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Sequential autotrophic-heterotrophic culture of *Scenedesmus* spp. for lipid production

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SEQUENTIAL AUTOTROPHIC-HETEROTROPHIC CULTURE OF *SCENEDESMUS* SPP. FOR LIPID PRODUCTION

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ABSTRACT

Algae are fascinating organisms that are receiving growing attention for their versatility on many fronts of research areas. Immense amounts of research is being conducted to find solutions to challenges in energy and climate change. A large portion of research is focused on algae as a potential solution to both of these issues. The focus of this research is to look at algae closer to home as a local, economical resource to aid in solving both of these problems. The algal genus *Scenedesmus* was tested for growth in a bi-phased scheme: photoautotrophically for carbon sequestration then heterotrophically on glycerol for lipid production. Lipids that could then be converted to biodiesel. The photosynthetic growth phase resulted in an increase in aqueous inorganic carbon concentrations and biomass growth. The heterotrophic phase did not result in glycerol consumption, however, there was an increase in lipid production.

DEDICATION

I'd like to dedicate this thesis to my parents Jerry and Ruth Taylor and my sister Alexis Scipio for their continuous support, encouragement, and celebration throughout the highs and lows that I've faced during this phase of my life. I am eternally grateful for the constant love and belief in me to impact the world in a positive way.

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CHAPTER ONE

LITERATURE REVIEW

1. Viewpoints and benefits

Two major issues facing the world today are: (1) finding a sustainable energy source and (2) retarding the effects of global climate change by atmospheric carbon reduction. The use of photoautotrophic organisms has been at the forefront of research to deal with both of these issues. The photosynthetic process is beneficial because it draws carbon dioxide from the atmosphere and incorporates it into biomass. Microalgae are photoautotrophic organism that are used as a source for biofuels.

Biofuels are a type of alternative fuel that is made from biologically renewable resources (Mutanda et al., 2011). Biofuels offer a way for utilizing native resources, locally or regionally. In this view biofuels are a means for eliminating the need for transport of finite resources like petroleum fuels and products. This alleviates the political and economical problems associated with petrofuel security and subsequently reducing emissions and energy usage in petro-product bulk transport from a few global locations (Demirbas, 2011).

Approaching algal biofuel production from the general prospective of a biorefinery can make the overall production process environmentally sustainable. The primary goal of a biorefinery is producing multiple valuable products and recycling valuable resources such as nutrients and water (Greenwell et al., 2010). In terms of valuable products, algae can serve as a multiuse feedstock in which different biomolecules may serve as the starting material for many products in addition to biofuels,

such as nutraceuticals, pigments and other chemicals (Mata et al., 2010). Algae can also serve multiple purposes including nutrient removal from wastewaters and serve as food sources in aquaculture (Mata et al., 2010). Furthermore, recycling post-lipid-extracted biomass (high in cell-bound nutrients) in anaerobic digesters can produce another energy source, methane, as well as release the nutrients so they may be available for reuse to grow more algae (Chowdhury et al., 2012). Use of these digested nutrients may also reduce dependence on fertilizers produced using petroleum and enhance the sustainability of the overall process.

Typical oilseed plants like sunflower or soybean have been compared to algae for the production of biofuel and biodiesel.Of the oil crops, algae have the most potential in terms of oil yield per area (Chisti, 2007). Microalgae are capable of growing in an array of environments leading to their preference over terrestrial oil seed crops (Mata et al., 2010). Algae also have higher oil yields per mass of biomass produced compared to other oilseed crops--sunflower 40% oil by weight, soybean 18% oil by weight and microalgae containing a medium oil content has 50% oil by weight (weight basis, wet versus dry, not specified)--(Mata et al., 2010).

A benefit of biodiesel over petroleum based diesel is its potential to be a carbon neutral fuel (Um and Kim, 2009); where the carbon that goes in to making the lipids balances the carbon released as the fuel is combusted. Petrodiesel (and other petroleum based fuels and products) utilizes buried carbon that, when brought to the surface is combusted thus adding carbon to the atmosphere. Dealing with the consequences of this

added carbon from petroleum--will come at a cost that is not present when dealing with bio-based fuels and products (Um and Kim, 2009).

2. Photoautotrophic growth and carbon dioxide use

Photoautotrophic organisms are means of carbon capture. Photoautotrophic growth of algae is environmentally useful in that it takes atmospheric CO_2 and incorporates it into biomass using light energy. Watson (2009) found that initial inorganic carbon concentrations of the media did not significantly affect the open systems ability to draw CO_2 out of the atmosphere. Mata et al., (2010) mentions a study that showed how waters in the alkaline range of pH saw faster absorption of CO_2 via one of two pH-dependent methods in which both end products were bicarbonate. Watson (2009) further explains that the open systems ability to draw in atmospheric CO_2 is dictated by the rate of algal growth.

Photoautotrophic algae are typically grown in open ponds or closed photobioreactors - each with their own advantages and drawbacks. Open pond raceways are less costly to construct and maintain, however the algal productivity is typically lower and contamination control is minimal compared to that of photobioreactors (Chisti, 2007; Jorquera et al., 2010). Despite having lower productivity and limited contamination control, open pond systems are as economically viable as current photobioreactors (Jorquera et al., 2010). Alternatively, an economic analysis by Davis et al. (2011) revealed that the cost of biodiesel produced using open ponds was half that of biodiesel produced via photobioreactors. Unfortunately, neither case was directly competitive with the cost of petroleum diesel. Open ponds are currently the easier of the two system types

to scale up for industrial applications and have been in use, industrially, for many years (Chisti, 2007; Borowitzka, 1999).

Drapcho and Brune (2000), reported on photoautotrophic algal growth in a partitioned aquaculture system (PAS)--a type of outdoor field-scale raceway--and the effects of inorganic carbon. Interestingly, they observed divisions of dominant algal species varied with an addition of inorganic carbon - prokaryotic cyanobacteria (genus *Merismopedia*) predominated when inorganic carbon was not added to the system and Chlorophyceae (genus *Scenedesmus*) predominated when inorganic carbon was added (Drapcho and Brune, 2000). Mata et al. (2010) cautions that the transfer of atmospheric CO_2, a form of inorganic carbon, into the water may limit the biomass growth rate because of its relatively low percentage in air. Continuous mixing of the culture would improve the gas transfer, both of CO_2 into the reactor as well as O_2 out of the reactor.

3. Heterotrophic growth and lipid synthesis

Heterotrophic metabolism is the utilization of organic substrates such as glucose, glycerol and acetic acid as a carbon and energy source (Mata et al., 2010; Lee, 2001; Chen and Walker, 2011).Glycerol is a substrate of interest when considering heterotrophic growth of algae because it is a by-product of the biodiesel production process. The use of the by-product glycerol can produce more biodiesel and continue the cycling of the perceived waste by-product instead of creating waste (Chowdhury et al., 2012). Chen and Walker (2011) studied the ability of the algal species, *Chlorella protothecoides,* to produce algal oil when grown on crude glycerol, co-produced with biodiesel, as a cheaper alternative to glucose. The algae grew better, with a higher oil

yield and specific growth rate of 0.74 day^{-1}, on the crude glycerol--unrefined glycerol with impurities--than it did on the pure glycerol, despite the impurities present (Chen and Walker, 2011).

In a study done by Das et al. (2011) the marine alga, *Nannochloropsis* produced a higher fatty acid yield when grown on glycerol, compared to growth on sucrose or glucose. Inexpensive substrates, like the crude, unrefined glycerol are ideal for an economically competitive product. There is an added economical and sustainable benefit to using a waste co-product like the glycerol to produce the needed feedstock--oil.

Lipids are synthesized or constructed starting with the biomolecule acetyl-CoA; acetyl CoA is a central part of lipid synthesis and regulation (Greenwell et al., 2010). The first significant step, in a series of steps that lead to the synthesis of fatty acids, is the formation of malonyl-CoA (shown in a figure from Greenwell et al. (2010); once this biomolecule is formed lipid synthesis will commence. In the synthesis of triacylglycerides (TAG), fatty acids are added to glycerol-3-phosphates (Greenwell et al., 2010). Greenwell et al. (2010) references two means of lipid production; *de novo* synthesis and the use of recycled fatty acids. They explain that the route chosen, new versus recycled, is dependent on the stage of algal growth as well as the nutritional conditions to which the algae are exposed; growing cells and stressed cells have more lipids produced by the *de novo* pathway over the use of recycled fatty acids (Greenwell et al., 2010).

4. Two-phased growth

Griffiths and Harrison (2009) discuss bi-phased algal growth from an optimization standpoint; the first phase optimizes the production of biomass and the second optimizes the production of lipids. Xiong et al. (2010) alluded to the notion that more biomass with less lipid production is generally achieved during normal photosynthetic growth and conversely, lipid accumulation with less biomass can be seen during heterotrophic growth. This idea is supported by Lee (2001) who mentions that the maximum specific growth rates of heterotrophic cultures are generally less than maximum specific growth rates attained during photosynthetic growth.

While the main focus of photoautotrophic growth is to provide for CO_2 uptake, the heterotrophic growth phase is desired to boost oil yield. The method of two-phased algal growth for lipid production was also part of the study conducted by Das et al. (2011) on *Nannochloropsis* in an open system growing off of glycerol during the heterotrophic growth phase. An increase in oil yield was observed in multiple studies of algal species grown in two phased process (Das et al., 2011; Zheng et al., 2012; Xiong et al., 2010). Grobbelaar (2000) suggested that algae accustomed to high levels of continuous illumination have higher photosynthetic rates in the light and higher respiration rates in the dark compared to algae grown with lower illumination levels. The dark respiration rates taper off with time. Two of the reviewed experimental studies were done in a closed system (at least for part of one study) and tests were conducted on a pure culture of species of *Chlorella* (Zheng et al., 2012; Xiong et al., 2010).

5. Algal cultures

An open system provides an opportunity to test a mixed culture of locally existing algae. Desired species of algae are those suited for local environmental conditions with high growth rate and productivity which increases yield per harvest and may subsequently lower cost. Suitability to local conditions and high growth rates may also prove promising for reducing in contamination issues (Griffiths and Harrison, 2009). When studying the influence of different water systems (stagnant versus moving) on algal productivity, Mohan et al. (2011) found that algal cultures from stagnant water bodies demonstrated higher degradation of their wastewater substrates and had a higher likelihood of producing more lipids compared to the running water algal culture tested. Abou-Shanab et al. (2011) isolated and grew locally collected (river and wastewater) algal species (primarily *Scenedesmus*). The *Scenedesmus* (from both water sources) showed good biomass and lipid productivity rates with fatty acid profiles that were reported as suitable for biodiesel production. In a similar fashion, Mohan et al. (2011) detailed the production of an array of fatty acids for use for biodiesel as well as other valuable products for nutrition, from ecologically diverse water-bodies. Stockenreiter et al. (2012) showed that algal species diversity enhanced lipid production, where the test samples with the most diversity resulted in higher productivity and higher oil yields.

If mixed cultures are not desired, Borowitzka (1999) and Lee (2001) suggested that pseudo-pure cultures can be achieved in open systems if the algae are grown in extreme environments that specifically select for the desired algae's niche. The hyper saline environment used in Das et al. (2011) prevented the growth and dominance of bacteria and other competitive species. Yoo et al. (2010) implied that microalgal

tolerance of CO_2 at higher concentrations may be a parameter for selection. This could be a method used to create a pseudo-pure, photosynthetic algal culture for bioproducts or building up biomass.

5.1 Scenedesmus

In previous studies conducted using local samples, *Scenedesmus* was the dominant algal genus in the open systems (Drapcho and Brune, 2000; Watson, 2009). The more diverse algal cultures studied by Mohan et al. (2011) resulted in greater lipid accumulation; various species of *Scenedesmus* were identified among the algae used in that study. *Scenedesmus* is capable of increased lipid production due to higher biomass productivity under higher carbon dioxide concentrations; at 10% CO_2 the *Scenedesmus* had a reported lipid productivity of about 20.65 mg/(L day) (Yoo et. al., 2010). Brennan and Owende (2010) credit *Scenedesmus* as a favorable choice for CO_2 mitigation. Mata et al. (2010) found a lipid productivity as high as about 54 mg/(L day) for *Scenedesmus* (mode of growth not specified). As shown by Watson (2009), higher dissolved carbon dioxide concentrations can result from the environment self-stabilizing under fairly high alkaline conditions caused by the algal (*Scenedesmus)* metabolism. Particular species of *Scenedesmus* have been known to survive in environments with low nutrient content, but they can also adjust their nutrient (nitrogen and phosphorus) uptake depending on nutrient availability (Xin et al., 2010). *Scenedesmus* can also grow heterotrophically; the species *Scenedesmus acutus* can utilize glucose (Ogawa and Aiba, 1981 in Lee, 2001). While Demirbas and Demirbas (2011) mentioned this as a drawback, *Scenedesmus* has been known to settle out of solution when not continuously mixed; this characteristic

could potentially provide an avenue for an innovative way to harvest these particular organisms.

6. External influences and oil production

On a broad scale view, the type and diversity of algae and the environmental conditions under which growth occurs makes profiling and charting the biosynthesis of specific lipids difficult. Furthermore, this inconsistency affects the conversion efficiency and quality of the biodiesel that may be produced (Greenwell et al., 2010). Algal lipids that are suitable for use as biodiesel need to possess specific characteristics based mostly on the type of fatty acids that make up the TAGs. Proper ratios of certain types of polyunsaturated fatty acids need to be maintained or regulated to yield the best quality biodiesel (Mutanda et al. 2011; Um and Kim, 2009). Weyer et al. (2010) presented a calculated conservative theoretical maximum yield of oil from algal of 354,000 L/(ha year) based on purely photosynthetic growth of an implied naturally existing alga (no mention of genetic or metabolic engineering).

Metabolic and /or genetic engineering have been suggested as a method for boosting oil production in algae (Mata et al., 2010; Brennan and Owende, 2010). Also stated in Mata et al. (2010), a threat to the natural environment is a possibility, if transgenic algae are used. Greenwell et al. (2010) also cautions that the balance between metabolism and energy storage could be negatively disrupted under genetic engineering; increased lipid production typically means a decrease in cell divisions.

Another means of increasing lipid yield is by depriving the algae of a key nutrient; limiting nitrogen (N) and silicon (Si) has been shown to enhance oil yield in

many species of Chlorophyta and Bacillariophyta, respectively (Griffiths and Harrison, 2009). Nutrient limitation results in the production of TAGs over free fatty acids, which is favorable if biodiesel is the desired product (Brennan and Owende, 2010; Greenwell et al., 2010). Mohan et. al. (2011) saw more lipids in algal cultures that were nitrogen limited. The cost, however, of improved lipid production under nutrient deficient conditions is a retardation of growth and therefore, biomass productivity (Griffiths and Harrison, 2009). In a study conducted on nitrate deprived algae, Mata et al. (2010) stated that the response is species-specific in terms of what types of biomolecules are accumulated; furthermore, in terms of neutral lipids under such conditions, single-chained, saturated fatty acids were less dominant.

7. Processing and analysis

When directly dealing with algal processing, efficient harvesting, extraction, separation and recovery of algal products are of great importance (Greenwell et al., 2010). In terms of biomass processing, harvesting the algae effectively is a challenge, especially when considering scale-up for commercial production (Demirbas and Demirbas, 2011). Of the many unit operations encountered during algal lipid processing, lipid extraction is one of the more difficult to perform efficiently, economically, and sustainably. Many methods have been developed and employed for this operation, but, as emphasized by Mercer and Armenta (2011), one method has yet to be developed to meet all of the aforementioned qualifications.

Many methods are used for quantifying and qualifying the algal lipids being produced. Many *in situ* measurements are done using fluorescent dyes like Nile Red or

BODIPY (Mutanda et al., 2011; Stockenreiter et al., 2012). For extraction, a variety of solvents are in use, the most common being chloroform and methanol or methanol combined with an acid (Das et al., 2011; Yoo et al., 2010; Xiong et al., 2011; Xin et al., 2010). To analyze and classify the lipids, gas chromatography, thin layer chromatography, and high pressure liquid chromatography are used (Chen and Walker, 2011; Mohan et al., 2011; Das et al., 2011; Xiong et al., 2010).

Procedures for bypassing the lipid extraction step have also been analyzed. The direct transesterification method is a simplified procedure for the analysis of the capability of total lipids to be converted to fatty acid methyl esters (FAME) without prior extraction; direct transesterification has even been known to improve yields of transesterfiable fatty acids (Wahlen et al., 2011). Granted, Wahlen et al. (2011) found that a greater excess of the alcohol (methanol) was needed for efficient conversion but stated it was financially compensated by the elimination of using an extracting solvent and by the possibility of reusing the excess alcohol. Sathish and Sims (2012) looked at direct transesterification compared to their developed wet lipid extraction procedure (WLEP). Their developed procedure produced fairly high yields, close to that of the direct transesterification (control), while reducing the problem of chlorophyll contamination of the biodiesel.

8. Conclusion and goal of research

Algae offer ecological benefits such as nutrient removal, product and resource opportunities in fuels and valuable bioproducts. If approached with the mind-set of establishing a multipurpose biorefinery, the prospects for algal utilization are high.

Efficient processing of algal biomass and algal products are still an obstacle despite the advances being made with technology and techniques.

Algal carbon caption by photosynthesis and subsequent storage by converting the carbon to lipids make algal-based fuels an attractive, ecologically resourceful form of energy. In addition, the heterotrophic utilization of waste products such as glycerol and the photosynthetic use of the free resource in sunlight enhance the algal growth process even further. The aim of this research is to employ these ideas as well as use what is locally available, in terms of algal species and an open system bioreactor, to produce algal oils that could potentially be used for the production of biodiesel.

CHAPTER TWO
MANUSCRIPT

Abstract

The focus of this research is to investigate a locally occurring algal genus as an economical source for carbon capture and lipid production. The algal genus *Scenedesmus* was grown in a bi-phased system designed to produce lipids that could be used to make biodiesel. A photoautotrophic phase was provided to sequester carbon. The algae were exposed to a light intensity of 8.5 W/m^2 during the photosynthetic growth phase (21-31 days), resulting in an average algal cell mass of 230 mg/L. Glycerol was provided as a substrate during the heterotrophic phase. However, there was no evidence of glycerol consumption during the 9 hour heterotrophic growth phase. This may have been due to the short time exposure to glycerol. The bi-phased system was run twice. Both runs did result in an increase in total lipid yield during the heterotrophic growth phase. The average initial and final oil yields--dry weight basis--were 2.5% and 9.4% for Run 1 and 11.7% and 37.5% for Run 2.

1. Introduction

Microalgae are a diverse group of unicellular organisms that are easily grown and present in a variety of natural environments (Greenwell et al., 2010; Mata et al., 2010). Compared to the vascular plants, the relatively simple structure of algae is what enables their versatility and growth in varied environments (Brennan and Owende, 2010).

Microalgae are extraordinary organisms that show great promise in various areas of research from nutrient removal to producing biofuels and biochemicals (Greenwell et al., 2010; Mata et al., 2010).

Algae, grown photoautotrophically, serve as carbon sinks for atmospheric carbon dioxide. It is an ideal characteristic considering the growing concern about rising levels of atmospheric carbon and its contribution to climate change. Algae have been shown to have a higher photosynthetic efficiency than terrestrial plants (2-8% compared to 1-2%), resulting in a higher solar to chemical energy conversion (Brennan and Owende, 2010).

Looking to local microalgal cultures as candidates for products, like lipids and biomass, is more economical. It removes the cost of either ordering and culturing pure algal species from culture banks or establishing and maintaining pure cultures. Utilizing a dominant local alga is also economical in that it will be best suited for growth in open systems and responding to environmental changes.

Scenedesmus is a fairly common algal genus found locally in upstate South Carolina. Previous research shows that this microalgae can flourish in open systems both in lab settings as well as in field-scale raceway ponds. Drapcho and Brune (2000), reported on algal growth in a partitioned aquaculture system (PAS). Interestingly, the division of dominant algal species varied with an addition of inorganic carbon - Cyanophyceae (genus *Merismopedia*) predominated when inorganic carbon was not added to the system and Chlorophyceae (genus *Scenedesmus*) predominated when inorganic carbon was added. A factor that promotes *Scenedesmus* dominant cultures is a higher inorganic carbon source (Drapcho and Brune, 2000; Watson, 2009).

Algae have been shown to utilize a variety of organic carbon substrates for hetetotrophic and or mixotrophic growth (Lee, 2001) such as glucose or acetate. Lee (2001) showed that the heterotrophic maximum specific growth rate is generally less than the photosynthetic maximum specific growth rate of algal species. This supports the claim that photosynthetic growth typically yields more biomass than heterotrophic (Griffiths and Harrison, 2009).

One point of inquiry is to investigate the ability of *Scenedesmus* to consume glycerol to produce lipids that can then be converted into biodiesel. Glycerol is a by-product of the transesterification process that is used to convert fatty acids/triacylglycerides (TAGs) into biodiesel (fatty acid esters). Glycerol is a slightly reduced carbon and energy source that can be consumed by some organisms. Considering that it is a by-product of the biodiesel production process, its direct use as a feedstock would be more economical and readily available than many other pure potential carbon sources like glucose. The species *Scenedesmus acutus* has been shown to utilize glucose for heterotrophic growth (Ogawa and Aiba, 1981 in Lee, 2001). El-Sheekh et al. (2013) observed heterotrophic glycerol utilization by *Scenedesmus obliquus* that resulted in a biomass increase when added at concentrations of 0.05 and 0.1 M; however a slight decrease in fatty acids was also observed. Chen and Walker (2011) showed that glycerol can be used as a substrate for lipid production in microalga *Chlorella protothecoides* via strictly heterotrophic growth. Their work resulted in an algal lipid yield of about 50% dry weight, when grown on pure glycerol.

The objective of this research is to investigate the growth and lipid production of a locally obtained *Scenedesmus* culture in an open system as a two-phased scheme--a long photosynthetic growth phase followed by a short heterotrophic phase. The photoautotrophic phase is primarily done to sequester atmospheric carbon and convert it into biomass. In the heterotrophic growth phase, light is excluded and the algae are given an organic carbon source in the form of glycerol. The heterotrophic growth phase is over a much shorter time frame (less than a day), in order to minimize the growth of bacteria in the culture.

2. Materials and Methods

2.1 Preliminary tests

Algae was collected from a pond in the South Carolina Botanical Gardens in Clemson, SC. Observation using a microscope confirmed that the sample was *Scenedesmus* dominant. This water sample was then cultured in lab, using BG11 media (see section 2.3). After an optical density (OD) of 0.05 was achieved, four 4 L reactors of BG11 were prepared and they were inoculated with 200 ml of the *Scenedesmus* dominant sample for the preliminary run.

2.2 Algae culturing for Runs 1 and 2

An algal culture originally collected from a local water body in 2007 and used in prior studies (Watson, 2009) and since refrigerated was obtained and identified as predominantly *Scenedesmus*. This pseudo-pure culture was used to inoculate a liter of prepared BG11 media to test viability. Once the viability of the culture had been

confirmed, the cells were cultured and used as an inoculum at optical densities of 0.069 and 0.161 at 750 nm for Runs 1 and 2, respectively.

2.3 Preparation of media for experimental runs

Media was prepared from stock solutions of the BG11 constituents: 1.5 g/L ($NaNO_3$), 0.04 g/L (K_2HPO_4), 0.075 g/L ($MgSO_4 \cdot 7H_2O$), 0.036 g/L ($CaCl_2 \cdot 2H_2O$), 0.006 g/L (Citric acid), 0.006 g/L (Ferric ammonium citrate), 0.001 g/L (EDTA disodium salt), 0.02 g/L (Na_2CO_3) and 1 ml of Trace metal mix A5 in one liter of distilled water. The trace metal mix A5 consisted of the following: 2.86 g/L (H_3BO_3), 1.81 g/L ($MnCl_2 \cdot 4H_2O$), 0.222 g/L ($ZnSO_4 \cdot 7H_2O$), 0.39 g/L ($NaMoO_4 \cdot 2H_2O$), 0.079 g/L ($CuSO_4 \cdot 5H_2O$), and 49.4 mg/L ($Co(NO_3)_2 \cdot 6H_2O$) in one liter of distilled water. The A5 metal mix and the organic component stock solutions were stored in a refrigerator when not in direct use. A modification from the original media was made using 10 times the carbon which resulted in a concentration of 0.2 g/L of Na_2CO_3 (Watson, 2009). A stock solution of the 0.2 g/L of Na_2CO_3 was made the day of media preparation for the runs to minimize the loss of carbon to the gaseous phase. The modified BG11 media was made in 2 L batches using 2 L volumetric flasks. Immediately after each 4 liter bioreactor was prepared parafilm was placed over the opening to minimize loss of carbon to the atmosphere while the other bioreactors were being prepared. The initial pH of each reactor was taken for both Run 1 and Run 2. For Run 2 the initial pH was adjusted to a pH of 10.3 (using either 0.02N H_2SO_4 or 1.0 N NaOH) before the inoculation of each reactor. Run 1was inoculated with 200 ml of the algal culture while Run 2 was inoculated with the 400 ml of the cultured algae to try and improve the final biomass concentration.

2.4 Photosynthetic autotrophic growth phase

For the experimental set up for photosynthetic autotrophic growth phase, the 4 bioreactors were on 4 stir plates, under four parallel 40 watt, 48 inch Ace Fluorescent F40 T-12 Universal tube lights in fixtures. The reactors received an average of 8.5 $W/m^2$of radiation measured with an MP-100 Apogee pyranometer. Each reactor had a 2 inch stir bar and was positioned on a stir plate for continuous mixing. Each of the reactors were inoculated with the lab-cultured algae. The starting day (day 0) alkalinity and pH were measured before inoculation and reactor pH and optical density (OD) were measured after inoculation.

2.4.1 Analytics

A Thermo Electron Corporation Spectronic 20D+ spectrophotometer was used to measure OD at a wavelength of 750 nm.

For measuring alkalinity 0.02 normal sulfuric acid (H_2SO_4) was prepared as described in Standard Methods (APHA, 1995) Titration Method (2320 B). A sample of 20 ml of culture liquid was taken from a reactor. The pH probe and meter used were the VWR SympHony probe (Cat. No. 14002-764) and the Thermo Electron Corporation Orion 2 Star pH Benchtop meter. Alkalinity, in units of equivalence per liter (Equ/L), was then as shown in equation 1.

These three measurements, pH, OD, and alkalinity titration, were taken daily for Run 1 and for the first 10 days and then every other day thereafter of Run 2. Total inorganic carbon (TIC) was calculated using the following equation 2, 3, and 4 from Stumm and Morgan (1981).

$$Alkalinity = \frac{Vol.of\ Titrant\ added\ (ml) \times Normality\ of\ Titrant\ (\frac{Equivalence}{L})}{Vol.of\ Sample\ (ml)} \quad (1)$$

$$TIC = \frac{[Alk] - [OH^-] + [H^+]}{\alpha_1 + 2\alpha_2} \quad (2)$$

$$\alpha_1 = \left[\frac{[H^+]}{K_1} + 1 + \frac{K_2}{[H^+]}\right]^{-1} \quad (3)$$

$$\alpha_2 = \left[\frac{[H^+]^2}{K_1 \times K_2} + \frac{[H^+]}{K_2} + 1\right]^{-1} \quad (4)$$

Where:

Total Inorganic Carbon (TIC); mol/L

Alkalinity (Alk); Equ /L

$K_1 = 10^{-6.3}$

$K_2 = 10^{-10.25}$

Total suspended solids (TSS) was also determined for each reactor using method 2540 D of Standard Methods (APHA, 1995). A 30 ml sample was taken. A vacuum filtration system was set up and 1 µm glass fiber filters were used. Samples were dried in an oven set to 103°C. The concentration of algae in each reactor was determined by dividing the samples mass by the volume of sample filtered after correcting for the change in mass of the blanks (filtered DI water).

To quantify the OD readings collected throughout experimental run, different concentrations of the algae were made. The OD of each solution was measured and the samples were run through the filtration system and dried, as described previously. A calibration curve corresponding algal TSS to OD was developed

2.5 Heterotrophic growth phase

A sample of 500 ml was taken from each reactor before the addition of glycerol and yeast extract for later lipid analysis. High performance liquid chromatography (HPLC) samples were also taken from each reactor before the addition of glycerol and yeast extract. From the determined algal densities, the amount of pure glycerol and yeast extract expected to be consumed during the 9 hour heterotrophic phase was calculated using the specific growth rate and biomass yield determined by Chen and Walker (2011).

$$Glycerol\ Vol.\ (ml) = \frac{((\frac{1}{Y_b} \times \mu \times X_b \times V) \times t)}{\rho glycerol} * 1000\ ml/L \quad (5)$$

$$Mass\ of\ yeast\ Extract\ (mg) = \frac{1}{Y_b} \times \mu \times X_b \times V \times t \times \frac{4\ mg\ yeast\ extract}{30\ mg\ glycerol} \quad (6)$$

Where:

Y_b - biomass yield 0.48 mg biomass/mg glycerol

μ - specific growth rate 0.029 hr^{-1}

X_b - Concentration of biomass (mg/L)

V - volume (L)

t - time (hrs)

$\rho_{glycerol}$ - 1,260,000 mg/L

The OD, pH, and dissolved oxygen (DO) concentration of each reactor was measured before glycerol and yeast extract addition.

The reactors were then wrapped in foil and the light source was removed. After the addition of the respective amounts of glycerol and yeast extract to each reactor,

samples were taken for the Time-0 HPLC analysis for Run 2. Air stones were added to keep the reactors adequately aerated. 100 ml samples were taken at every hour and a half interval for 9 hours for later analysis. Other measurements taken during these intervals include OD, pH, and DO concentrations. Small samples were also taken and filtered into HPLC vials for glycerol analysis. The 100 ml samples and HPLC samples were frozen for later use.

2.6 HPLC

Standards of glycerol were made at the following concentrations 0.5, 0.4, 0.3, 0.2,0.1, 0.05, 0.02, 0.01 ml glycerol/L DI water, and filtered through 0.45 μm nylon membrane syringe filters into HPLC vials. Samples were frozen until analyzed by HPLC. The HPLC analysis was done on an Aminex HPX-87H column, with 0.01 N sulfuric acid (H_2SO_4) as the mobile phase, set to a temperature of 60°C with a flow rate of 0.6 ml/ min and using a refractive index detector (RI detector). The reactors samples were again run in the perceived ascending order (not sorted by reactor), starting with the samples taken the latest.

2.7 Preparing frozen samples for lipid extraction

The 100 ml samples were frozen in two 50 ml centrifuge tubes. These tubes were taken out of the freezer to thaw at room temperature. After thawing the samples were placed into a centrifuge (Eppendorf Centrifuge 5702) set at 3,500 rpm for 2.5 minutes. Once a pellet was formed, the supernatant was removed and the algal pellets were combined into one tube and centrifuged again. Once a pellet was formed in this tube, the supernatant was again decanted and distilled water was added to the pellet as a washing

step to remove the glycerol that was in the sample. The sample was again centrifuged and washed. For Run 1 and half of Run 2 the samples were then placed in the oven set at 103°C for additional water removal. Lyophilization was used instead of oven drying the samples for better preservation of lipids. Therefore, the remaining samples of Run 2, about half of the pellets, were lyophilized

2.8 Lipid extraction and quantification

To conduct lipid extraction, 15 ml of hexane was added to the centrifuge tubes containing the dried algal pellets. The solvent and pellets were then homogenized for 5 minutes using a Polytron PT-1200 homogenizer. The slurry was then placed in a water bath set at a temperature of 55°C for 5 minutes before being homogenized again for an additional 5 minutes. The centrifuge tube was then placed in a centrifuge to run at 3,200 rpm for 5 minutes. The hexane/lipid supernatant was decanted into a preweighed centrifuge tube and the extraction procedure was repeated, using 5 ml of hexane on the remaining biomass, to retrieve any remaining lipids. The supernatant after centrifugation was added to the already decanted supernatant from the previous run. The procedure using 5 ml of hexane was repeated for additional lipid extraction if the hexane had a tinted appearance. The collected supernatant transferred to pre-weighed aluminum dishes to dry the extracted lipids-hexane solution in an oven set to 50°C for 35 minutes and reweigh the tins.

3. Results and Discussion

3.1 Preliminary run

Shown in Figure 3.1.1 is the photomicrograph (taken at 40x) from the preliminary run. For the preliminary run, despite the inoculum being *Scenedesmus* dominant, all reactors experienced a culture shift from the green alga *Scenedesmus* to the blue-green algae (cyanobacteria) *Oscillatoria* over the course of the photosynthetic growth phase when BG11 medium was used. While *Scenedesmus* was still present, their appearance was low in number. The *Oscillatoria*, on the other hand, thrived. As can be seen in Figure 3.1.1, *Scenedesmus* is still present in very low numbers.

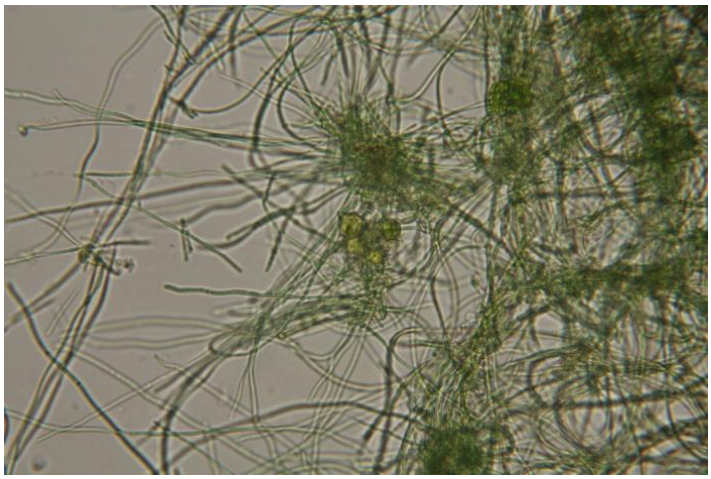

Figure 3.1.1 Photomicrograph of the preliminary run taken around day 20 at 40x magnification.

3.2 Photosynthetic growth phase -- Run's 1 and 2

Figure 3.2.1 shows the photographs of the cultures. *Scenedesmus* was confirmed to be the dominant species throughout both experimental runs.

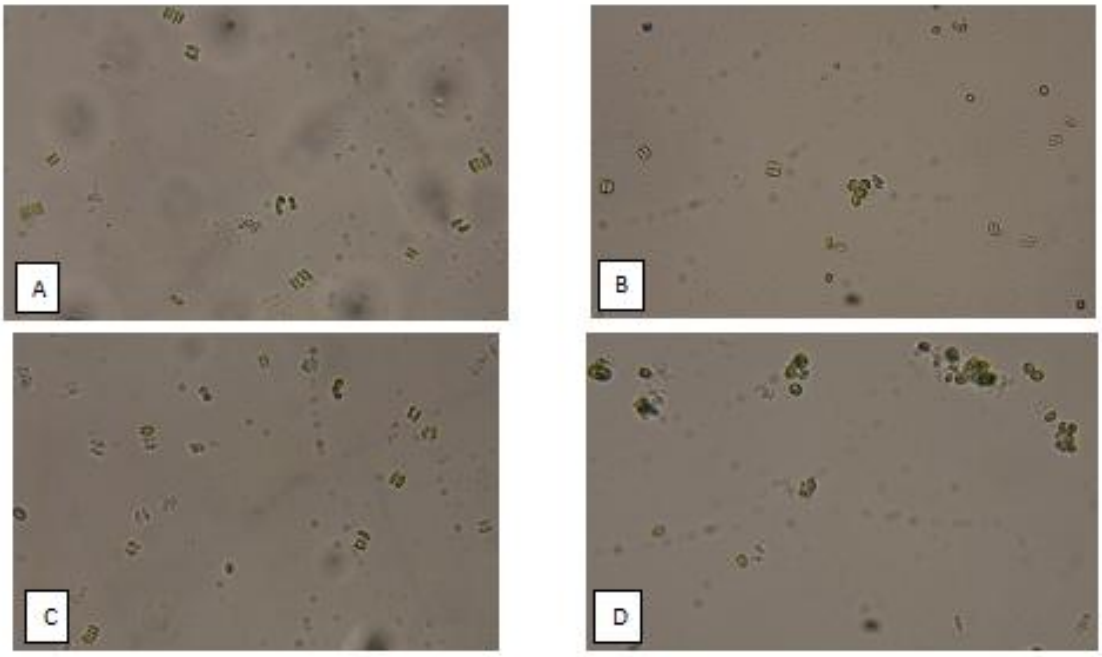

Figure 3.2.1 Micro graphs of algae (40x). A. day 5 of Run 1, B. day 5 of Run 2, C. day 20 of Run 1, D. day 20 of Run 2

Figure 3.2.2 shows the first and last day of the photosynthetic growth phase for both Run 1 and Run 2. The inoculum for Run 1 was 5% of the reactors working volume. Run 2 was inoculated with 10% of the reactors working volume. The inocula for both runs were *Scenedesmus* dominant cultures and *Scenedesmus* dominated the cultures throughout the growth phase for both runs. In these runs modified BG11 medium containing 0.2 g/L Na_2CO_3 was used. The photosynthetic phase of Run 1 was carried out for 21 days and that of Run 2 was carried out for 31 days. The purpose of the longer time frame and larger inoculum volume was to acquire more biomass by the end of the run.

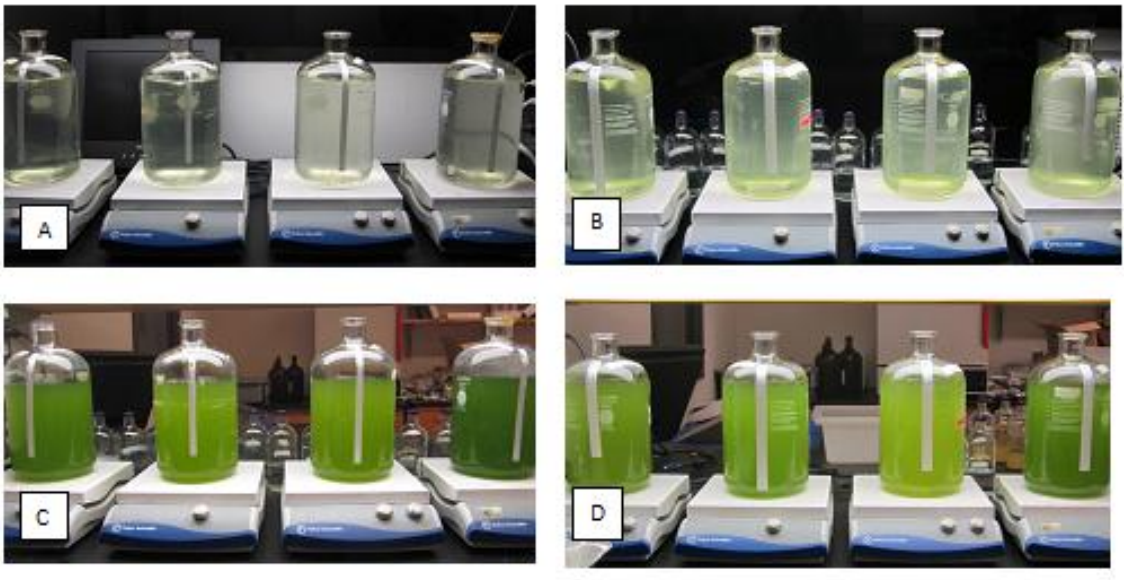

Figure 3.2.2 Reactors A. Run 1 at day 0, B. Run 2 at day 0, C. Run 1 at day 20, D. Run 2 at day 20

Throughout the photosynthetic growth phase the optical density (OD) showed a steady increase in all reactors (Figures 3.2.3 and 3.2.4). Reactors were rotated on the stir plates throughout Run 2, on the stir plates approximately once every five days to equalize the light received over the course of this experimental run (Table 3.2.1). In general, the reactors on the outer edges received less intense light than the reactors in the center. Figure 3.2.2 C displays the brighter coloring of the reactors in the center compared to the darker color of the reactors on the outer edge of the light source. Figure 3.2.2 D show less of a color variation among the reactors.

Table 3.2.1 Pyranometer readings over rotation of reactors for Run 2 (spectrum range of instrument was 360-1120 nm). Letters correspond to the reactor replicate identification. Upper measurements were taken at the top of the liquid level, lower at the base of the reactors. Light measurements taken between reactors and at the outer edges (edges designated by a dash "-")

Day 0	lab lights off	-,A	A,B	B,C	C,D	D,-	Average
W/m^2	Upper	7	16	18	16	5	12.4
	Lower	3	6	6	6	3	4.8
Day 1	lab lights on	-,A	A,B	B,C	C,D	D,-	Average
W/m^2	Upper	7	15	19	17	5	12.6
	Lower	4	6	7	6	3	5.2
Day 5	lab lights on	-,B	B,C	C,D	D,A	A,-	Average
W/m^2	Upper	7	16	17	16	6	12.4
	Lower	3	6	6	6	2	4.6
Day 10	lab lights off	-,C	C,D	D,A	A,B	B,-	Average
W/m^2	Upper	7	15	17	16	5	12
	Lower	3	5	6	5	2	4.2
Day 15	lab lights off	-,D	D,A	A,B	B,C	C,-	Average
W/m^2	Upper	7	15	17	16	6	12.2
	Lower	2	5	6	5	2	4
Day 20	lab lights on	-,A	A,B	B,C	C,D	D,-	Average
W/m^2	Upper	7	15	16	15	5	11.6
	Lower	3	6	6	5	2	4.4
Day 25	lab lights on	-,B	B,C	C,D	D,A	A,-	Average
W/m^2	Upper	7	14	16	16	5	11.6
	Lower	3	5	6	6	2	4.4
Day 31	lab lights on	-,B	B,C	C,D	D,A	A,-	Average
W/m^2	Upper	6	13	15	13	5	10.4
	Lower	3	6	7	6	3	5

As shown in Figure 3.2.4, this rotation routine had little influence on the growth and subsequent OD measurements after the tenth day. Reactors C and D consistently had higher OD readings, despite their positioning under the light.

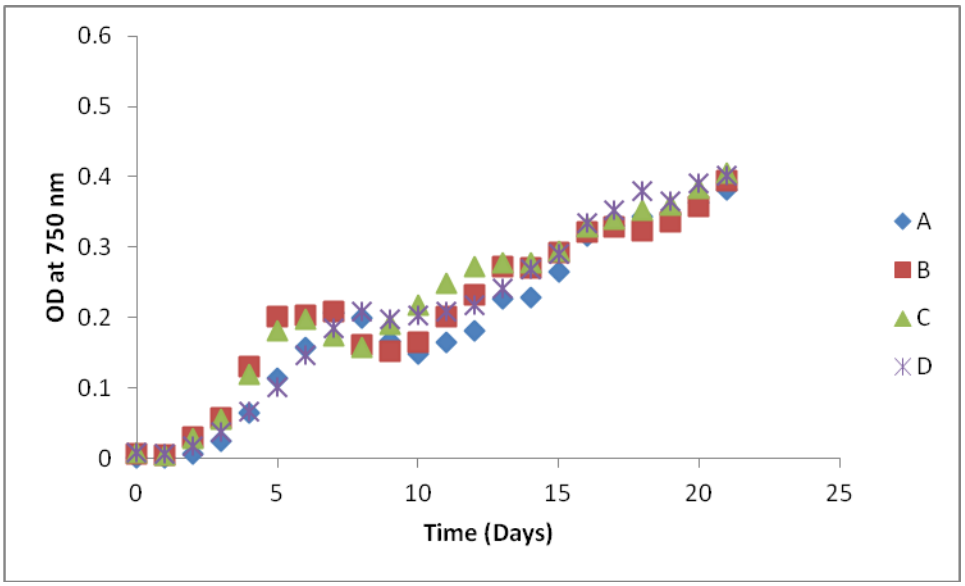

Figure 3.2.3 Optical density measured at 750 nm over photosynthetic growth, Run 1

A trend of rapidly increasing OD from days 0-5, followed by a plateau then further increase is observed in both runs (figures 3.2.3, 3.2.4, and 3.2.5). This trend was also observed in Watson (2009) in the early phase of growth.

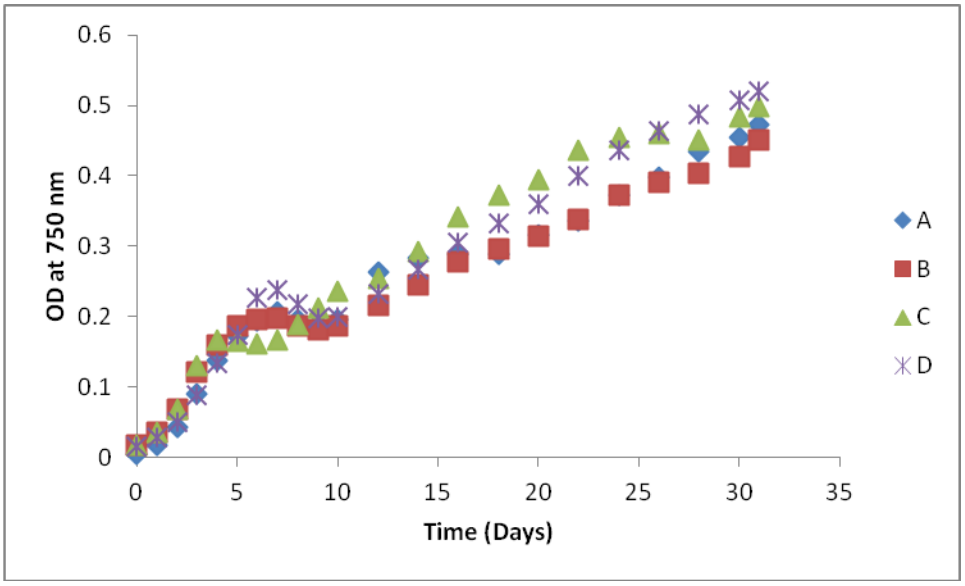

Figure 3.2.4 Optical density over photosynthetic growth phase, Run 2

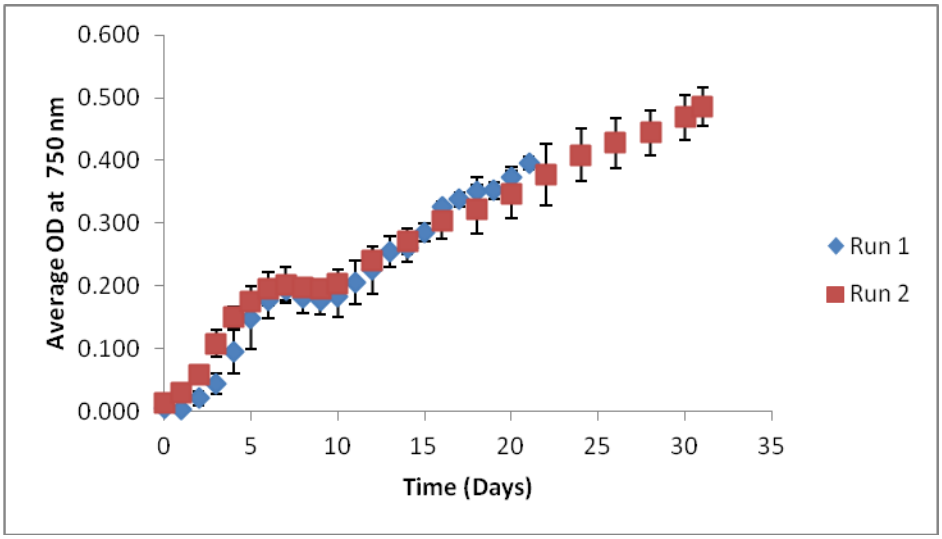

Figure 3.2.5 Comparison of average OD for both Runs 1 and 2 for photosynthetic growth phase

To get a more direct measure of biomass in the reactors a correlation between OD and TSS was created. Figure 3.2.6 shows the standard curves of TSS for Run 1and Run 2. The lower OD of Run 2 compared to Run 1 resulted in a corresponding lower total suspended solids (TSS) for Run 2 compared to Run 1 (Figure 3.2.7).

The difference between the average TSS values of the two runs is magnified by the differences in the slopes of each run on the standard curve.

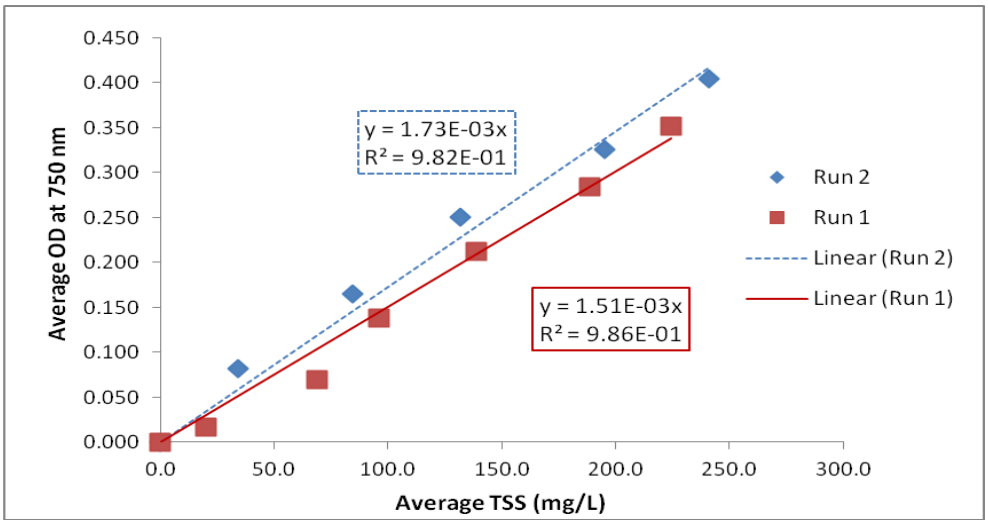

Figure 3.2.6 Standard curve for quantifying biomass concentration during the photosynthetic phase

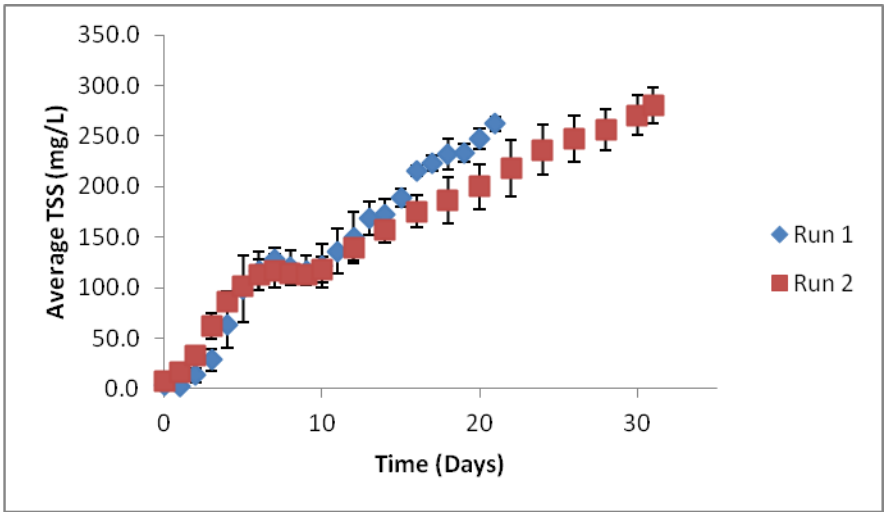

Figure 3.2.7 Comparison of average TSS for both Runs 1 and 2 for photosynthetic growth phase

Expressed as TSS, Run 1 had an average of 249 mg/L and Run 2 had an average of 203 mg/L at day 20. This compares fairly well with Watson's (2009) biomass concentration at 20 days reflecting a value of about 200 mg/L for the algae in the same

modified media. Abou-Shanab et al. (2011) reported biomass concentrations of different strains of *Scenedesmus obliquus* species ranging 1.57-1.98 g dw/L for photosynthetic growth over a 21 day time period in Bold's Basal Medium.

Figures 3.2.8 and 3.2.9 present the changes in pH over the photosynthetic growth phase. The pH for both runs stabilized around 11, indicating a fairly alkaline solution.

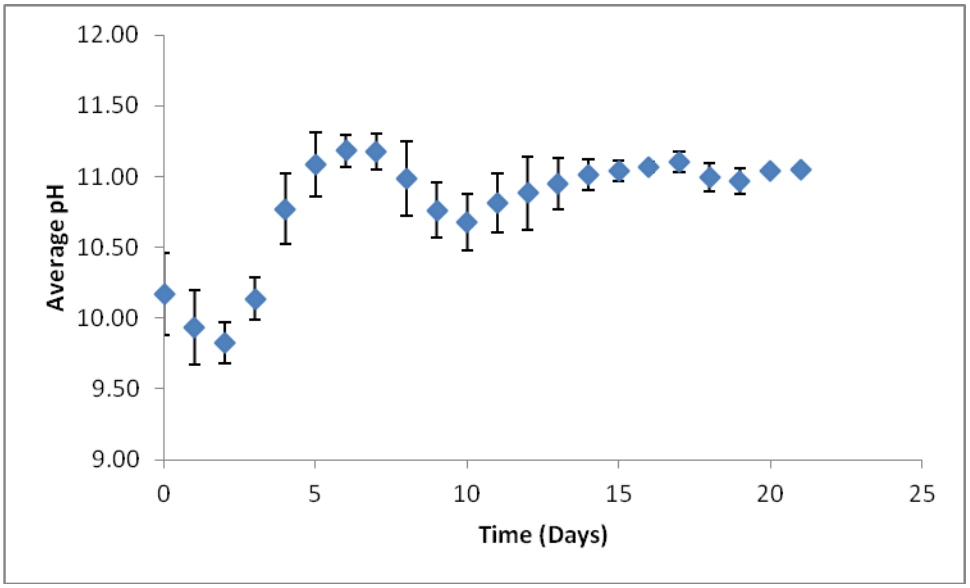

Figure 3.2.8 Average pH during the photosynthetic phase of the Run 1

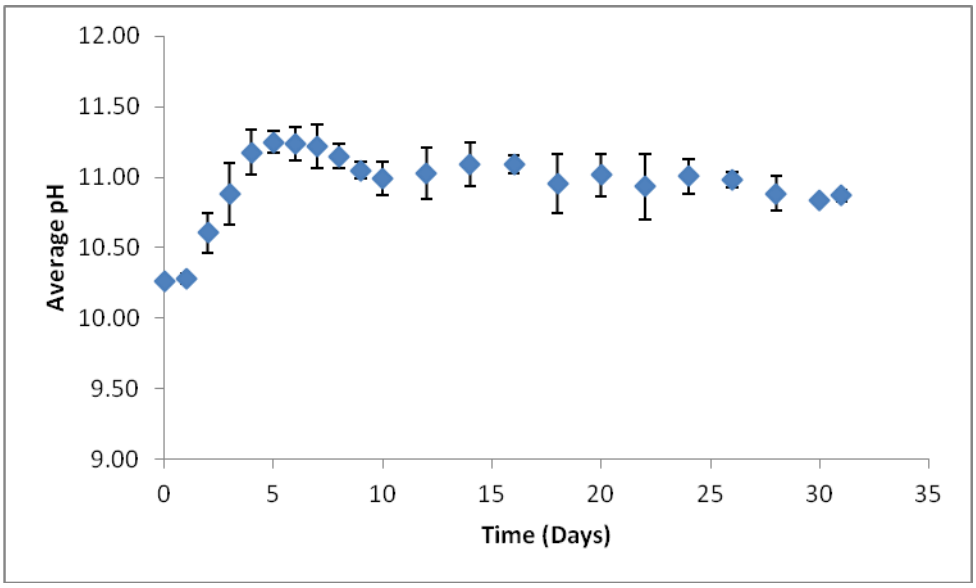

Figure 3.2.9 Average pH during the photosynthetic phase of Run 2

A characteristic that is important for natural systems is the ability to resist changes in pH. This is known as the alkalinity, or buffering capacity of the water source. Both runs saw steady increases in alkalinity as the photosynthetic phase proceeded (Figures 3.2.10 and 3.2.11). The increase observed in alkalinity is due to increases in the primary contributors, carbonate and bicarbonate in the water (APHA, 1995).

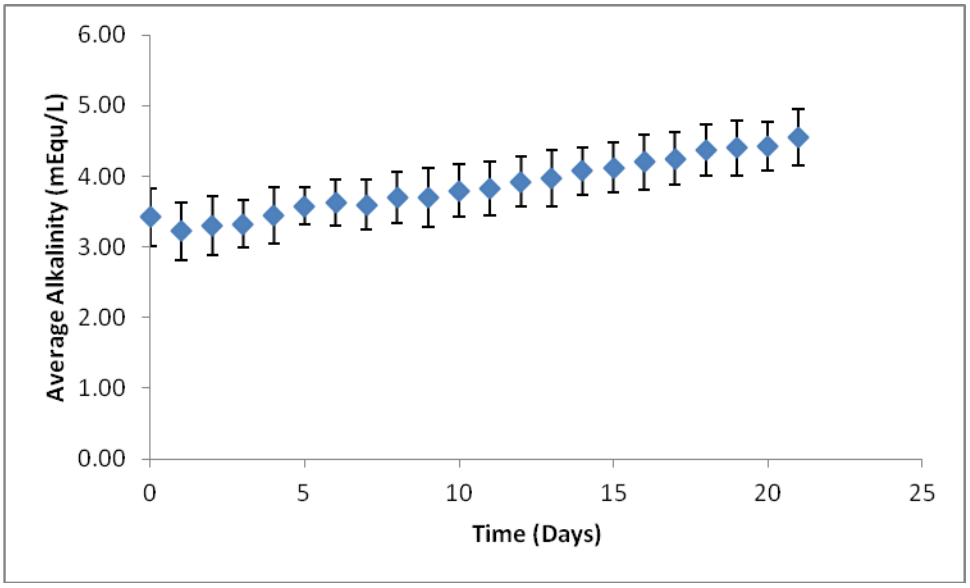

Figure 3.2.10 Average alkalinity during the photosynthetic phase of the Run 1

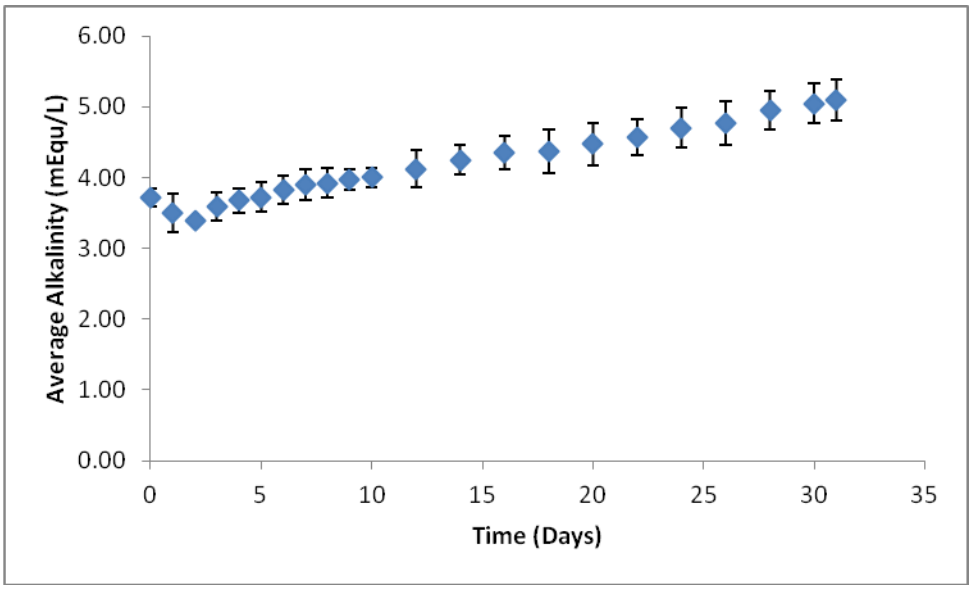

Figure 3.2.11 Average alkalinity during the photosynthetic phase of Run 2

Finally, the total inorganic carbon was calculated in each reactor during both runs. This value reveals the amount of atmospheric carbon that diffused into solution. Figures

3.2.12 and 3.2.13 represent the total inorganic carbon (TIC) present in the media. The TIC consists of carbonic acid, bicarbonate, and carbonate. In both cases (Runs 1and 2), carbonate makes up the majority of the inorganic carbon present in the reactors. Furthermore, the fairly high starting pH of the reactors is also linked to the higher concentration of sodium carbonate, which is the modification from the original BG-11 media. The original media, with 0.02 g/L Na_2CO_3, had a pH of about 8.5.

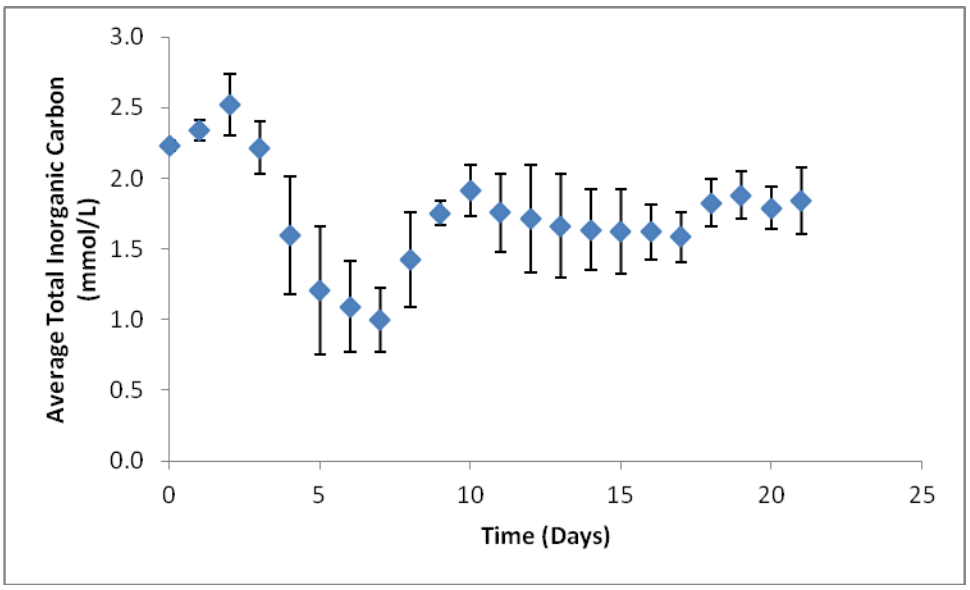

Figure 3.2.12 Average TIC levels during the photosynthetic growth phase, Run 1

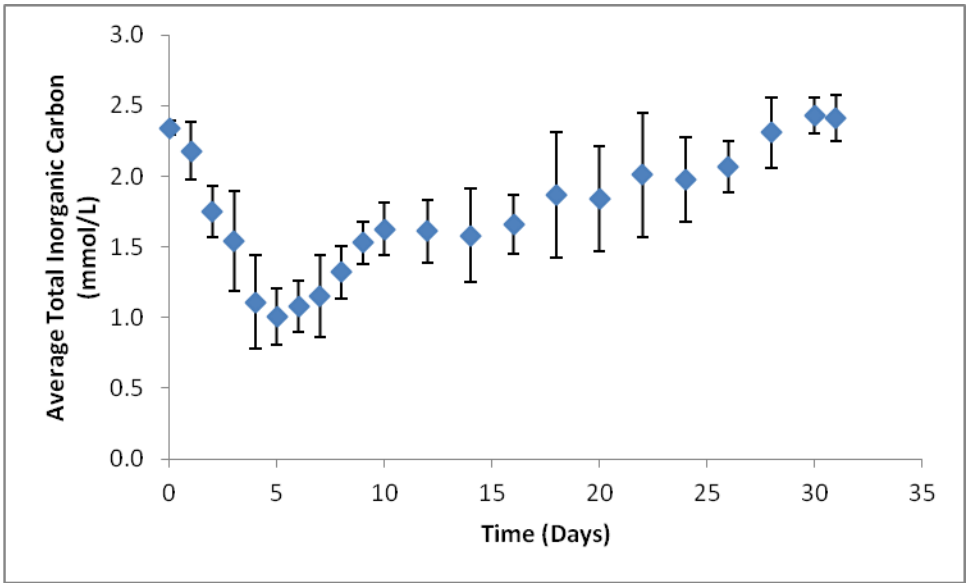

Figure 3.2.13 Average TIC levels during the photosynthetic growth phase, Run 2

3.3 Heterotrophic Growth Phase

Based on the final biomass concentrations in each reactor, the calculated amounts of glycerol and yeast extract were added to each reactor. Concentrations of the carbon and nitrogen source are shown in Table 3.3.1.

Table 3.3.1 Amounts of glycerol and yeast extract added to reactors for heterotrophic growth phase

Run 1	Reactors	A	B	C	D	Average
Glycerol	mg gly/L media	108	129	133	123	123.2
Yeast extract	mg yeast extract/L media	14.3	17.3	17.7	16	16.3
Run 2	Reactors	A	B	C	D	Average
Glycerol	mg gly/L media	138	118	147	109	128.0
Yeast extract	mg yeast extract/L media	18.4	15.8	19.6	14.5	17.1

An initial measurement of dissolved oxygen was taken before the glycerol feeding and implementation of the air stones. After, DO readings were taken regularly at

1.5-- hour intervals (Figure 3.3.1). Time 0 represents measurements at the very end of the photosynthetic growth phase.

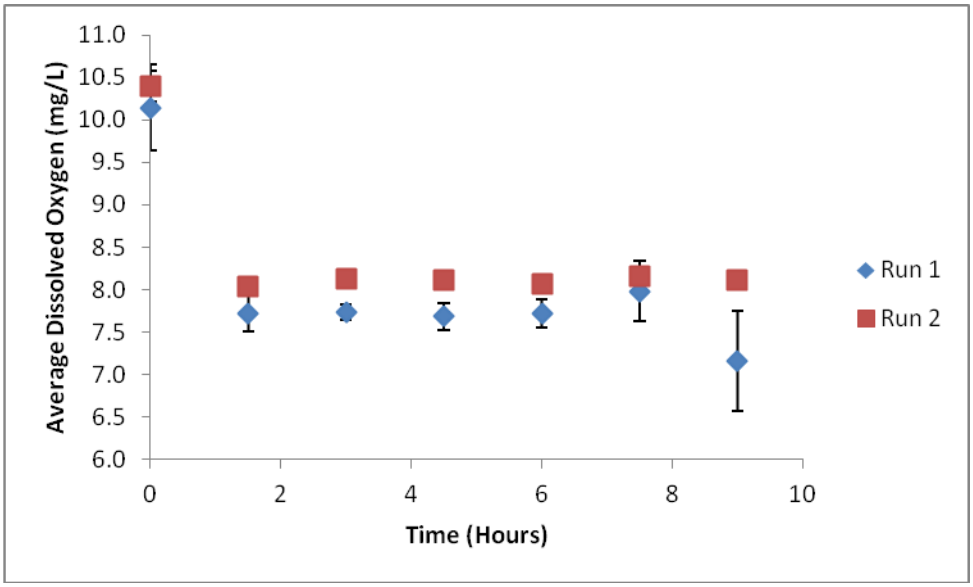

Figure 3.3.1 Average dissolved oxygen readings over the course of the heterotrophic phase

For Figure 3.3.1, there is an immediate drop in the oxygen levels after the initial reading. Because DO values were above saturation, the addition and use of the air stones would have bubbled out oxygen originally present in the water.

Figure 3.3.2 reflect the average changes in pH within the reactors. Grobbelaar (2000) proposed the idea that continued high light exposure may lead to increased respiration rates in the dark. These respiration rates level off with extended exposure to the darkness (Grobbelaar, 2000), which may explain the phenomenon in Run 2 shown in Figure 3.3.2. While both runs received the same ratios of glycerol and yeast extract. The resulting pHs over the course of the runs were quite different.

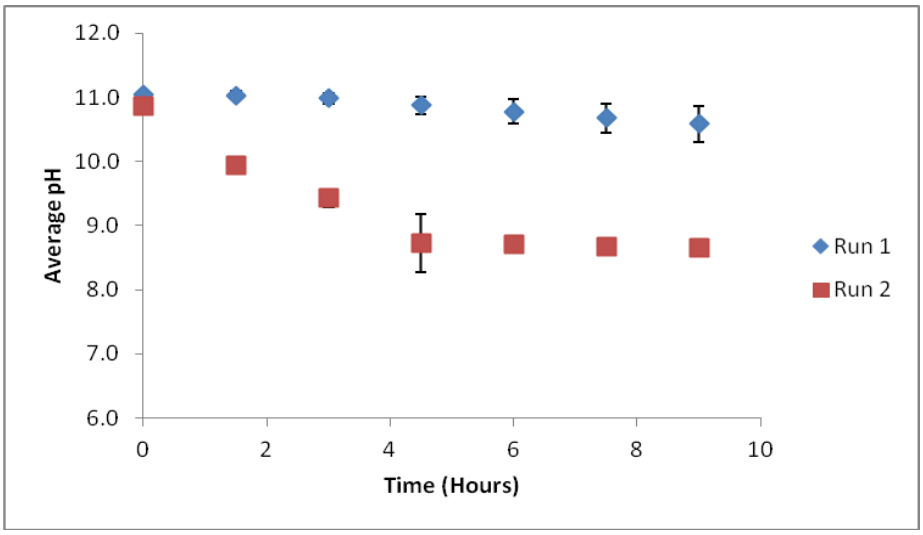

Figure 3.3.2 Average changes in pH during heterotrophic growth phase

Similar to the reactor pHs, the reactor OD values displayed more variance in Run 2 over Run 1 (Figures 3.3.3 and 3.3.4). Interestingly, the varying observed color of the algae in Run 1 did not appear to have much of an effect on the resulting OD, in that each reactor displayed uniform readings on the spectrophotometer (Fig. 3.3.3). Conversely, Run 2, which appeared to have a fairly uniform algal color among all reactors, displayed a more varied OD reading initially and throughout the heterotrophic growth phase (Fig. 3.3.4).

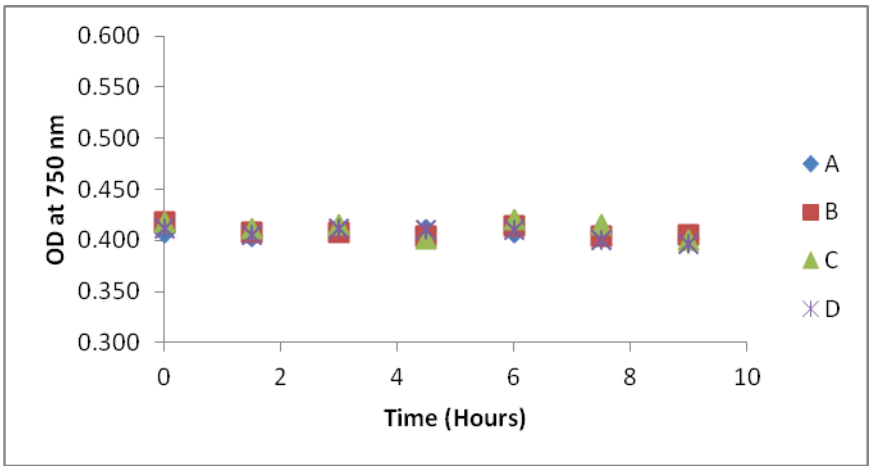

Figure 3.3.3 Varying OD over Run 1 heterotrophic growth phase

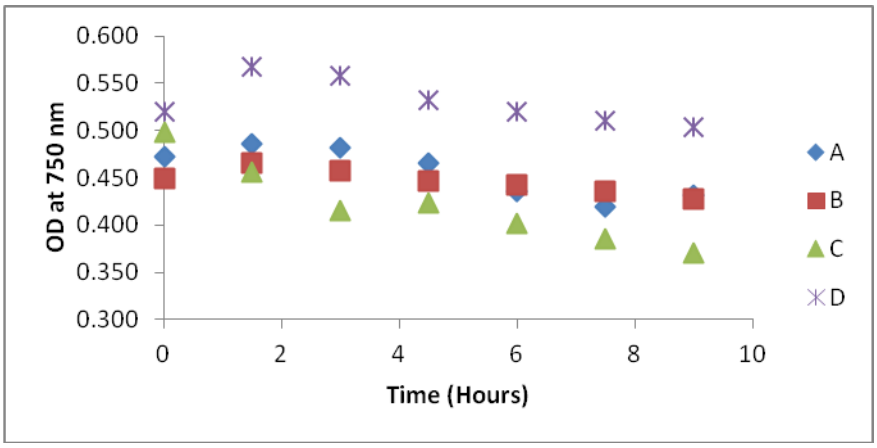

Figure 3.3.4 Varying OD over Run 2 heterotrophic growth phase

Table 3.3.2 shows the average starting and ending biomass concentration for both runs. There was an increase in biomass concentration during the heterotrophic growth phase in Run 2 (Table 3.3.2) despite the observed decrease in OD for Run 2 (Figure 3.3.4). The apparent decrease in OD may be due to a reduction in chlorophyll over the course of the heterotrophic phase. A degradation of chlorophyll, signified by a fade in color, was also detected in a two-phased photosynthetic-fermentation growth scheme on

Chlorella conducted by Xiong et al. (2010); as the growth scheme shifted from autotrophic to heterotrophic.

Table 3.3.2 Average TSS values for time 0 and the end of the heterotrophic growth phase

Average Heterotrophic TSS Values		
mg/L		
Run	Initial	Final
1	262.3	270.8
2	280.3	317.7

Figure 3.3.5 depicts the standard curves used for the individual experimental runs and how they compare to each other. The corresponding equations for the trend lines were used to correlate the HPLC readings to glycerol concentration when analyzing the glycerol present in each reactor.

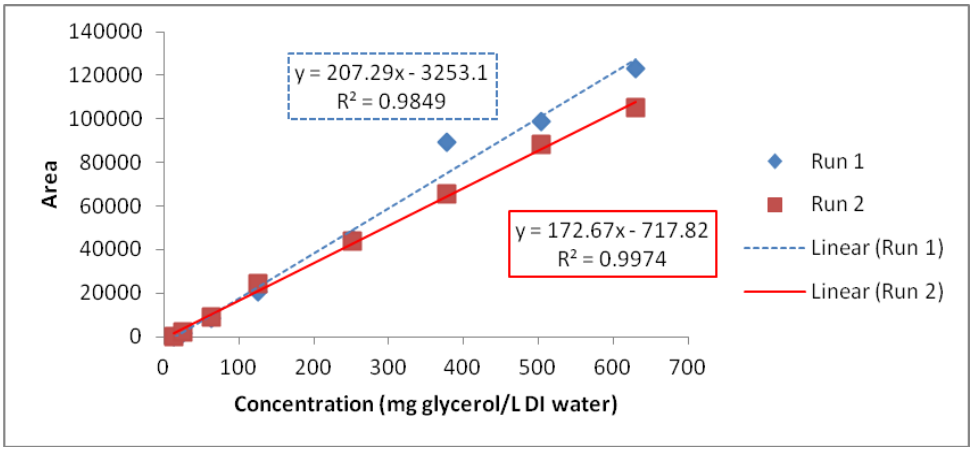

Figure 3.3.5 Glycerol standard curve for HPLC results (comparing Run 1 and Run 2)

Initial media samples were taken from each reactor before glycerol addition (not shown in Figures), to make sure other compounds that were present did not elute during the same time frame as the glycerol. Figure 3.3.6 shows the glycerol concentrations during the heterotrophic growth phase of Run 1, with samples starting at the first sampling time (1.5 hours in to the run). A sample (labeled for time 0) was taken immediately after the glycerol and yeast addition in all the reactors (Fig. 3.3.7); in conjunction with the regular sampling done in the previously described time intervals, for Run 2. This time 0 sample was done to make sure there was no initial glycerol consumption as the calculated glycerol amount added to each reactor was a significantly larger than the HPLC values at the first sampling time.

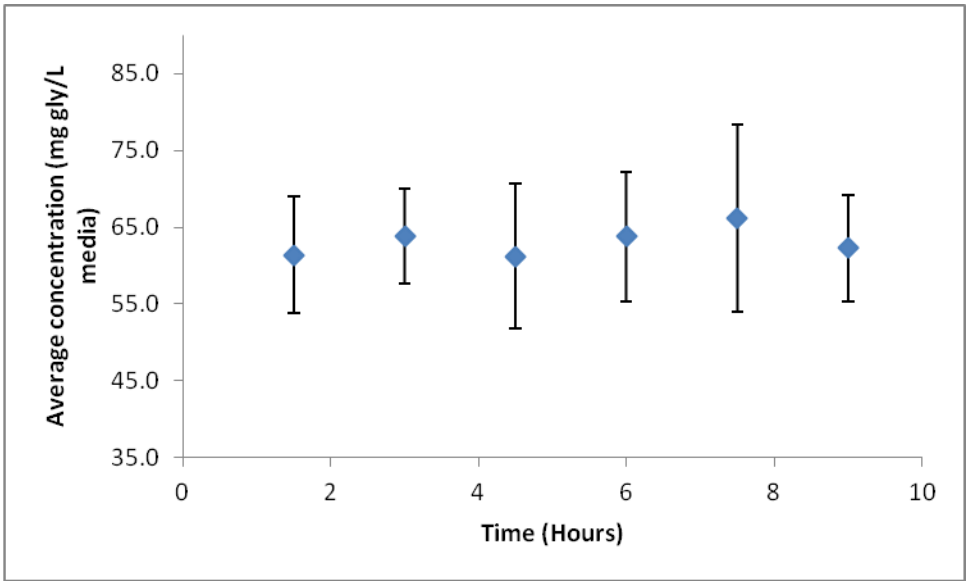

Figure 3.3.6 Average HPLC results of glycerol content over Run 1 heterotrophic growth phase

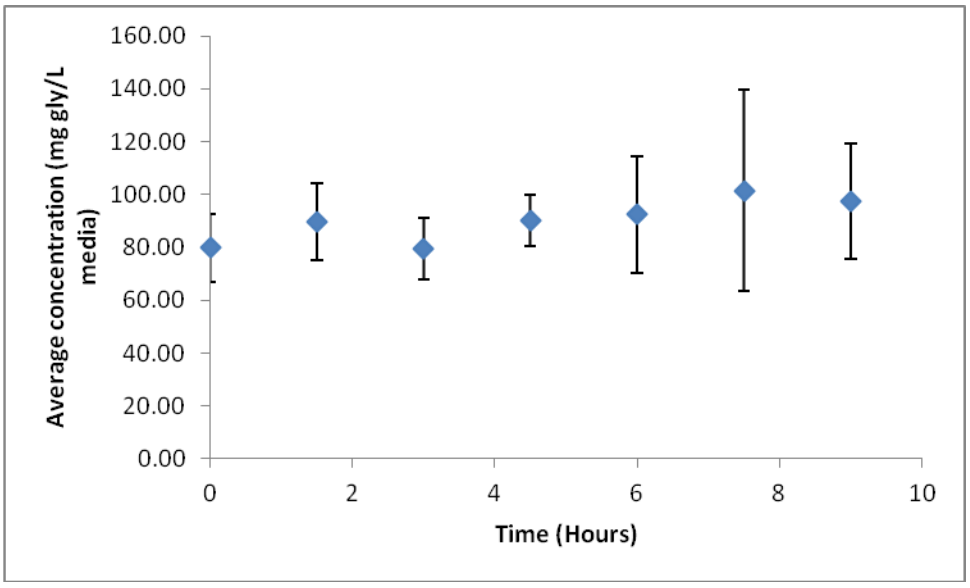

Figure 3.3.7 Average HPLC results of glycerol content over Run 2 heterotrophic growth phase

The concentration of glycerol did not decline with time (Figures 3.3.6 and 3.3.7). Therefore under the conditions of this experiment-physical environment, nutrients available and the time frame of the two phases--the *Scenedesmus* did not consume the glycerol in the heterotrophic growth phase. During the photosynthetic growth phase, the algae were not exposed to dark periods but were kept under lights during the entire length of the first growth phase. During the 20-30 days of the photosynthetic phase, there may have been a retardation in the signaling for the development of necessary enzymes that would enable the cells to carry out glycerol metabolism and a longer time frame may have been needed. Lee (2001) poses the possibility that microalgae maintained under photosynthetic conditions may not possess the ability to efficiently metabolize organic carbon substrates; this would be the case if the necessary enzymes are not in place to catabolize glycerol into the needed intermediate for lipid synthesis. The particular species

of *Scenedesmus* -used in these experiments- may not have the ability to consume glycerol. The apparent trend displayed in Run 2 in Figure 3.3.2, does seem to reflect that some type of respiration did occur in that particular run, for all reactors.

3.4 Total Lipids Quantification

For Run 2 there was an observable presence of non-polar bio-molecules, such as lipids and chlorophyll as seen in Figure 3.4.1, in the hexane solvent. There was a change in appearance of the extracted materials as a function of time, starting with the autotrophic extract and continuing through the heterotrophic growth phase extractants (Fig. 3.4.1). This observation further supports the possibility for chlorophyll degradation as evidenced in the drop in OD shown in Figure 3.3.4 and the apparent fade in extractant color shown in Figure 3.4.1.

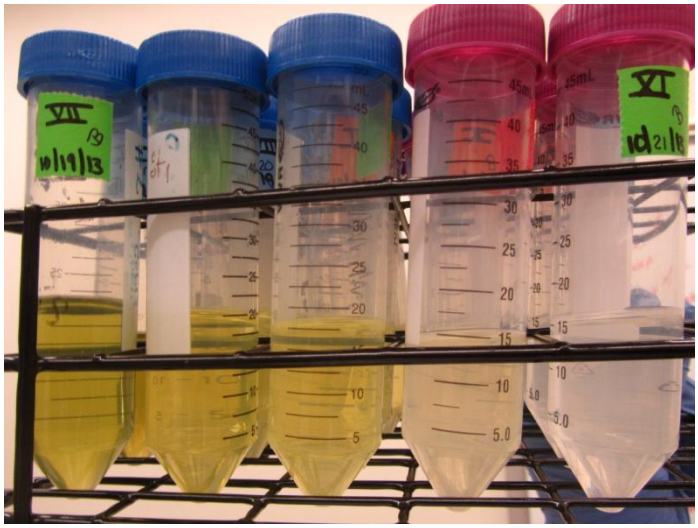

Figure 3.4.1 Extracted lipids in hexane solvent for Run 2. From left to right: 0, 1.5, 3, 6, and 9 hour interval heterotrophic growth lipid extractions

Using the measured values of lipid masses and the calculated volumes of sample used for extraction, oil concentrations could be determined (Tables 3.4.1 and 3.4.2).

Table 3.4.1 shows an increase in lipids from the autotrophic to heterotrophic growth phase; however on average there is a decrease in lipids as the heterotrophic phase continues. In Table 3.4.2, the increased lipids near the end indicate that lipid anabolism did occur in all reactors. Chen and Walker (2011) reported an oil yield of about 0.5 g lipid/g biomass for their *Chlorella protothecoides* heterotrophic culture on pure glycerol. Das et al. (2011) gave photosynthetic and mixotrophic (using glycerol) oil yields of about 14.7% and 19%, respectively, for their alga, *Nannochloropsis*. Different strains of *Scenedesmus obliquus* grown photosynthetically in Abou-Shanab et al. (2011) had lipid concentrations that range from 0.4-.91 g/L, which correspond to 21-58% lipid yields. On average, however, the photosynthetic lipid yield was about 20% of the biomass of *Scenedesmus* sp. (Griffiths and Harrison, 2009).

Table 3.4.1 Values of oil concentrations from Run 1 heterotrophic phase (time 0 represents values from autotrophic growth phase)

Oil concentration (mg total lipids/ L sample)					
Time (hours)					
Reactor	0	1.5	3	6	9
A	5.8	41.0	36.0	30.0	60.0
B	6.2	36.0	49.0	56.0	11.0
C	9.0	44.0	31.0	22.0	10.0
D	5.6	28.0	18.0	27.0	21.0
Average	6.7	37.2	33.5	33.8	25.5
Standard Deviation	1.6	7.0	12.8	15.2	23.5

Table 3.4.2 Values of oil concentrations from Run 2 heterotrophic phase (time 0 represents values from autotrophic growth phase)

Oil concentration (mg total lipids/ L sample)					
Time (hours)					
Reactor	0	1.5	3	6	9
A	29.4	40.0	36.0	122.0	118.0
B	37.8	33.0	31.0	119.0	127.0
C	25.2	27.0	30.0	150.0	116.0
D	39.0	48.0	36.0	131.0	116.0
Average	32.9	37.0	33.3	130.5	119.3
Standard Deviation	6.7	9.1	3.2	14.0	5.3

Table 3.4.3 shows the increase in average oil yield of the *Scenedesmus* in heterotrophic growth phase of both runs. Run 2 was more dynamic in most of the measurements taken during the heterotrophic growth phase, signifying the occurrence of metabolism. This may account for the greater difference between the starting and ending lipid yields shown in Table 3.4.3 for Run 2 compared to Run 1. These lipid values also compare fairly well with those obtained by Abou-Shanab et al. (2011).

Table 3.4.3 Average starting and ending oil yields from the heterotrophic growth phase

Average oil yield		
mg lipid/mg biomass		
Run	Initial	Final
1	0.025	0.094
2	0.117	0.375

When considering the occurrence of photosynthesis during the day for algae in natural systems the Calvin cycle is typically displayed as CO_2 being converted to a simple sugar like glucose (Brennan and Owende, 2010). During the night, aerobic respiration is occurring as the algae metabolize their sugars for biomass (biomolecules)

and energy needs. With regards to this work, the algae were exposed to continuous light during the photosynthetic growth phase, leading to the idea that for the most part sugars were being formed. Without the diurnal pattern of light and dark, respiration may have been reduced. The presence of acidic products from metabolic respiration caused a drop in pH (Figure 3.3.4). During the metabolism of these stored sugars, additional biomolecules, like lipids, could be synthesized (Table 3.4.1) and the bubbling of oxygen with the air stones may have balanced the consumption of oxygen by the algae (Figure 3.3.1). Within the 9 hours given to algae for heterotrophic growth, the algae may have been preferentially consuming stored sugars for metabolism, and therefore not utilize the glycerol.

Additional time may be necessary to determine if the *Scenedesmus* sp. used can metabolize glycerol. Mata et al. (2010) cites photoheterotrophic metabolism as a type of metabolism performed by the algal species *Scenedesmus acutus*. In this type of metabolism light is required for the use of organic compounds (Mata et al., 2010), which may be another possible explanation for the lack of glycerol utilization in this case.

4. Conclusion

The photosynthetic growth phase produced an average of 225 mg/L algal cell mass in 20 days. Given the results of the heterotrophic test runs, the data does not fully support a conclusion that Scenedesmus can or cannot consume glycerol. Lipids were produced and an increase in lipid production was seen when the cultures were switched from the photoautotrophic growth phase to the heterotrophic growth phase in all reactors tested despite having no apparent glycerol uptake. Future studies could be done to

investigate the possibility that removing light without adding an external organic carbon source would increase lipid production in algal biomass.

CHAPTER THREE

CONCLUSIONS AND FUTURE RESEARCH

Bi-phased algal growth can provide many benefits for culturing algae for extraction of lipids. Photosynthetic growth provides a means for CO_2 capture and helps build up biomass. Heterotrophic (or even mixotrophic) growth allows for lipid accumulation in the biomass and the utilization of substrate; preferably a substrate that is a product from another source that normally would be discarded as waste. The use of local algae in a raceway open pond is also a very viable option especially when considering the utilization of the cultures already present. A possibility exists to select for a culture to dominate by changing some aspect of the immediate environment, in terms of nutrient or carbon concentrations.

Many possibilities exist for where the next step may result from the work presented in this research. If the use of *Scenedesmus* cultures is desired, it may be useful to alternate light and dark periods during the photosynthetic growth phase or lengthen the heterotrophic growth phase to examine glycerol utilization. Better data should be obtained on the lipid profile during the photosynthetic growth phase, either by collecting a larger sample size and analyzing total lipids or by performing a FAME analysis.

Other algae could also be analyzed in a similar fashion to how this research was conducted while incorporating some of the changes/adjustments suggested. Locally, one of the dominant cultures in a PAS unit was a species of *Euglena. Oscillatoria* is a genus of cyanobacteria that has fared quite well in a lab setting, at the very least, as a very dominant culture without purposeful purification and isolation. Even conducting the

experiments on a mixed culture and watching for indications of a species domination over time may be worthwhile.

Additional ideas for future research may include the use of nutrients, nitrogen and phosphorus, from wastewater or anaerobically digested nutrients instead of fertilizer and chemically synthesized nutrient forms for algal growth. Use of CO_2 rich industrial flue gas for incorporating inorganic carbon into solution for photosynthetic growth is an option. The CO_2 can subsequently be used for mixing by agitation by the bubbles if desired. The use of substrate rich wastewater or another "waste" material from a process for heterotrophic growth is also favorable. The incorporation of these resources may also serve as a means for selecting the species best suited for growth under such culture conditions.

Finally, from a biorefinery view point, information is needed for feasible uses of the products obtained from the algae. Meaningful uses for the carbohydrate, protein and lipid components could include fermentation substrates, nutritional supplements and biodiesel. Many of the possible uses of algae have been looked at on a one product basis. Applying an approach for obtaining multiple products from processed algae and following through to the trial phase has yet to be fully developed. The more utility and versatility that can be gained from the use of algae the more profitable and economical its use will be. Algae are a clean, sustainable, and ecologically viable resource that have great promise in contributing to the arsenal of possible solutions to many of the present problems.

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