbon source. Although both strains lack cell walls, sta6 lacks a key starch-producing enzyme. Because of this, it is known that sta6 primarily incorporates the exogenous acetate into triacylglycerides (TAGs) while cw15 primarily produces starch under nitrogen-starvation. The acetate was labeled with 1-^{13}C acetate and tracked using nuclear magnetic resonance spectroscopy. It was found that the acetate is not incorporated into lipids and starch directly through typical metabolic pathways but through a bicarbonate system.

APPROVED BY DIRECTOR OF HONORS THESIS:

_____

Dr. Sung Joon Kim, Ph.D, Department of Chemistry and Biochemistry

APPROVED BY THE HONORS PROGRAM

_____

Dr. Andrew Wisely, Director

DATE: _____

CARBON METABOLISM IN LIPID-PRODUCING, NITROGEN-STARVED, *CHLAMYDOMONAS REINHARDTII*

A Thesis Submitted to the Faculty of

Baylor University

In Partial Fulfillment of the Requirements for the

Honors Program

By

Angeline Nguyen

Waco, TX

May 2014

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iii.

CHAPTER 1 - INTRODUCTION 1.

Section 1.1 - Formulation 1

Section 1.2- Synthesis of Energy Storage in *Chlamydomonas reinhardtii* 3

Section 1.3 – Carbon Utilization in *Chlamydomonas reinhardtii* 8

Section 1.4 - Goal 12

CHAPTER 2 – MATERIALS AND METHODS 14.

Section 2.1 – Cultivation of algae 14

Section 2.2 – Preparation for Solution NMR 17

Section 2.3 – Measurement with Nuclear Magnetic Resonance Spectroscopy 18

Section 2.4 – Data Processing 19

CHAPTER 3 - RESULTS 20.

Section 3.1 – Growth 20

Section 3.2 – NMR Data 22

CHAPTER 4 – DISCUSSION AND CONCLUSION 35.

Section 4.1 – Growth of *Chlamydomonas reinhardii* with Exogenous Acetate 35

Section 4.2 – NMR Results 36

Section 4.3 – Outside Application 38

Section 4.4 – Final Conclusions 41

REFERENCES 44.

ACKNOWLEDGEMENTS

I would like to express my gratitude for Dr. Sung Joon Kim and James Chang for their continuous guidance throughout the writing process of this thesis. In addition, I would like to thank Dr. Christina Chan-Park and Dr. Sung-Kun Kim for serving on my defense committee. Thank you all for providing me with the support to complete this process successfully.

CHAPTER ONE

Introduction

1.1. Formulation

In a society that thrives upon an ever-shrinking supply of fossil fuels, there exists the interest for the discovery and utilization of more environment-friendly biofuels. In nature, there are two classes of molecules that serve explicitly to provide energy for organisms. Carbohydrates, or sugars, provide quick energy; while lipids, such as fats or oils, provide long-term energy. While carbohydrates provide approximately four Calories per gram of energy upon consumption, fats provide approximately nine Calories per gram.[4] In organisms, the increased energy density provided by lipid offers advantages in both storage and transportation of the energy per unit volume. Therefore, studies have shown that cultivating these lipids from living organisms can produce sustainable energy. In particular, mutants of the unicellular green algae *Chlamydomonas reinhardtii* have been proven to produce copious amounts of lipids after being nitrogen-starved.[19] Researchers have determined this species to be a viable option for future energy among other autotrophic organisms due to its high per-acre productivity when growing, its non-food based feedstock, and its potential to recycle carbon dioxide.[17] As a eukaryotic unicellular organism, this alga is certainly a model organism.

Model organisms are used in order to understand certain biological and biochemical processes. *Chlamydomonas reinhardtii* is one such organism (Figure 1). This is due to the fact that is unicellular, haploid, and contains three genomes (nuclear,

chloroplastic, and mitochondrial) that are all susceptible to transformation. In addition, the alga can grow either photoautotropically or heterotrophically, making it a model system for studies in processes such as chloroplast biogenesis, energy production, photosynthesis, and flagellar structure and assembly.[5]

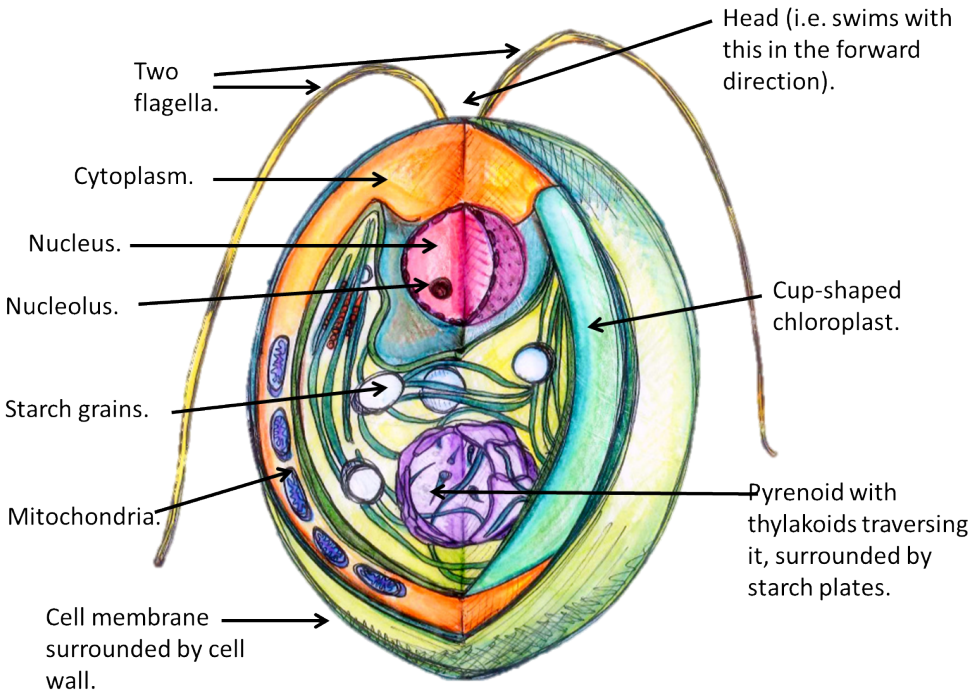

Figure 1. A Three-Dimensional Sketch of *Chlamydomonas reinhardtii* [3]

Despite the fact that there is a known mechanism for lipid production in this organism and other algae, the process by which algae decide to produce lipids as the main source of energy under stress is unclear. Tracking the carbon pathways within these cells will provide a better understanding of the metabolism of the cells and perhaps be applicable to other organisms in future studies.

Comparable to plants, *Chlamydomonas reinhardtii* creates starch as its first line of energy storage. It has been shown that the starch synthesis and lipid synthesis pathways within the cell are not always simultaneous, but rather competitive based upon carbon availability.[5] Since starch biosynthesis is dominant over lipid production, high rates of triacylglyceride synthesis only occurs in mutant strains or when carbon supplies exceed the capacity for starch synthesis. Therefore, an exogenous carbon source is necessary for excessive lipid production.

1.2 Synthesis of Energy Storage in Chlamydomonas reinhardtii

1.2a Starch Production in Algae

In wild-type algae, starch production is the primary form of energy storage. Starch is a polysaccharide made of glucose subunits. This process begins with the sugar products of photosynthesis. While the main product of photosynthesis is glucose, $C_6H_{12}O_6$, it is typically converted to sucrose, a disaccharide comprised of glucose and fructose. Therefore, the process of starch synthesis often times begins with sucrose. However, this sucrose must be converted back into monosaccharides before starch can be synthesized.[6]

First, sucrose is broken down into fructose and UDP-glucose by sucrose synthase. Both of these products are converted into glucose-1-phosphate through different enzymes. For fructose, hexokinase phosphorylates the carbon at the six position using ATP. Mutase and isomerase then assist fructose-6-phosphate in isomeric changes to become glucose-1-phosphate. As for UDP-glucose, UDP glucose pyrophosphorylase converts UDP-glucose into glucose-1-phosphate by phosphorylation.

Once glucose-1-phosphate is formed, ADP glucose pyrophosphorylase converts it into ADP-glucose, which is then transported from the cytoplasm into an amyloplast. Starch synthase then links the ADP-glucose together to form the linear α-1,4-Glucan. Branching enzyme branches these chains with α-1,6 bonds to form α-1,4 α-1,6 glucan. Amylopectin 1,6-glucosidase converts this molecule into amylopectin, one of the two major components of starch. Various enzymes then combine this branched sugar with amylose, a linear α-1,6 glucose chain to form starch (Figure 2).

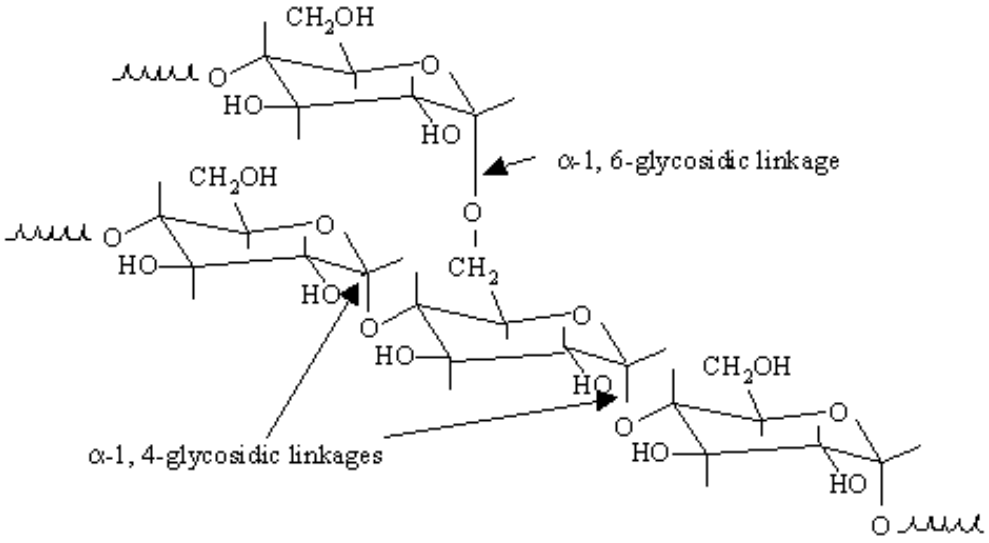

Figure 2. Glycosidic bonds formed between glucose monomers in starch[6]

1.2b Lipid Production in Algae

Fatty acids are long chains of carbon molecules with a carboxylic acid end. Unsaturated fatty acids are fatty acids with at least one double bond in the carbon chain, or tail, while saturated fatty acids do not contain any double bonds. These molecules are the most basic building block of lipids and are used to form triacylglycerides, larger

molecules that carry an immense amount of energy. Each triacylglyceride is made of three fatty acids connected by a three-carbon glycerol backbone.

Despite the fact that fatty acid and triacylglyceride synthesis in microalgae has not been extensively and explicitly studied, it has been noted that the processes are extremely similar to that of higher plants and other eukaryotes.[2] The main difference is that lipid production in plants occurs across various organs, whereas all activity in microalgae occurs within a single cell. In both plants and algae, the thylakoid membrane of the chloroplasts is directly responsible for preliminary fatty acid synthesis. However, only some of the fatty acids produced are directly assembled and incorporated into the thylakoid membrane. The other fatty acids are transported to the endoplasmic reticulum for further assembly into larger lipid structures, while a very small fraction is retransferred into the chloroplast for thylakoid lipid synthesis. Lipid structures produced by the cell are incorporated into organelle and cell membranes and can be incorporated into photosynthetic complexes.[19]

1.2c Mechanism

In general, lipid production starts with de novo synthesis of fatty acids, the most basic building block of triacylglycerides (Figure 4).[4] Fatty acid is comprised of an aliphatic chain attached to a carboxylic acid end group. Fatty acid synthesis begins with acetyl-CoA, the product of the decarboxylation of pyruvate by the enzyme pyruvate dehydrogenase.[18] This process begins within the chloroplasts of plants and algae. The first committed step of fatty acid biosynthesis is then the biotin-dependent reaction of acetyl-CoA carboxylase (Figure 3). This involves activation of CO_2 followed by a carboxylation reaction in order to yield a three-carbon malonyl-CoA.

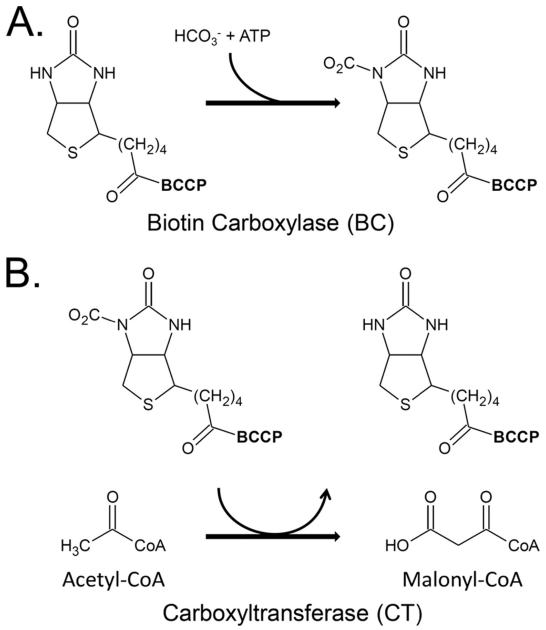

Figure 3. First committed step in fatty acid synthesis[14]

After this, the fatty acid is elongated two carbons at a time. This is accomplished by fatty acid synthase, a complex of various enzymes that carry out separate reactions. The first of these reactions involves hydrolysis of the thioester bond in acetyl-CoA to release the acetyl group, which is transferred to acyl-carrier protein (ACP). A malonyl group from malonyl-CoA is also transferred to ACP. After that, the acetyl group is transferred to an enzyme Cys residue of B-ketoacyl-ACP synthase. Then, malonyl-ACP is decarboxylated and attacks the acetyl-thioester to form a four-carbon acetoacetyl-ACP through a condensation reaction. Next, after two reductions and a dehydration reaction the B-keto group of acetoacetyl-ACP is converted to an alkyl group, resulting in an elongation of two carbon units. This process is repeated six more times in order to form palmitoyl-ACP. The thioester bond is hydrolyzed by palmitoyl thioesterase in order to yield palmitate, the most common saturated fatty acid.

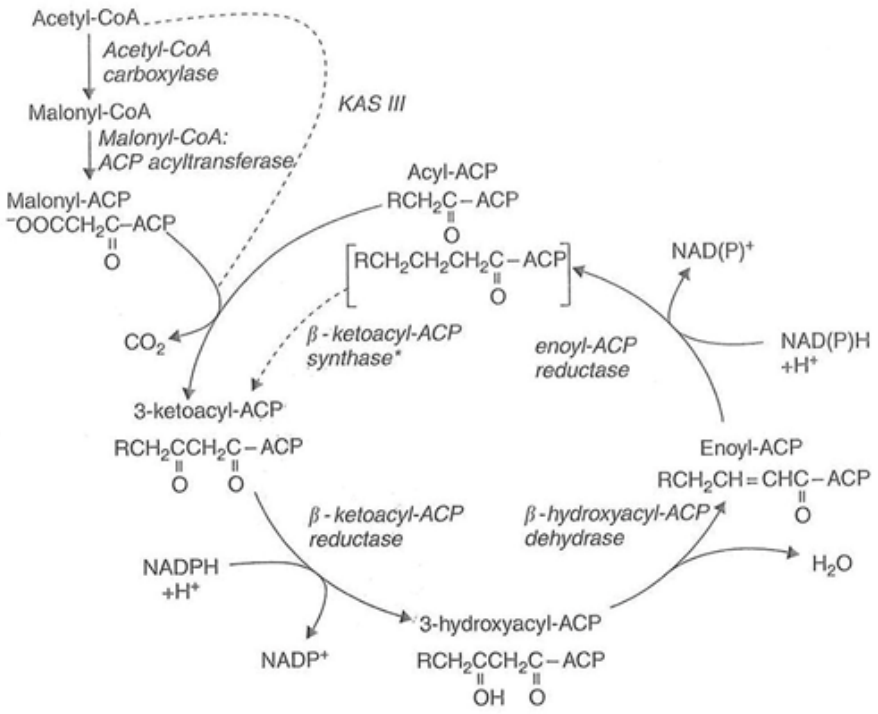

Figure 4. General Schematic of Fatty Acid synthesis[16]

1.2d Triacylglyceride Formation

Glycerol-3-phospate, an intermediate in glucose production, is acylated in order to form lysophosphatidic acid while a fatty acid coenzyme A ester attaches a fatty acid onto carbon 1 of the three-carbon structure.[16] Then, acylglycerophosphate acyltransferase acts in conjunction with fatty acid coenzyme A ester in order to attach another fatty acid at the carbon 2 position of the lysophosphatidic acid in order to form phosphatidic acid. Phosphatidic acid phosphohydrolase removes the phosphate group from the phosphatidic acid to form diacylglycerol. Finally, diacylglycerol acyltransferase and fatty acid coenzyme A ester add the final fatty acid to the three-carbon backbone to form triacylglyceride (Figure 5).

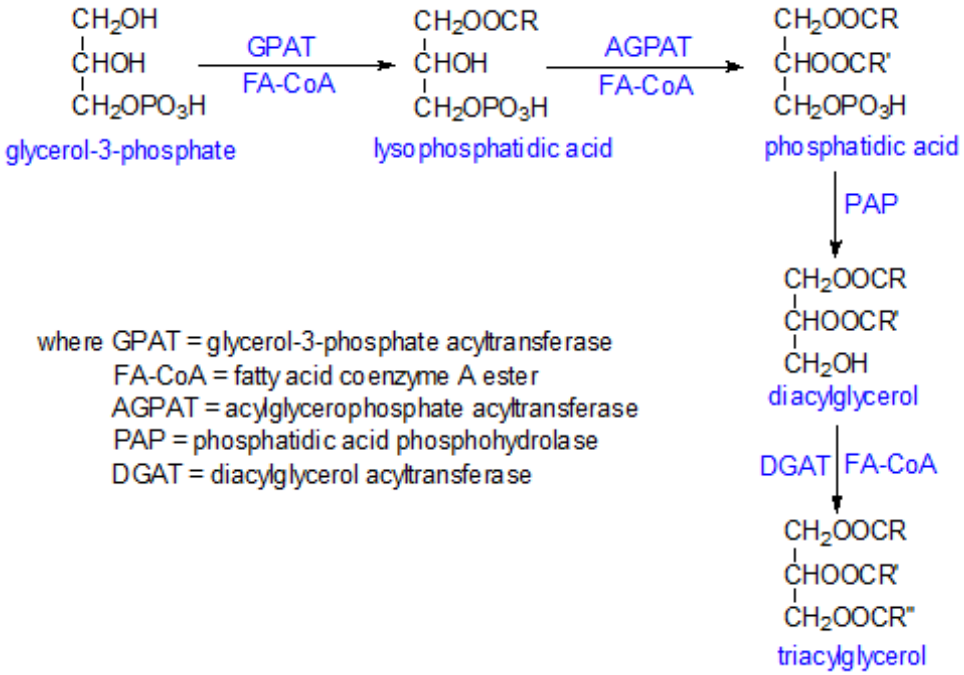

Figure 5. Formation of triacylglyceride from glycerol-3-phosphate and fatty acids[16]

1.3 – Carbon Utilization in Chlamydomonas reinhardtii

1.3a cw15 & sta6 Mutant Strains

In studies such as those conducted by the Goodenough laboratory at Washington University in Saint Louis, two *Chlamydomonas reinhardtii* mutants strains, the *cw15* and *cw15 sta6* (referred to as *sta6*), have mainly been used for comparison and the maximization of lipid production.[20] Both strains lack cell walls, and are therefore easily lysed for lipid purification. In addition, both lack flagella and grow at the same rate as wild-type strains. Despite these similarities, a key difference exists between the mutant strains. The strain *cw15* produces starch. However, *sta6* cannot because the gene that controls starch production has been deleted.

Although the processes of starch production and lipid production produce very energetically different storage molecules, they are sometimes related through a particular

metabolite of glycolysis and gluconeogenesis, glyceraldehyde-3-phosphate. In gluconeogenesis, glyceraldehyde-3-phosphate is an intermediate in the production of glucose. Then, chains of glucose and enzymes produce starch. However, this intermediate is also the precursor for the glycerol backbone in triacylglycerides during glyclolysis. When cells commit to creating lipids, synthesis of ADP-glucose, one of the first precursors for starch, is inhibited. Therefore, since the *sta6* strain lacks ADP-pyrophosphorylase, the enzyme that creates ADP-glucose and is therefore a key enzyme in starch formation, it cannot produce starch and only creates energy storages in the form of triacylglycerides. For this reason, *sta6* often produces approximately twice the amount of lipids as *cw15* after nitrogen-starvation.[20]

1.3b Nitrogen-starvation and increase in triacylglycerides

As with other autotrophs, a limiting factor in algal growth is sunlight. Although some strains of *Chlamydomonas reinhardtii* has been found to be able to grow in the absence of light, the organism cannot grow to a vital abundance without light energy. However, the absence of certain nutrients will create stress on the organism but still allow for relatively normal growth rate. An example of such nutrient is nitrogen. Removing it from the algal metabolic system will signal a stress response pathway, specifically the production of lipids over starch in order to store more energy. Deficiencies in other nutrients such as phosphorus, magnesium, and sulfur will also cause stress upon the organism, but result in an increase in starch, rather than lipid, production.[4]

Nitrogen can be metabolized in cells in several different molecules such as: NO_3^-, NO_2^-, NH_4^+, and N_2. The order of preference in unicellular species is $NH_4^+ > NO_3^- > N_2$, however, the form that nitrogen takes is also temperature and pH dependent.[10] While

nitrogen is most often associated with proteins within the cell, it is also a key component in photosynthetic activity. This may explain a preference for carbohydrate production over lipid production under normal nitrogen-replete conditions. At excessive levels of exogenous nitrogen, photosynthetic activity is inhibited, and cells utilize the nitrogen to create proteins.

1.3c Key Factor in Lipid Production

While it is evident that there is a nitrogen trigger in lipid production, the absence of nitrogen is not the only key factor in production of lipid bodies. It has been shown that lipid production in algae only occurs when carbon supplies exceed the amount required for starch production or in starch less mutants. A series of experiments was conducted in the Goodenough laboratory that tested the variables of nitrogen starvation, exogenous acetate input, and time.[11] When cells of *Chlamydomonas reinhardtii* grow, they undergo an exponential growth stage followed by a logarithmic phase and eventual stationary phase in which growth considerably slows down. In the first set of experiments, cells taken from the mid-logarithmic phase were placed into a nitrogen-less media. The cell count continued to increase after nitrogen-starvation. This effectively proves that the cells continue to proliferate in the absence of nitrogen.

The next experiment demonstrated that cells transferred into the media during the log phase had a much smaller tendency to lyse due to autophagy after developing lipid bodies than cells transferred during the stationary phase. Once the optimal time to transfer cells became evident, the importance of the addition of exogenous acetate was tested. Cells from both the *cw15* strain and *sta6* strain were grown in nitrogen-less media without any addition of acetate. It was shown that while these cells do not require acetate

to grow, they do not produce lipid bodies without the presence of acetate in the media. A boost of 20 mM acetate after growth in acetate-less media produced ample lipid bodies within the cells (Figure 6).[8] Therefore, exogenous input of carbon, and furthermore the exceeding of carbon limits for starch, is critical for lipid production.

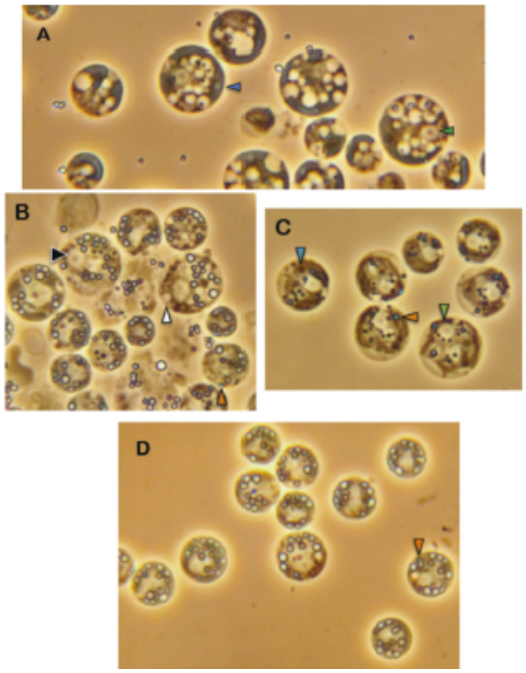

Figure 6. sta6 algae cells filled with lipid bodies after N-starvation following an acetate boost in order to achieve an "obese" state[7]

1.3d – Acetate Incorporation into Chlamydomonas reinhardtii cells

Algal cells use acetate as their carbon source for metabolites such as starch and lipids.[7] However, the carbon derived from acetate is not reserved exclusively for these metabolites. When acetate is first incorporated into these cells, it is broken down and incorporated into a bicarbonate buffering system. This is because this system provides carbon for photosynthesis. Since the algal cells have autotrophic capacities, it is through photosynthesis that storage molecules are created.

Unicellular photosynthetic organisms have the ability to use inorganic carbon from their environment. This inorganic carbon is concentrated into carbon dioxide (CO_2) and used intracellularly in the form of bicarbonate (HCO_3^-). Living systems attempt to maintain equilibrium between CO_2 and HCO_3^- through a buffering system that is presided over by the enzyme carbonic anhydrase.

$$CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+ \quad (1)$$

Carbon is then provided for photosynthesis through a series of reactions. This leads to the eventual production of glucose, which is later incorporated, into starch.

$$2HCO_3^- \Leftrightarrow CO_3^{2-} + H_2O + CO_2 \quad (2)$$

$$HCO_3^- \Leftrightarrow CO_2 + OH^- \quad (3)$$

$$CO_3^{2-} + H_2O \Leftrightarrow CO_3^- + 2OH \quad (4)$$

The formation of the intermediate carbonate (CO_3^-) creates a more acidic environment, however, the subsequent formation of hydroxide (OH^-) raises the pH, effectively stabilizing the pH. At higher pH levels, biological systems tend to favor carbonate (CO_3^-) over HCO_3^-. This happens when the pH exceeds approximately 10.5 although in most organisms, biomass productivity tends to decrease under high pH values.

1.4 Goal

The first goal that must be reached before proving the carbon pathways in the algae cells is to grow healthy, viable cells. Because the cells grow in a logarithmic fashion, they must undergo an exponential state and a subsequent stable phase before being utilized for experimentation. Measuring the optical density of the cells monitors this. Then, the metabolism of carbon, specifically the usage of acetate by the cells, will be

observed using nuclear magnetic resonance spectroscopy. This will elucidate the pathway that carbon from exogenous acetate takes within the cell during nitrogen starvation in order to form lipids. The hypothesis is that acetate is directly incorporated into storage macromolecules through typical lipid and starch synthesis pathways and their intermediates. If this is true, then the acetate will be almost entirely incorporated into starch and lipid in *cw15* and only lipids in s*ta6*.

CHAPTER TWO

Materials and Methods

2.1. Cultivation of Algae

2.1a. Sueoka's High Salt Medium

In order to ensure optimal growth of *Chlamydomonas reinhardtii*, Sueoka's high salt medium (HSM) was used.[12] These media consists of three parts: a salts solution, a phosphate solution, and Hutner's trace elements. Stock solutions of all three were prepared prior to growth media preparation. One liter of the salts solution contains a combination 100 g of NH_4Cl, 4.0 g of $MgSO_4 \bullet 7H_2O$, and 2.0 g of $CaCl_2 \bullet 2H_2O$ brought up to one liter with deionized water. One liter of the phosphate solution contains 288.0 g K_2HPO_4 and 144.0 g of KH_2PO_4 brought to one liter with deionized water.

Hutner's trace elements contains the following compounds with their respective solid amounts in specific mL of water:

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

The final solution is prepared by first mixing all solutions except EDTA. Then, the solution is brought to a boil and EDTA is added. 85 mL of hot 20% KOH solution is then added. Once the solution is brought to a final volume of 1 liter, the flask must be stoppered and allowed to stand for one to two weeks. The solution should begin as a clear green, but eventually turn purple with a brown precipitate. The precipitate should be filtered out using Whatman#1 filter paper until the solution is clear.

One liter of the final grown media should contain 5 mL of the salts solution, 5 mL of the phosphate solution, and 1.0 mL of the trace element solution. As an exogenous source of acetate, 1.64 g/liter of sodium acetate is added. This particular experiment used 100 mL of growth media and therefore 500 μL of the salts solution, 500 μL of the phosphate solution, 100 μL of the trace elements, and 0.164 g of sodium acetate. Since the sample was used in nuclear magnetic resonance analysis, the sodium acetate used was tagged with ^{13}C at the 1 position for initial growth.

2.1b Growth

Using cw15 and sta6 strains from the Goodenough laboratory at the University of Washington in St. Louis, streak plates and slanted agar tubes were made using Sueoka's HSM media and agar (Figure 7). Samples from these plates and tubes after growth were used in order to inoculate 100 mL of HSM Media. The algae were grown in this media for five days before being used to inoculate two 100 mL bottles of fresh media. Two bottles, one containing cw15 and another containing sta6 were grown for five days. All plate and media cultures were grown underneath fluorescent lighting in constant room temperature. Media cultures were constantly shaken on shaker (Figure 8).

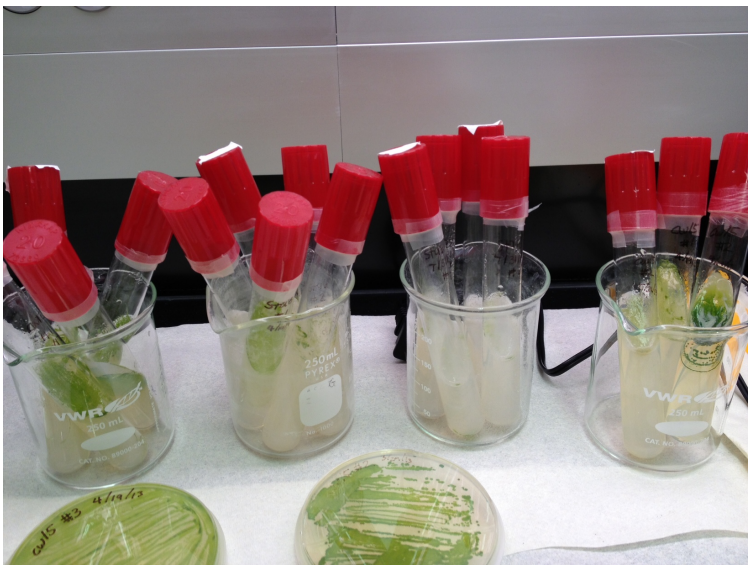

Figure 7. Algae Cultivation on Slanted Agar and Agar Plates

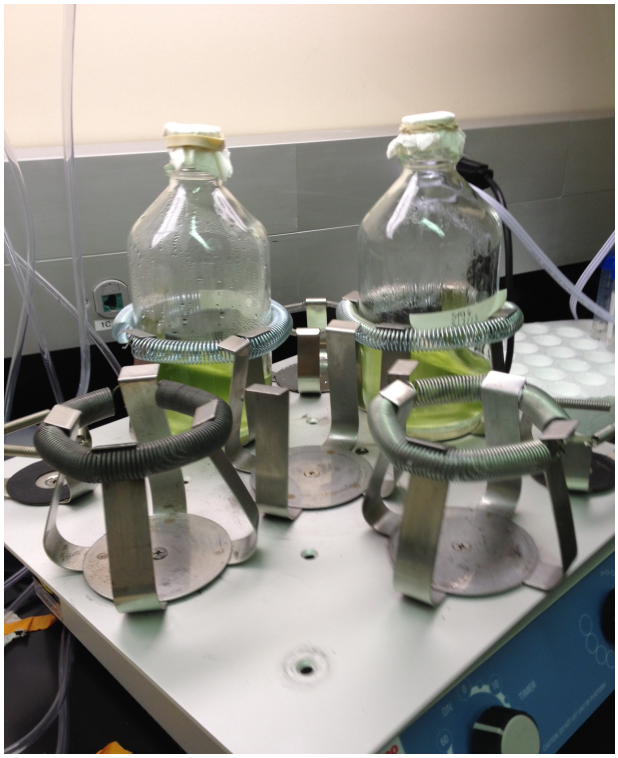

Figure 8. *cw15* and *sta6* growing under constant lighting, temperature, and shaking

2.1c Monitoring of Growth

In order to monitor the growth of the algae, 1 mL samples of both media cultures were taken and the optical density was measured in 24-hour increments using a UV-Vis spectrophotometer set at 660 nm. A sample of HSM media was used as a blank. A total of four mL of each strain were taken each day and frozen in 1 mL Eppendorf tubes in a -80°C freezer. These frozen samples were used for solution-state nuclear magnetic resonance measurements.

2.1d Media Exchange

Since *C. reinhardtii* produces lipids under nitrogen-starvation stress, a nitrogen-less environment was achieved by exchanging the media. The nitrogen-absent media was identical to the HSM media that was previously used except for the fact that NH_4Cl was excluded from the salts solution. This exchange was accomplished by centrifuging the media culture using a Beckman centrifuge at 7.5k g for 10 minutes. The cultures were distributed into Falcon tubes and balanced prior to centrifugation. A pellet of algae at the bottom of each tube resulted from centrifugation. The supernatant was discarded from each tube, and the pellet was re-suspended in the nitrogen-absent media. The algae were then allowed to grow for 5 days under the same conditions as previously mentioned.

2.2 Preparation for Solution NMR

The samples used for NMR measurements were not fresh samples, but rather samples that had been frozen in -80°C for at least a day. However, measurements from every day of the experiment were taken. In order to have a more concentrated sample, the four eppendorf tubes that were taken each given day were centrifuged in a small

centrifuge then combined. Since each tube contained 1 mL of sample, 750 μL of the supernatant was discarded from each tube. The cells were re-suspended in the remaining media in the tubes. As a result, upon combining the samples, there was then just one 1 mL, though very concentrated, sample for each day. Using the newly concentrated samples, one mL of sample was micropipetted into an NMR tube. 200 mL of D_2O were added for signal locking and shimming purposes. This was done for both the *cw15* and *sta6* strains resulting in the preparation of one tube of each sample each day.

2.3 Measurement with Nuclear Magnetic Resonance Spectroscopy

In order to analyze the carbon flux, and specifically the incorporation of acetate into *Chlamydomonas reinhardtii*'s metabolic system, 1-^{13}C labeled acetate was used in solution-state NMR. There were two sets of experiments that were carried out. In the first experiment, the nitrogen-replete media contained acetate while the nitrogen-starved media contained unlabeled acetate. The reverse is true for the second set of experiments. This was done using a Bruker 360 MHz as well as a Bruker 500 MHz NMR apparatus. Analysis using both magnetic strengths was done in order to validate results and to obtain higher resolution peaks. Spectra were obtained for both strains for every day of the experiment.

Prior to running the experiment, the Bruker 360 was shimmed in order to ensure homogeneity of the internal magnetic field. Using the following parameters, the time required for each sample was 10 hours and 22 minutes. For each sample 10,000 scans were completed through a three-time accumulation loop. The relaxation delay, or the time between each pulse, was 0.8 seconds while the acquisition time was 0.38 seconds. In addition the degree pulse was 60°.

2.4 Data Processing

MestRenova was the program used to analyze and edit the NMR spectra. The baseline was corrected through phase correction. This eliminated any negative peaks caused by invisible or imaginary values resulting from chemical shifts. The spectra were stacked according to specific strain and whether the data was obtained prior to or after N-starvation. Magnified portions of spectra were also created with MestRenova. The program Prism was used in order to create all other figures.

CHAPTER THREE

Results

3.1. Growth

3.1a. Growth Pattern

Both the sta6 and cw15 samples were grown for five days in Sueoka's high salt medium containing nitrogen in the form of NH_4^+Cl. Growth in both strains resulted in a logarithmic pattern in which an exponential phase was followed by a stationary phase (Figure 9). This is the expected growth pattern for *Chlamydomonas reinhardtii*. The *cw15* cells proliferated to higher concentrations than sta6 cells as evidenced by higher absorbance values. It is assumed that an increase in optical density corresponds to an increase in cellular concentration.

After media exchange into nitrogen-less mediums, the concentration of algal cells continued to increase in both samples (Figure 10). Both samples were grown and observed in this media for four days. The *cw15* strain continued to proliferate more than the sta6 strain. Despite a rather significant increase in absorbance in both samples, the growth following media exchange appears to be continuous with the stationary phase of growth. The relative concentration of *cw15* cells continuously increased throughout the entire experiment while the concentration of *sta6* cells decreased starting at 96 hours and again at 72 hours post-starvation.

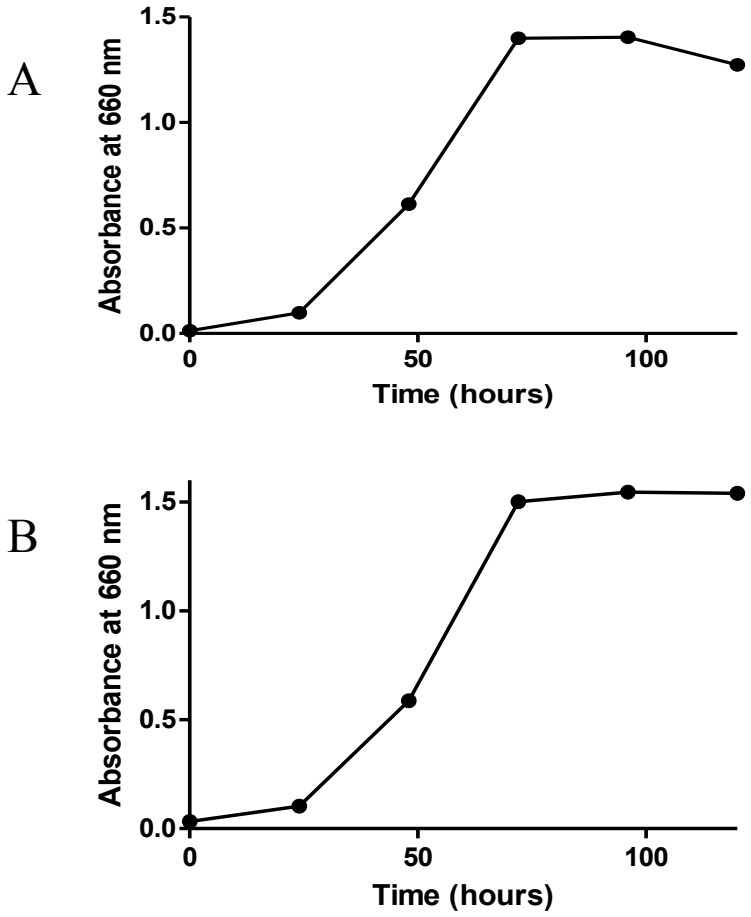

Figure 9. Growth of *sta6*(A) and *cw15*(B) Before N-Starvation. Measurements were taken at 24-hour increments. The total time period prior to N-Starvation was 120 hours.

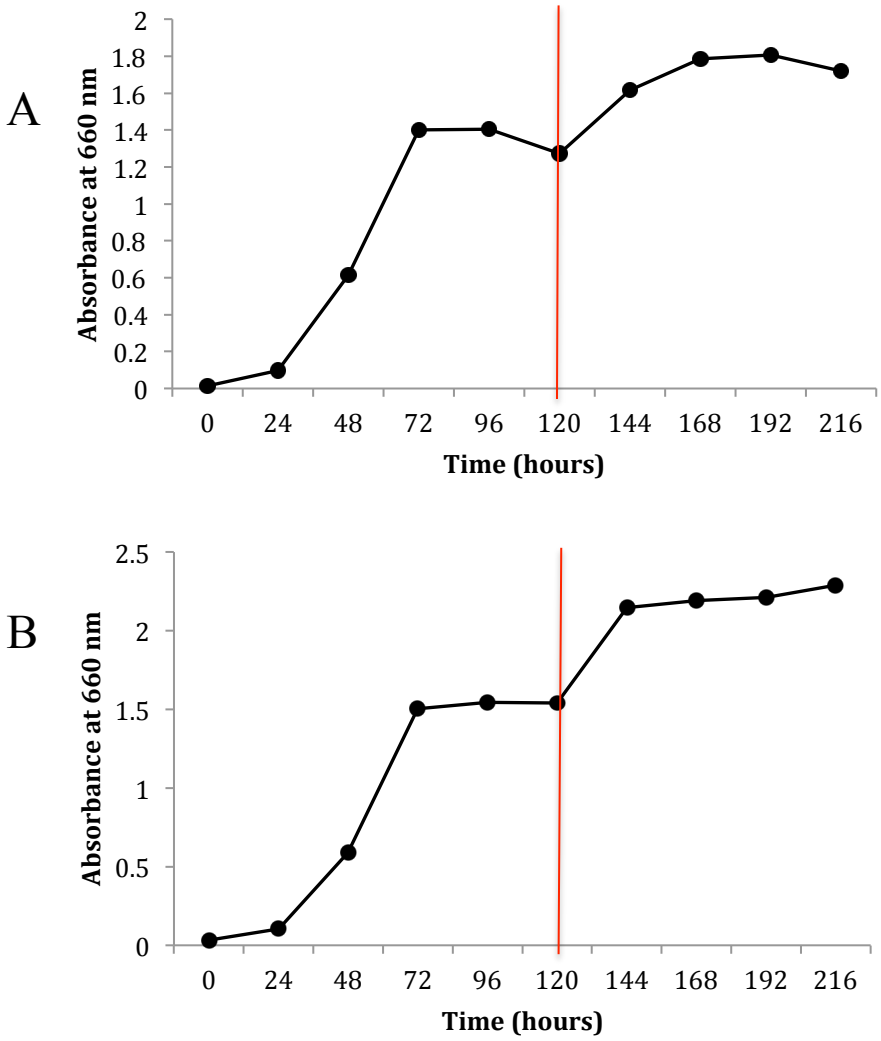

Figure 10. Growth of *sta6*(A) and *cw15*(B). The vertical line indicates media exchange to N-starvation conditions. Media exchange occurred 120 hours, or 5 days, after inoculation. Measurements were taken at 660 nm because this is within the optimal range for chlorophyll absorption. It is assumed that growth is indicated by an increase in chlorophyll absorption

3.2. NMR Data

3.2a. Chemical Shifts

Consistent with the hypothesis that exogenous acetate is the main carbon source under nitrogen starvation for lipid production in *sta6* and *cw15*, the 1-^{13}C acetate was shown to be incorporated into various molecules within the cell (Figure 11, 12). The

acetate peak appears in the spectra for both strains around 182 δ (182.29 δ in *sta6* and 182.23 δ in *cw15*). This is within the chemical shift range for ester carbonyls. As the acetate peak begins decreasing in intensity, a peak at 161 δ (161.21 δ in *sta6* and 161.77 δ in *cw15*) begins to appear in both spectra. This chemical shift is representative of the bicarbonate buffering system, and therefore is indicative of bicarbonate or carbonic acid. Lipid peaks appear in both spectra from 21-30 δ (21.19 δ, 23.47 δ, 25.59 δ, 26.33 δ, 27.96 δ, 29.79 δ, 30.19 δ, 30.60 δ in sta6 and 23.50 δ, 25.62 δ, 27.74 δ, 27.98 δ, 30.24 δ, 30.63 δ in *cw15*). These are chemical shifts for the alkyl carbons in the lipid tails (Figure 11, 12).

While it was expected for the acetate to be incorporated greatly into starch molecules in *cw15*, only a small portion of the labeled acetate appeared in the peaks that correspond with the secondary hydroxyl groups found in starch (62.62 δ). Interestingly, there is a visible peak in this range for sta6 as well despite the fact that this mutant lacks a necessary enzyme required for starch production (62.62 δ). Other visible peaks that appear on both spectra are peaks that correspond with carbons in double bonds (127.88 δ, 128.80 δ, 130.33 δ in sta6 and 127.81 δ, 129.64 δ, 130.42 δ in cw15). These double bonds are most likely those that are found on the tails of triacylglycerides. A peak at 172 δ (172.59 δ in sta6 and 172.53 δ in cw15) that appears in the nitrogen-starved spectra is also indicative of protein production, and is most likely from α-carbons on peptide chains (Figure 11, 12).

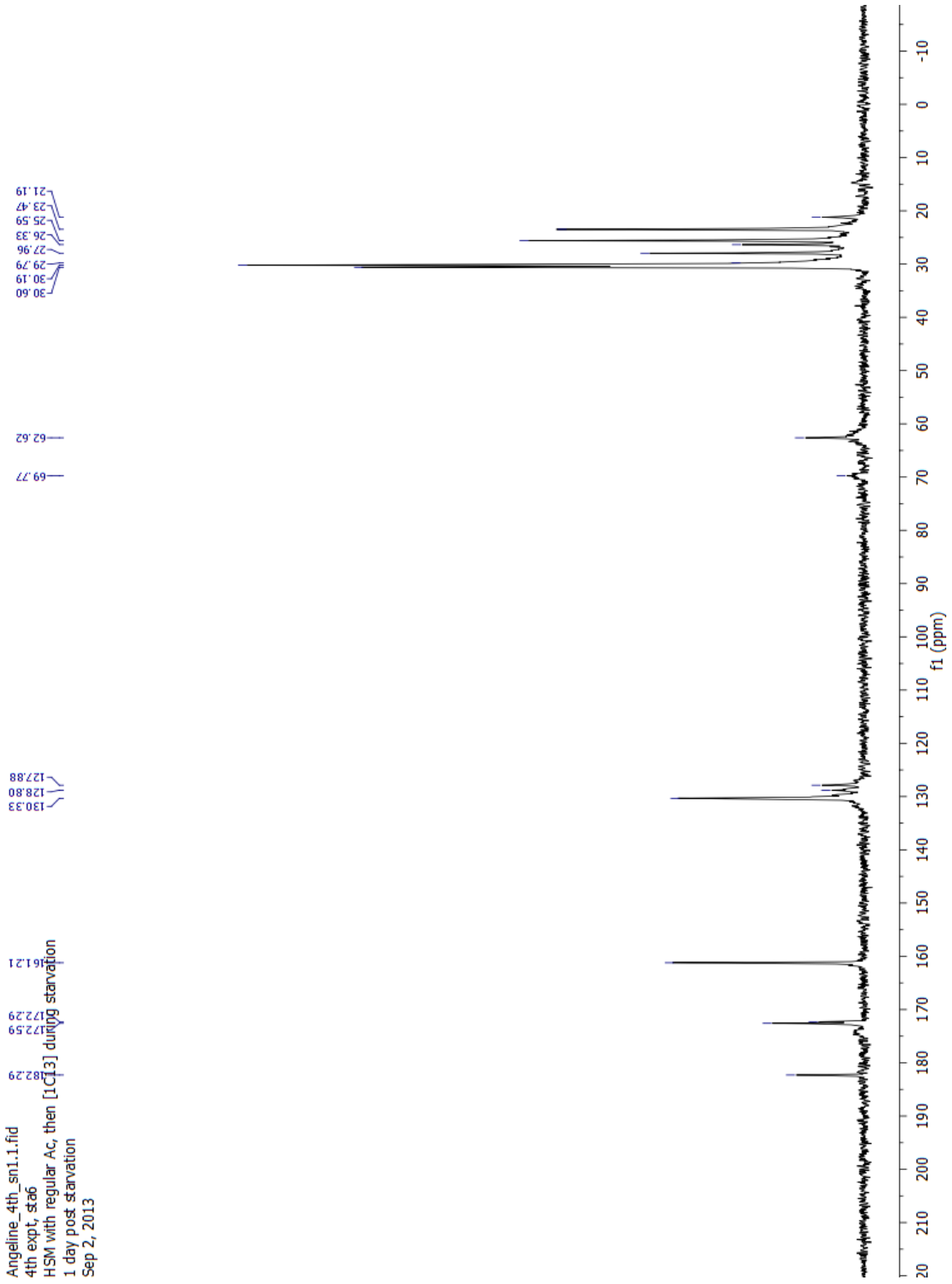

Figure 11. ^{13}-C NMR spectrum of Nitrogen-starved *sta6*

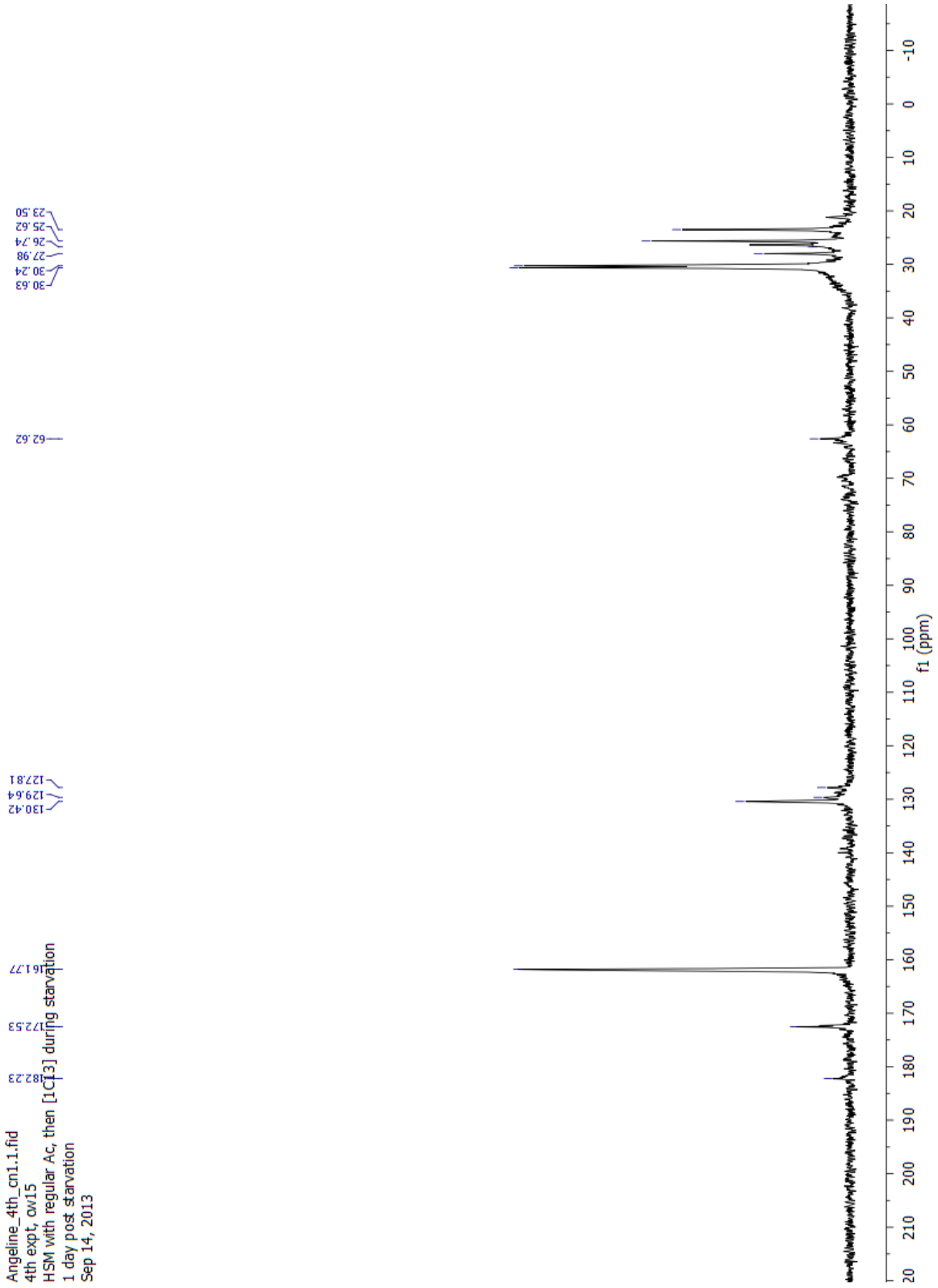

Figure 12. ^{13}-C NMR spectrum of Nitrogen-starved *cw15*

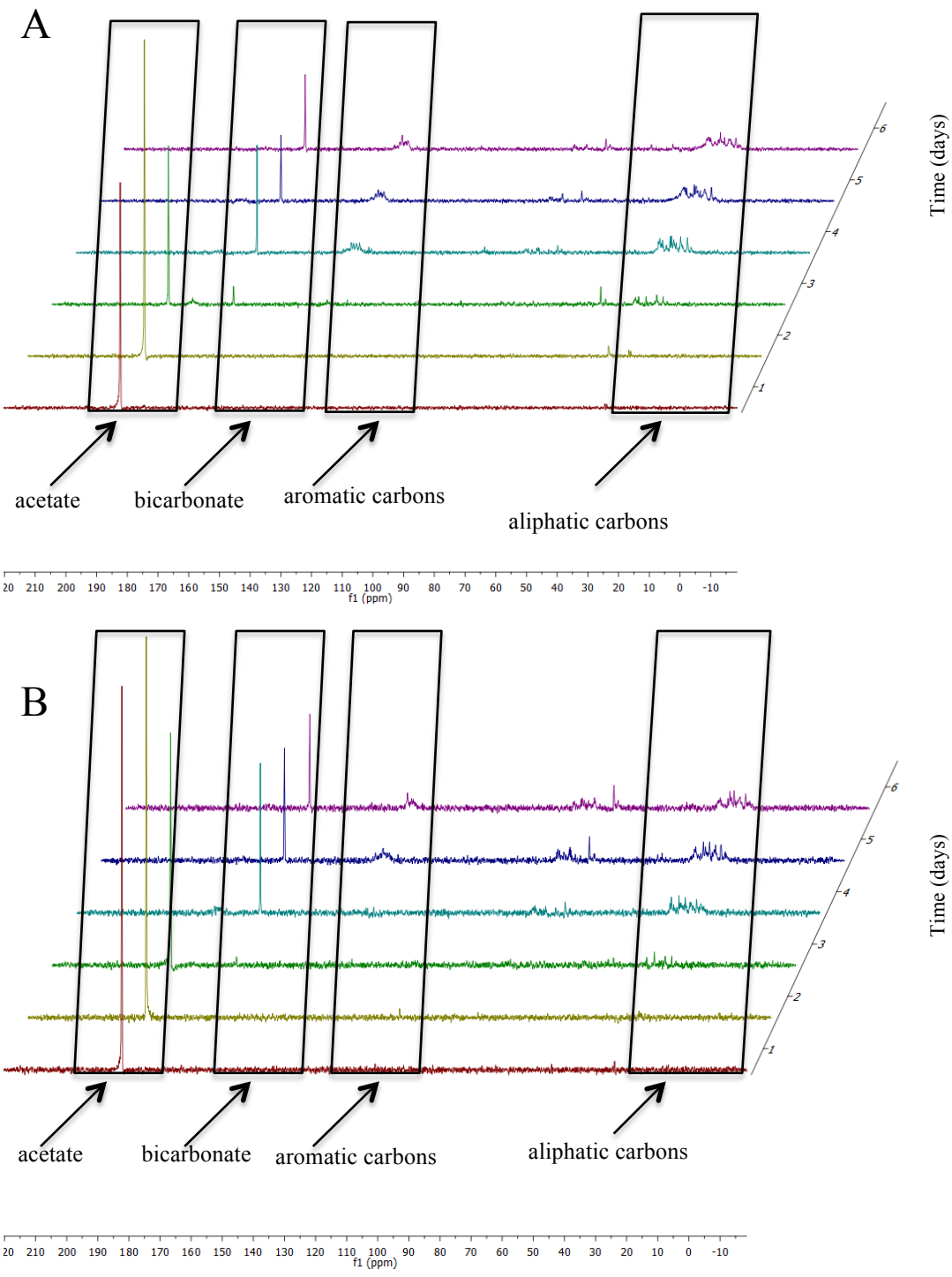

Figure 13. *sta6* (A) and *cw15* (B) ^{13}C-NMR spectrum prior to N-starvation. The spectra were taken in 24-hours increments. The first spectrum was taken 0 hours after inoculation and only shows a peak for acetate. Peaks that appear in the spectra on subsequent days show where the acetate was either incorporated in the cell or what it was converted to.

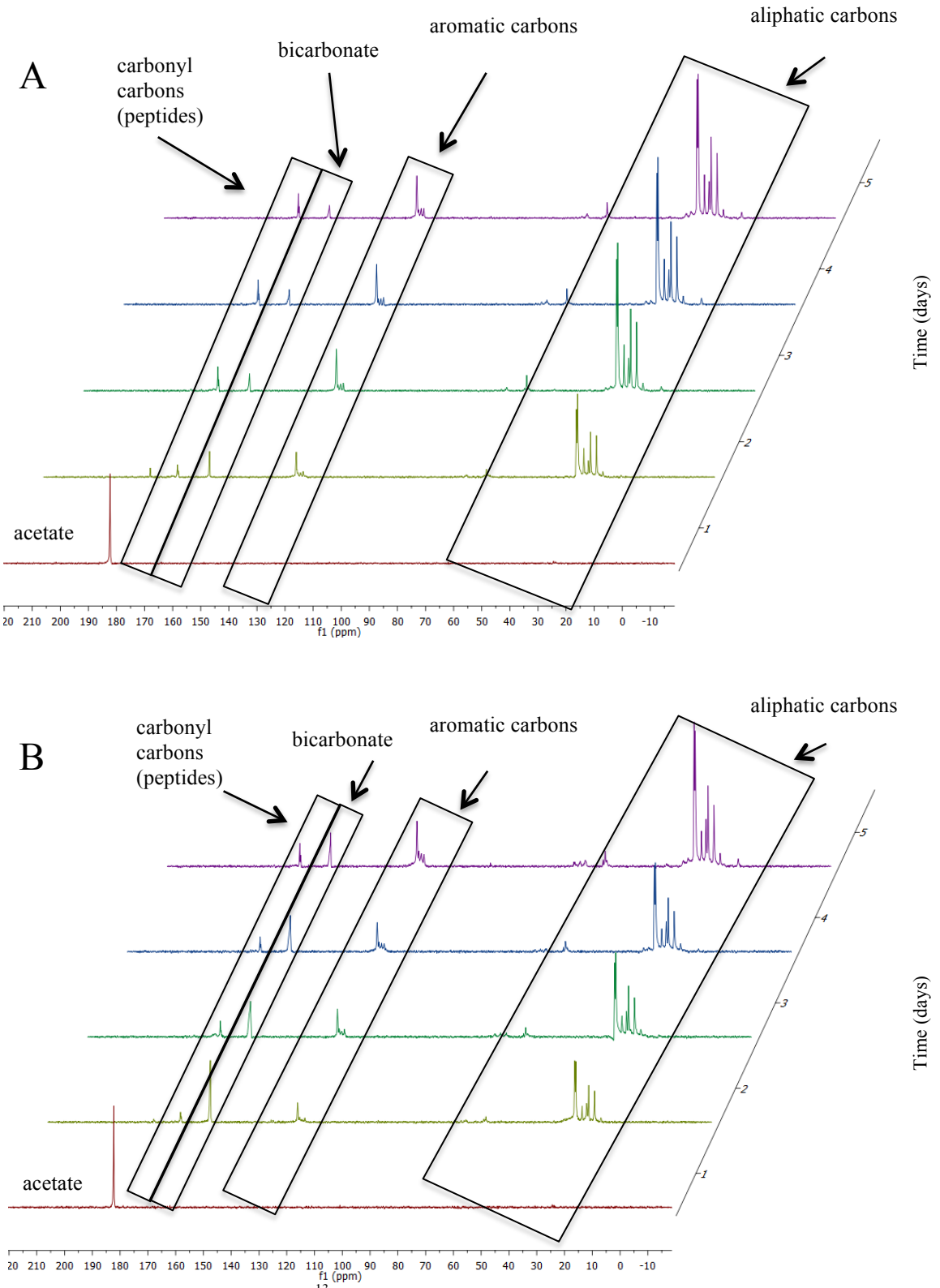

Figure 14. *sta6* (A) and *cw15* (B) ^{13}C-NMR Spectrum in which the acetate was not labeled until after N-Starvation. The spectra were taken in 24-hour increments after media exchange. The first spectrum is a reference peak that only shows an acetate peak. Initially, the only peak that appeared was the peak for acetate. The subsequent spectra show where in the cell the acetate was incorporated after N-starvation.

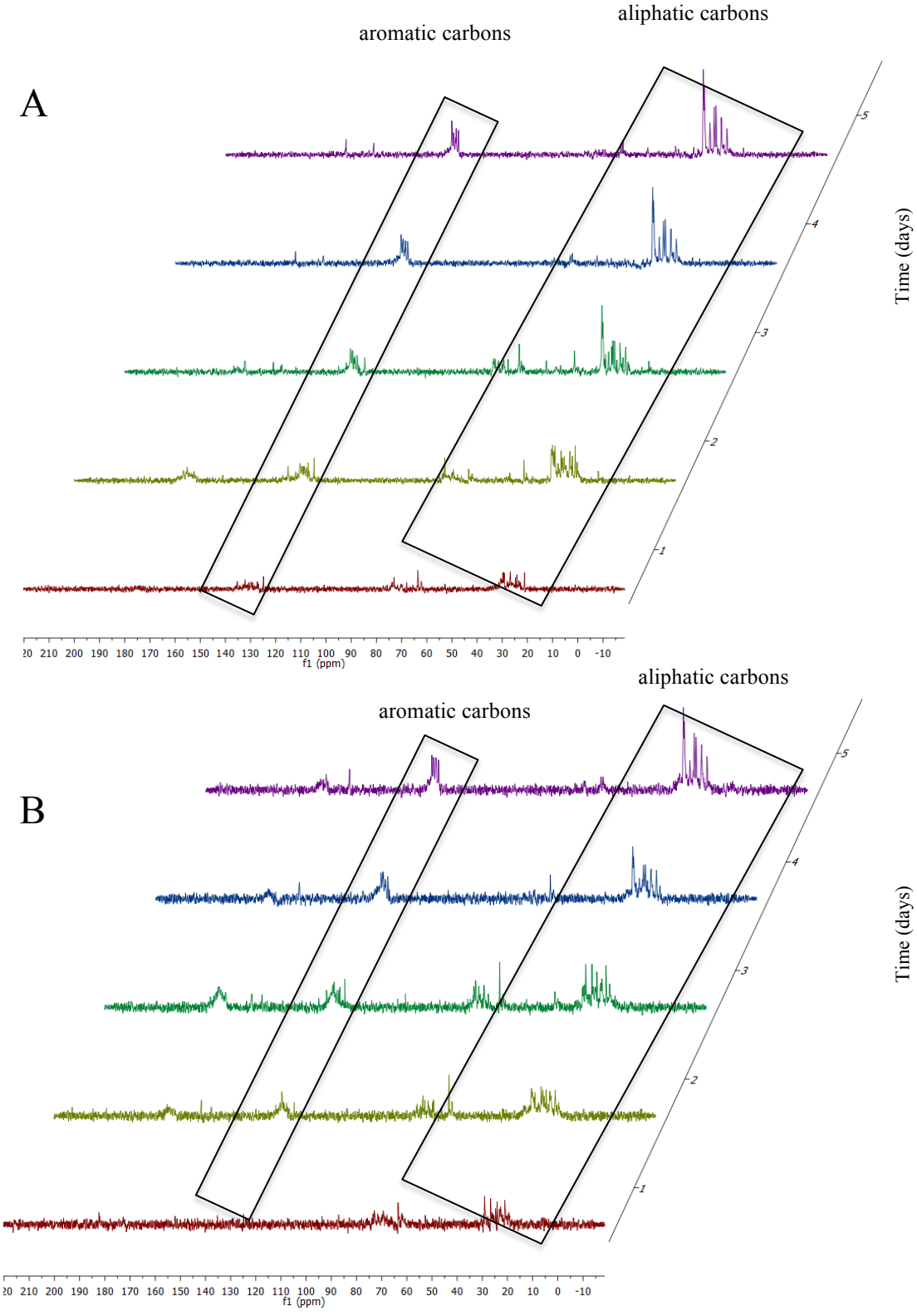

Figure 15. These spectra are the results of labeling the acetate during growth but not during N-starvation. These spectra were taken after N-starvation. Both *sta6* (A) and *cw15* (B) show peaks that are much less defined and conclusive in comparison to those in Figure 14.

3.2b. Analysis of Acetate Consumption Compared to Bicarbonate Appearance

In both spectra for cw15 and sta6, there is a noticeable incorporation of carbon from acetate into bicarbonate prior to starvation (Figure 13). These bicarbonate peaks are the first to appear in both spectra. For both *sta6* and *cw15*, acetate levels truly begin falling 48 hours after the initial algal introduction into nitrogen-replete media. Bicarbonate also begins to appear at 48 hours (Figure 13). The bicarbonate peaks begin decreasing after 72 hours in both strains.

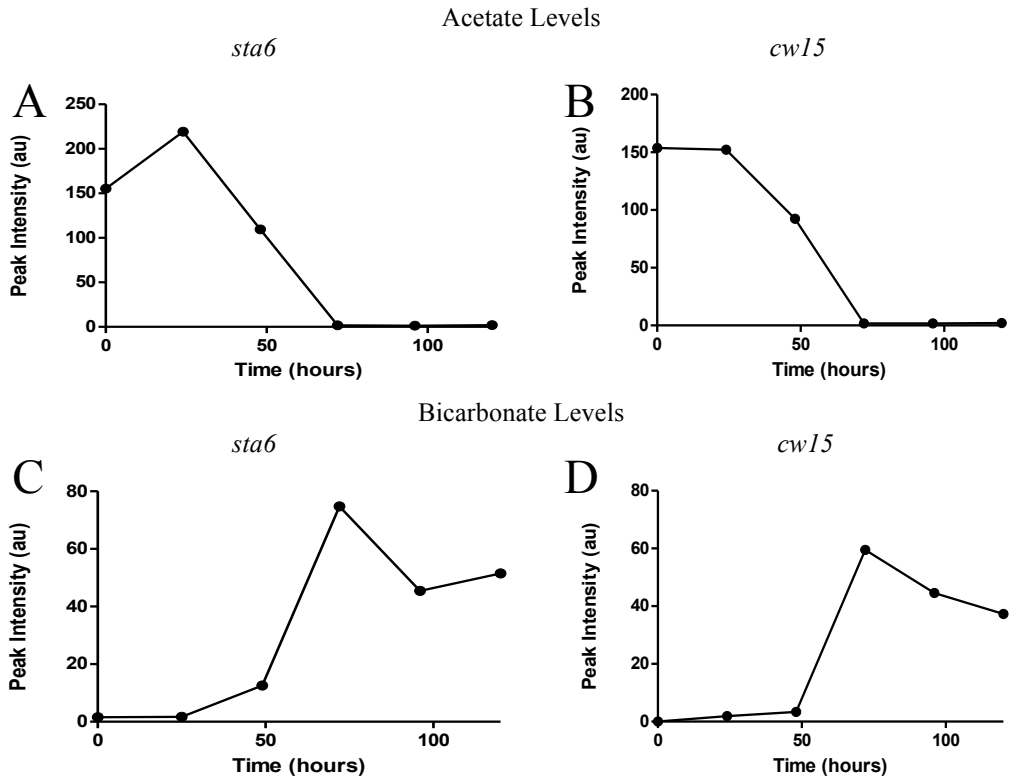

Figure 16. Disappearance of Acetate in *sta6*(A) and *cw15*(B) and the appearance of bicarbonate in *sta6*(C) and *cw15*(D) over time prior to N-Starvation. The values for peak intensity are relative values obtained from both the sta6 and cw15 NMR spectra. Quantitative values for peak intensity were determined using MestRenova and are in the unit of atomic units. These values are not molar values.

While there appears to be an increase in acetate at 24 hours after growth, most noticeably in sta6, this is most likely due to a lack in proper normalizing of the spectra.

The acetate level certainly continues to decrease after this apparent increase to almost undetectable levels (Figure 13). After the algae cells were transferred to nitrogen-less media, the acetate peak is barely visible, while the bicarbonate persists in a very small amount in comparison to other peaks (Figure 14). In addition, the bicarbonate level continues to decrease in subsequent days after nitrogen-starvation. The first line in Figure 14 shows a reference peak for acetate. In the post-starvation spectra in which acetate was only labeled during growth and not after nitrogen-starvation, it is evident that there is activity around the chemical shift of acetate and bicarbonate, but the peaks are hidden in the large amount of noise (Figure 15).

The decrease in acetate along with the simultaneous increase in bicarbonate prior to nitrogen-starvation is quantified in Figure 16. Although there is a similar pattern for acetate consumption and bicarbonate appearance in both strains, it is noticeable that more bicarbonate is produced after nitrogen-starvation in *cw15* in comparison to *sta6* (Figure 17). This is well defined when the acetate was labeled after nitrogen-starvation (Figure 18).

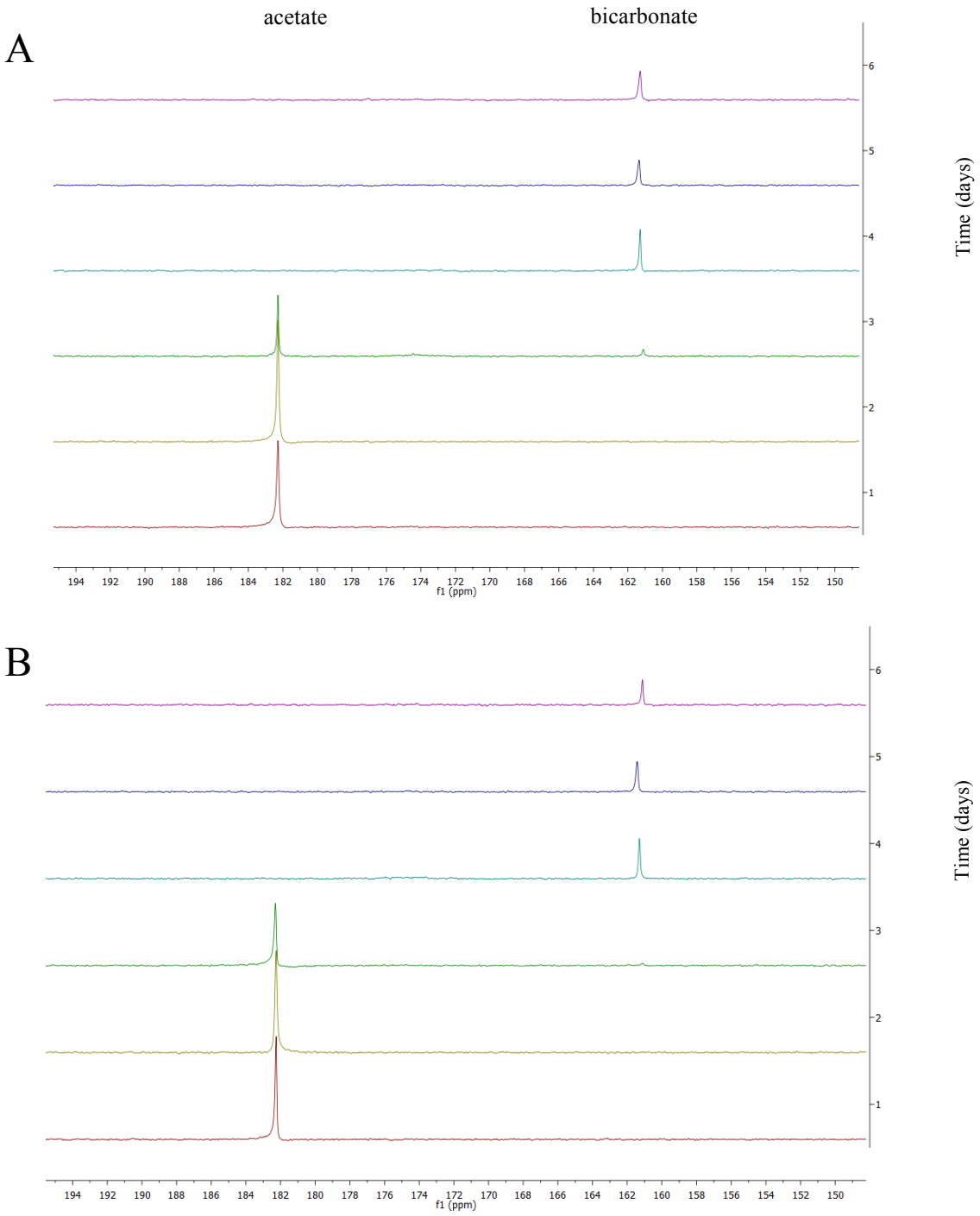

Figure 17. Acetate and Bicarbonate Peaks Over Time in days in *sta6*(A) and *cw15*(B) prior to N-Starvation. The first spectra for both strains was taken 0 hours after inoculation. Subsequent spectra were obtained in 24- hour increments. The acetate peak (182 δ) is almost completely gone after 72 hours, while the bicarbonate peak (161 δ) is clearly visible at 72 hours, indicating a relationship between acetate disappearance and bicarbonate appearance.

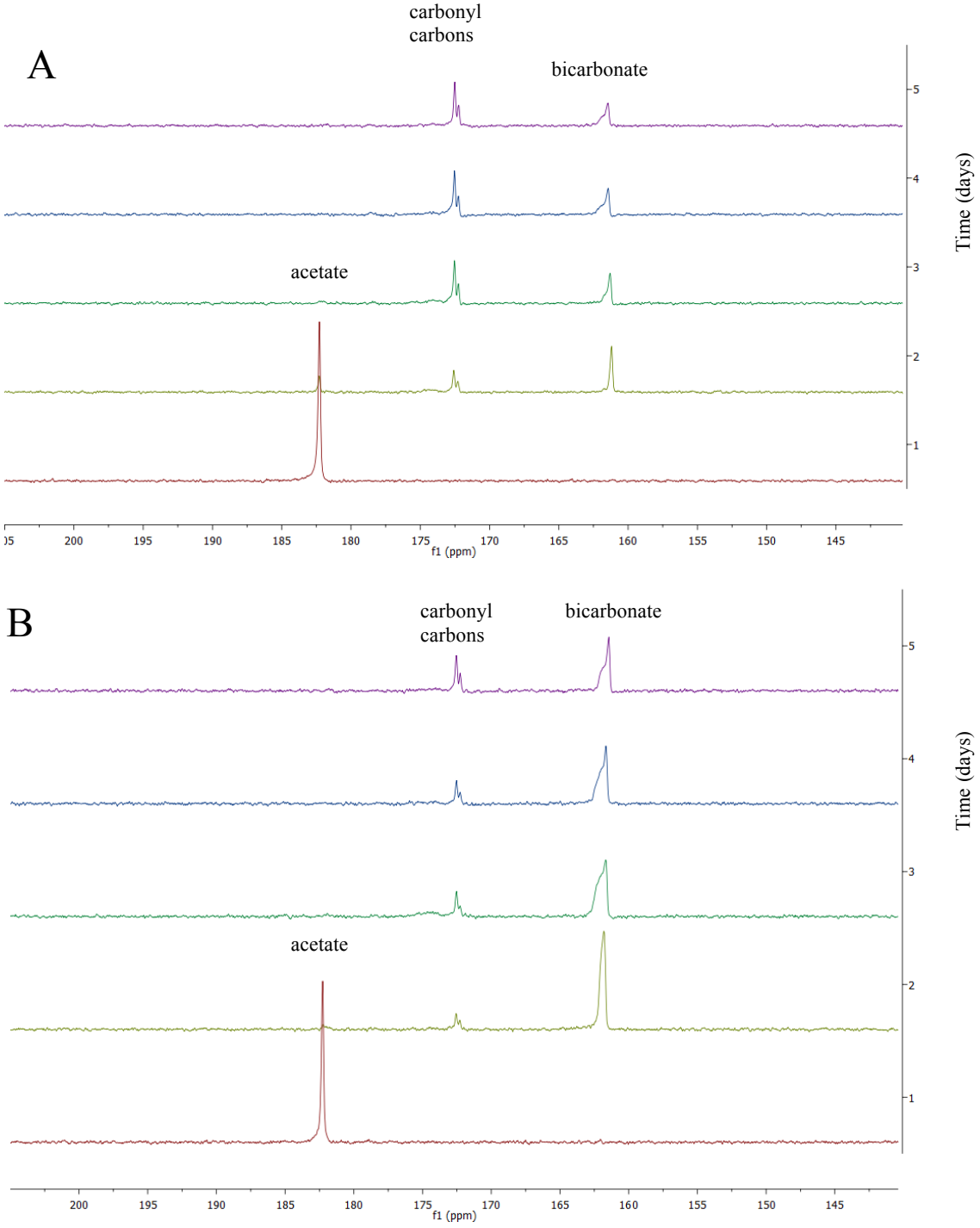

Figure 18. Acetate and Bicarbonate Peaks Over Time in days in *sta6*(A) and *cw15*(B) after N-Starvation from 0 hours after starvation to 4 days post-starvation. The first spectra for both strains are references that show the acetate peak after inoculation. This peak is not visible after N-Starvation indicating that the cell used all of the acetate.

3.2c. Incorporation of Labeled Acetate into Lipids

In the five days prior to N-Starvation in both strains, very little of the labeled acetate was incorporated into lipids. After N-starvation, the peaks that correspond to the alkyl carbons of the lipids appear very intensely in both strains. These peaks appear more intensely than any other peaks on the spectra. In addition, the peaks increase in intensity over time (Figure 19). While the quantity of the lipids certainly increased daily in both strains, it seems that the lipid concentration increased at a greater rate in *sta6*. This is apparent at 48 hours and 72 hours after N-starvation.

Since *cw15* has the necessary enzymes to create starch, it was expected that a large amount of the acetate would be incorporated into starch compared to lipids. However, the *cw15* strain appears to create the same amount of lipids as *sta6*, while the peak that corresponds to starch is very small. The *sta6* spectrum also shows a small, but presumably starch peak despite lacking starch-making enzymes. These peaks in both *cw15* and *sta6* also appear to increase over time, though with far less intensity than the lipid peaks. Despite the amount of noise in the spectra that did not use labeled acetate after starvation, it is still evident that the acetate was incorporated into lipids in an increasing amount per day (Figure 19).

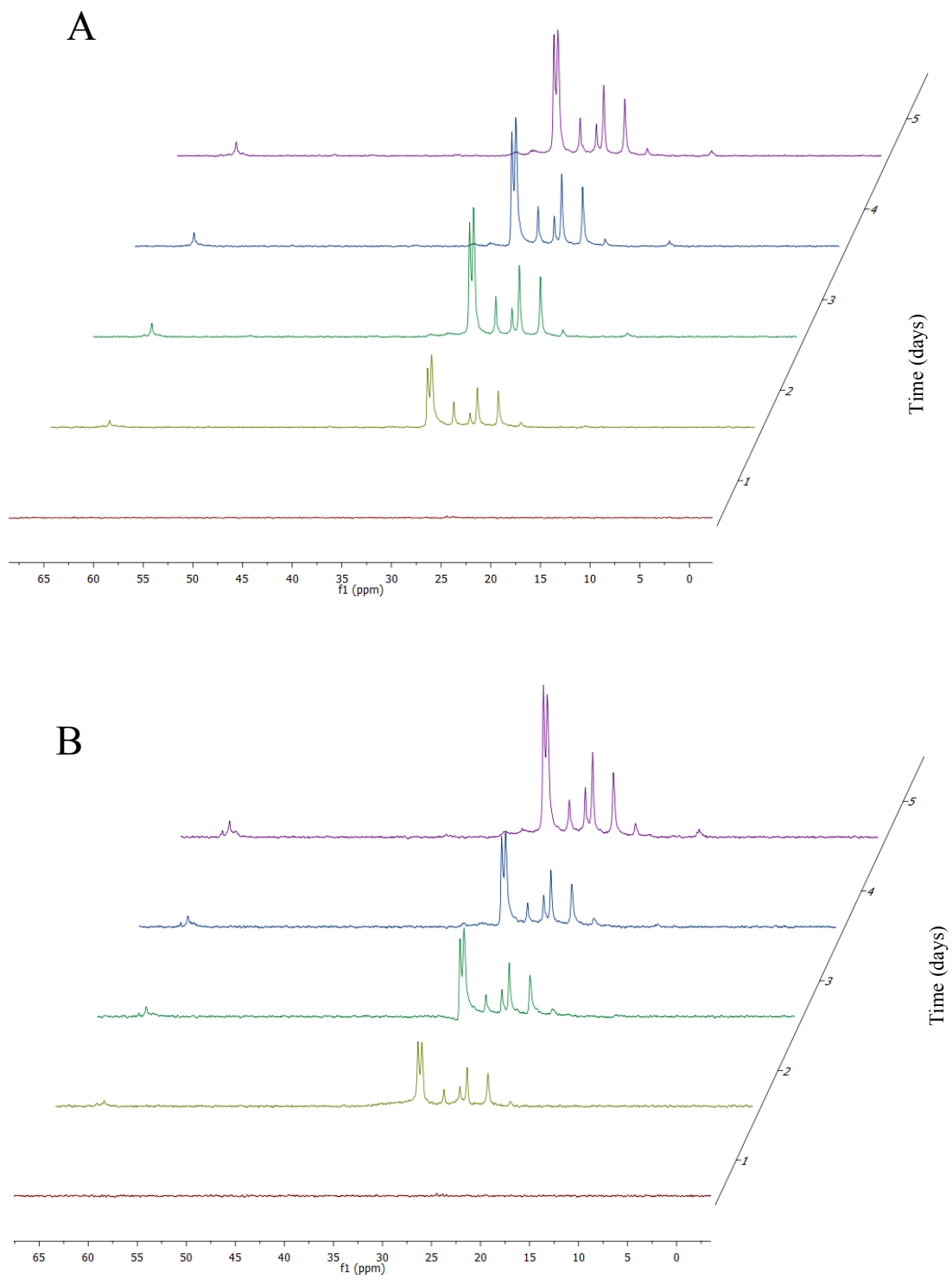

Figure 19. Lipid Peaks in *sta6*(A) and *cw15*(B) beginning at 0 hours after N-Starvation to 4 days after N-Starvation. While peaks that correspond to lipids were evident prior to N-starvation (Figure 10), these peaks greatly increased in intensity over time after starvation.

CHAPTER FOUR

Discussion and Conclusion

4.1. Growth of Chlamydomonas reinhardtii with Exogenous Acetate

Assuming that an increase in optical density is indicative of an increase in concentration of cells, both *C. reinhardtii* strains increased in concentration over time. Both exhibited an overall logarithmic pattern and continued growing after media exchange, though at a rate that was much slower than prior to starvation. An exception in this pattern is shown in the *sta6* strain. The optical density of the strain decreases after 96 hours after inoculation. While this could be attributed to the fact that a different sample of algae/media was measured every day per strain, the optical density also decreases 72 hours after starvation. The fact that this pattern was not observed in the *cw15* strain suggests that mutations in the sta6 strain genome may inhibit growth along with preventing starch formation.

As the optical density of the samples increased, the consumption of acetate from the media increased as well. It is assumed that an increased optical density corresponds to an increase in cellular concentration. It appears that the rate of consumption also increased over time, though this does not necessarily correspond to the rate of bicarbonate appearance. This is due to the fact that bicarbonate also exists in the environment in the form of CO_2 through the actions of carbonic anhydrase.[10]

4.2 NMR Results

4.2a. Acetate and Bicarbonate Peaks

In general, the acetate peaks prior to starvation continually decrease prior to N-starvation. However, the acetate level appears to increase in *sta6* 24 hours after inoculation. Since no additional exogenous acetate was added, it can only be assumed that the labeled 1-^{13}C either dissociated from the rest of the acetate then became bound to another molecule that produces the same chemical shift as acetate, or that proper normalization in the NMR machinery was not achieved. This apparent increase could also be caused by the fact that while the algae samples used each day for NMR results came from the same source, they were not the same exact sample. Therefore, there is a possibility that there was a higher concentration of algal cells in the 24-hour sample despite the fact that the bottles containing the samples were shaken constantly.

The first peak to appear in the NMR spectra once the acetate peak began to decrease was one that corresponded with bicarbonate. While the acetate level appears to decrease at a relatively constant rate, the bicarbonate level seems to be more sporadic. The bicarbonate level in both strains increase dramatically after 48 hours of growth and began decreasing soon after. The level increased in sta6 after 96 hours. This fluctuation can be attributed to the bicarbonate buffering system that is present in algae cells. This entails an equilibrium conversion between bicarbonate and gaseous CO_2 and therefore accounts for the fluctuating bicarbonate level. This is consistent with the belief that the bicarbonate system serves as an important carbon source for photosynthesis and the building of nutrients.[10]

However, this also disproves the initial hypothesis. Since acetate was expected to incorporate its carbons into starch and lipid biosynthesis intermediates, there should only be peaks that indicate starch, lipids, and their intermediates in the spectra. The fact that bicarbonate appeared before any other products, while decreasing in quantity after 48 hours post-starvation, suggests that acetate is not directly incorporated into starch and lipid but rather depends on bicarbonate. This is most likely due to the fact that both starch and lipids require carbon dioxide during their synthesis processes, as seen in the cases of pyruvate carboxylase in starch and acetyl-CoA carboxylase in lipids. In addition, since *cw15* produces more bicarbonate after N-starvation than *sta6*, this suggests that *sta6* incorporates acetate into lipids more readily than *cw15*.

4.2b. Production of Storage Macromolecule

Since the *cw15* mutant strain simply lacks a cell wall and has completely functional metabolic machinery, it was expected to utilize the acetate to make copious amounts of starch in addition to lipids under nutrient starvation conditions. However, while the strain did produce an increasing amount of lipids when nitrogen-starved, very little starch appeared in the NMR spectra (Figure 10,15). This is due to the fact that starch exists as a solid, and solution NMR is unable to recognize such molecules. While it is possible that the algae cells could have utilized a different carbon source other than the labeled acetate to make starch, further experiments would need to be carried out in order to confirm that this. For sta6, large amounts of lipids were visible in the NMR spectra, while the peaks that would correspond with starch are very small (Figure 15). Since the sta6 mutant strain is devoid of the key enzyme to create starch, there should not be a

starch peak in the spectra at all. The peak that appears at 62.62 δ in the sta6 spectra may then be another molecule that contains functional groups similar to that of starch.

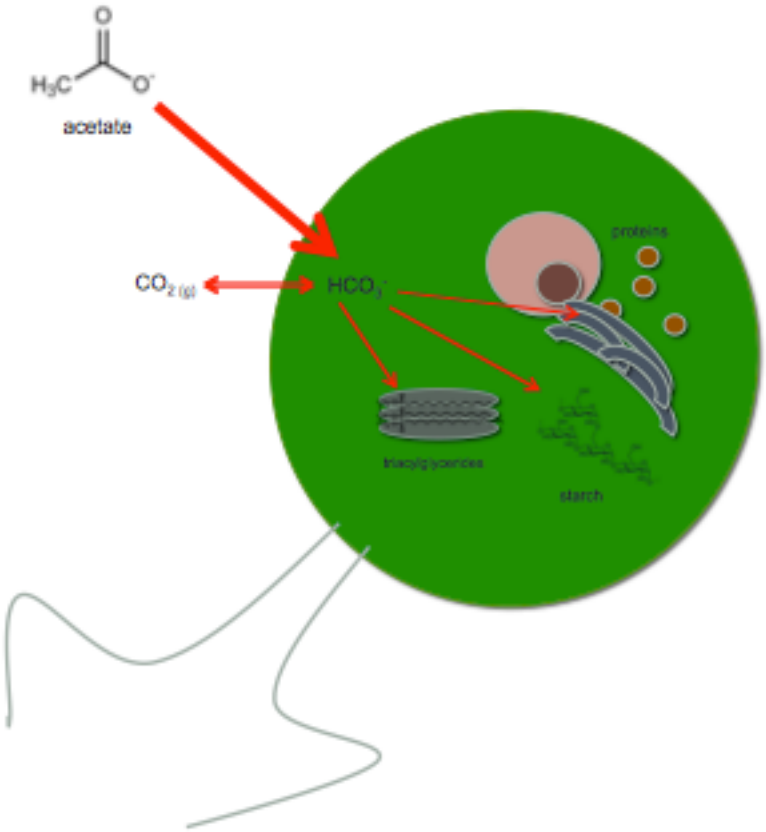

Figure 20. A summary of the incorporation of acetate into a normal algal cell

4.3 Outside Application

4.3a. Biofuel and other Ecological Functions

Knowledge concerning the metabolism of carbon in these algae cells could serve as key points in the utilization of algae in biofuel production. The large amount of fatty acids that are esterified into triacylglycerides combined with *Chlamydomonas reinhardtii*'s high production rate make this species of algae an ideal candidate for biofuel production.[10] The use of these organisms would eliminate the environmental

pollution that plagues the globe. In addition to being more ecologically favorable than fossil fuels, the algae would be relatively inexpensive to produce. Since algae such as *C. reinhardtii* are aquatic, the "crops" would be grown in cultivating ponds (Figure 21). This excises the cost for fertilizer that is needed for conventional crops. Furthermore, the water used in these cultivation ponds would not have to be purified, effectively reducing the need for funding prior wastewater treatment. In fact, if the algae are grown in wastewater, they can reduce the inorganic and organic load in the water, becoming a wastewater treatment system themselves.[10]

Figure 21. An illustration of a proposed algae field with cultivation ponds[1]

Despite this, there would be several complications involved in cultivating mass quantities of algae. As demonstrated in this particular experiment, harvesting or

collecting the algae could result in algal cells that may not be viable. This is because the cells might be too concentrated or too dilute upon removal from the water system. A larger issue is that the algae need to be nitrogen-starved before producing a viable amount of triacylglycerides. Further research into the metabolic pathways of this organism could result in a more cost-efficient method than media exchange in order to obtain high levels of triacylglycerides.

4.3b. Metabolism

Beyond using *Chlamydomonas reinhardtii* as a source of fuel, understanding the carbon and other nutrient pathways within the algal cells could result in the application of this research to other metabolic systems, including humans. As a model organism, the algae provide researches with a template for eukaryotic metabolism at the simplest level. Therefore, studying the carbon metabolism with nutrient starvation can certainly be applied to human metabolic studies. Several theories can be derived from analyzing data from these cells. Although the experiment was not carried out in this particular study, when algae cells receive a 20 mM boost of acetate after N-starvation, the cells become "obese" and produce an excessive amount of triacylglycerides.[6] This could possibly serve as a basis for studies of obesity in humans in which a deficiency of certain nutrients (such as nitrogen in proteins) from a poor diet could play a key part in an increase of lipids in body cells.

4.3c. *NMR in Healthcare*

Analysis of cells using labeled media and NMR could serve an important role not only in studying metabolic flux, but also in the detection of diseases. For example, there

have been studies that propose that a microscopic chip implanted into tissues can serve as a biomarker in a modified NMR system.[9] This system could then identify specific bacteria strains, provide a profile of circulating cells, and identify various cancer biomarkers. While this is much more complex than analyzing single-celled organisms in a liquid media, studies such as algal metabolic studies serve as frameworks for biological detection in multi-cellular, tissue-composed organisms.

4.4 Final Conclusions

This study aimed to determine whether or not exogenous acetate is incorporated into lipids directly through typical metabolic pathways. It was expected that the starch-less *sta6* mutant strain would create copious amounts of lipids after being starved while the *cw15* mutant strain would create lipids, but incorporate more of the exogenous acetate into starch. After carrying out the NMR experiments and analyzing the date, it was found that both strains created a very large amount of lipids. Qualitatively, it appears that sta6 creates more lipids than *cw15* based upon the height of the lipid peak, but this is not certain since the scale on the NMR spectra is relative. However, starch peaks are not visible in *cw15*. This is most likely due to the fact that this experiment used solution-state NMR, but starch exists in a very solid state. Therefore, *cw15* probably still uses acetate to make starch, but it is not measurable with this type of NMR. Furthermore, the carbon flux derived from exogenous acetate follows the course of bicarbonate/CO_2 production, protein production, and lipid production. This is enough to conclude that acetate is not incorporated into lipids through typical metabolic processes because peaks for intermediates would have been visible. In particular, because so many of the starch and

lipid synthesis intermediates contain ketones and carboxyl groups, there would have been peaks in those regions (around 200 δ for ketones and 70 δ for carboxyls) if typical metabolic pathways were utilized. The surplus in CO_2 in the form of bicarbonate is also evidence that the carbon flow from acetate to storage molecules is heavily dependent upon carbon derived from a CO_2 system instead of direct intermediates of synthesis pathways.

In order to test whether or not the lipids produced were energy-filled triacylglycerides, a mass spectrometry experiment could be carried out in order to distinguish fatty acids from TAGs. This would be valuable information for researchers seeking to use algae-produced lipids for sustainable energy. Nevertheless, it was demonstrated in this study shows that carbon from exogenous acetate is indeed incorporated into a copious amount of lipids. Subsequent experiments in this laboratory will determine whether or not those lipids were primarily composed of TAGs. In addition, the pH of the solution will be monitored in order to confirm the presence of the bicarbonate buffering system.

REFERENCES

[1] Algae Industry Magazine. AIM Interview: C.J. Warner and Tim Zenk. http://www.algaeindustrymagazine.com/aim-interview-cjwarner-timzenk-page2/ (accessed March 21 2014).

[2] Cagliari, A.; Margis, R; Dos Santos Maraschin, F; Carina Turchetto-Zolet, A; Loss, G; Margis-Pinheiro, M. Biosynthesis of Triacylglycerols (TAGs) in Plants and Algae. *International Journal of Plant Biology*. **2011**.

[3] CAPP. Algae. http://cambridgecapp.wordpress.com/improving-photosynthesis/algae/ (accessed Jan 5 2014).

[4] Eggling, S. Carbohydrates vs. Fats. http://dl.clackamas.cc.or.us/ch106-07/carbohyd1.htm (accessed Oct 27, 2013).

[5] Fan, J.; Yan, C.; Andre, C; Shanklin, J; Schwender, J; Xu, C; Oil Accumulation Is Controlled by Carbon Precursor Supply for Fatty Acid Synthesis in Chlamydomonas Reinhardtii. *Plant and Cell Physiology* 53, **2012**, *8, 1380–1390.*

[6] Funes, S.; Franzén, L.; González-Halphen, D.; *Chlamydomonas reinhardtii*: The Model of Choice to Study Mitochondria From Unicellular Photosynthetic Organisms. *Methods in Molecular Biology*. **2007**. *372, 137-149*.

[7] Goodson, C.; Roth, R; Wang, Z.T.; Goodenough, U. Structural Correlates of Cytoplasmic and Chloroplast Lipid Body Synthesis in Chlamydomonas Reinhardtii and Stimulation of Lipid Body Production with Acetate Boost. *Eukaryotic Cell* **2011**, *10, 1592–1606.*

[8] Iowa State University. "Starch Pathway." http://www.public.iastate.edu/~pkeeling/Pathway.htm (accessed Oct 28, 2013).

[9] Lee, H.; Sun, E. Chip-NMR Biosensor for Detection and Molecular Analysis of Cells. *Nature Medicine*. **2008**, 14, 869-874.

[10] Markou, G.; Georgakakis, D. Cultivation of Filamentous Cyanobacteria (blue-green algae) in Agro-industrial Wastes and Wastewaters: A review. *Applied Energy*, **2011**, 88, 3389-3401.

[11] National Renewable Energy Laboratory. Glossary of Biomass Terms. http://www.nrel.gov/biomass/glossary.html (accessed October 27, 2013).

[12] Seuoka, N. Mitotic Replication of Deoxyribonucleic Acid in Chlamydomonas Reinhardi. *Proceedings of the National Academy of Sciences*, **1960,** *46, 83-91.*

[13] Shevela, D.; Eaton-Rye, J.; Shen, J.; Gonvindjee. Photosystem II and the unique role of bicarbonate: A historical perspective. *Biochimica et Biophysica Acta*. **2012**, 1817, 1134-1151.

[14] Smith, A. C.; Cronan, J. E. Dimerization of the Bacterial Biotin Carboxylase Subunit Is Required for Acetyl Coenzyme A Carboxylase Activity. *Journal of Bacteriology*. **2012**. *194, 72-78*.

[15] The AOCS Lipid Library. Plant Fatty Acid Synthesis. http://lipidlibrary.aocs.org/plantbio/fa_biosynth/index.htm (accessed Oct 20, 2013).

[16] The AOCS Lipid Library. Triacylglycerols Biosynthesis and Metabolism. http://lipidlibrary.aocs.org/Lipids/tag2/index.htm (accessed Oct 20, 2013).

[17] U.S. DOE. National Algal Biofuels Technology Roadmap. U.S. Department of Energy, Office of Energy Efficiency and Renewable Energy, Biomass Program. **2010**.

[18] Voet, D.; and Voet, J. G.; Pratt, C. W. *Fundamentals of Biochemistry*, 3rd ed.; Wiley: 2008.

[19] Wang, Z.; Benning, C. Chloroplast Lipid Synthesis and Lipid Trafficking through ER–plastid Membrane Contact Sites. *Biochemical Society Transactions* **2012**. *40, 457–463.*

[20] Wang, Z.T.; Ullrich, N.; Joo, S.; Waffenschmidt, S.; Goodenough, U. Algal Lipid Bodies: Stress Induction, Purification, and Biochemical Characterization in Wild-Type and Starchless Chlamydomonas Reinhardtii. *Eukaryotic Cell* **2009**. *8, 1856-1868.*