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Investigation of Techniques and Their Application for the Cryopreservation of Algal Species

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INVESTIGATION OF TECHNIQUES AND THEIR APPLICATION FOR THE CRYOPRESERVATION OF ALGAL SPECIES

A Thesis

by

JAZMINE LEIJA

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ABSTRACT

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An alternative source for petroleum-based crude oils are algae derived biofuels. Fossil fuels are harmful for the environment, expensive and becoming scarce. There has been an increase in research on environmentally sustainable energy, using lipids derived from microalgae, which can be converted into biofuel. The use of microalgae as a source for biofuels has many benefits including decreasing greenhouse gas emissions, rapid fuel production, absorption of carbon dioxide, and production of a renewable source of energy.

Algae cryopreservation aids in the maintenance of the best algae strains selected for producing lipids for biofuels. Cryopreservation will help minimize genetic drift, as well as facilitating strain and clone exchange. Overall, culture maintenance, labor and costs will be reduced. Most algae cryopreservation methods require storage in liquid nitrogen which is expensive and inconvenient due to the need of specialized containers and a constant liquid nitrogen supply. Research is needed on faster, more cost effective, and less technically complex cryopreservation methods that can be used for a wide variety of algae. In this study, we applied four different methods to optimize the cryopreservation of algae in a -80 ºC freezer. The most effective method was the GeneArt cryopreservation technique at 36 hours (96.13% cell viability), and the least effective was the Glycerol at 84 hours (86.37% cell viability).

DEDICATION

For my family, who have supported me endlessly throughout my graduate studies. Thank you for all that you do, for all your love, and all your patience. Lastly, thank you for motivating me to work hard, and inspiring me to accomplish this degree.

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CHAPTER I

INTRODUCTION

Statement of Research Purpose

There is a growing interest in the use photosynthetic organisms called microalgae for use in biofuel production due to their unique characteristics and properties. Microalgae have cellular and biochemical characteristics that are similar to plants, except that microalgae are single-celled organisms and are easier to extract lipids from. Microalgae are found in wide range of environments which include the saltwater and freshwater, as well as soils (Slade and Bauen, 2013).

Fossil fuels are nonrenewable resources of energy, that are also causing a buildup of greenhouse gasses, which is what has led to the increase on the demand for a substitute that would be both cost effective and environmentally sustainable (Saharan *et al.,* 2013). Biodiesel has been successfully produced by using commercial oil crops, however, they have some disadvantages such as low production that results in small quantities of final product. Some species of microalgae can produce a high quantity of lipids and oils, which exceeds the levels in oil yielding crops such as palm oil and soybeans (Saharan *et al.,* 2013). Oils produced by microalgae are chemically comparable to fossil fuel oils, therefore microalgae have been considered an alternative source to produce biofuels. (Lyon *et al.,* 2013).

The labor required to successfully maintain an algal culture by subculturing in either liquid media or on agar can be rather intensive, time consuming, and can be very expensive. There is also a high risk of contamination due to constant handling and passage of the cultures (Bui *et al.*, 2013). Genetic drift can also occur, which defeats the purpose to maintain the genetic consistency of algal cultures. For example, if a high lipid producing algal strain was found, it would be very important to have that trait for lipid production maintained. The loss of this desired trait would result in a loss of an algal strain that would be useful for biofuel production. Genetic drift can affect small populations in which a natural allele can potentially become extinct. (Boddy, 2016). Specific characteristics from chosen strains are targeted for preservation and having an optimal cryopreservation technique will stop the algae from losing traits that are desirable (Gwo *et al.*, 2005).

Due to the previously mentioned factors, the need for cost effective and rapid culture conservation is now increasing, therefore needing a cryopreservation approach to preserving algal species. This is to prevent issues from occurring such as contamination, human error, and regrowth failure. (Kapoore *et al.*, 2019)

Overview of *Chlamydomonas reinhardtii*

Chlamydomonas reinhardtii has become a model organism due to several characteristics such as, a short life cycle, a small size, and the ease of culture (Harris, 2001). *C. reinhardtii* is a photosynthetically active single- celled green algae that is motile due to its two flagella. This microalgae species can be found in a variety of habitats such as temperate soils, and in fresh and salt waters. *C. reinhardtii* primarily exists in a vegetative growth form, which is haploid. The haploid nature of this organism also allows the discovery of phenotypic mutations by observation. It grows rapidly when under favorable conditions, doubling its numbers

approximately every 8 hours (Sasso *et al.,* 2018). One of the main reasons that has allowed this species to be selected as a model organism is its non-photosynthetic ability to grow, by supplementing the media with acetate as a source of carbon.

Cryopreservation of Microalgae. Cryopreservation has the ability to preserve living cells at very low temperatures without causing any changes to the organism's physiology, morphology, and genetics. For practical purposes, it is important to have a strain with consistent characteristics for each replicate experiment. Cryogenic preservation of plant and animal cells has been of interest to researchers has been practiced since the early 1960s (Taylor and Fletcher, 1998). A variety of organisms such as soil microalgae and cyanobacteria can be successfully cryopreserved and thawed with a high percentage of the cells that retain their viability. Cryopreservation of other algal species such as aquatic microalgae do not result in a high percentage of surviving cells after thawing, but they can still be cryopreserved with a few cells surviving the process (Day, 2007).

Cryopreservation Procedures and Cryoprotectants. Every organism may respond differently to the various types of freezing techniques, so it is difficult to find a single technique that will work universally with every kind of cell type and organism (Taylor and Fletcher, 1998). The techniques used to successfully cryopreserve algal cultures for the long term require an immense amount of initial effort, but in the long term, the genotype can be retained, contamination probabilities are reduced, and labor requirements are decreased significantly (Gwo *et al.,* 2005).

Methods such as DMSO cryopreservation in liquid nitrogen for animal cells, and freezing at -80 ºC in Glycerol for bacteria, are traditionally used for animal cells and bacteria, but do not

work as well when these techniques are applied to algae. One technique is rapid cooling, in which the algae cells are immediately plunged in liquid nitrogen after pre-treatment with a series of steps. This process allows the possibility for ice crystals to form which can damage the cells. The ice formation can be prevented by the two-step cooling technique in which the culture is cooled at a temperature about -30 ºC, before plunging it into the liquid nitrogen. (Taylor and Fletcher, 1998).

Cells undergoing freezing and thawing procedures have a greater viability when they are aided by a cryoprotectant during freezing and recovery. Cryoprotectants help prevent or reduce the amount of ice crystal formation when freezing the cells. The most commonly used cryoprotectants are glycerol, dimethyl sulfoxide (DMSO), and methanol. DMSO and methanol are highly preferred because they pass through the cell membranes much easier and removal from the cells is more efficient. If glycerol is used as the cryoprotectant for algal cells, it may be more difficult to remove the glycerol and contamination can occur. The concentration of cryoprotectants used is also an important factor, as having too high of a concentration can also damage algae cells (Taylor and Fletcher, 1998).

Thawing processes should be conducted as fast as possible upon removal from the liquid nitrogen storage. To thaw out organisms in cryovials, a typical technique is to place the vials in a warm water bath for approximately 1 to 2 minutes, then dilute out or remove the cryoprotectants and inoculate a flask containing media for regrowth. (Taylor and Fletcher, 1998).

Cost Efficiency of Cryopreservation. The rapid cooling technique can be rather costly as it requires the use of liquid nitrogen. Liquid nitrogen must be purchased or generated by a machine and the cells must be preserved under liquid nitrogen in a Dewar for long term storage. The cost of this infrastructure can be very expensive. (Abreu *et al.,* 2012). Cylinder rentals are

often required for storage and delivery of the liquid nitrogen. Nitrogen gas is also wasted within each cylinder, as it is suggested to discard the remaining 10% of gas which may contain impurities. Therefore, customers will be paying for gas that they will not be using up completely. Installations of large bulk storage tanks may also require monthly rental and maintenance, which will add more to the costs of liquid nitrogen usage. Customers will sometimes commit to multi-year contracts with bulk nitrogen suppliers, which will prevent them from switching to a more cost-effective alternative (Peak Gas Generation, 2019).

Other freezing protocols utilize the two-step method in which a cooling canister containing isopropyl alcohol is required to initially cool the algal material inside of some cryovials. This cooling canister is then placed inside of a -80 ºC freezer until it reaches the desired temperature, then the cryovials are immediately plunged into a liquid nitrogen storage system (Abreu *et al.,* 2012).

Study Objective

This study examines the overall cost of four cryopreservation methods, the efficiency of various cryoprotection techniques for *C. reinhardtii,* and which assays work best to determine cell viability once the cells have been thawed and recovered. Four different cryoprotectants, DMSO, glycerol, methanol, and a proprietary GeneArt cryopreservation kit from ThermoFisher scientific were tested using a -80 ºC freezer as substitute for the use of liquid nitrogen.

Two different assays were utilized to examine the cell viability just after thawing, as well as for the long-term recovery of the algae. The determination of cell viability right after thawing was the uptake and staining of the cells with Neutral Red. The long-term recovery and quantitation of the cells was attempted by inoculating the cells onto petri dishes of appropriate medium.

Prior to freezing trials, the *C. reinhardtii* cultures were observed every twelve hours for one week to determine the time points for optimal growth in order to collect the samples at those optimal time points for cryopreservation. This was done by measuring cell counts and lipid content over the course of the growth curve for the algae.

Hypothesis

One of more out of the four modified cryopreservation protocols will be able to replace the liquid nitrogen method for algae cryopreservation and result in a higher post cryopreservation cell viability.

One of more out of the four modified cryopreservation protocols will be more cost effective than liquid nitrogen cryopreservation.

CHAPTER II

METHODOLOGY

Algae Strain

The living algal strain of *C. reinhardtii* used in this project was purchased from the University of Texas at Austin (UTEX Algae Culture Collection, 2021).

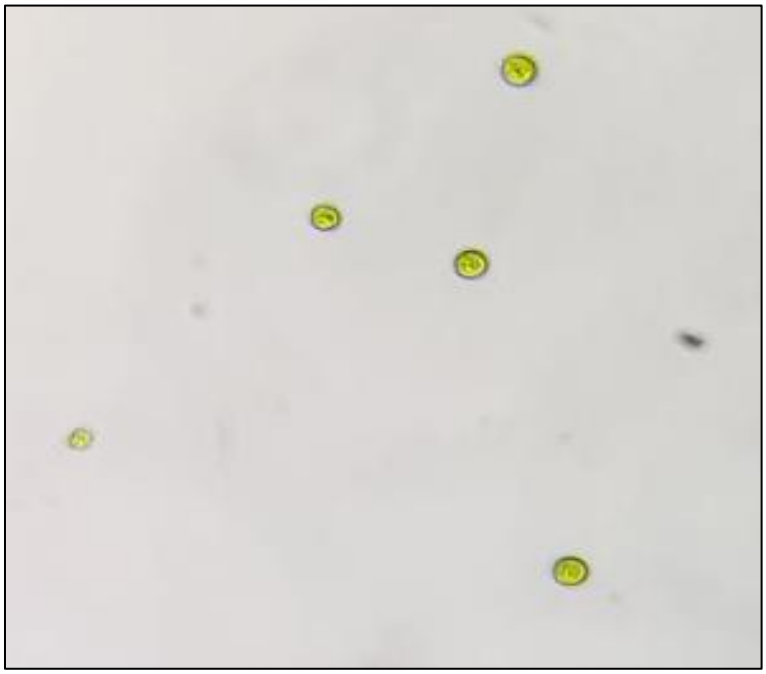

Figure 1. *Chlamydomonas reinhardtii* algae cells observed at 400X on a hemocytometer (Photo provided by Jazmine Leija).

Preparation of TAP media

All *C. reinhardtii* cultures were grown on TAP medium (UTEX Algae Culture Collection, 2021). Two liters of TAP medium were prepared by adding approximately 1,600 mL

of deionized water to a 2L flask and then adding four different solutions in the following order. The first solution added was 100 mL of Beijernick's solution followed second by 17 mL of Phosphate Buffer stock solution, then third by 2 mL of Hunter's Trace stock solution, and lastly 20 mL of Tris Acetate stock solution (UTEX Algae Culture Collection, 2021). Once all the solutions were added, the final volume was raised with the deionized water to 2 liters. The media were autoclaved at 120 ºC, 15 lbs/sq in for 20 minutes then allowed to cool to room temperature (22 ºC) and stored at 4 º C.

Optimal Growth. The lag, log, and stationary phases were observed from preliminary cultures in order to determine the optimal time frames in which *C. reinhardtii* grew and the best points for sampling the cells for cryopreservation. Initially, the cultures were monitored from 0 to 160 hours. After analysis, the optimal time period for sampling was determined to be at 16, 36, 66, 84, 144-hour intervals over the course of five days (144 hours total). Once optimal sampling times were determined, the cultures were analyzed via absorbance (A600) (Figure 4), cell counts (cell/mL) (Figure 6), and average lipid fluorescence units per cell (Figure 7).

Algae Culture Growth Conditions. From a Tris-acetate-phosphate (TAP) agar slant culture provided by UTEX was the source material to culture single colonies on TAP plates. *C. reinhardtii* were streaked for single colonies on TAP plates and one single colony was chosen for use in further studies. From TAP plates, algae were inoculated with an inoculation loop into 50 mL of TAP for use as a starter culture. This culture was then grown for approximately 5 days under the conditions below. Experimental algae cultures were inoculated with a total number of 2.5 E+07 cells (final concentration of 5.0 E+05 cells/mL) in 50 mL of TAP media in 125 mL

flasks. Four replicates were prepared. The algae cultures were then placed on a Labnet Orbit™ 1900 shaker (Labnet International, Edison, NJ) and shaken at a speed of 100 rotations per minute under a Sun Blaze® 48-120 Volt fluorescent light fixtures with T5HO fluorescent bulbs (Sunlight Supply Incorporated and Vancouver, WA). The light output of the fixtures ranged from 250 to 350 µEinsteins in light intensity. The lights were on a timer to provide of 8-hours of light and 16-hours of darkness, and the cultures were grown at 30 ºC when illumination was present and at 22 ºC during the dark cycle.

Experimental cultures were grown for a total period of 5 days and samples were taken at hour 16, hour 36, hour 66, hour 84, and hour 144 time periods. Algae sampled at these time points were then treated with the modified cryopreservation protocols.

Cell Growth Measurements. Absorbance and cell counting measurements of the experimental cultures were done at hour 16, hour 36, hour 66, hour 84, and hour 144 time periods. Cell culture absorbance was measured via spectrophotometry at a wavelength of 600 nanometers (A600) by using a Thermo Scientific Genesys 10S UV-Vis spectrophotometer (ThermoFisher Scientific, Waltham, MA). This was accomplished by pipetting 1 mL of algae culture into 1.5 mL cuvettes (ThermoFisher Scientific, Waltham, MA), and by preparing a blank using TAP media.

Cell counts were performed on each experimental culture by using a Hausser Scientific Bright-Line hemocytometer (Hausser Scientific, Horsham, PA). Using a Nikon YS2-T light microscope (Nikon USA, Melville, NY) at 400X magnification, five large squares on the hemocytometer were counted to obtain the number of cells/mL. The number of cells/mL was calculated by dividing the total number of algae cells counted in the five hemocytometer squares,

then divided by five, multiplied by a dilution factor (if any), and then multiplied by 10,000 to determine the total number of cells/mL in each culture.

Quantification of Cellular Lipids. Four replicate 1mL samples of each of the four culture flasks were taken at hour 16, hour 36, hour 66, hour 84, and hour 144 time periods for lipid quantification. To a blank solution (1mL TAP + 20µL of a 10% v/v Triton X-100) without Nile Red (Kodak, Rochester, NY) was added 1mL of culture sample in a 4mL cuvette with mixing. For fluorescence measurements to a 4mL cuvette were added 1mL of culture sample, 1mL of TAP media, 20µL of a 10% v/v Triton X- 100 solution and 2µL of a 100µg/mL Nile Red solution with mixing.

A BioRad VersaFluor Fluorometer (BioRad Laboratories, Hercules, CA) was then used to measure the lipid fluorescence by reading the samples for 30 seconds over the course of 2 minutes with four total readings. The lipid fluorescence/cell value was calculated by dividing the average lipid fluorescence readings (four readings each 30 seconds over 2 minutes) by the number of cells per milliliter (total cells in assay).

Cryopreservation Techniques. Three replicate trials were conducted to test four different cryoprotectants using modified protocols from the University of Texas at Austin and ThermoFisher Scientific's GeneArt cryopreservation kit, as well as two self-devised protocols using Glycerol and Dimethyl sulfoxide (DMSO). Each sample with the different cryoprotectants were prepared in 2.0 mL cryovials (ThermoFisher Scientific, Waltham, MA) via sterile technique. Samples were frozen at the predetermined optimal growth points which were at the hour 16, hour 36, hour 66, hour 84, and hour 144 time periods. Once all the samples were prepared, the cryovials were all placed inside of a Mr. Frosty freezing container (ThermoFisher Scientific, Waltham, MA) and were frozen simultaneously inside of the -80º C freezer.

For the modified GeneArt cryopreservation kit (Invitrogen ThermoFisher Scientific, Waltham, MA) protocol, 950 µL of each algae culture (labeled C1-C4) were added into four individual 1.5 mL sterile microfuge tubes (ThermoFisher Scientific, Waltham, MA). Each microfuge tube then had 50 µL of cryopreservation reagent "A" added, then sat underneath LED lights (35-40 µEinsteins) for one hour. After the one-hour mark, the microfuge tubes were placed into the Beckman Coulter Microcentrifuge 18 (Beckman Coulter, Indianapolis, IN) and were spun at 4,000 rotations per minute for five minutes. Once that was complete, the supernatant was removed by pouring off the supernate and pipetting off the remainder of the supernate. The cells were resuspended gently in 250 µL of cryopreservation reagent "B" and were left to sit at room temperature for 45 minutes before transferred into the cryovials and being placed into the Mr. Frosty freezing container.

The next modified protocol followed was provided by the University of Texas at Austin, in which 0.5 mL of each algae culture was added to four different cryovials. Then 0.5 mL of 10% methanol (HPLC grade, ThermoFisher Scientific, Waltham, MA) was also added into the cryovials and mixed well.

For the self-devised protocols, 800 µL of algae culture and 200 µL of the 80% Glycerol (Molecular Biology grade, Millipore Sigma, St. Louis, MO) were added into each of the four cryovials and mixed well resulting in a final concentration of 16% Glycerol. Nine hundred and fifty µL of algae culture and 50 µL of DMSO (HPLC grade, Millipore Sigma, St. Louis, MO) were added into another set of four cryovials and mixed well resulting in a final concentration of 5% DMSO.

Preparation of Mr. Frosty Container and Cell Freezing. The Mr. Frosty Freezing container was prepared by filling the lower chamber with 100% Isopropanol (ACS Reagent

grade, Millipore Sigma, St. Louis, MO). The upper chamber which contained the cryovials was placed into the lower chamber and the cap screwed on. Then the entire Mr. Frosty Freezing container was placed in a -80 ºC freezer (So-Low, Cincinnati, OH) overnight. Finally, the cryovials were removed from the Mr. Frosty Freezing container and placed in cardboard freezer boxes (ThermoFisher Scientific, Waltham, MA) for permanent storage.

Once the samples had been frozen for 13 weeks, they were individually thawed out by the following process. The cryovials containing the frozen algae cells were removed from the -80º C freezer and were immediately placed in a Styrofoam container containing dry ice to transport the vials from the freezer to the water bath. Each cryovial was then instantly placed inside of a warm water bath which was at 34º C for approximately one to two minutes. After the cryovials had been thawed, the contents were transferred into 1.5 mL microfuge tubes, and were then centrifuged at 5,000 RPM for five minutes. After the supernatant was removed by pipetting, 1 mL of fresh TAP media was added to resuspend the algae cells.

Cell Viability Measurements. Neutral red (Cell culture grade, Millipore Sigma, St. Louis, MO) is a dye that allows for quantitative estimations of cell viability and has low toxicity levels in comparison to other dyes used for plant systems. It can penetrate biological membranes easily, staining lysosomes in animal cells, and the vacuoles in plant cells. (Stefano, G. *et al.*, 2017)

Cell viability was quantified by Neutral Red staining in combination with cell counts from the cryovials post thawing. Six point one µL of Neutral Red (0.33%) was added to 100 µL of each thawed algae sample in a 1.5mL microfuge tube to detect the cells that remained alive after the thawing process (0.055% final concentration of Neutral Red). Simultaneously, cell counts were also performed using a hemocytometer.

The percent viability was determined by dividing the number of cells that stained red with the neutral red, divided by the total number of cells counted (red and non-red cells).

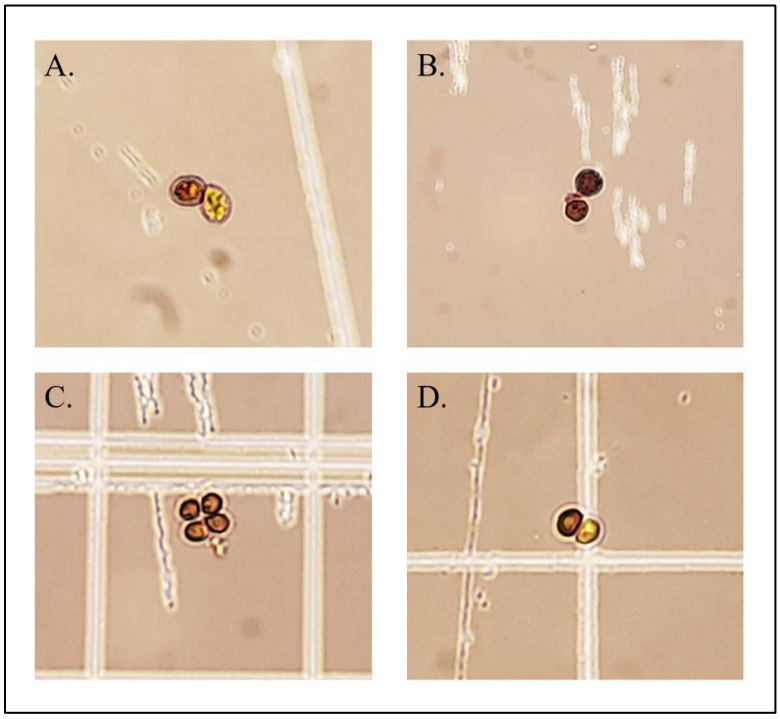

Figure 2. *C. reinhardtii* cells post thawing and stained with Neutral Red to quantify cell viability. (A.) DMSO cryopreservation technique. (B.) Modified UTEX cryopreservation technique. (C.) Modified GeneArt cryopreservation technique. (D.) Self-devised Glycerol cryopreservation technique (photo provided by Jazmine Leija).

Cell Plating on TAP Media Plates. The thawed algae cells were diluted to 40,000 cells/mL and spread on 150mm x 10mm TAP agar petri plates (ThermoFisher Scientific, Waltham, MA). Each experimental sample was plated on three replicate plates via sterile technique, by adding 250 µL of the diluted algae sample to the center of each petri plate. Using a sterile glass cell spreader and a turntable (ThermoFisher Scientific, Waltham, MA), the 250 µL was evenly spread over the surface of the petri plate. The petri dishes were then left under the low intensity fluorescent lights, receiving 8 hours of light and 16 hours of darkness at 55-60

µEinsteins to allow the cells to rejuvenate and grow for approximately 12 weeks. Samples were plated from the hour 16, hour 36, hour 66, hour 84, and hour 144 time periods.

Petri Dish Quantification. After recovered cells were spread on TAP agar plates, they were grown under low intensity fluorescent lights (50 µEinsteins) (Envirolite, Troy, MI) with T8 bulbs for 12 weeks, the three replicate petri dishes for each trial were visually counted to quantify the number of colonies present on the plates. The percentage of viable cells was determined by the number of colonies counted and divided by 10,000.

CHAPTER III

RESULTS

C. reinhardtii Preliminary Absorbance Measurements at 600 nm

The average absorbance at the culture starting point was 0.10 ± 0.00 AU, which increased to 0.12 ± 0.00 AU by the 12th hour, 0.20 ± 0.01 AU at hour 24, which further increased to 0.28 ± 0.02 AU by the 36th hour. At hour 48 the absorbance was 0.59 ± 0.00 AU, then increased to 0.79 ± 0.06 AU at hour 60, and 1.05 ± 0.02 AU at 72 hours. After the 72 hours, there was a decrease in the average absorbance, dropping to 1.01 ± 0.06 AU at the 84th hour. Then at hour 96 the culture reached a higher absorbance, which was at 1.13 ± 0.00 AU, but then dropped again at hours 115.5 and 136.5. Hour 115.5 had an absorbance of 1.09 ± 0.03 AU, while hour 136.5 had 1.01 ± 0.03 AU. At 160.5 hours, the absorbance levels had increased to a final absorbance of 1.19 ± 0.01 AU (Figure 3).

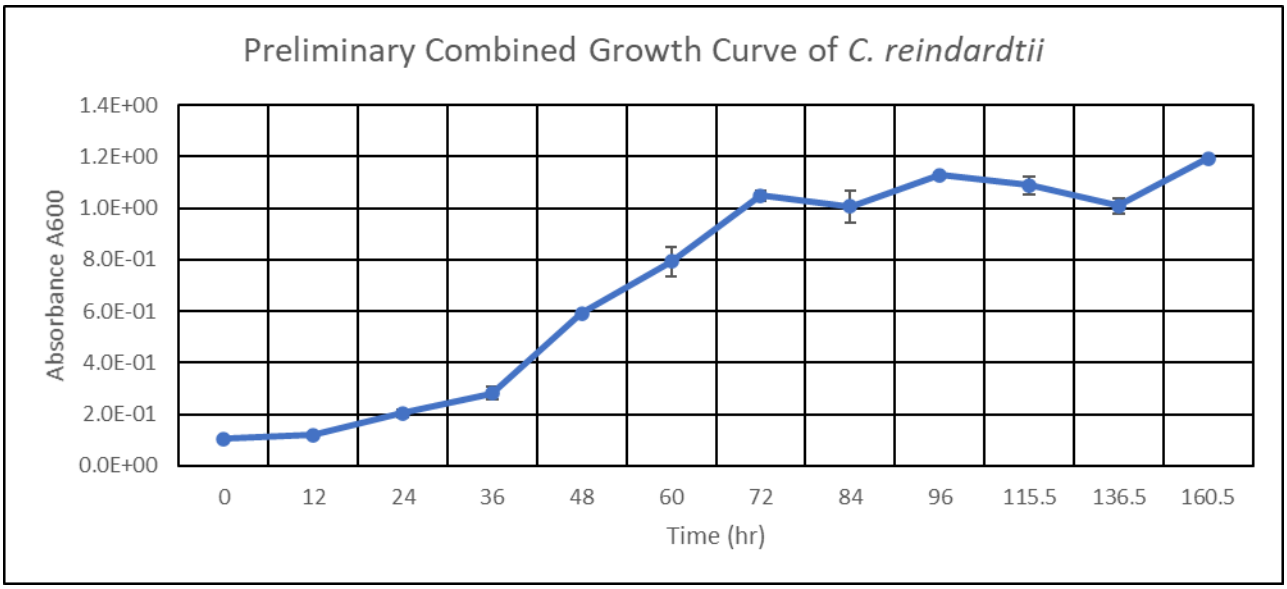

Figure 3. Average absorbance (A600) measurements of preliminary *C. reinhardtii* cultures observed at 12-hour intervals.

Culture Average Absorbance Measurements for Cell Freezing

The average absorbance levels from the four internal replicate cultures from two trials were also analyzed. The average absorbance levels demonstrated a steady increase throughout the five selected growth time points. There was an average absorbance of 0.05 $\pm$ 0.03 AU at the 16th hour of growth. The 36th hour time point was 0.12 $\pm$ 0.06 AU, which increased to 0.57 $\pm$ 0.06 AU by the 66th hour. By the 84th hour, the absorbance has increased to 0.80 $\pm$ 0.04 AU, and then to 1.06 $\pm$ 0.06 AU at the 144th hour (Figure 4).

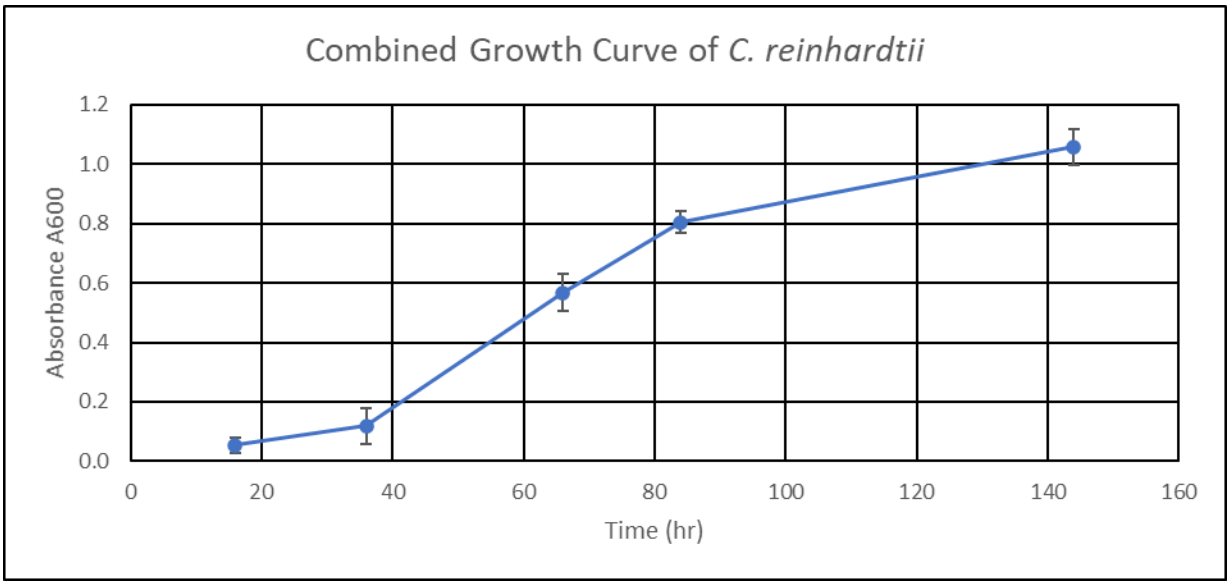

Figure 4. Average absorbance (A600) measurements of experimental (internal replicates) *C. reinhardtii* cultures observed at 12-hour intervals.

Preliminary cells per mL. On day 0, the cultures started with an average cell count of 5.00 E+05 ± 0.00 which then decreased slightly to 3.43 E+05 ± 1.41 E+03 cells/mL on hour 12, and at hour 24 there was an increase in the average of cell counts to 3.29 E+05 ± 5.52 E+03 cells/mL. By the 36th hour, the cell counts had increased to 1.23 E+06 ± 2.69 E+05 cells/mL, followed by increases at hours 48, 60, 72 and 84. At hour 48 there were 2.48 E+06 ± 2.26 E+05 cells/mL present, and at hour 60, a total of 5.22 E+06 ± 9.33 E+05 cells/mL were present. At hour 72 it was found that the average cell counts had increased to 5.45 E+06 ± 1.34 E+06 cells/mL, and at the 84th hour the average cell concentration had reached 1.06 E+07 ± 2.90 E+06 cells/mL. At hour 96, the average cell concentration had decreased to 7.28 E+06 ± 1.98 E+06 cells/mL, followed by a further decrease to 5.74 E+06 ± 3.96 E+05 cells/mL at 115.5 hours. The average cell counts then increased once more to 7.14 E+06 ± 0.00 E+0 cells/mL at 136.5 hours, followed by another increase to 8.75 E+06 + 1.48 E+06 cells/mL at 160.5 hours (Figure 5).

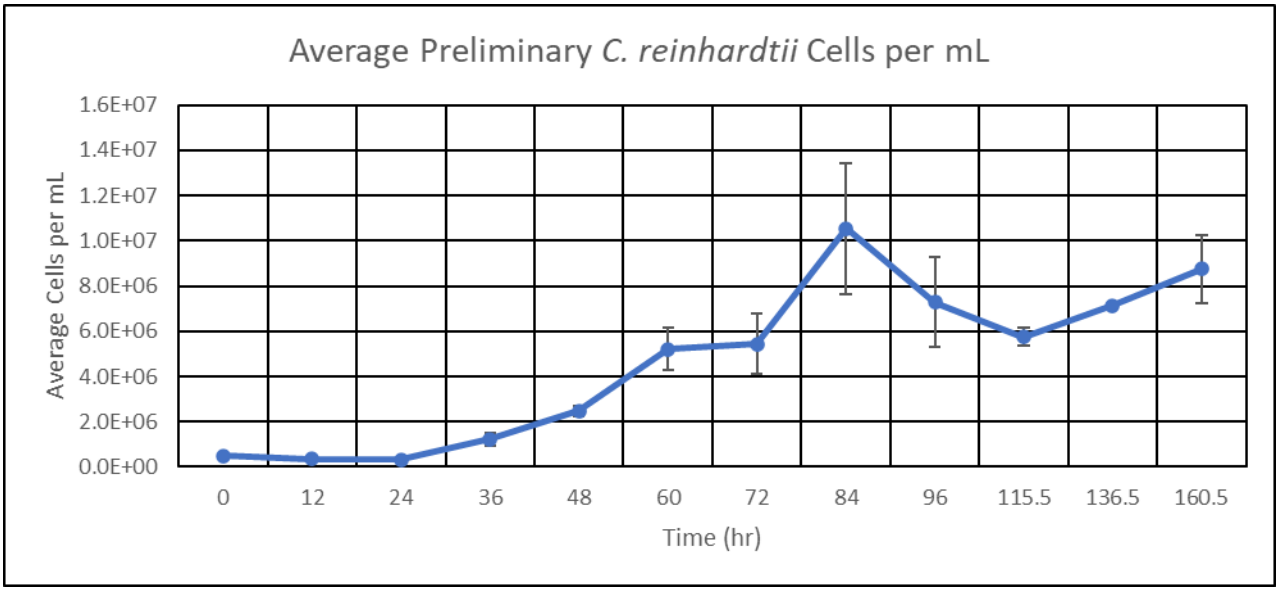

Figure 5. Average cell density measurements of preliminary *C. reinhardtii* cultures observed at 12-hour intervals.

Average Cell Density Prior to Cell Freezing. The four 50mL internal replicate cultures (four each of the two trials) were inoculated with 2.5 E+07 cells total (5.00 E+05 cells/mL) and grown on shakers under high intensity fluorescent lights. Samples were taken for use with the freezing techniques at the five designated time points previously mentioned (16, 36, 66, 84, 144 hours). The average cells per mL from all cultures at hour 16 was 2.35 E+05 $\pm$ 8.79 E+04 cells/mL, which then slowly increased to 3.56 E+05 $\pm$ 1.34 E+05 cells/mL at the 36th hour. At hour 66, the average cells/mL had a larger increase to 5.63 E+06 $\pm$ 1.24 E+06 cells/mL, and then further increasing to 9.28 E+06 $\pm$ 1.43 E+06 cells/mL at the 84th hour. At hour 144 there was a decrease, in which the average cells/mL dropped to 8.27 E+06 $\pm$ 2.76 E+06 cells/mL (Figure 6).

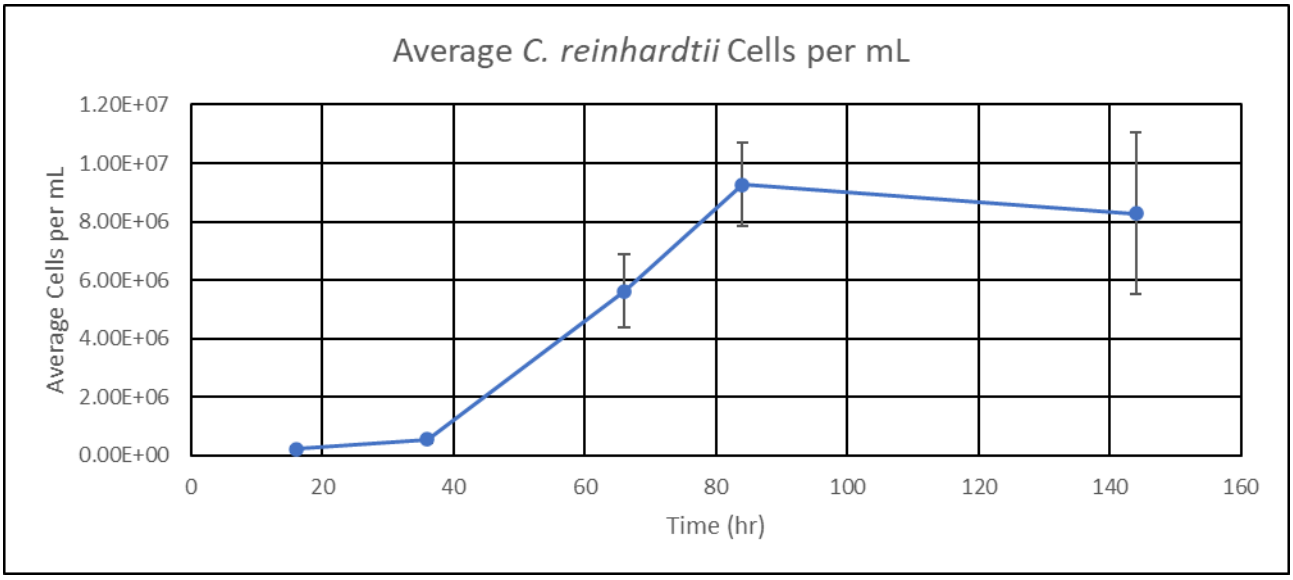

Figure 6. Average cell density measurements of experimental (internal replicates) *C. reinhardtii* cultures observed at 12-hour intervals.

Total Average Lipids per Cell. Lipid fluorescence per cell were analyzed through fluorometric analysis using Nile Red at each time point (16, 36, 66, 84, and 144 hours). The average lipid fluorescence per cell had an initial value of 2.64 E-02 ± 2.38 E-02 lipid fluorescence units/cell at the 16-hour mark, which then decreased to 9.63 E-03 ± 9.04 E-03 lipid fluorescence units/cell at the 36th hour time point. The lipid fluorescence units/cell then decreased slightly during hours 66, 84 and 144. At hour 66, there was an average of 3.19 E-04 ± 3.91 E-04 lipids fluorescence units/cell, and hour 84 had an average of 1.68 E-04 ± 6.33 E-05 lipids fluorescence units/cell at the 84th hour. At hour 144, the average resulted in 2.19 E-04 ± 2.07 E-04 lipids fluorescence units/cell (Figure 7).

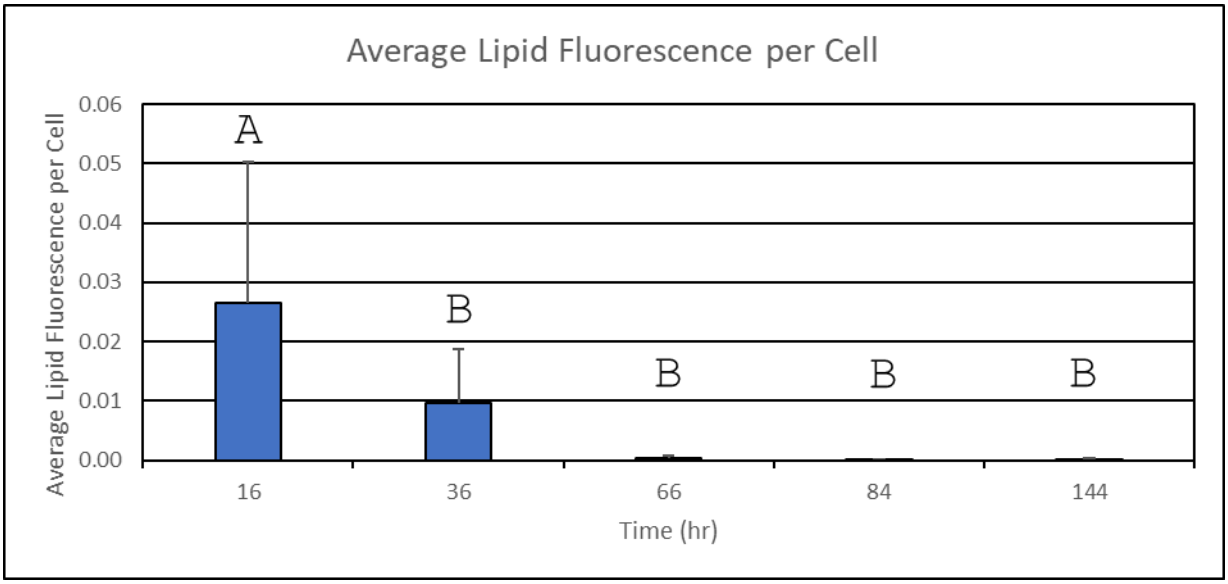

Figure 7. Average lipid fluorescence units per cell measurements of experimental (internal replicates) *C. reinhardtii* cultures at the hours of observation. Statistical analysis was applied by conducting an ANOVA and a post hoc (Tukey- HSD) in which hour 16 was statistically significant when compared to hours 36, 66, 84, and 144.

Average Cell Viability Per Treatment Via Neutral Red Stain. Upon recovery from freezing, cell counts were performed using a neutral red vital stain to determine the cell viability percentages from each of the different treatments (DMSO, UTEX, Glycerol, and GeneArt Kit).

The DMSO technique demonstrated a high survival rate immediately after the samples had been thawed, resulting in a viability of 93.92% $\pm$ 2.89% from the cells that were frozen at the 16th hour time point. Hour 36 had a cell viability of 94.27% $\pm$ 0.56%, and at 66 hours, 96.25% $\pm$ 1.60%. At the 84- and 144-hour time points, the cell viability percentages decreased to 90.85% $\pm$ 7.89% and 91.61% $\pm$ 1.65% respectively (Figure 8).

The UTEX protocol had 92.59% $\pm$ 3.14% cell viability after thawing the cells at hour 16. There was a slight increase at the 36th hour which resulted in 93.99% $\pm$ 1.29% of viability. At hour 66 the cell viability further increased to 96.69% $\pm$ 0.73%, but then there was a decrease to

an average of 90.85% ± 3.10% cells/mL at the 84th hour. At hour 144, the cell viability increased to 94.91% ± 4.15% (Figure 9).

Glycerol also demonstrated that it could work efficiently as a cryoprotectant. At hour 16, there were 92.77% ± 1.65% viable cells. The 36th and 66th hour samples decreased to 91.23% ± 1.13% and 91.29% ± 6.27% respectively. The average cell viability further decreased 86.37% ± 2.63% at hour 84, and then increased to 86.68% ± 2.63% at hour 144 (Figure 10).

The GeneArt Kit technique also had a high cell viability post thawing and cell recovery. At hour 16 there was a cell viability of 94.84% ± 3.91% which increased to 96.13% ± 2.73% at 36 hours. There was a decrease at hour 66, which resulted in 94. 22% ± 0.30% cell viability, and there was a further decrease to 94.45% ± 1.08% at the 84th hour. At 144 hours, the cell viability remained approximately the same with a percentage of 94.91% ± 2.53% (Figure 11).

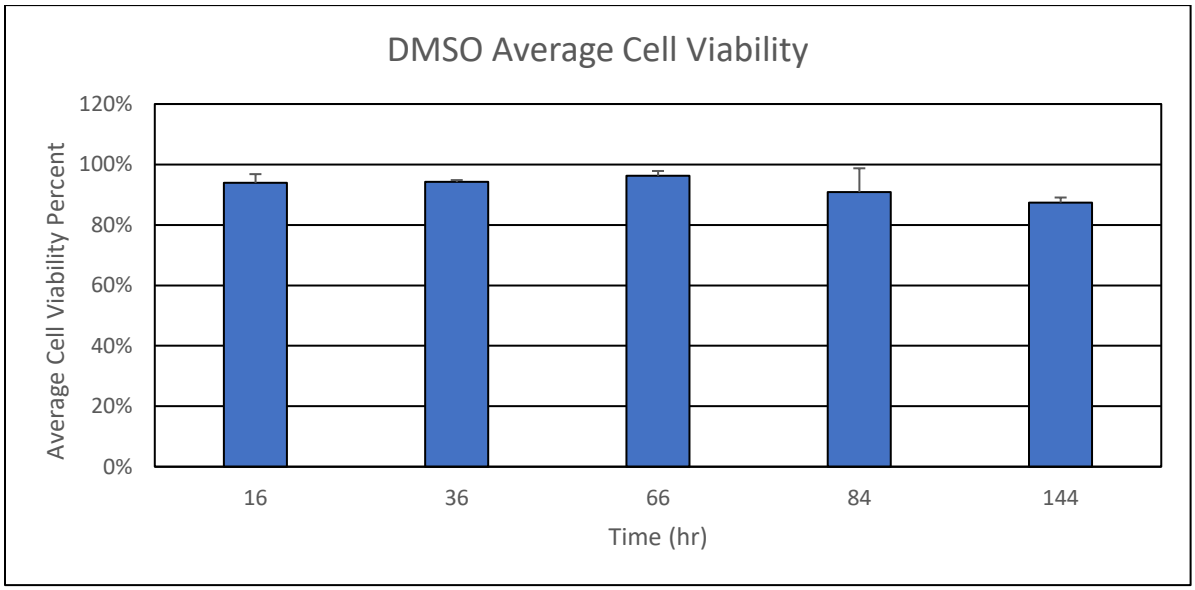

Figure 8. Average cell viability percent of experimental (internal replicates) *C. reinhardtii* cultures with the DMSO cryopreservation technique at hours of observation after thawing and Neutral Red staining.

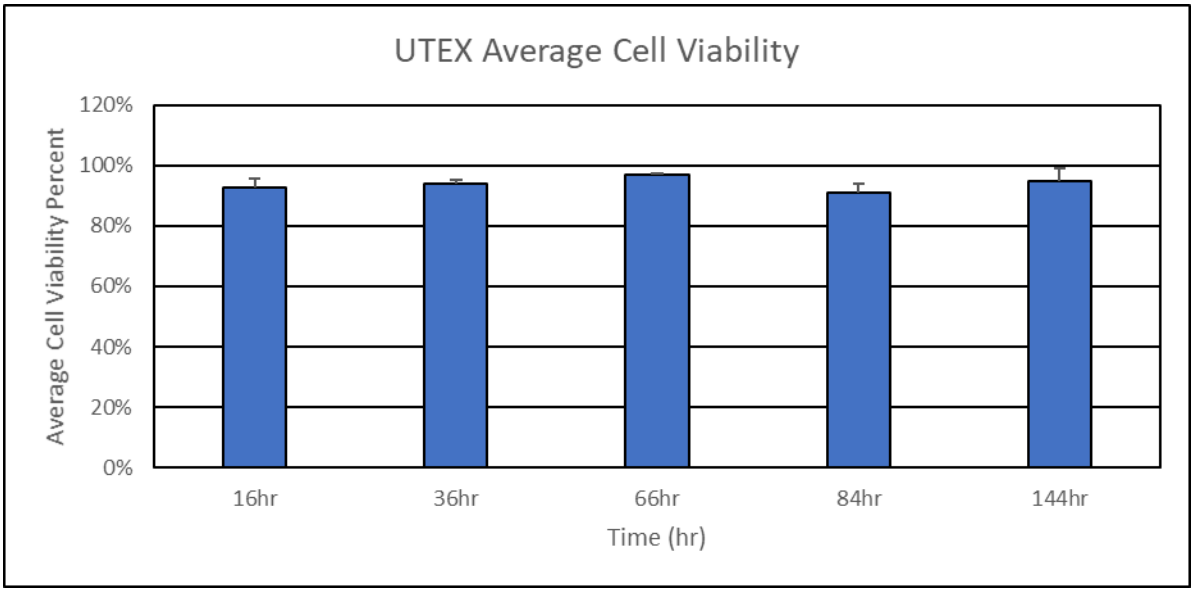

Figure 9. Average cell viability percent of experimental (internal replicates) *C. reinhardtii* cultures with the UTEX cryopreservation technique at hours of observation after thawing and Neutral Red staining.

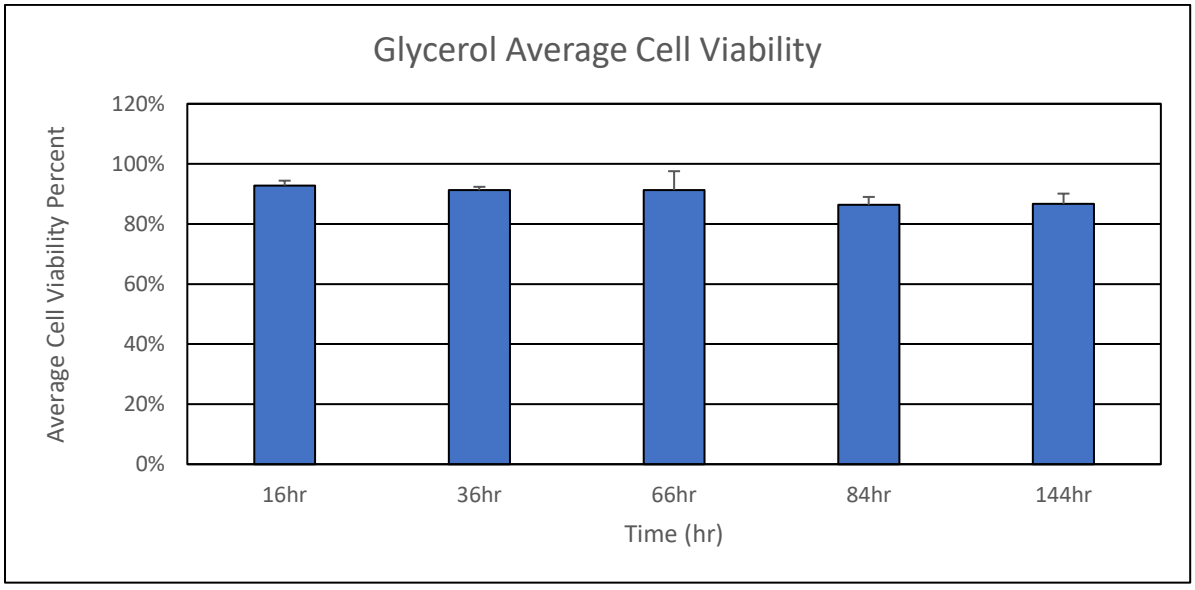

Figure 10. Average cell viability percent of experimental (internal replicates) *C. reinhardtii* cultures with the Glycerol cryopreservation technique at hours of observation after thawing and Neutral Red staining.

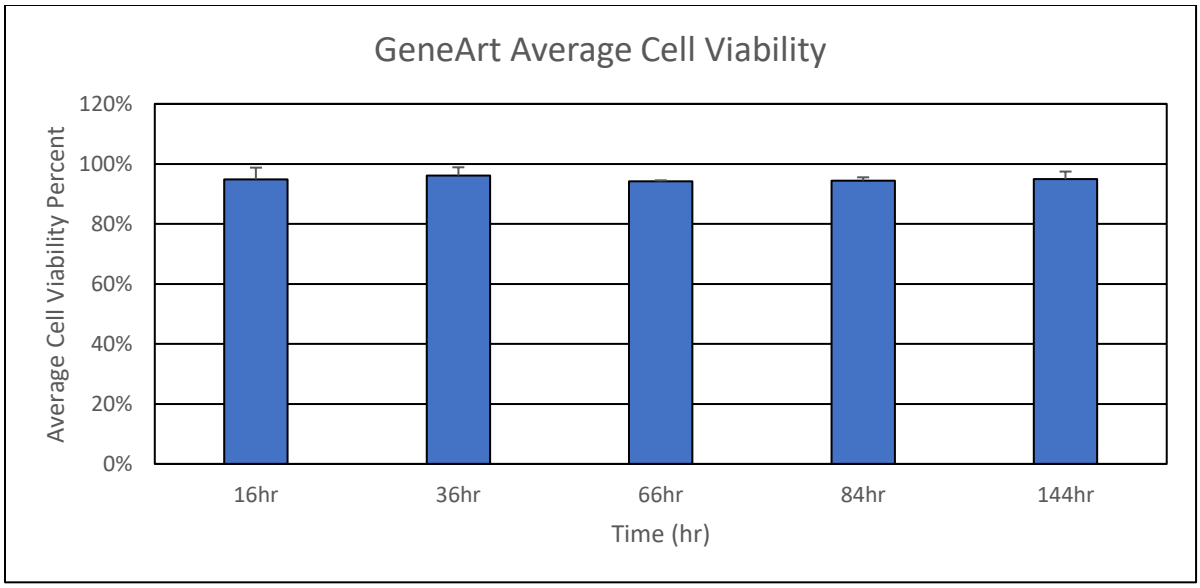

Figure 11. Average cell viability percent of experimental (internal replicates) *C. reinhardtii* cultures with the GeneArt cryopreservation technique at hours of observation after thawing and Neutral Red staining.

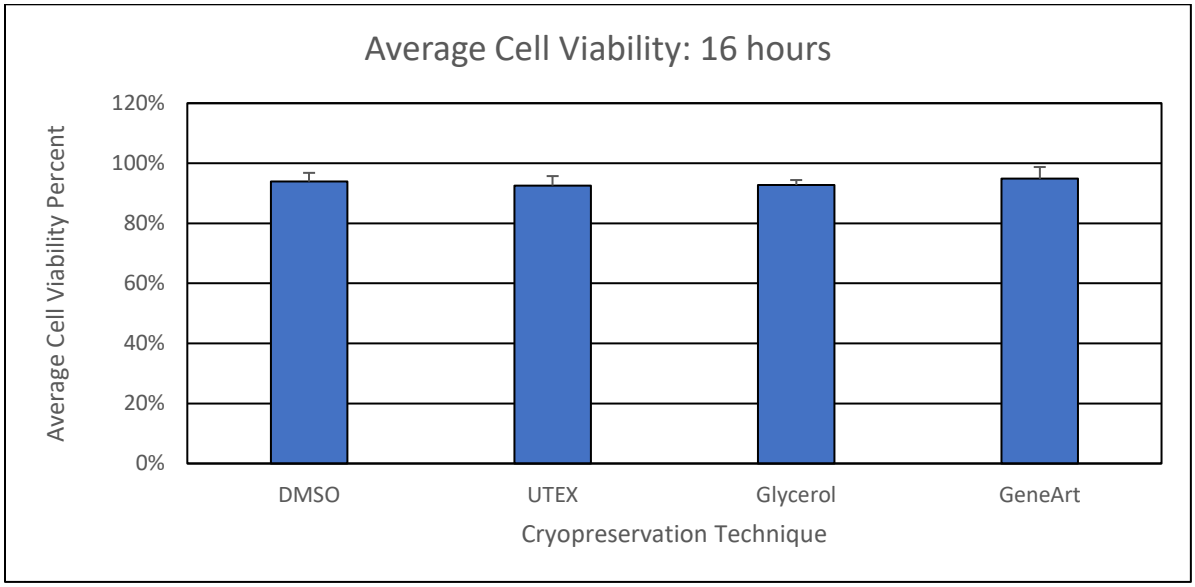

Figure 12. Average cell viability percent of experimental (internal replicates) *C. reinhardtii* cultures comparing the four cryopreservation techniques at 16 hours of observation after thawing and Neutral Red staining.

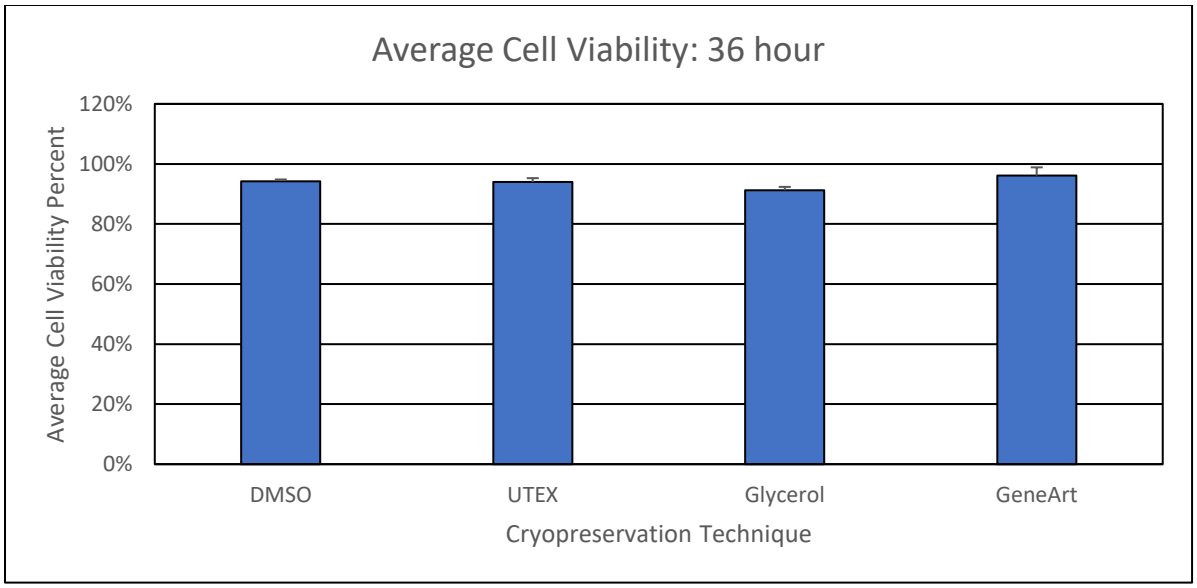

Figure 13. Average cell viability percent of experimental (internal replicates) *C. reinhardtii* cultures comparing the four cryopreservation techniques at 36 hours of observation after thawing and Neutral Red staining.

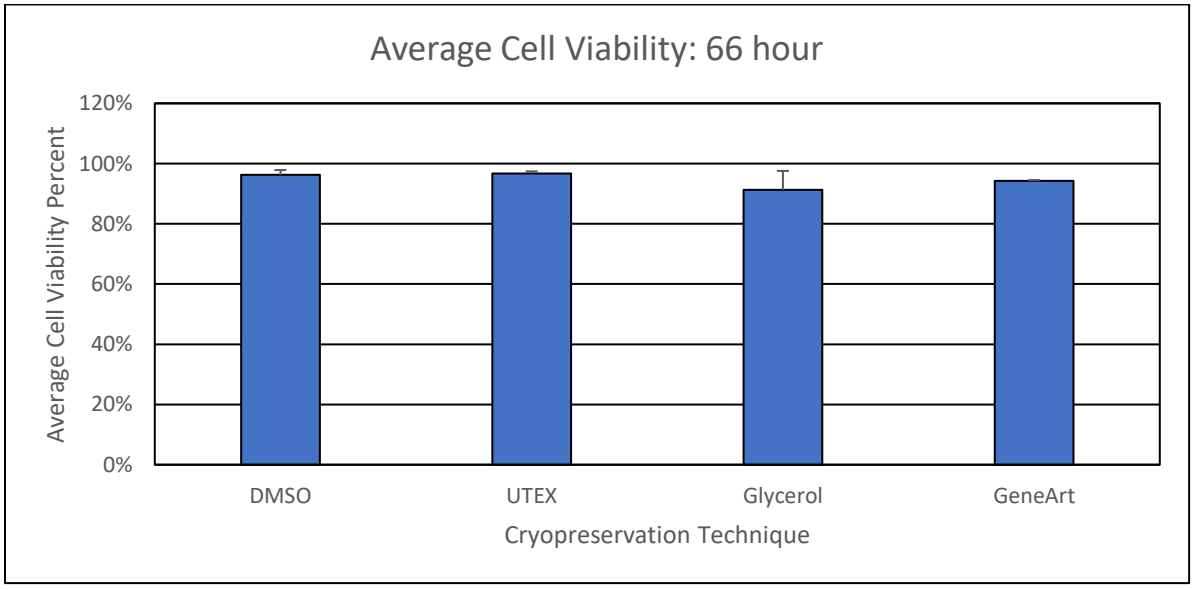

Figure 14. Average cell viability percent of experimental (internal replicates) *C. reinhardtii* cultures comparing the four cryopreservation techniques at 66 hours of observation after thawing and Neutral Red staining.

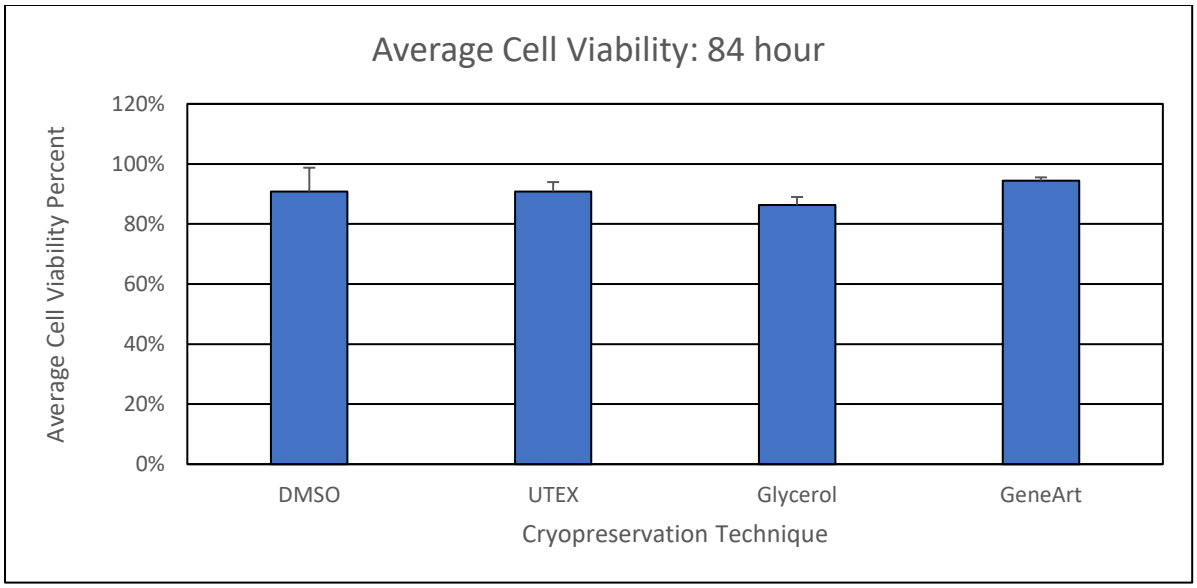

Figure 15. Average cell viability percent of experimental (internal replicates) *C. reinhardtii* cultures comparing the four cryopreservation techniques at 84 hours of observation after thawing and Neutral Red staining.

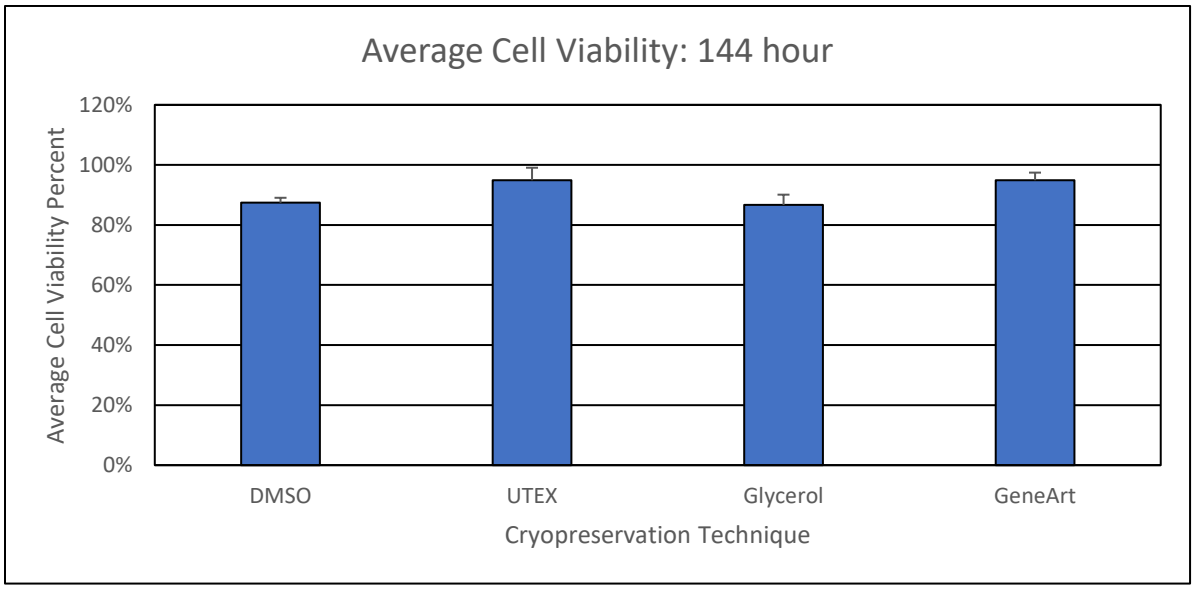

Figure 16. Average cell viability percent of experimental (internal replicates) *C. reinhardtii* cultures comparing the four cryopreservation techniques at 144 hours of observation after thawing and Neutral Red staining.

Hour	Average ± SD Cells Frozen	Average ± SD Percent Cell Survival
DMSO 16	2.35E+05 ± 1.41E+05	93.92 ± 2.89%
DMSO 36	5.56E+05 ± 3.74E+05	94.27 ± 0.56%
DMSO 66	5.63E+06 ± 3.01E+06	96.25 ± 1.60%
DMSO 84	9.28E+06 ± 2.86E+06	90.85 ± 7.89%
DMSO 144	8.27E+06 ± 1.11E+06	87.41 ± 1.65%

Table 1. DMSO cryopreservation technique. Average number of cells frozen versus average percent of viable cells at 16, 36, 66, 84, and 144.

Hour	Average ± SD Cells Frozen	Average ± SD Percent Cell Survival
UTEX 16	2.35E+05 ± 1.41E+05	92.59 ± 3.14%
UTEX 36	5.56E+05 ± 3.74E+05	93.99 ± 1.29%
UTEX 66	5.63E+06 ± 3.01E+06	96.69 ± 0.73%
UTEX 84	9.28E+06 ± 2.86E+06	90.85 ± 3.10%
UTEX 144	8.27E+06 ± 1.11E+06	94.91 ± 4.15%

Table 2. UTEX cryopreservation technique. Average number of cells frozen versus average percent of viable cells at 16, 36, 66, 84, and 144.

Hour	Average ± SD Cells Frozen	Average ± SD Percent Cell Survival
Glycerol 16	2.35E+05 ± 1.41E+05	92.77 ± 1.65%
Glycerol 36	5.56E+05 ± 3.74E+05	91.23 ± 1.13%
Glycerol 66	5.63E+06 ± 3.01E+06	91.29 ± 6.27%
Glycerol 84	9.28E+06 ± 2.86E+06	86.37 ± 2.63%
Glycerol 144	8.27E+06 ± 1.11E+06	86.68 ± 3.42%

Table 3. Glycerol cryopreservation technique. Average number of cells frozen versus average percent of viable cells at 16, 36, 66, 84, and 144.

Hour	Average ± SD Cells Frozen	Average ± SD Percent Cell Survival
GeneArt 16	2.35E+05 ± 1.41E+05	94.84 ± 3.91%
GeneArt 36	5.56E+05 ± 3.74E+05	96.13 ± 2.73%
GeneArt 66	5.63E+06 ± 3.01E+06	94.22 ± 0.30%
GeneArt 84	9.28E+06 ± 2.86E+06	94.45 ± 1.08%
GeneArt 144	8.27E+06 ± 1.11E+06	94.91 ± 2.53%

Table 4. GeneArt cryopreservation technique. Average number of cells frozen versus average percent of viable cells at 16, 36, 66, 84, and 144.

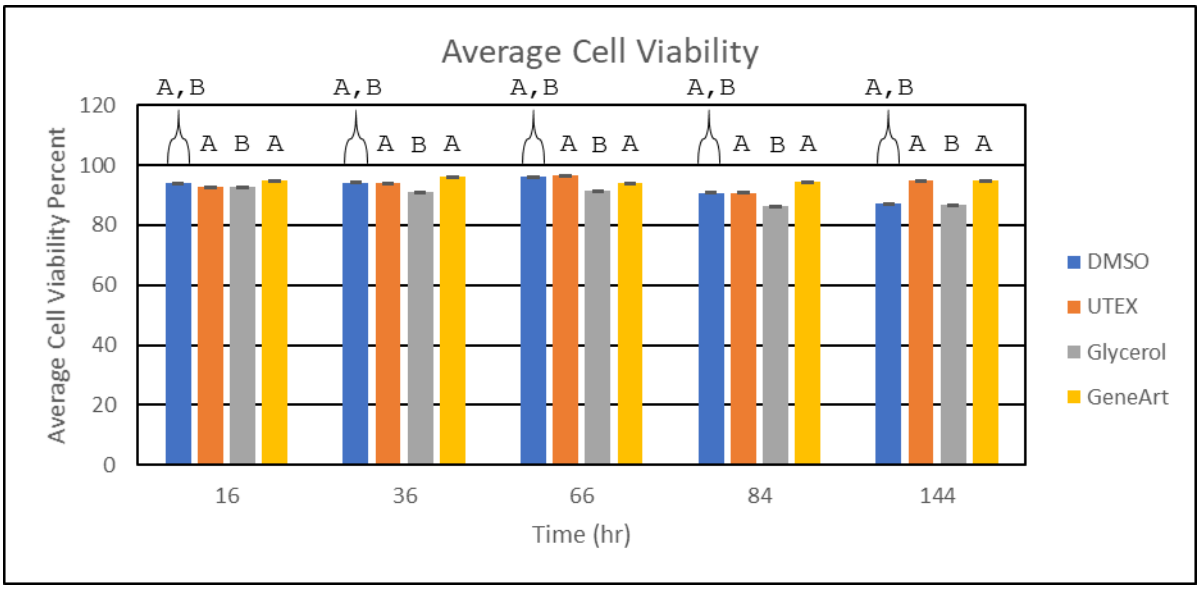

Figure 17. Average cell viability percent of experimental (internal replicates) *C. reinhardtii* cultures comparing the four cryopreservation techniques at hours of observation after thawing and Neutral Red staining. Statistical analysis was applied by conducting a MANOVA and a post hoc (Tukey- HSD) in which the GeneArt and Glycerol techniques are statistically significant when compared to one another, and the UTEX and Glycerol techniques are also significant when compared to each other. The DMSO, UTEX, and GeneArt techniques on the other hand are not significant when compared to each other, and the DMSO and Glycerol techniques were not significant when compared to each other.

CHAPTER IV

DISCUSSION

Growth of *C. reinhardtii* Preliminary Control Cultures

Control cultures C3 and C4 were used to determine a preliminary growth curve for the microalgae. After observing the growth of the algae by performing absorbance, cell counts, and lipid fluorescence for approximately five days. The time points in which the microalgae grew the best were at 16, 36, 66, 84, and 144 hours of growth. It was found that these time periods covered the entire growth curve from the lag phase, to the log phase, and to the stationary phase. The optimal growth properties of the preliminary cultures were determined by using absorbance and cell counts (Figure 3).

The absorbance levels demonstrated that the microalgae were getting denser as the time passed, starting at an average of 0.10 $\pm$ 0.00 AU of the two cultures at the 0-hour mark, and increasing continuously up until 72 hours of growth which resulted in an absorbance of 1.05 $\pm$ 0.02 AU. At 84 hours there was a slight decrease in the absorbance to 1.01 $\pm$ 0.06 AU, however by the end of the observed time period (160.5 hours) the absorbance increased back to 1.19 $\pm$ 0.01 AU. This result indicates that the algae entered at a stationary phase at about 84 hours (Figure 3).

Growth of *C. reinhardtii* Experimental Cultures

Four experimental microalgae cultures (internal replicates) were grown over the same period of five days with samples taken for freezing at hours 16, 36, 66, 84, and 144. Absorbance readings

were also taken at each time point to monitor the cell density for the cryopreservation process. The results indicate that the algae were in the lag phase from hours 16 to 36, entering the log phase from hours 36 to 84, and then entered a stationary phase after hour 84. The pattern was very similar to the average absorbance levels of the preliminary culture, which remained at a high absorbance the more time the cultures had for growth (Figure 4).

Average Preliminary Control Culture cells per mL. Preliminary cell counts were taken from control cultures C3 and C4 at hours 16, 36, 66, 84, and 144. These time points were selected to cover the lag phase, log phase, and stationary phase. At 0 hours, the cultures were inoculated with an average of 5.00 E+05 $\pm$ 0 cells/mL which increased in number very slowly until hour 36. Between hours 36 and 84 there was a much larger increase in the average cells/mL recorded. After hour 84 the cell counts began to slightly decrease. These results indicated that the microalgae were in the lag phase from hours 0 to 36, entered the log phase after hour 36 up until hour 84, and finally the stationary phase was then reached between 84 and 144 hours. Upon reaching stationary phase, the cells per mL counts seemed to decrease the older the culture was (Sasso *et al.*, 2018) (Figure 5).

Average Experimental Culture cells per mL. The four experimental microalgae cultures (internal replicates) were inoculated with 2.5 E+07 cells total (5.00 E+05 cells/mL starting concentration) had fluctuations in the average of cells/mL present in each culture, at each of the five time points (16, 36, 66, 84 and 144), however the overall trend was for the cell number to increase. The progression in the average cells/mL of the four cultures were very similar to the growth characteristics of the preliminary cultures. At the start of growth, the

cultures had a low cells/mL average at hour 16, which was 2.35 E+05 ± 8.79 E+04 cells/mL, up until hour 36 (3.56 E+05 ± 1.34 E+05 cells/mL), indicating that this was the lag phase. Similar to the preliminary culture, the experimental culture cells/mL average had a large increase in the cells/mL counts between hours 36 and 84 demonstrating that the algae culture had entered the log phase. After hour 84, the cell counts steadily decreased to hour 144 and this indicated the entry into the stationary phase (Sasso *et al.,* 2018) (Figure 6).

Average Lipid Production of Experimental Cultures. The fluorometric analysis of average lipids per cell at the five time points demonstrated that as more time passed, the more the lipid per cell average decreased. At the 16 hours there was an average of 2.64 E-02 ± 2.38 E-02 lipid fluorescence units/cell, which continuously decreased and stayed approximately the same up until hour 144 which had 2.19 E+04 ± 2.07 E+04 lipid fluorescence units/cell. The maximum lipid production appears to be at 16 hours and steadily decreased over time. At 16 hours, the culture appeared to produce the most lipids.

Based on the results from the one-way ANOVA comparing the average lipid fluorescence units/cell by time, results show that lipid production was statistically significantly different. After running the post hoc (Tukey-HSD) for further analysis, it was shown that at hour 16 lipids fluorescence units/cell were significantly higher than hours 36, 66, 84, and 144. The analysis showed that hour 16 had the greatest amount of lipids/cell ($P= 0.0001$). Hours 36, 66, 84 and 144 were not significantly different from each other regarding the lipid fluorescence units/cell. These results indicate that more lipids were present the younger the cultures were (hour 16) (Figure 7).

Cell Viability per Cryopreservation Technique. Post thawing, the experimental samples were diluted and stained with Neutral Red to determine how many cells survived the freezing and thawing process of each technique, by counting the cells with the hemocytometer. (Stefano, G. et al., 2017)

Data from trial 1 was not utilized because two out of the four techniques (GeneArt and UTEX) were significantly different in comparison to trials 2 and 3. This was due to slightly different methodology used in trials 2 and 3, after trial 1 resulted in contamination post-thawing and regrowth. In trial 1, the cells from the thawed samples had been diluted and washed multiple times to obtain a fixed number of 40,000 cells/mL to spread 250 µL of the sample onto three TAP agar plates for regrowth and visual quantification of the colonies that grew, leaving the remaining 250 µL for neutral red staining and cell counts. However, the remaining 250 µL was already heavily diluted, which resulted in very minimal numbers of cells present on the hemocytometer to count. In trial 1, there were only approximately 2 to 5 cells, versus trials 2 and 3 which had approximately 20 to 30 cells. This cell inconsistency increased the probability of dead cells not being counted because they were not seen due to the heavy dilutions. As a result of this observation and other contaminating factors, the dilution and washing process step was then eliminated from trials 2 and 3. Therefore, slightly changing the methodology for trials 2 and 3 which affected the statistical analysis of the cell viability in trial 1, therefore, resulting in a statistical difference between trial 1 and trials 2 and 3. The conclusion was made that trial 1 was an outlier and eliminated from the analysis.

Comparing the different cryopreservation techniques with each other and at each time point with regard to percent viability, a determination was made as to what hour of growth would

be used for the cryopreservation experiment, so that the most successful technique could be found.

Using the four cryopreservation techniques, at 16 hours 2.35 E+05 ± 1.41 E+05 cells, at 36 hours 5.56 E+05 ± 3.74 E+05, at 66 hours 5.63 E+06 ± 3.01 E+06, at 84 hours 9.28 E+06 ± 2.86 E+06, and at 144 hours 8.27 E+06 ± 1.11 E+06 cells on average were frozen. With regard to the total number of cells frozen, within each technique (DMSO, UTEX, Glycerol, or GeneArt) do not appear to have a significant difference between the time periods and the number of cells frozen in the range of 2.35E +05 ± 1.41E +05 to 9.28E +06 ± 2.86E +06 total cells frozen (Tables 1- 4).

At the 16th hour, all four cryopreservation techniques resulted in a high cell viability, but the highest number of viable cells counted immediately after thawing came from the modified GeneArt cryopreservation technique at 94.84%. At hour 36, the technique that had the highest cell viability was the modified GeneArt technique once again at 96.13%. At hour 66 the UTEX technique and the DMSO technique were very close to each other in cell viability, but the highest was UTEX at 96.69%. The GeneArt technique also displayed the highest cell viability with 94.45% at hour 84. At hour 144 the UTEX and GeneArt techniques both resulted in 94.91% viable cells. Based on the results, the modified GeneArt cryopreservation technique was most successful, showing the highest percentages of live cell recovery at four of the five selected time points (Figures 12-16).

The DMSO cryopreservation technique had its highest cell viability (96.25%) at hour 66, with its lowest percentage of viable cells (87.41%) at 144 hours. The UTEX cryopreservation technique also demonstrated that its highest cell viability at hour 66 was 96.69% of viable cells, and its lowest percentage of viable cells was at 84 hours with 90.85%. The Glycerol

cryopreservation technique on the other hand, had its highest cell viability at hour 16 with 92.77% of the cells staining with neutral red. The lowest percentage of viable cells for the Glycerol technique was 86.37% at 84 hours. Lastly, with regard to the GeneArt technique, it was most successful at 36 hours with 96.13% of viable cells and was least successful at 66 hours with 94.22% viable cells. These results indicate that the most successful time point for cryopreservation was at hour 66, and the least successful time point was at hour 144 for all of the studied techniques (Figures 8-11).

After analyzing the cell viability at each time point of the different cryopreservation techniques by performing a MANOVA, it can be determined that the factor of time was statistically significant (P= 0.0114), and the technique used was also statistically significant (P= 0.0014). Additionally, when performing a post hoc test (Tukey-HSD), When analyzing the cryopreservation techniques, GeneArt and Glycerol were significantly different when compared to each other, as well as UTEX and Glycerol techniques when compared to each other. The UTEX, DMSO and GeneArt techniques were not significant in comparison to one another, and DMSO and Glycerol were not significant when compared to each other. These results indicate that the technique with the highest cell viability after the thawing process was the modified GeneArt, and the one with least cell viability was Glycerol over all the time points (Figure 17).

Upon analysis of the time points, hours 66 and 144 were found to be significant when compared to each other. Hours 16, 36, and 84 were found to be not significant when compared to one another. These results indicate the time point in which cells were most viable after thawing and recovery was at 66 hours, and the least viable at 144 hours (Figure 17).

Regrowth and Cell Quantification on Petri Dishes. After the microalgae samples had been thawed out, they were counted for viable cells, and the samples diluted to a concentration of

40,000 cells/mL. Then the cells were spread (250 µL containing a total of 10,000 cells) onto three replicate petri dishes from the cultures that were sampled at the same optimal time points previously mentioned, using a cell spreader, and placed under low intensity fluorescent lights (50 µEinsteins) for approximately 12 weeks.

The intent was to quantify how many cells were successfully recovered from the cryopreservation process by calculating the number of colonies present and dividing it by 10,000, which would give the percentage of the number of cells that survived the cryopreservation process. After 12 weeks of growth on the petri dishes it was observed that no viable algae cells survived the plating process, and that various species of bacteria and fungi were growing on the plates instead. The cell plating process was repeated multiple times, but contamination still occurred, the viable algae cells did not regrow, resulting in no usable data for these experiments.

To further determine the source of contamination, the TAP media being used in the experiments was tested for contamination by adding 200µL of the media to three TAP agar plates. They were placed under the fluorescent lights for approximately one week and were then observed to see if the contamination was coming from the liquid TAP media. After the one-week growth period, the bacteria and fungi grew once again, leading to the conclusion that the liquid TAP media or the TAP plates had been exposed to an unsterile environment at some point during the experimentation process.

As a result of this, fresh sterile TAP media and TAP agar plates were made, and 200µL of the new liquid TAP media was spread on three TAP agar plates, and they were incubated underneath the low intensity lights for one week, as to observe for any fungal or bacterial growth. No fungal or bacterial growth was observed, and the new media was determined to be

sterile. However, when repeating the cell plating process with the new agar plates and fresh TAP media, contamination occurred once again, therefore the contamination was determined to be coming from the microalgal culture cryopreserved samples. Until the contamination issues can be resolved, this technique will not be in a functional form and therefore with regard to this thesis was unable to be utilized as a technique to determine algal cell viability post cryopreservation.

Conclusion

The lag, log, and stationary phased for both the preliminary cultures and the internal replicate experimental cultures showed that the lag phase started at inoculation and persisted through hour 36. The log phase then followed starting at hour 36 and progressed up until hour 84, after which the cultures then reached the stationary phase at 84 hours and this continued up to the last time point taken which was 144 hours. The average cells/mL for the cultures also resulted in similar lag, log, and stationary phase time points. Although the absorbance levels became higher the more that time passed, the average cells/mL for the cultures reached a maximum at 84 hours. This indicates that probably cell debris is collecting in the culture as time passes, causing the cultures to have a higher absorbance, but with a lower average cells/mL (Sasso *et al.*, 2018).

The lipid fluorescence units/cell average values of the experimental cultures were highest when the cultures were at hour 16. The lipid fluorescence units/cell values dropped significantly at hours 36, 66, 84, and 144. There was a statistically significant difference between hours 16 and 36, 66, 84, and 144, therefore hour 16 would be the most ideal time point to collect the microalgae samples for lipid production.

Four different cryopreservation techniques were tested by using a -80º C freezer and a Mr. Frosty freezing container instead of using liquid nitrogen and a storage Dewar. The techniques tested were a modified GeneArt preservation kit protocol, a modified UTEX protocol, a self-generated DMSO protocol, and a self-generated Glycerol protocol. All techniques are very inexpensive compared to liquid nitrogen, the most inexpensive one would be Glycerol, followed by UTEX (methanol), then DMSO, and finally the GeneArt protocol (Taylor and Fletcher, 1998).

Optimal cell viability post cryopreservation was found to be from the 66-hour sample time point for all four techniques. Samples taken at hour 144 were found to have the lowest post cryopreservation cell viability. The technique that had the highest cell viability overall post-thawing and recovery (at all sampled time points) was the modified GeneArt cryopreservation kit technique. The least successful cryopreservation technique at all sampled time points was the self-generated glycerol protocol. However, given all the protocols at all sampling time points, the lowest cell viability was 86.37% (Glycerol at 84 hours), which is still a very good post cryopreservation cell viability, and still a useful level of viable cells for culture initiation.

The infrastructure required for freezing and maintaining cryopreserved cells in liquid nitrogen is very expensive. The storage Dewars and the constant need for liquid nitrogen supplies result in a high initial cost as well as high maintenance cost. If any of the four cryopreservation techniques which store the algae in a -80 ºC freezer are useful, then costs will be cut significantly. The only major cost is that of the -80 ºC freezer itself. However, the long-term costs are just the electricity to power the freezer. (Abreu *et al.,* 2012)

This study assisted future research related to the cryopreservation of microalgae with the application of different cryopreservation techniques. This project aided in providing more

information on a faster and more cost-efficient method of cryopreservation, which can facilitate the cryopreservation of microalgal cultures for use in biofuel production.

Dr. Schonna Manning from the University of Texas at Austin was contacted for guidance with the cryopreservation technique cell recovery process. She advised giving the post cryopreservation cultures more time (30 days) to remain in the cryovials under low light after thawing.

To make future experiments more successful, instead of plating the cells immediately after thawing the cryopreserved samples, or inoculating them into liquid TAP media, it is suggested to quickly thaw the samples and incubate them under fluorescent lights for a prolonged amount of time, to allow the cells to recuperate from the cryopreservation stress. Once the cells have had an extended time to recover, then they can then be transferred to liquid media or spread on TAP agar plates for regrowth and quantification of viable cells post cryopreservation.

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APPENDIX

APPENDIX

Algae Media Protocol

Solution #	Solution	Amount (2 L)	Amount (1 L)	Amount (0.5 L)	Amount (0.25 L)
1	Beijerinck's Solution	100 mL/2 L	50 mL/1 L	25 mL/0.5 L	12.5 mL/0.25 L
2	Phophate Buffer Stock Solution	17 mL/2 L	8.5 mL/1 L	4.25 mL/0.5 L	2.13 mL/0.25 L
3	Hunter's Trace Stock Solution	2 mL/2 L	1 mL/1 L	0.5 mL/0.5 L	0.25 mL/0.25 L
4	Tris Acetate Stock Solution	20 mL/2 L	10 mL/1 L	5 mL/0.5 L	2.5 mL/0.25 L

Beijernick's Solution

#	Component	Amount	Final Concentration
1	Ammonium chloride (NH_4Cl)	8 g/L	7.5 mM
2	Calcium chloride dihydrate ($CaCl \cdot 2H_2O$)	1 g/L	0.35 mM
	*Can substitute with calcium chloride anhydrous (0.755 g/L)		
3	Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	2 g/L	0.4 mM

Phosphate Buffer Solution			
#	Component	Amount	Final Concentration
1	Sodium phosphate dibasic (Na_2HPO_4)	11.62 g/L	7.5 mM
2	Potassium phosphate monobasic (KH_2PO_4)	7.26 g/L	0.35 mM

Hunter's Trace Stock Solution			
#	Component	Amount	Final Concentration
1	Ethylenediaminetetraacetic acid disodium salt dihydrate ($Na_2EDTA•2H_2O$)	50 g/L	0.134 mM
2	Zinc sulfate heptahydrate ($ZnSO_4•7H_2O$)	22 g/L	0.077 mM
3	Boric acid (H_3BO_3)	11.4 g/L	0.184 mM
4	Manganese(II) chloride tetrahydrate ($MnCl_2•4H_2O$)	5.1 g/L	0.026 mM
5	Iron(II) sulfate heptahydrate ($FeSO_4•7H_2O$)	5 g/L	0.018 mM
6	Cobalt(II) chloride hexahydrate ($CoCl_2•6H_2O$)	1.6 g/L	0.007 mM
7	Copper(II) sulfate pentahydrate ($CuSO_4•5H_2O$)	1.16 g/L	0.005 mM
8	Ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}•4H_2O$)	1.1 g/L	0.0008 mM

Tris Acetate Stock Solution			
#	Component	Amount	Final Concentration
1	Trizma Base	121 g/500 mL	0.02 M
2	Glacial Acetic Acid (CH_3CO_2H)	50 mL/500 mL	0.017 M

Table 5. Protocol for the preparation of 2L of TAP media.

BIOGRAPHICAL SKETCH

Jazmine Leija was born on the 29th of September in 1997 in Lemoore, California. Jazmine began her undergraduate studies at the University of Texas Rio Grande Valley, in Edinburg Texas, in August 2016 and graduated with a bachelor's degree in Biology in the Fall of 2018. In her time as an undergraduate student, she worked part-time at the McAllen Public Library, as well as participated in Dr. Michael Persans' research lab in the Biology Department at UTRGV.

Jazmine started her graduate studies at the University of Texas Rio Grande Valley in June 2019. She worked as a graduate teaching assistant for Anatomy and Physiology I from August 2019 to July 2021 with the department of Biology at UTRGV. Jazmine received her Master of Science degree in Biochemistry and Molecular Biology from the University of Texas Rio Grande Valley in August 2021.

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