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Prospecting for and Isolation of Microalgae in South Texas

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PROSPECTING FOR AND ISOLATION OF MICROALGAE IN SOUTH TEXAS

A Thesis

by

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ABSTRACT

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Microalgae are a very important microorganisms that can be used for beneficial purposes. One way that algae are important to Earth is the contribution of oxygen that they provide to the atmosphere, which many aerobic organisms rely on. Carbon dioxide pollution of the atmosphere has been one of the greatest possible causes for global warming, a large proportion of this pollution is due to the use of fossils fuels. One of the ways to reduce the carbon footprint of fossil fuels is to replace the use of those fuels in automobiles and industry. Microalgae can produce lipids that can be used to synthesize biofuels. These biofuels leave a smaller carbon footprint since they are a source of green energy. By replacing fossil fuels with biofuels, the overall contribution of carbon dioxide to the atmosphere can be reduced. It is necessary to find a microalgae that produces a high yield that can be converted into desired biofuels. Many microalgae found in the environment might be great candidates to produce biofuels. Therefore, we conducted a survey of five different algal species found in surface fresh water in the area of Edinburg Texas. Two microalgal isolates, SB and 18A, were found to have the most amendable characteristics for biofuel production when grown on MS media. SB, 18A, 2/4A, and 4/4B were grown in HS, they had some traits for increased lipid production that would make them useful for biofuel production.

DEDICATION

All my efforts were made for my family, who always believed in me, and never doubted that I could do this. Mom, dad, I finally did it! Thank you for all your trust, cheers and all the things you taught me which made me be the person that I am today. To my grandparents who are watching me from somewhere else who always told me to keep studying. Grandma Gabby, thank you for all the support you gave me and everyone else. I would not be here if it wasn't for you. To my siblings that always looked at me like I was the smartest person they knew, although I have a long way to become close to what you think I am. Galan, my dog, my old little brother. Every single day I told you that this was temporary and after this I would have more time to play and be with you. The time finally came. I am sorry for making you wait for so long. Thank you friends, for believing in me without doubt, and to all the professors that always believed that I would go farther than expected. You all mean the world to me.

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CHAPTER I

INTRODUCTION

Climate change has been one of the potential greatest problems in recent years that affects the environment of the Earth. One of the biggest contributors to this problem is the use of fossil fuels, which cause carbon dioxide to build up in the atmosphere, having a negative impact for humans and other living organisms. Almost 80% of the World's energy demand comes from these fossil fuels, and if there is no alternative, such as biofuels or other types of green energy, global warming will only worsen (Medeiros *et al.,* 2015). Biofuels are a more carbon neutral source of energy than the fossil fuels, aside from the carbon generated in the production of biofuels, they are mostly carbon neutral.

There have been studies on which species of organisms can be used to produce green energy. Microalgae can be considered to be one of the most important organisms on Earth due to their ability to reduce the amount of carbon dioxide in the atmosphere and produce oxygen. Most of the Earth is covered by ocean, and almost 50% of the oxygen that aerobic organisms breathe is comes from the algae that is present in the ocean (Chapman, 2010). It is estimated that there may be over 200,000 number of species of algae, however only about 32,500 species have been taxonomically identified and accepted as distinct species. (Guiry, 2012).

One of the greatest advantages that microalgae have is their ability to fix carbon dioxide and to produce lipids, which then can be converted onto biofuels, resulting in a negative carbon

footprint. This can be a great mechanism for the replacement of fossil fuels that are being used today for daily needs, such as for transportation (Chapman, 2010). There are different approaches for minimizing the emission of carbon dioxide from carbon combustion. One of the approaches used is the use of pellets which are created from microalgae. These pellets can be burnt with the help of machines, such as a circulating fluidized bed, on which by the combustion of these products will lead to generation of energy by the hot gases produced form the combustion of the microalgae's biomass. This is one alternative to use a greener energy source rather than use fossil fuel (Golachowska *et al.,* 2017). Not only are pellets an alternative source of energy, but another alternate source is also to use extracted lipids to generate liquid biofuels as well. Microalgae are being studied to produce a better lipid yield so that production of biofuels can result in lower costs (Chang and Su, 2010).

Environmental stresses can be applied to microalgae to increase the yield of lipids. Stress can have effects on the accumulation and synthesis of the lipids inside the cell for survival purposes. Finding the right stress factors will make the biofuel production more efficient, however, more research must be done to find the best stressor for the algae to be induced to over produce lipids. Some of the environmental factors that can be manipulated are the type of medias on which the algae are grown, the pH level or nutrient availability, as well as other factors such as temperature, light exposure, and aeration of the culture (Chen *et al.,* 2017).

In addition to the manipulation of the environment to stress the algae to enhance the production of lipids, each microalgal species can also have different base amounts of lipids present. Not all the microalgae are the same, depending on their individual metabolism, survival mechanisms, morphology or even Genus, some species will be better suited for biofuel production (Chen *et al.,* 2017).

Even though microalgae are green and have chlorophyll, it can turn from an autotrophic lifestyle to a heterotrophic one depending on environmental conditions. Microalgae that can live both ways can be considered to be photoheterotrophic. These microalgae can be grown in the light; however, the addition of fixed carbon can possibly enhance the overall lipid production of the cells (Lowery *et al.,* 2014).

Since autotrophic microalgae will be more light-dependent and can be shaded in a dense culture, this has effects on lipid production. The stationary phase of a culture is defined as the point on where the growth is limited by environmental factors and causes the culture to reach the saturation point (Lowery *et al.,* 2014). By knowing this value, lipid production may be enhanced by testing mixotrophic cultures with media that contains fixed carbon. Therefore, the microalgae may be able to produce a higher lipid content in reduced lighting conditions. Further research experiments should be conducted in this area to determine what tradeoffs, if any, will be needed to implement a possible more efficient way to produce biofuels (Lowery *et al.,* 2014).

Different environmental factors, such as the interactions with other species, temperature changes on the area, or availability of nutrients have differing impacts on living organisms. Microalgae can be found in many different aqueous environments with varying properties. South Texas has extremely high temperatures (greater than 32ºC) for most of the year, as well as brackish water sources which may contain a higher level of salts due to evaporation. This may result in stress on the microalgae which may force them to produce more lipids. Water samples from bodies located in the Edinburg area of South Texas are good places to prospect for microalgae that can be isolated and tested for lipid productions in order to determine if there are any potential microalgal candidates that will contain a high level of lipids. By prospecting for the different species found in the samples it may be possible to find several new algal species that

may be well suited for biofuel production. Properties such as fast growth, the ability to grow with a high density and have a high lipid production are desired characteristics for a microalga that would be useful for biofuel production (Nielsen-Gammon, 2019).

It is hypothesized that at least one of the five microalgal strains that were isolated from the sample sites Edinburg Scenic Wetlands & World Birding Center and Edinburg Municipal Park area will have positive characteristics of a microalgae that can be used for biofuel production.

It is also hypothesized that one of the two medias studied (1/2 X MS + Gamborg's B5 Vitamins or 1.0 X Hoagland's solution + Fe + Gamborg's B5 Vitamins) will result in faster growth, high density, and high levels of lipids when the microalgae are grown.

CHAPTER II

METHODOLOGY

Collection of samples from local ponds, a park, and irrigation water sources

Samples used for the experiments were collected from four different bodies of water from places very close to the University of Texas Rio Grande Valley, in Edinburg, TX. Two samples were from local ponds at the Edinburg Scenic Wetlands & World Birding Center, one was from the Edinburg Municipal Park located very close to the ponds, and the fourth sample was from an irrigation canal located approximately 100 meters away from the Edinburg Municipal Park.

The four samples (in 250 mL bottles) were labeled as 1, 2, 3, and 4, and 250 mL of water was collected from each location above. Sample 1 was labeled as Big Pond (26°17'31.7"N 98°08'09.1"W), sample 2 was labeled as Small Pond (26°17'30.5"N 98°08'06.8"W), sample 3 was labeled as Park (26°17'21.1"N 98°07'51.2"W), and sample 4 was labeled as Irrigation Canal (26°17'23.6"N 98°07'60.0"W).

In addition, algae samples were taken from the shaded side of the HVAC condensation tank exterior located next to the Science building on the UTRGV campus (26°18'24.5"N 98°10'17.3"W) This sample was labeled as SB.

Preparation of media: 1/2 X MS + Gamborg's B5 Vitamins (and variations)

To prepare 500 mL of 1/2 X MS + Gamborg's B5 Vitamins, 1.1 g of Murashige & Skoog powder (CAISSON, Smithfield, UT) and 0.25 g of MES (Sigma, St. Louis, MO) was added to 450 mL of distilled deionized water in a 1 L flask. The solution was stirred using a stir bar and a stir plate until the solids were dissolved. Then, the solution was brought to a final pH of 5.7 by adding drops of a 1 M KOH solution and distilled deionized water to make a final volume of 500 mL. For media containing agar, 7.5 g of plant tissue culture agar (Milipore Sigma, St. Louis, MO) (final agar concentration of 1.5%) was added to the media. The media solution was autoclaved at 120ºC 15 lbs/sq in for 35 min.

For the microalgae cultures, 500 mL of media was prepared and 50 mL aliquoted in 125 mL flasks that were then sealed with a foam rubber plug and flask cap. The liquid media solution was autoclaved at 120ºC at 15 lbs/sq in for 35 min.

Media with agar was poured into 100 mm X 15 mm petri-dishes (ThermoFisher Scientific, Waltham, MA). and once the agar solidified, the plates were dried in the laminar flow hood until the lids had no more condensation present.

Preparation of medial: Hoagland's Solution + Fe (and variations)

Hoagland's solution was prepared as a 4.0 X stock solution. To prepare, 9.5 L of water were added into a 10 L container. To prepare the solution, 46 g of Ammonium dihydrogen phosphate, 26.44 g of Calcium nitrate, 19.64 g Magnesium sulfate heptahydrate (9.56 g if Anhydrous), and 24.28 g of Potassium Nitrate were added until dissolved into the 9.5 L. Once those components were dissolved, 40 mL of each following stock solutions were added: Zinc sulfate heptahydrate, Dihydrogen borate, Manganese (II) chloride tetrahydrate and Molybdenum

trioxide (MoO3). Once everything was mixed into the solution, DDH_2O was added to have a final volume of 10 L of solution.

To prepare the Fe Solution that is added to the Hoagland's Solution, a stock solution of Diethylenetriaminepentaacetic acid Iron (III) disodium salt hydrate, 98% (Fe DTPA) was prepared. For a 4 L stock solution, 2.104 g of Fe DTPA was added to 3.95 L in a 4 L bottle and mixed. After mixing, the solution was brought up to 4 L with DDH_2O and filter sterilized using a 0.22 μM filter sterilization unit (Corning, Corning, NY).

From the 4.0 X Hoagland's Solution stock, various dilutions were made depending on the experiments that were conducted.

To prepare 500 mL of 1.0 X Hoagland's solution + Fe + Gamborg's B5 vitamins, 125 mL of 4.0 X Hoagland's Solution was mixed with 375 mL of DDH_2O. Once the diluted solution was mixed, 0.5 mL of Gamborg's B5 vitamins X 1000 Solution (CAISSON, Smithfield, UT) were added, then 2.5 mL of the Fe DTPA stock solution was added, then the whole solution was filtered, or media was allocated in 50 mL portions into 125 mL Erlenmeyer's flasks, covered with foam insert and flask cap, then autoclaved for 35 minutes at 120ºC at 15 lbs/sq in. Once the media was autoclaved and at a room temperature, 0.25mL of the filtered Iron was added to each flask.

Experimental setup

Microalgae were grown on 100 mm X 15mm agar plates (ThermoFisher Scientific, Waltham, MA), 24-well plates (Becton Dickinson Labware, Franklin Lakes, NJ), and 125 mL Erlenmeyer flasks. for culture isolation. For the isolation and maintenance of cultures for initial screening, these were grown on 100 mm X 15mm agar plates containing either 1/2 X MS +

Gamborg's B5 Vitamins or 0.1 X, 0.25 X, 0.5 X, or 1.0 X Hoagland's solution + Fe with or without 1.0 X antibiotic/antimycotic solution (CAISSON, Smithfield, UT). These microalgae were grown with a light timing that was set to 12 hours of light (28ºC, 25 µmol m^{-2} s^{-1}) and 12 hours of darkness (25ºC).

For maintenance of isolated microalgal cultures, these were grown on 1/2 X MS + Gamborg's B5 Vitamins or 1.0 X Hoagland's solution + Fe + Gamborg's B5 Vitamins. The light cycle was at 8 hours of light (50 µmol m^{-2} s^{-1}) and 16 hours of darkness with a day temperature of 28ºC and a night temperature of 22ºC.

For experiments conducted on purified microalgae in the 125 mL Erlenmeyer flasks, these were grown at a light intensity of between 250 and 350 µmol m^{-2} s^{-1}, with an 8-hour light and 16-hour dark schedule shaking at 100 rpm on an orbital shaker (Labnet International, Edison, NJ). The day temperature was 30ºC with a night temperature of 22ºC.

Light was provided by low intensity fluorescent lamps (Envirolite, Troy, MI) for the initial screening and for the maintenance of cultures, however, experiments in the 125 mL Erlenmeyer flasks, the light was provided by high intensity fluorescent lamps (Sunlight Supply Incorporated and Vancouver, WA).

Isolation of algae from samples collected

Different isolation methods were used to get a culture of a pure single type of algae. One of the methods used serial dilution in 24-well plates, while the other method was streaking for single colonies on 100 mm X 15mm petri plates. Also, two different medias were used to isolate microalgal colonies, Murashige and Skoog or Hoagland's Solution.

For the serial dilution method, 24-well plates (4 rows X 6 columns) were used, in which each well was filled with 1800 µL of liquid media and 200 µL of the microalgal sample. Each row represented each unique sample, and the columns represented replicates of each unique sample. Four different 24-well plates were inoculated, one contained 1/2 X MS + Gamborg's B5 vitamins, and the other three contained 0.25 X, 0.5 X, and 1.0 X Hoagland's solution + Fe respectively.

Four replicates of each of the four different 24-well plates were generated. The serial diluted plates were grown for 30 days, then another serial dilution was made for each plate into a new 24-well plate until a pure microalgae culture was isolated successfully.

For the single colony streaking technique, 1 mL of microalgal samples were spread on 25 cm X 25 cm large petri dishes containing 1/2 X MS + B5 Vitamins + 0.05% sucrose. Four replicates for samples from bottles 1, 2, 3 and 4 were made and grown for 30 days. Visual inspection of the plates resulted in many different colony morphologies of microalgae. Single colonies of nine different algal morphologies were streaked on 100 mm X 15mm petri plates containing 1/2 X MS + Gamborg's B5 Vitamins or 0.1 X, 0.25 X, 0.5 X, or 1.0 X Hoagland's solution + Fe with or without 1.0 X antibiotic/antimycotic solution. Through a process of continued re-streaking of the algae on the petri plates, eventually single isolated and purified colonies of each algal morphology were obtained.

Once all the microalgal samples were selected and purified using these methods, then they were subjected to growth curve, cell counting, and lipid analysis.

Labeling of microalgal isolates

Each microalgae strain was labeled based upon its point of origin. The microalgae strains 1/4A, 2/4A, and 4/4B came from the water samples 1, 2 and 4 respectively. These were obtained from dilution of the samples using the 24-well plates. Once isolated, 1/4A, 2/4A and 4/4B were spread on petri-dishes with 1/2 X MS + Gamborg's B5 vitamins and a variety of concentrations of Hoagland's Solution + Fe. Microalgal strain 18A came from sample 3 and was directly spread on big petri plates (250 mm X 250 mm) (Corning, Corning, NY) containing 1/2 X MS + Gamborg's B5 vitamins + 0.05% sucrose for isolation. SB was sampled from the exterior of the HVAC water recovery tank outside the Science building at UTRGV. SB was isolated through spreading on small petri-plates (100 mm X 15 mm) containing 0.5 X Hoagland's Solution + Fe.

Identification of the microalgae strains

Taxonomy books (Presscott, 1982, Wehr and Sheath, 2015) and a database for microalgae (https://utex.org) were used to identify the microalgal isolates. Notes about the morphology, shape, size, arrangement of the cells, and locations on where they can potentially be found were made. The microalgal isolates then were compared to the microalgal Genera from the books and databases for taxonomic identification.

Growth experiments

Each microalgal sample was inoculated into a 125 mL Erlenmeyer flask which contained 50 mL of either 1/2 X MS + Gamborg's B5 Vitamins or 1.0 x Hoagland's solution + Fe + Gamborg's B5 Vitamins. The flasks had 50 mL of autoclaved media with a foam plug and plastic flask cap. Four internal replicates of each microalgae sample were made, and three trials were conducted.

Starter cultures were inoculated from samples grown on the petri-plates until the cell density was at least 2.5 E+07 cells/mL to start a lipid assay. Those starter cultures were grown under the same way as the experimental cultures for the lipid assays.

The flasks were inoculated with the volume of media from a starter culture necessary to have a final concentration of 5.0 E+05 microalgae per mL in 50 mL (2.5 E+07 total microalgal cells). The microalgae cultures were grown for 21 days for both 1/2 X MS + vitamins and 1.0 X HS.

For the microalgae growing in 1/2 X MS + Gamborg's B5 Vitamins, the experimental assays were conducted every 3 days (1/4A, 2/4A, 4/4B, and 18A) on day 0, 3, 6, 9, 12, 15, 18, and 21. For SB, experimental assays were conducted every day for a total of 7 days on day 0, 1, 2, 3, 4, 5, 6, and 7.

For the microalgae growing in 1.0 x HS + Fe + Gamborg's B5 vitamins, the experimental assays were conducted every 7 days (1/4A, 2/4A, 4/4B,18A and SB), on day 0, 7, 14, and 21.

Quantitating cell growth using absorbance at 600 nm

Spectrophotometry was used to record the density increase in the microalgal cultures. Two different blanks were created and labeled depending on which media was used in the experiment. One blank contained 1 mL of 1/2 X MS + Gamborg's B5 vitamins and the other contained 1 mL of 1.0 x H.S. + Fe + Gamborg's B5 vitamins. The spectrophotometer (ThermoFisher Scientific, Waltham, MA) was set at 600 nm and zeroed by using the corresponding blanks before taking the absorbance values of the experimental samples. Absorbance values were used to make a growth curve graph.

Cell counts using hemocytometer

Microalgal cells are counted under the microscope (ThermoFisher Scientific, Waltham, MA) by using a hemocytometer (Hausser Scientific, Horsham, PA) with a hemocytometer coverslip (Propper manufacturing, Long Island City, NY). The cells were counted at a magnification of 400 X. Five large squares were counted and the number of cells in each large square was noted. The microalgal cells were diluted from the original culture as to result in a count of 50 to 250 cells per one large square. The cells per milliliter value was determined by the mathematical equation:

Number of cells total in 5 large squares divided by 5 times the dilution factor, then multiplied by 10,000 and this will equal the number of cells per milliliter. These cell count data were used to construct a growth curve graph for each experimental trial.

Lipid Assay using Nile Red Fluorescence

To determine the lipid fluorescence units per cell, the diluted microalgae cultures from the cell counting were used. To 4 mL cuvettes (ThermoFisher Scientific, Waltham, MA) were added 1 mL of media, 1 mL of microalgal diluted culture and 20 µL of a 10% solution v/v of Triton X 100 (final Triton X 100 concentration of 0.01%) (Hausser Scientific, Horsham, PA). These served as blanks to zero the fluorimeter (BioRad Laboratories, Hercules, CA) For experimental samples, to 4 mL cuvettes were added 1 mL of media, 1 mL of microalgal diluted culture, 20 µL of a 10% solution v/v of Triton X 100 and 2 µL of a 100 µg/mL solution of Nile Red (Kodak, Rochester, NY).

The final working solution in the cuvettes was incubated at room temperature (22ºC) for 10 minutes. Then, the cuvettes were sealed with parafilm, mixed by inversion 5 times, then

placed in the fluorimeter and read every 30 seconds for a total of 2 minutes (4 readings total per experimental sample). The four readings were averaged and used to create a bar graph of the lipid fluorescence units per cell by dividing the average lipid fluorescence units by the cells/mL cell count.

Statistical analyses

Averages and standard deviation were calculated by using Microsoft Excel (Microsoft, Redmond, WA). The data was used to create the graphs for the growth curves for absorbance levels and cell counts, and lipid assays in Microsoft Excel.

The statistical software package JMP (JMP, Cary, NC) was used to conduct a Tukey's HSD test to group the different strains of algae, different days and the different type of media related to the days where lipids are analyzed.

CHAPTER III

RESULTS

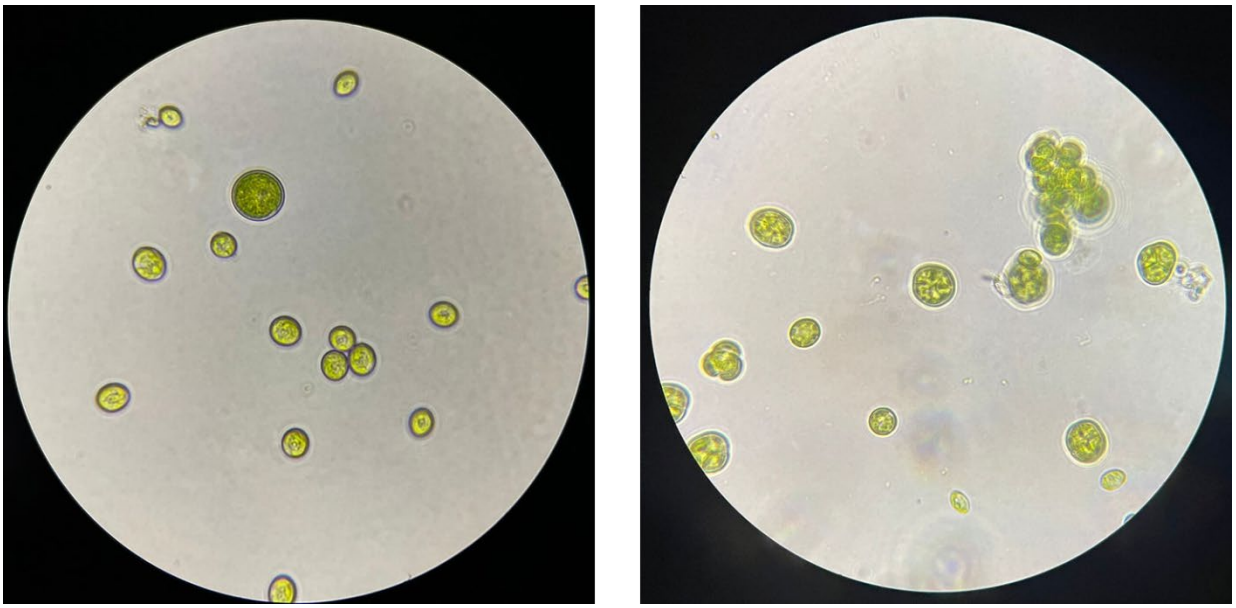

Figure 1. Microalgal isolate SB, preliminarily determined to be *Bracteococcus sp.* (1000X). (Photo provided by: Mauricio A. Peña)

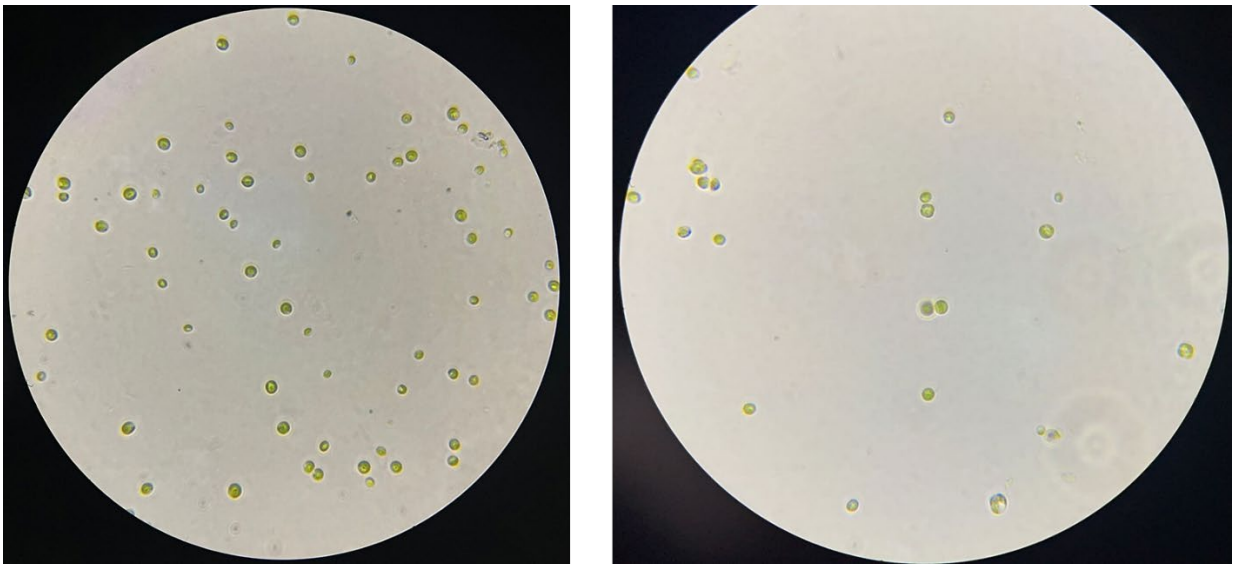

Figure 2. Microalgal isolate 18A, preliminarily determined to be *Chlorella sp.* (1000X). (Photo provided by: Mauricio A. Peña)

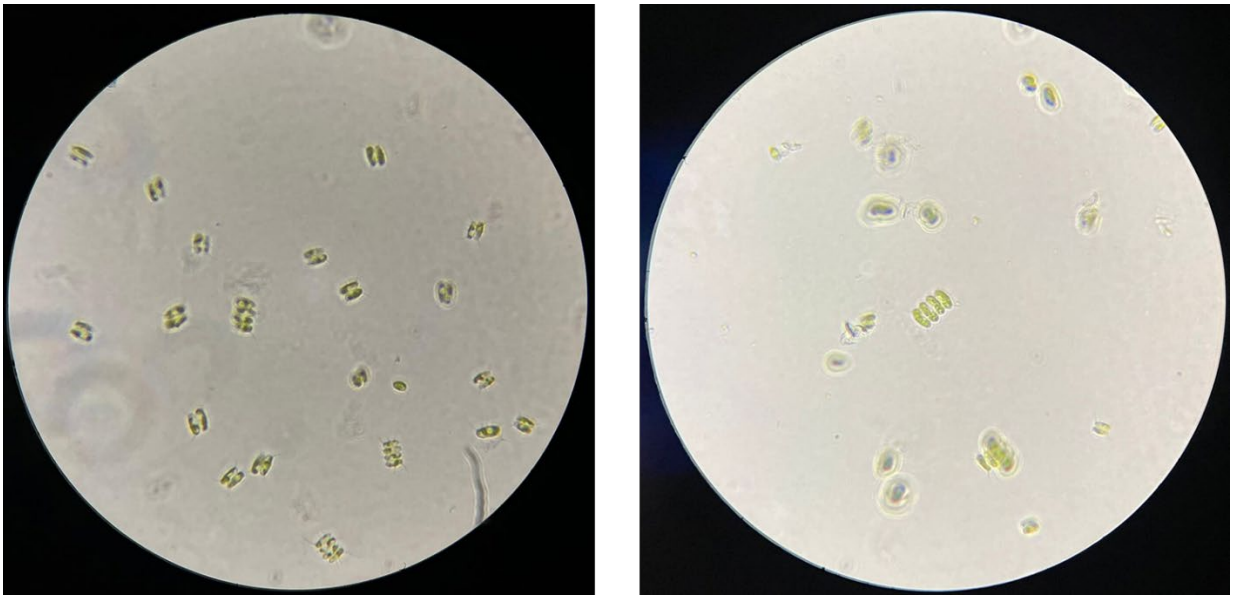

Figure 3. Microalgal isolate 1/4A, preliminarily determined to be *Scenedesmus sp.* (1000X). (Photo provided by: Mauricio A. Peña)

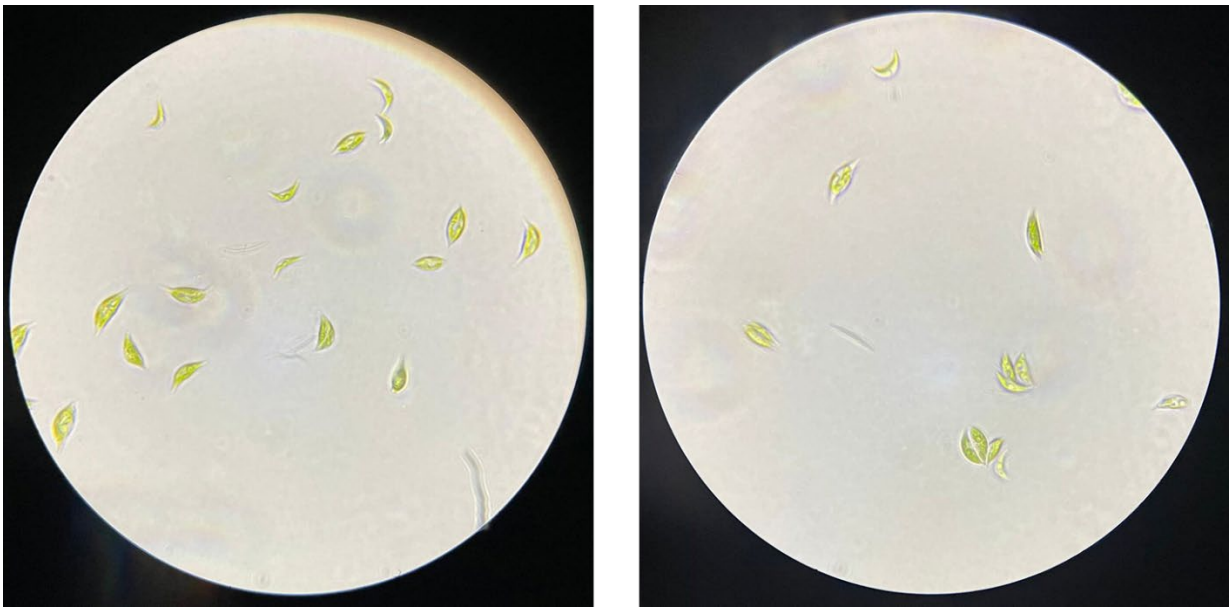

Figure 4. Microalgal isolate 2/4A, preliminarily determined to be *Scenedesmus sp.* (1000X). (Photo provided by: Mauricio A. Peña)

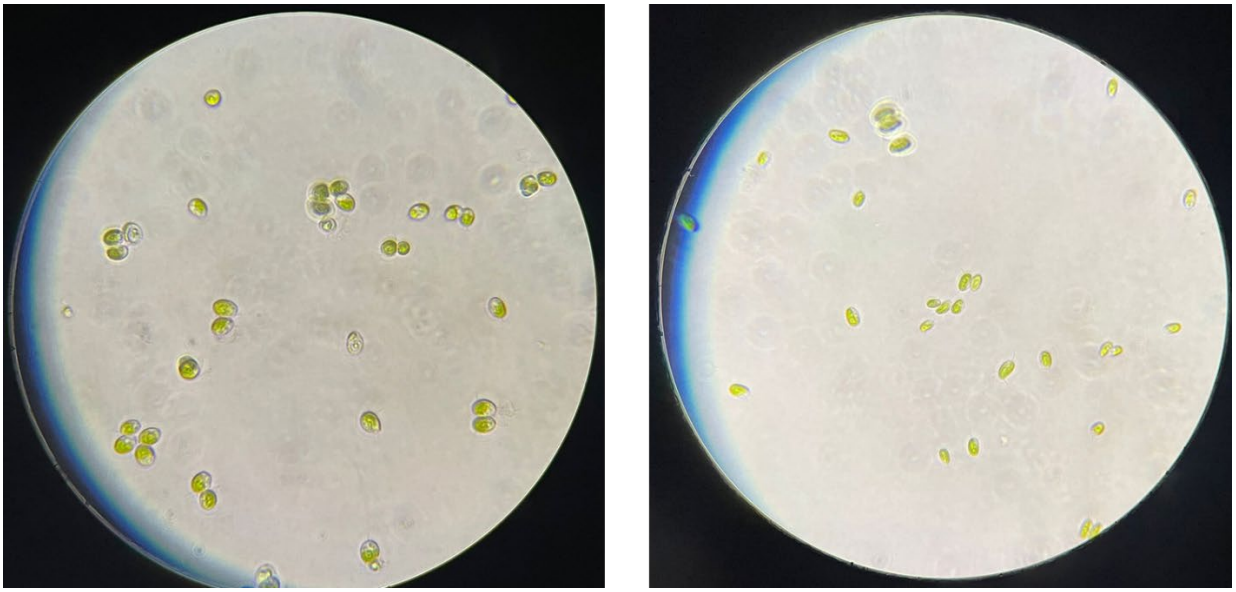

Figure 5. Microalgal isolate 4/4B, preliminarily determined to be *Tetrastrum sp.* (1000X). (Photo provided by: Mauricio A. Peña)

Five microalgal isolates were found from water samples taken at the Edinburg Scenic Wetlands & World Birding Center, Edinburg Municipal Park area, and the HVAC water recovery tank at the University of Texas Rio Grande Valley Science Building. These isolates were designated SB (water reclamation tank), 18A (Park), 1/4A (Big Pond), 2/4A (Small Pond), and 4/4B (Irrigation Canal).

Preliminarily identifications of microalgal isolate were made using dichotomous keys (Presscott, 1982, Wehr and Sheath, 2015), and the UTEX image database (https://utex.org/). SB was preliminarily identified as *Bracteococcus sp.*, isolate 18A was preliminarily identified as *Chlorella sp.*, isolate 1/4A was preliminarily identified as *Scenedesmus sp.*, isolate 2/4A was preliminarily identified as another separate *Scenedesmus sp.*, and isolate 4/4B was preliminarily identified as *Tetrastrum sp.*.

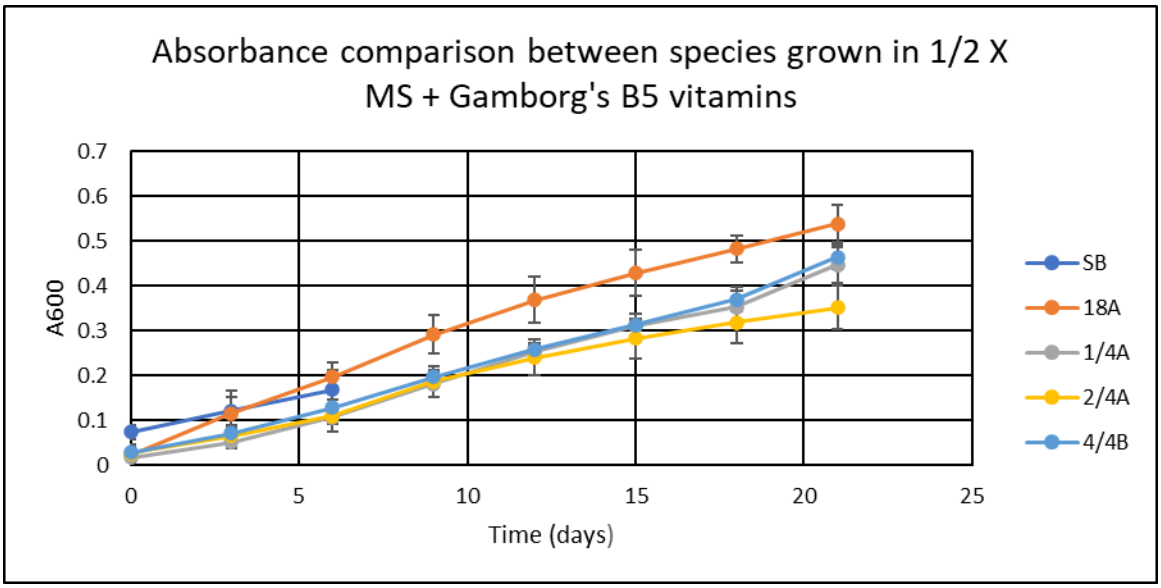

Figure 6. Absorbance comparison between microalgae species grown in 1/2 X MS + Gamborg's B5 vitamins.

Microalgal cultures were grown for 21 days (SB grown for 7 days) until the cells reached the saturation point. After 7 days of growth, SB reached a final average of 0.192 ± 0.044 Absorbance units. After 21 days of growth, 18A, 1/4A, 2/4A and 4/4B reached final averages of 0.538 ± 0.042, 0.446 ± 0.039, 0.351 ± 0.049, and 0.464 ± 0.028 Absorbance units respectively.

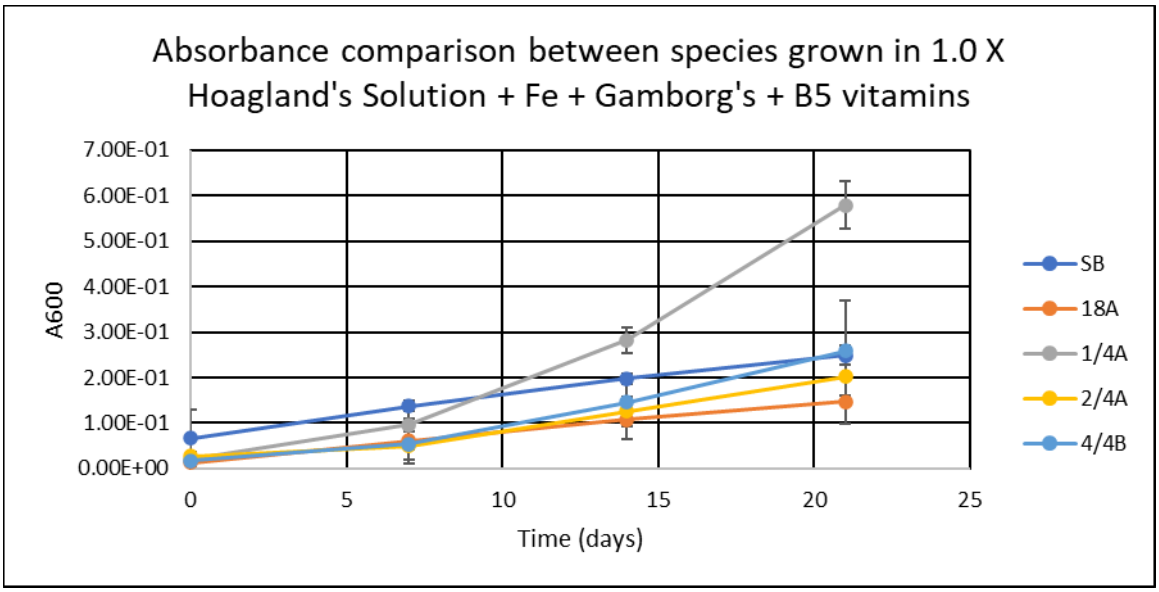

Figure 7. Absorbance comparison between microalgae species grown in 1.0 X Hoagland's Solution + Fe + Gamborg's B5 vitamins.

Microalgal cultures were grown for 21 days until the cells reached the saturation point. After 21 days of growth, SB, 18A, 1/4A, 2/4A and 4/4B reached final averages of 0.249 ± 0.021, 0.147 ± 0.049, 0.579 ± 0.053, 0.202 ± 0.041, and 0.258 ± 0.011 Absorbance units respectively.

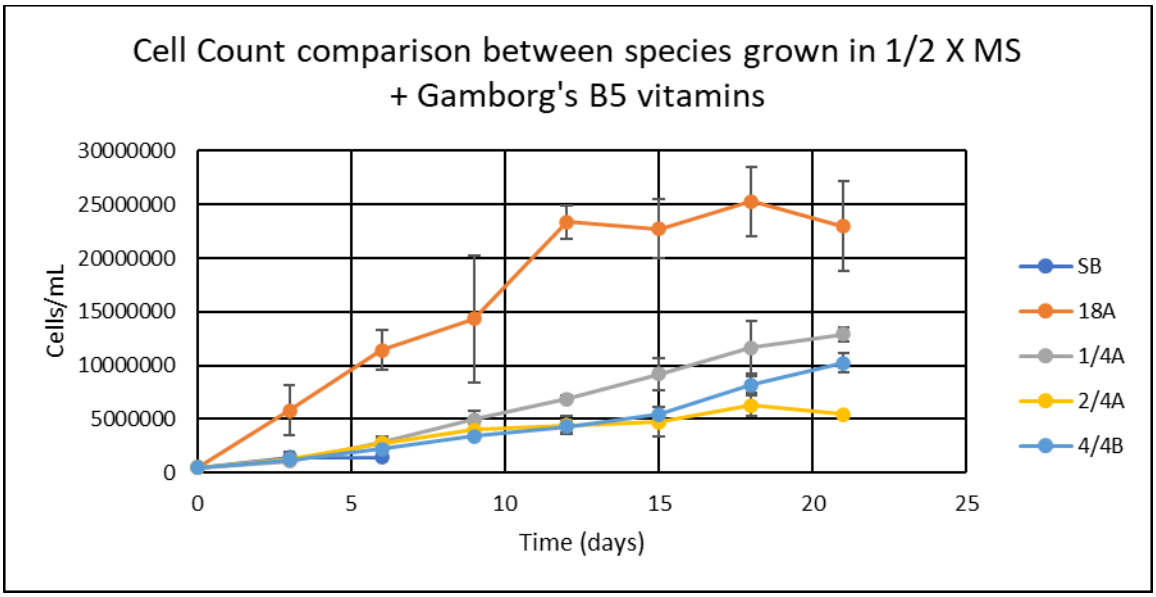

Figure 8. Cell Count comparison between microalgae species grown in 1/2 X MS + Gamborg's B5 vitamins.

Microalgal cultures were grown for 21 days (SB grown for 7 days) until the cells reached the saturation point. After 7 days of growth, SB reached a highest average cells/mL of 1.60E+06 ± 4.22E+05 on day 7. After 18 days of growth, 18A reached a highest average cells/mL of 2.53E+07 ± 3.18E+06 and 2/4A reached a highest average cells/mL of 6.29E+06 ± 9.71E+05. After 21 days of growth, 1/4A, and 4/4B reached a highest average cells/mL of 1.29E+07 ± 6.61E+05 and 1.02E+07 ± 9.10E+05 cells/mL respectively.

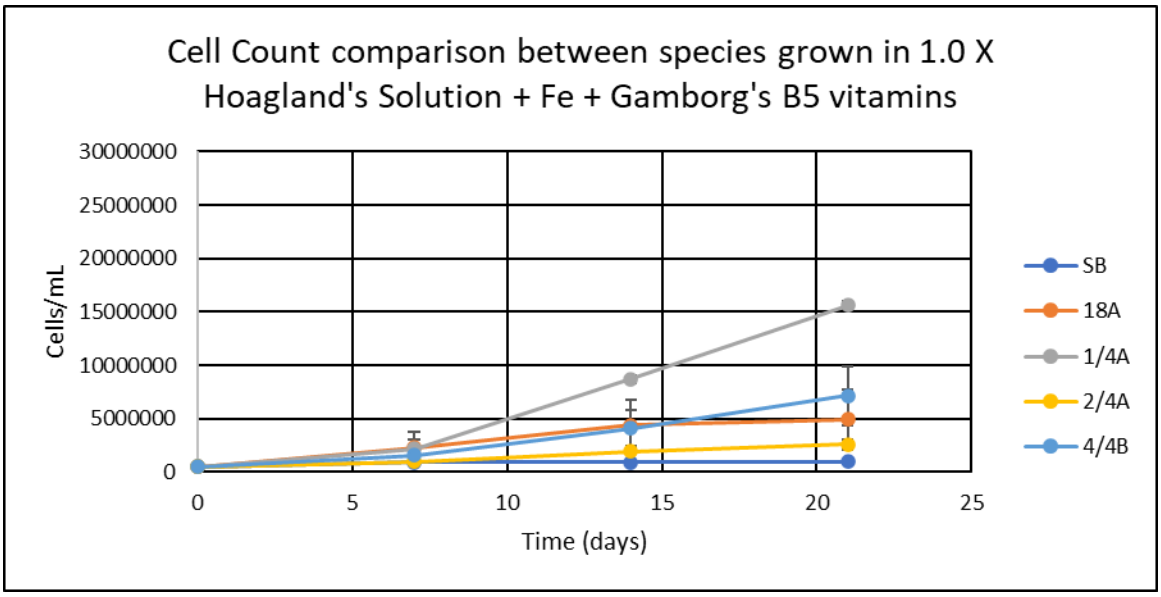

Figure 9. Cell Count comparison between microalgae species grown in 1.0 X Hoagland's Solution + Fe + Gamborg's B5 vitamins.

Microalgal cultures were grown for 21 days until the cells reached the saturation point. After 21 days of growth, SB, 18A, 1/4A, 2/4A, 4/4B reached a highest average cells/mL of 9.83E+05 ± 1.38E+05, 4.90E+06 ± 2.82E+06, 1.56E+07 ± 3.91E+05, 2.58E+06 ± 4.71E+05 and 7.12E+06 ± 2.78E+06 cells/mL respectively.

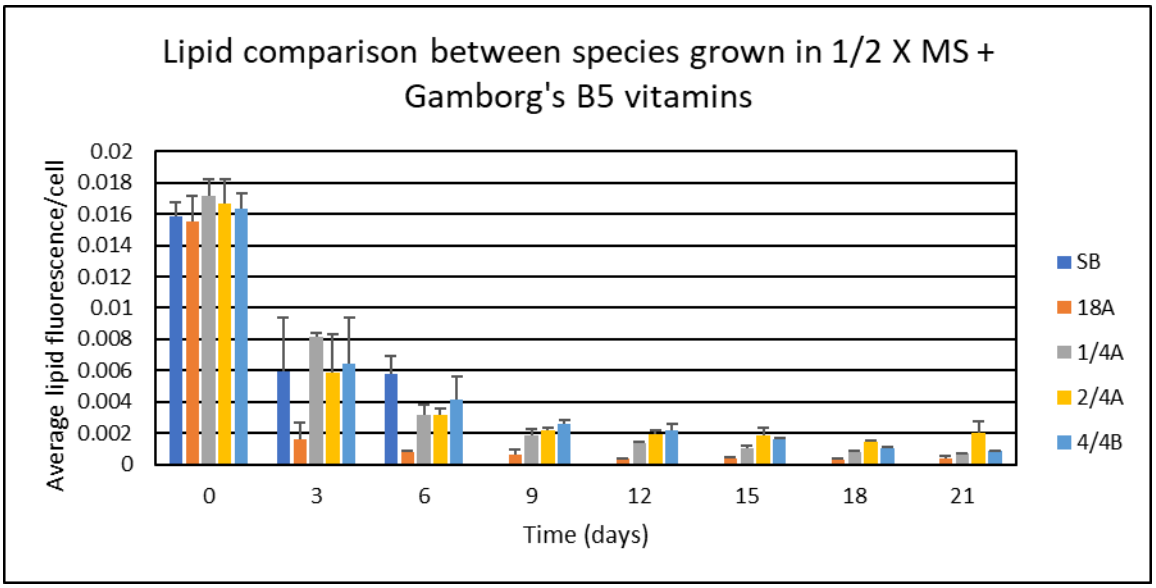

Figure 10. Lipid comparison between microalgae species grown in 1/2 X MS + Gamborg's B5 vitamins.

The average lipid fluorescence per cell on day 0 for SB, 18A, 1/4A, 2/4A and 4/4B were 1.58E-02 ± 9.51E-04, 1.55E-02 ± 1.61E-03, 1.72E-02 ± 1.00E-03, 1.67E-02 ± 1.52E-03, and 1.63E-02 ± 9.81E-04 respectively. On day 3, the average lipid fluorescence per cell for SB, 18A, 1/4A, 2/4A and 4/4B were 5.97E-03 ± 3.38E-03, 1.58E-03 ± 1.06E-03, 8.12E-03 ± 2.82E-04, 5.89E-03 ± 2.44E-03, and 6.44E-03 ± 2.93E-03 respectively. The average lipid fluorescence per cell on day 6 for SB, 18A, 1/4A, 2/4A and 4/4B were 5.78E-03 ± 1.13E-03, 7.71E-04 ± 1.13E-04, 3.16E-03 ± 6.63E-04, 3.18E-03 ± 4.14E-04, and 4.18E-03 ± 1.44E-03 respectively. The average lipid fluorescence per cell on day 9 for 18A, 1/4A, 2/4A and 4/4B were 6.70E-04 ± 2.59E-04, 1.85E-03 ± 4.29E-04, 2.17E-03 ± 1.97E-04, and 2.59E-03 ± 2.59E-04 respectively. The average lipid fluorescence per cell on day 12 for SB, 18A, 1/4A, 2/4A and 4/4B were 3.85E-04 ± 3.12E-05, 1.40E-03 ± 4.52E-05, 1.93E-03 ± 2.46E-04, and 2.15E-03 ± 4.29E-04 respectively. On day 15, the average lipid fluorescence per cell for 18A, 1/4A, 2/4A and 4/4B were 3.91E-04 ± 5.71E-05, 1.01E-03 ± 2.22E-04, 1.90E-03 ± 4.65E-04, and 1.60E-03 ± 5.81E-05 respectively. The average lipid fluorescence per cell on day 18 for 18A, 1/4A, 2/4A and 4/4B

were 3.46E-04 ± 5.49E-05, 7.79E-04 ± 1.27E-04, 1.43E-03 ± 1.46E-04 and 1.07E-03 ± 8.11E-05 respectively. On day 21, the average lipid fluorescence per cell for 18A, 1/4A, 2/4A and 4/4B were 4.07E-04 ± 1.09E-04, 6.90E-04 ± 6.38E-05, 2.04E-03 ± 7.26E-04 and 8.50E-04 ± 4.43E-05 respectively.

SB,0,MS	A								0.015812
18A,0,MS	A								0.015523
1/4,0,MS	A								0.017174
2/4,0,MS	A								0.016693
4/4B,0,MS	A								0.016305
SB,3,MS			C	D					0.005967
18A,3,MS						F	G	H	0.001582
1/4,3,MS		B							0.008117
2/4,3,MS			C	D					0.005888
4/4B,3,MS		B	C						0.006437
SB,6,MS			C	D					0.00578
18A,6,MS							G	H	0.000771
1/4,6,MS					E	F			0.003156
2/4,6,MS					E	F			0.003177
4/4B,6,MS				D	E				0.004181
18A,9,MS								H	0.00067
1/4,9,MS						F	G	H	0.001851
2/4,9,MS						F	G	H	0.002174
4/4B,9,MS					E	F	G		0.002588
18A,12,MS								H	0.000385
1/4,12,MS						F	G	H	0.001404
2/4,12,MS						F	G	H	0.001934
4/4B,12,MS						F	G	H	0.002155
18A,15,MS								H	0.000391
1/4,15,MS							G	H	0.001011
2/4,15,MS						F	G	H	0.001895
4/4B,15,MS						F	G	H	0.001599
18A,18,MS								H	0.000346
1/4,18,MS							G	H	0.000779
2/4,18,MS						F	G	H	0.001426
4/4B,18,MS							G	H	0.001072
18A,21,MS								H	0.000407
1/4,21,MS								H	0.00069
2/4,21,MS						F	G	H	0.002042
4/4B,21,MS							G	H	0.00085

Table 1. Tukey's HSD of average lipid fluorescence/cell for microalgae grown on 1/2 X MS + Gamborg's B5 vitamins. The first column represents species, media, and time point. Letters A through H indicate groups that are significantly different. The last column represents the least square means calculation.

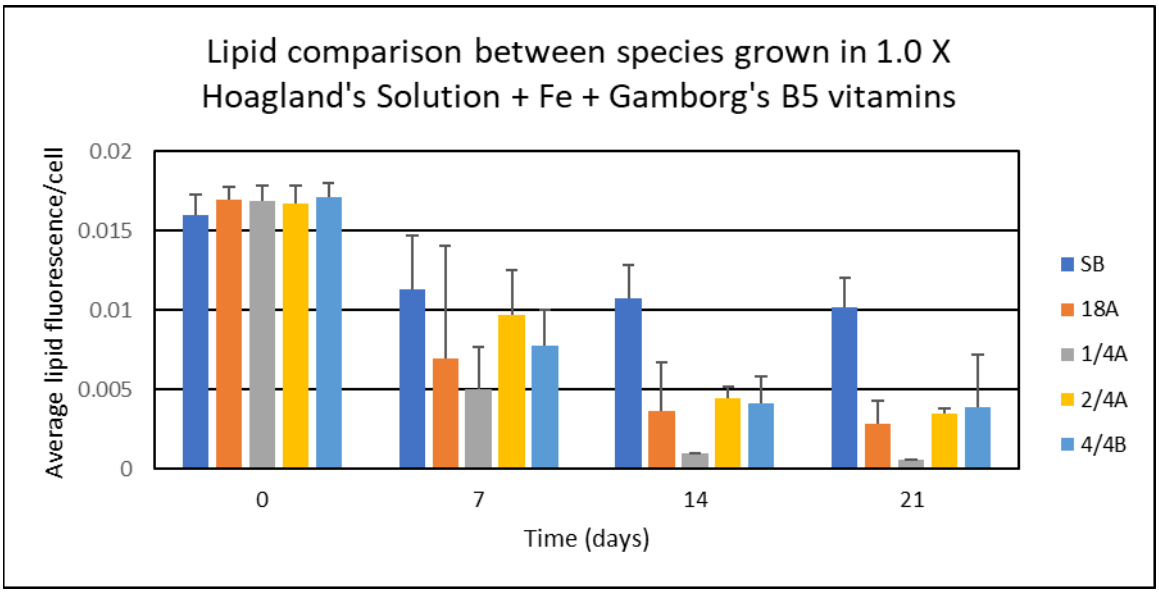

Figure 11. Lipid comparison between microalgae species grown in 1.0 X Hoagland's Solution + Fe + Gamborg's B5 vitamins.

The average lipid fluorescence per cell on day 0 for SB, 18A, 1/4A, 2/4A and 4/4B were 1.59E-02 ± 1.31E-03, 1.70E-02 ± 8.12E-04, 1.68E-02 ± 1.02E-03, 1.67E-02 ± 1.10E-03, and 1.71E-02 ± 8.95E-04 respectively. On day 7, the average lipid fluorescence per cell for SB, 18A, 1/4A, 2/4A and 4/4B were 1.13E-02 ± 3.38E-03, 6.93E-03 ± 7.11E-03, 5.02E-03 ± 2.69E-03, 9.67E-03 ± 2.83E-03, and 7.78E-03 ± 2.24E-03 respectively. The average lipid fluorescence per cell on day 14 for SB, 18A, 1/4A, 2/4A and 4/4B were 1.07E-02 ± 2.13E-03, 3.63E-03 ± 3.04E-03, 9.86E-04 ± 3.18E-05, 4.46E-03 ± 6.94E-04, and 4.14E-03 ± 1.66E-03 respectively. The average lipid fluorescence per cell on day 21 for 18A, 1/4A, 2/4A and 4/4B were 1.01E-02 ± 1.84E-03, 2.80E-03 ± 1.48E-03, 5.56E-04 ± 1.97E-05, 3.48E-03 ± 3.11E-04, and 3.87E-03 ± 3.34E-03 respectively.

SB,0,HS	A	B						0.015949
18A,0,HS	A							0.016953
1/4,0,HS	A							0.016844
2/4,0,HS	A							0.016688
4/4B,0,HS	A							0.017085
SB,7,HS		B	C					0.011286
18A,7,HS			C	D	E	F		0.006932
1/4,7,HS				D	E	F	G	0.005015
2/4,7,HS			C	D				0.009675
4/4B,7,HS			C	D	E			0.007777
SB,14,HS			C					0.010698
18A,14,HS					E	F	G	0.003629
1/4,14,HS							G	0.000986
2/4,14,HS					E	F	G	0.004456
4/4B,14,HS					E	F	G	0.004142
SB,21,HS			C					0.010149
18A,21,HS						F	G	0.002803
1/4,21,HS							G	0.000556
2/4,21,HS					E	F	G	0.003483
4/4B,21,HS					E	F	G	0.003874

Table 2. Tukey's HSD of average lipid fluorescence/cell for microalgae grown on 1.0 X Hoagland's Solution + Fe + Gamborg's B5 vitamins. The first column represents species, media, and time point. Letters A through G indicate groups that are significantly different. The last column represents the least square means calculation.

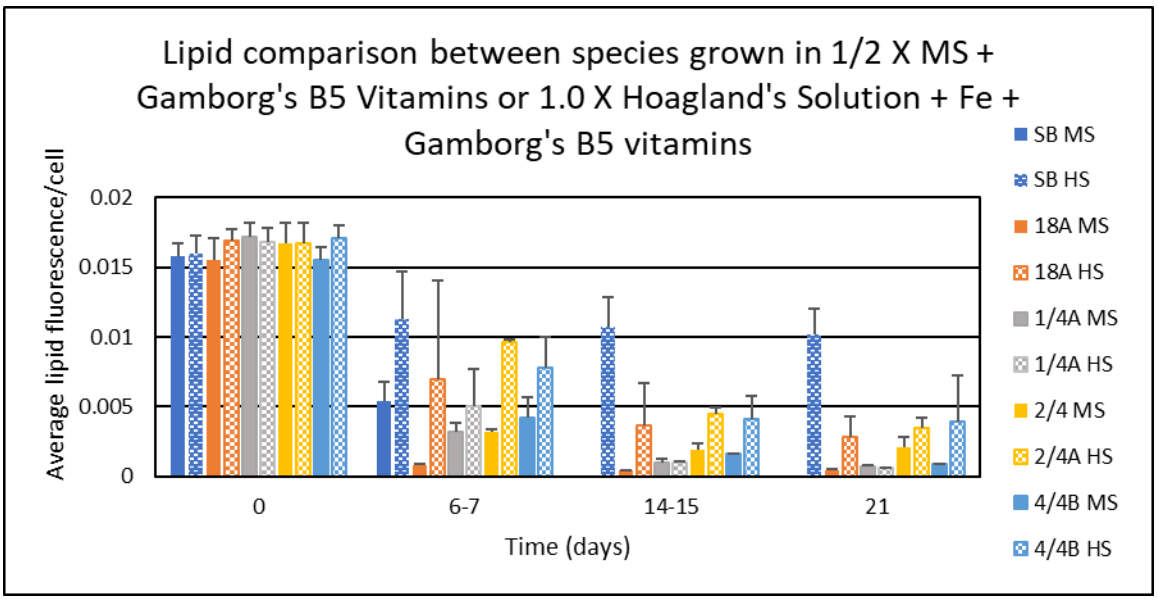

Figure 12. Lipid comparison between microalgae species grown in 1/2 X MS + Gamborg's B5 vitamins or 1.0 X Hoagland's Solution + Fe + Gamborg's B5 vitamins.

At day 0, SB had an average lipid fluorescence per cell of 1.58E-02 + 9.51E-04 and 1.59E-02 + 1.31E-03 when grown on 1/2 X MS + Gamborg's B5 vitamins (MS), or 1.0 X Hoagland's Solution + Fe + Gamborg's B5 vitamins (HS) respectively. SB had an average lipid fluorescence per cell of 5.78E-03 ± 1.13E-03 (MS) at day 6 and 1.13E-02 ± 3.38E-03 (HS) at day 7.

At day 0, 18A had an average lipid fluorescence per cell of 1.55E-02 ± 1.61E-03 and 1.70E-02 ± 8.12E-04 when grown on MS, or HS respectively. 18A had an average lipid fluorescence per cell of 7.71E-04 ± 1.13E-04 (MS) at day 6 and 6.93E-03 ± 7.11E-03 (HS) at day 7. 18A had an average lipid fluorescence per cell of 3.63E-03 ± 3.04E-03 (HS) at day 14 and 3.91E-04 ± 5.71E-05 (MS) at day 15. At day 21, 18A had an average lipid fluorescence per cell of 4.07E-04 ± 1.09E-04 and 2.80E-03 ± 1.48E-03 when grown on MS, or HS respectively.

At day 0, 1/4A had an average lipid fluorescence per cell of 1.72E-02 ± 1.00E-03 and 1.68E-02 ± 1.02E-03 when grown on MS, or HS respectively. 1/4A had an average lipid

fluorescence per cell of 3.16E-03 ± 6.63E-04 (MS) at day 6 and 5.02E-03 ± 2.69E-03 (HS) at day 7. 1/4A had an average lipid fluorescence per cell of 9.86E-04 ± 3.18E-05 (HS) at day 14 and 1.01E-03 ± 2.22E-04 (MS) at day 15. At day 21, 1/4A had an average lipid fluorescence per cell of 6.90E-04 ± 6.38E-05 and 5.56E-04 ± 1.97E-05 when grown on MS, or HS respectively.

At day 0, 2/4A had an average lipid fluorescence per cell of 1.67E-02 ± 1.52E-03 and 1.67E-02 ± 1.10E-03 when grown on MS, or HS respectively. 2/4A had an average lipid fluorescence per cell of 3.18E-03 ± 4.14E-04 (MS) at day 6 and 9.67E-03 ± 2.83E-03 (HS) at day 7. 2/4A had an average lipid fluorescence per cell of 4.46E-03 ± 6.94E-04 (HS) at day 14 and 1.90E-03 ± 4.65E-04 (MS) at day 15. At day 21, 2/4A had an average lipid fluorescence per cell of 2.04E-03 ± 7.26E-04 and 3.48E-03 ± 3.11E-04 when grown on MS, or HS respectively.

At day 0, 4/4B had an average lipid fluorescence per cell of 1.63E-02 ± 9.81E-04 and 1.71E-02 ± 8.95E-04 when grown on MS, or HS respectively. 4/4B had an average lipid fluorescence per cell of 4.18E-03 ± 1.44E-03 (MS) at day 6 and 7.78E-03 ± 2.24E-03 (HS) at day 7. 4/4B had an average lipid fluorescence per cell of 4.14E-03 ± 1.66E-03 (HS) at day 14 and 1.60E-03 ± 5.81E-05 (MS) at day 15. At day 21, 4/4B had an average lipid fluorescence per cell of 8.50E-04 ± 4.43E-05 and 3.87E-03 ± 3.34E-03 when grown on MS, or HS respectively.

SB,0,MS	A									0.015762
SB,0,HS	A									0.015949
18A,0,MS	A									0.015523
18A,0,HS	A									0.016953
1/4,0,MS	A									0.017174
1/4,0,HS	A									0.016844
2/4,0,MS	A									0.016693
2/4,0,HS	A									0.016688
4/4B,0,MS	A									0.016305
4/4B,0,HS	A									0.017085
SB,6 and 7,MS				D	E	F				0.005436
SB,6 and 7,HS		B								0.011286
18A,6 and 7,MS								H	I	0.000771
18A,6 and 7,HS			C	D	E					0.006932
1/4,6 and 7,MS					E	F	G	H	I	0.003156
1/4,6 and 7,HS				D	E	F	G			0.005015
2/4,6 and 7,MS					E	F	G	H	I	0.003177
2/4,6 and 7,HS		B	C							0.009675
4/4B,6 and 7,MS				D	E	F	G	H	I	0.004181
4/4B,6 and 7,HS		B	C	D						0.007777
SB,14 and 15,HS		B	C							0.010698
18A,14 and 15,MS									I	0.000391
18A,14 and 15,HS					E	F	G	H	I	0.003629
1/4,14 and 15,MS							G	H	I	0.001011
1/4,14 and 15,HS							G	H	I	0.000986
2/4,14 and 15,MS						F	G	H	I	0.001895
2/4,14 and 15,HS				D	E	F	G	H		0.004456
4/4B,14 and 15,MS						F	G	H	I	0.001599
4/4B,14 and 15,HS				D	E	F	G	H	I	0.004142
SB,21,HS		B	C							0.010149
18A,21,MS								H	I	0.000407
18A,21,HS						F	G	H	I	0.002803
1/4,21,MS								H	I	0.00069
1/4,21,HS								H	I	0.000556
2/4,21,MS						F	G	H	I	0.002042
2/4,21,HS					E	F	G	H	I	0.003483
4/4B,21,HS				D	E	F	G	H	I	0.003874
4/4B,21,MS								H	I	0.00085

Table 3. Tukey's HSD of average lipid fluorescence/cell for microalgae grown on 1/2 X MS + Gamborg's B5 vitamins or 1.0 X Hoagland's Solution + Fe + Gamborg's B5 vitamins. First column represents species, media, and time point. Letters A through I indicate groups that are significantly different. The last column represents the least square means calculation.

Strain/Media	Initial average lipid fluorescence/cell	Sustained average lipid fluorescence/cell	Higher cells/mL	Higher growth rate	Total
SB in MS	1	1	0	1	3
SB in HS	1	1	0	0	2
18A in MS	1	0	1	1	3
18A in HS	1	1	0	0	2
1/4A in MS	1	0	0	0	1
1/4A in HS	1	0	0	0	1
2/4A in MS	1	0	0	0	1
2/4 in HS	1	1	0	0	2
4/4B in MS	1	0	0	0	1
4/4B in HS	1	1	0	0	2

Figure 13. Evaluation of favorable characteristics for biofuel production (Scale: 1 point for each characteristic).

CHAPTER IV

DISCUSSION

Microalgal species isolation and identification

The isolated microalgae (SB, 18A, 1/4A, 2/A, and 4/4B) were identified at the Genera level. The various Genera found were: *Bracteococcus sp.* (SB), *Chlorella sp.*(18A)*, Scenedesmus sp.* isolate 1 (1/4A)*, Scenedesmus sp*. isolate 2 (2/4A)*, and Tetrastrum sp*. (4/4B). The only Genera which have been studied for biofuel production that were isolated in this screen were *Chlorella sp.* and *Scenedesmus sp.* (Singh and Dhar, 2011, Nascimiento *et al*., 2012). However, the microalgae isolates, *Bracteococcus sp.* and *Tetrastrum sp*., have not been previously studied for biofuel production on an industrial scale. Other microalgae were partially isolated also in this screen but were not characterized (Water tank sun side "A", 18A2) (Figures 1-5.).

For microalgae isolation purposes, 1.0 X Hoagland's Solution + Fe media worked better than 0.1 X, 0.25 X, 0.5 X Hoagland's Solution + Fe, 0.1 X, 0.25 X, 0.5 X, 1.0 X Hoagland's Solution + Fe + Gamborg's B5 vitamins, and 1/2 X MS + Gamborg's B5 vitamins. On lower concentrations of Hoagland's Solution, the microalgae did not grow well, and this was probably due to the lack of sufficient nutrients for the growth of the organisms. Also, 1/2 X MS + Gamborg's B5 vitamins encouraged bacteria and fungi to grow, thus, making their separation from the microalgae more difficult. Antibiotic and antimycotics (penicillin, streptomycin, and amphotericin B) were added to both medias, but this did not have a significant influence on the isolation of the microalgae. The bacteria and fungi associated with the microalgae in this samples

were largely resistant to the antibiotic/antimycotic solution that was added to the medias. Therefore, it was still very difficult to separate the bacteria and fungi from the microalgae despite using the antibiotic/antimycotic solution.

There were several microalgae that could not be isolated away from the bacteria or fungi even though many different techniques were tried. This is possibly due to the bacteria and fungi being tightly associated with the cell wall or growing in between the cell membrane and cell wall of the microalgae. This may indicate that a strong mutualistic, commensalistic, or parasitic relationship between the bacteria, fungi, and microalgae.

Ultimately, five microalgal strains were able to be purified (SB, 18A, 1/4A, 2/4A, and 4/4B), and used for further study in the lipid assays. Other microalgae were purified (Water tank, sun side "A", 18A2), but not selected for further study at the time. Several others were unable to be separated from their associated bacteria and fungi flora (Little Pond, 12A, 13A1, 13A2), and were not chosen for further study at this time (Figures 1-5.).

One point of note is that the 18A microalgal cultures lost their green color after 30 days growing under the high light intensity (250 – 350 μmol m^{-2} s^{-1}) with a color change from green to white. When moved to lower light intensity (50 μmol m^{-2} s^{-1}), the cultures started to regain the green coloration after about 20 days. This color change could indicate that these microalgae can potentially become heterotrophic under nutrient poor conditions and high light level.

Cell growth monitored by absorbance

The absorbance levels for all species of microalgae were higher in 1/2 X Murashige and Skoog + Gamborg's B5 vitamins (MS) compared to the cultures grown in 1.0 X Hoagland's Solution + Fe + Gamborg's B5 vitamins (HS). When the microalgae were grown in MS, on day

21, 18A had the highest absorbance readings, and 2/4A had the lowest absorbance readings (Figure 6.). However, when the microalgae were grown in HS, on day 21, 1/4A had the highest absorbance readings, while SB had the lowest absorbance (Figure 7.).

Cell growth monitored by cell counts

It was observed that at day 21, the cells/mL of the SB, 18A, 2/4A and 4/4B cultures were higher when grown in MS versus HS media (Figure 8.). However, microalgal isolate 1/4A had a higher cells/mL on day 21 when grown in HS vs MS media (Figure 9.). The microalgal isolate that consistently had the highest cell density was 18A in MS, while microalgal isolate SB consistently had the lowest cell density (Figure 8.) when grown in MS for the entire time period. In HS media, microalgal isolate 1/4A grew more quickly than all the other microalgal species with SB growing the least (Figure 9.).

Lipid fluorescence/cell assay

All the microalgal species grown in MS showed the highest average lipid fluorescence/cell on day 0, and after day 0, there was a steady decline in the overall lipid production. Microalgal isolates SB and 2/4A seemed to sustain the best lipid production over time during the experiment (Figure 10.). The Tukey's HSD test showed that all microalgal species, at day 0, were not significant different from each other in the lipid fluorescence/cell value. For the other time points, the microalgal species were found to have significantly different lipid fluorescence/cell values (Table 1.).

All the microalgal species grown in HS showed the highest average lipid fluorescence/cell value on day 0 as well and were not significantly different from each other (Table 2.). With regard to day 7, day 14, and day 21, it was found that the microalgal species had

significantly different lipid fluorescence/cell values compared to each other at those time points. Compared to day 0 at the rest of the time points (day 7, day 14, day 21), SB maintained a relatively high lipid fluorescence/cell value (Figure 11.).

At day 0, the lipid fluorescence/cell values amongst all the microalgal species were determined to be not statistically significant via a Tukey's HSD test (Table 3.). When days 6-7, 14-15, and 21 were compared to each other, it was found that the microalgal species had significant differences in the lipid fluorescence/cell values. Overall, the lipid fluorescence/cell values on of all the microalgal species were higher when the cells were grown on HS media compared to MS media after day 0. However, comparing days 14-15 and day 21, microalgal isolate 1/4A had lower lipid fluorescence/cell values when grown in HS media compared to MS media (Figure 12.).

Evaluation of microalgal isolates for biofuel production

According to and Singh and Dhar (2011), and Nascimiento et al (2012), there are many characteristics that algae possess that make them amendable for use in biofuel production. Among these are: initial average lipid production, sustained average lipid production, growth rate, and high cell density. The microalgal isolates were scored using these four parameters (1 point per characteristic). It was found that when SB and 18A were grown on MS that they had a score of 3 out of 4 positive characteristics for lipid production (Figure 13.). When SB, 18A, 2/4A, and 4/4B were grown in HS, they had a score of 2 out of 4 positive characteristics for lipid production (Figure 13.).

Future experimental directions

Different species of algae will grow differently on different types of media. More extensive experiments using different media types should be conducted. Measurements where nutrient levels are recorded would be useful in order to determine which stress factors that the microalgae may be subjected to as the culture density increases. Nutrient availability may have a large impact on lipid production due to the observation that the microalgae, when diluted, had an increased lipid fluorescence/cell value (Figure 12.). When the microalgal cultures are diluted into new fresh media, it is possible that the lipid production efficiency increases drastically over a very short period of time after dilution. If the dilution of microalgae into new media does have a large effect on the lipid production after the first hours of culture, this would be very helpful information for the purpose of increasing lipid production. A new strategy to maximize the lipid production of microalgae may be to grow the microalgae to a very high cell density in culture, then dilute the culture to a low cell density, and then within a relatively short period of time, harvest the microalgae for lipids.

Microalgal isolate 18A appeared to have a heterotrophic growth state. Lipid assays could be conducted in the heterotrophic state to see if there is a better lipid yield per cell. 18A cultures in both the auxotrophic and heterotrophic states (green and white phenotypes) could be inoculated together to also see if there is a better production of lipids over time.

Barcoding of the isolated microalgal species will result in determining the identity of the microalgal isolates at the species level. This information would be beneficial for future use of any of these microalgal isolates for biofuel production. Once the species are identified, they can be compared to known species that are used in biofuel production. Novel microalgae species could be identified in this screen and be considered for use in biofuel production.

Conclusions

At least four of the five microalgal strains that were isolated from the sample sites Edinburg Scenic Wetlands & World Birding Center and Edinburg Municipal Park area had positive characteristics of a microalgae that can be used for biofuel production. SB and 18A had the most positive characteristics for biofuel production when grown on MS media. SB, 18A, 2/4A and 4/4B had the most positive characteristics for biofuel production when grown on HS media.

Of the two medias studied (1/2 X MS + Gamborg's B5 Vitamins or 1.0 X Hoagland's solution + Fe + Gamborg's B5 Vitamins), MS media resulted in faster growth, higher cell density and initial average lipid fluorescence/cell for all species. HS media resulted in better sustained lipid fluorescence/cell for all species.

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APPENDIX

APPENDIX

1.0 X Murashige and Skoog + Gamborg's B5 Vitamins

Components	mg/L
Ammonium Nitrate (NH_4NO_3)	1650.0000
Boric Acid (H_2BO_3)	6.2000
Calcium Chloride, Anhydrous ($CaCl_2$)	332.2000
Cobalt Chloride, Hexahydrate ($CoCl_2$ $6H_2O$)	0.0250
Cupric Sulfate, Pentahydrate ($CuSo_2$ $5H_2O$)	0.0250
EDTA, Disodium Salt, Dihydrate ($C_{10}H_{14}N_2Na_2O_8$)	37.2600
Ferrous Sulfate, Heptahydrate ($FeSO_4 \cdot 7H_2O$)	27.8000
Magnesium Sulfate, Anhydrous ($MgSO_4$)	180.7000
Manganese Sulfate, Monohydrate ($MnSo_4 \cdot H_2O$)	16.9000
Molybdic Acid Sodium Salt, Dihydrate ($Na_2MoO_4 \cdot 2H_2O$)	0.2500
Myo-Inositol ($C_6H_{12}O_6$)	100.0000
Nicotinic Acid ($C_6H_5NO_2$)	1.0000
Potassium Iodide (KI)	0.8300
Potassium Nitrate (KNO_3)	1900.0000
Potassium Phosphate (KH_2PO_4)	170.0000
Pyridoxine, Hydrochloride ($C_8H_{11}NO_3 \cdot HCL$)	1.0000
Thiamine, Hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCL$)	10.0000
Zinc Sulfate, Heptahydrate ($ZnSO_4 \cdot 7H_2O$)	8.6000

Gamborg's vitamins X 1000 Solution

Components	mg/L
Myo-Inositol ($C_6H_{12}O_6$)	100000.0000
Nicotinic Acid ($C_6H_5NO_2$)	1000.0000
Pyridoxine, Hydrochloride ($C_8H_{11}NO_3 \cdot HCL$)	1000.0000
Thiamine, Hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCL$)	10000.0000

Hoagland's Solution (10 Liter): (4.0 X)

Components	mg or mL added	Stock Solution concentration
Ammonium dihydrogen phosphate ($NH_4H_2PO_4$)	46000.0000	
Calcium nitrate ($Ca(NO_3)_2$)	26440.0000	
***Magnesium sulfate* heptahydrate (MgSO4 • $7H_2O$)**	19640.0000 (9560.0000 if Anhydrous)	
Potassium Nitrate (KNO_3)	24280.0000	
Copper (II) sulfate pentahydrate ($CuSO_4$ • 5H2O)	40 mL	(0.075 g/L stock solution)
Zinc sulfate heptahydrate ($ZnSO_4$ • $7H_2O$)	40 mL	(0.23 g/L stock solution)
Dihydrogen borate (H_2BO_3)	40 mL	(2.844.075 g/L stock solution)
Manganese (II) chloride tetrahydrate ($MnCl_2$ • $4H_2O$)	40 mL	(1.01 g/L stock solution)
Molybdenum trioxide (MoO_3)	40 mL	(0.016 g/L stock solution)
Diethylenetriaminepentaacetic acid Iron (III) disodium salt hydrate, 98% (Fe DTPA)		5 mL/L in 1.0 X Hoagland's solution (0.526 g/L Stock Solution)

Media components in common between Hoagland's Solution + Gamborg's B5 Vitamins and Murashige and Skoog + Gamborg's B5 Vitamins**:**

Media Component
NH4
NO3
SO4
PO4
BO3
K
Ca
Mg
Mn
Mo
Fe
Cu
Zn
Gamborg's B5 vitamins

Media components that are different between Hoagland's Solution + Gamborg's B5 Vitamins and Murashige and Skoog + Gamborg's B5 Vitamins**:**

Media components unique to MS
Co
EDTA
I

BIOGRAPHICAL SKETCH

Mauricio A. Pena was born in Monterrey, Nuevo Leon in Mexico. He completed school there and moved to the United States in 2006 where started middle school and continued his studies in McAllen, Texas. He completed his Associate of Science in Biology at the end of 2016 at South Texas College and started his bachelor's degree in science in Biology with a minor on Chemistry on January 2017, on which he graduated on December 2018 at the University of Texas Rio Grande Valley (UTRGV). On January 2019 he started his Master of Science (MS) in Biology at UTRGV and finished it in December 2021. Mauricio can be contacted through his email account Maulex95@gmail.com