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THE INFLUENCE OF MEDIA INGREDIENTS AND PH ON THE GROWTH OF *CHLOROMONAS ROSAE VAR. PSYCHROPHILA*

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THESIS

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science in Biosystems and Agricultural Engineering in the College of Engineering at the University of Kentucky

By

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ABSTRACT OF THESIS

THE INFLUENCE OF MEDIA INGREDIENTS AND PH ON THE GROWTH OF *CHLOROMONAS ROSAE VAR. PSYCHROPHILA*

In order to utilize algae to fix CO_2 and reduce Greenhouse gas emissions, large-scale, year-round algae cultivation will be required. This will require alga species that thrive in colder weather with media recipes based on commercially available fertilizers. The objectives of this study were to evaluate the effect of using commercial based media and the effect of pH when growing the cold-temperature algae *Chloromonas rosae var. psychrophila*. Commercial grade media ingredients urea, potash, triple superphosphate, and Sprint 330® were used to provide nitrogen, potassium, phosphorus, and EDTA with iron, respectively. The commercial grade media supported growth similar to the growth found with the lab-based media typically used. Additionally, tests were done to determine the effect of the pH of the media. Testing with initial pH targets of 5, 6, 7 and 8 using the laboratory media, showed that growth was not sufficient at a pH around 8. However both the laboratory and commercial grade media performed well when the initial pH was between 5 and 7, and no significant difference in algal growth was measured within this pH range.

KEYWORDS: *Chloromonas rosae var. psychrophila*, urea media, pH, Bold's Basal Media

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CHAPTER 1: INTRODUCTION

1.1 General Background

Global warming has been cited as the cause of the Earth's on-going climate change. More than 90% of the global warming effect can be attributed to the increasing concentrations of greenhouse gases produced by human activities (IPCC, 2013). Greenhouse gases include carbon dioxide (CO_2), water vapor, ozone, and methane.

The burning of coal to produce electricity, combustion of transportation fuels, and industrial air emissions are cited as the main human activities, which raise atmospheric CO_2 concentrations (EPA, 2015a). In 2013, the IPCC concurred that the key factor in global warming was CO_2 emissions derived from fossil fuel combustion, cement production, and land use changes (IPCC, 2013).

Many countries in the world are working to reduce their greenhouse gas emissions and consequently reduce the rate at which the concentration of atmospheric CO_2 is increasing. Carbon dioxide emission can be reduced by 1) reducing the amount of fossil fuels being used or 2) recapturing the CO_2 when it is emitted during combustion of fossil fuels (EPA, 2015a). While advancements have been made, these alternative fuels are still a very small segment of the country's energy portfolio (REN21, 2014). In recent years, the US has invested in the advancement of biofuels made from lignocellulose, and also in wind and solar energy technology (REN21, 2014). Although reducing the amount of fossil fuels being used will eventually become a necessity, the main energy resources in use are still fossil fuels such as petroleum and coal (EPA, 2015a).

When coal is burned, the exhaust gases produced include mainly carbon dioxide (CO_2), sulfur dioxide (SO_2), and NOx, where x represents the number of oxygen molecules bound to the nitrogen, where x typically varies between 1 and 4.

When coal is combusted the organic matter reacts with oxygen and changes into volatiles and giving off energy (Shen, 2009). During this process proceeds through three or four distinct steps: 1) heating and drying; 2) devolatilization and volatile combustion; 3) swelling and primary fragmentation (for some types of coal); and 4) burning of char

(Basu, 1999). During the devolatilization stage, with increasing temperature, different amount of gases and heavy tarry matter are released depending on coal types and combustion conditions. The remaining solid after devolatilization stage is called char, which consists of carbon, ash, nitrogen and sulfur. When heating temperature reaches above 750 °C, char oxidizes to CO, CO_2, SO_2, NO, NO_2, and N_2O while ash is leftover and does not participate in the combustion reaction (Basu, 1999).

1.2 CO_2 Capture

In nature, most CO_2 mitigation relies on photosynthesis, while few depend on chemical weathering of rocks, soil, and sand or ocean sequestration (EPA, 2015b). Moreover, CO_2 mitigation can be classified into two types directed by humans: chemical or biological based technology. They both are developed from natural CO_2 fixation with added human activity to make them much easier to control and conduct.

As the most common method to separate CO_2 in industry, chemically-based CO_2 capture technologies have focused on using different solvents to capture CO_2, such as amines and ammonia (Hanak et al., 2015). In order for these reactions to proceed, or to proceed at a rate which is commercially viable, energy is required (Lam et al., 2012). It is reported that about 3.8 MJ of energy is needed to capture 1 kg of CO_2 using monoethanolamine (MEA) solution (an amine) (Knuutila et al., 2009). In another words, if the energy used to capture CO_2 with MEA solution comes from coal-fired plant, it will emit 352 kg of CO_2 to absorb 1 ton of CO_2 (Lam et al., 2012).

Unlike other CO_2 fixation technologies developed by humans, it is more difficult to control ocean systems to capture CO_2. Pressures and temperatures through “self-sealing” gravitational and hydrate-formation mechanisms would catch CO_2 after it is injected into marine sedimentary strata (Eccles & Pratson, 2012). Additionally, marine systems absorb CO_2 directly from the atmosphere, and global oceans can capture about 2 billion tons of carbon every year (Canu et al., 2015).

Another common method to fix CO_2 is biological based CO_2 capture. Biological CO_2 mitigation involves using organisms to use CO_2 in the process of photosynthesis to produce carbohydrates, which represent stored energy for the organism. Both terrestrial

and aquatic vegetation can use CO_2 from their environment. Skjanes et al. reported that 36% of fossil fuel emissions could be used by plants in agriculture (2007). This percentage would be difficult to increase and is limited mainly by the relatively slow growth rate of plants (Skjanes et al., 2007).

Aquatic vegetation may be an alternative to CO_2 fixation by terrestrial plants. Some species of microalgae are known to be fast-growing and are able to absorb CO_2 for photosynthesis with an efficiency 10-50 times superior to plants (Murakami & Ikenouchi, 1997; Usui & Ikenouchi, 1997). The International Panel on Climate Control stated that the use of microalgae for CO_2 mitigation appears to be a promising strategy (IPCC, 2013) since microalgae offers the following advantages: 1) higher growth rates and better abilities to capture CO_2 than terrestrial plants (Kennedy, 2010); 2) has the ability to recycle CO_2 completely through photosynthesis (Baringer et al., 2010); and 3) is easier to adapt to extreme living conditions (IPCC, 2013). Subsequent use of the microalgae could result in beneficial biofuel products (Skjanes et al., 2007), which might make the process more economical and sustainable (Wang et al., 2008).

1.3 Characteristics of Algae and Strain Selection

Microalgae CO_2 fixation systems typically must consider the following: 1) limited land resources; 2) influence of climate or weather conditions on an open photobioreactor; 3) nutrient requirements to cultivate the microorganisms; and 4) disposal of the medium and cell mass. Algae is robust, therefore environmental conditions in which the algae can thrive are not as limited as for many microorganisms and plants. For example, algae have been found in freshwater, saltwater, and even wastewater.

However, each strain of algae has its own ideal growth conditions. In order to cultivate algae year-round in colder climates, algae strains are needed that can withstand the harsher winter conditions. The annual temperature range of Lexington, KY (where this study was conducted) can extend below 0 °C during winter, and not all algae can survive those temperatures.

In the literature, another *Chloromonas* algae *(Chloromonas pichinchae)* was found to grow well at lower temperatures, specifically 5 °C (Rezanka et al., 2014). C*hloromonas*

rosae var. psychrophila, has been shown to be suitable for low-temperatures in previous strain screening (*unpublished, Dawson, 2012*). In the screening study more than 60 strains were evaluated in both BBM and urea media at pH values of 5.5 and 7.5 at 4 °C. The results of this study showed that *C. rosae* had the highest growth rate and was therefore selected for further testing.

1.4 Project Objectives

The overarching objective of this study is to determine the optimal conditions for C. *rosae* growth at a lower temperature (4 °C). Temperature, pH, light levels, feed CO_2 concentrations, and medium components have the greatest effect on algae growth, however, temperature and light levels will not be controlled in a commercial system. Medium components and pH will be controllable. Therefore this study focused on the following specific objectives:

1. Evaluate the effect of simplifying the typically used BBM media recipe, ending with an evaluation of a simplified media based on urea with all of the ingredients coming from commercial sources. The goal is to track changes in growth, while making incremental changes to the media recipe:
    a. BBM - based on the recipe from literature.
    b. MI - BBM without micronutrients, and using a more readily available EDTA source.
    c. MII - MI with the N source ($NaNO_3$) replaced by a less expensive N source (urea), while keeping the amount of N available the same.
    d. MIII – MII using tap water instead of DI water, without adding $CaCl_2$ (it should be available in the tap water) and less EDTA (to reduce costs).
    e. MIV – MIII using only one (less expensive) K source and less EDTA (to reduce costs).
    f. UI – MIV using all commercially available ingredients for N, P, K, and EDTA.
    g. UII – UI without EDTA (to reduce costs).
    h. UIII – UI with additional $MgSO_4$ (to see if the addition of this micronutrient results in enhanced growth).

2. Evaluate the effect of various pH levels on the optimum recipe based on using lab and commercial grade ingredients on the growth rate.

CHAPTER 2: EXTENDED BACKGROUND

2.1 Microalgae

Microalgae are eukaryotic autotrophs that can be divided into four species according to the light-harvesting photosynthetic pigments they possess (Richmond, 2004): *Rhodophyta* (red algae), *Phaeophyceae* (brown algae), *Chrysophyceae* (golden algae) and *Chlorophyta* (green algae). There are a large number of microalgae species, which can grow under a variety of environmental conditions. *Chloromonas rosae* of the *Chlorophyta* species is a strain classified as snow algae, meaning this microalgae can survive at lower temperatures than most algae. Snow algae may make algal cultivation possible during cold weather.

Microalgae have been increasingly studied in the past decade, predominantly because their oils can be used to produce bioproducts, specifically biodiesel based on the high content of oils (up to 80% of the biomass by dry weight). Microalgae have also been used to produce proteins or pigments.

Microalgal cell growth is quantified by an increase in total cell biomass, and not in the number and/or size of the individual organisms. Growth rate quantifies the change in total cell mass in a culture per unit time (Richmond, 2004) and is a useful quantity for designing larger cultivation systems. Nakagawa and colleagues report on microalgae's high tolerance to extreme environments and fast growth rates (Nakagawa et al., 1995).

2.2 CO_2 Fixation

In terms of the global carbon cycle, aquatic microalgae has a much smaller role when compared to terrestrial plants, but aquatic microalgae are still candidates for carbon dioxide fixation because of their unique features.

High-density microalgae cultures are therefore attractive for their potential to reduce atmospheric CO_2. Though several other CO_2 mitigation technologies have been suggested in the literature, most are not feasible for scaling up to an industrial scale, due to their high cost of operation. Unlike chemical utilization and ocean sequestration, biological CO_2 fixation can use flue gases directly when certain microalgae are available,

which means the whole mitigation process may cost very little, while for the other two methods the CO_2 separation can cost $170 per ton of carbon (tC), which raises the total cost of ocean sequestration up to $230 /tC. Thus, CO_2 separation is a big portion (70%) of total sequestration (Figure 2-1). In addition, the carbohydrates and lipids produced as byproducts of growth are available following the cultivation for conversion to bioproducts (Lee & Lee, 2003).

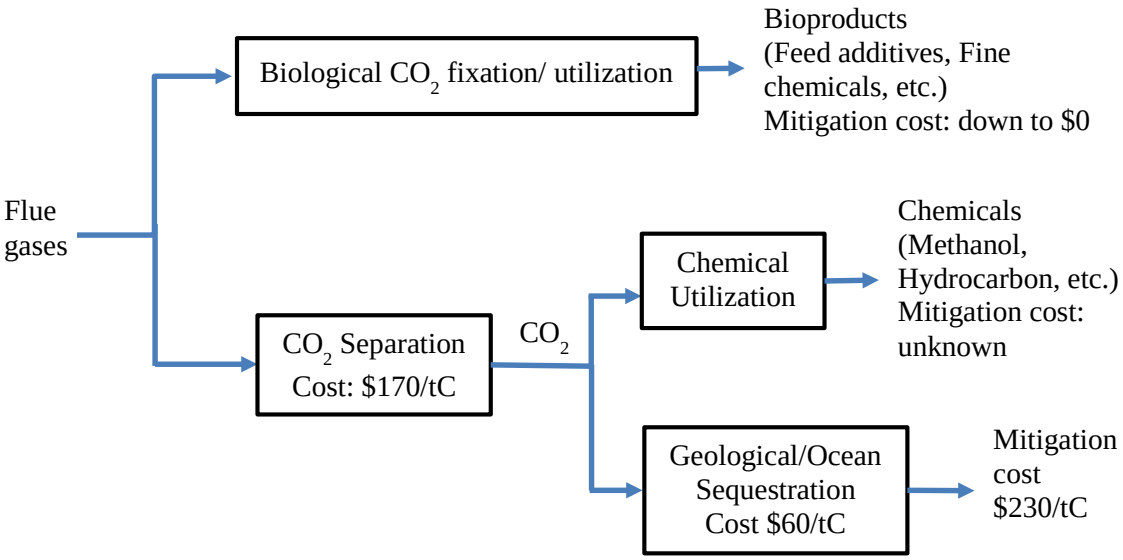

Figure 2-1 Overview of flue-gas CO_2 mitigation technologies (Lee & Lee, 2003) and their associated costs per ton of carbon (tC).

Microalgae only have access to dissolved CO_2. CO_2 enters the aqueous phase through equilibrium with the atmospheric CO_2. A small portion of the aqueous carbon dioxide reacts with water, forming carbonic acid (H_2CO_3). Aqueous CO_2 will also react with calcium carbonate ($CaCO_3$) or sodium carbonate, forming bicarbonate (HCO_3^-) and calcium or sodium ions. Both carbonic acid and bicarbonate can be used by the microalgae as carbon sources. Therefore, both CO_2 gas (either atmospheric or industrial) and sodium bicarbonate salt can used in the algal cultures as inorganic carbon sources (Jeong et al., 2003).

There is evidence that bicarbonate (HCO_3^-) is more effective biologically at providing carbon than the other forms (Sultemeyer et al., 1995). Under all conditions studied, the

net HCO_3^- transport was nearly equal to the net O_2 evolution for cells grown at low HCO_3^- concentrations in a 1995 study by Sultemeyer et al. As HCO_3^- concentration rose, the rate of net O_2 evolution increased and the differences between net O_2 evolution and net HCO_3^- absorption were larger in cultures supplied with high concentrations of inorganic carbon than cultures supplied with low concentrations of inorganic carbon for *Synechococcus* PCC7002 (Sultemeyer et al., 1995). The relationship between CO_2 and HCO_3^- in solution changes with pH (Goldman et al., 1982):

$$CO_2 + H_2O \rightarrow H_2CO_3 \longrightarrow HCO_3^- + H^+ \qquad (2\text{–}1)$$

This indicates that there is a maximum amount of HCO_3^- that can exist in solution (Figure 2-2). Moreover, most microalgal cultures are probably CO_2-limited, as a result of the physicochemical properties of freshwater, including other limitations (Moheimani, 2013). At normal freshwater pH (pH=7), less than 30% of dissolved inorganic carbon is CO_2 and more than 70% occurs in the form of HCO_3^- (Figure 2-2). According to the efficiency of microalgae CO_2 mitigation, a suitable flow rate of CO_2 would reduce waste and provide a better condition for algae growth.

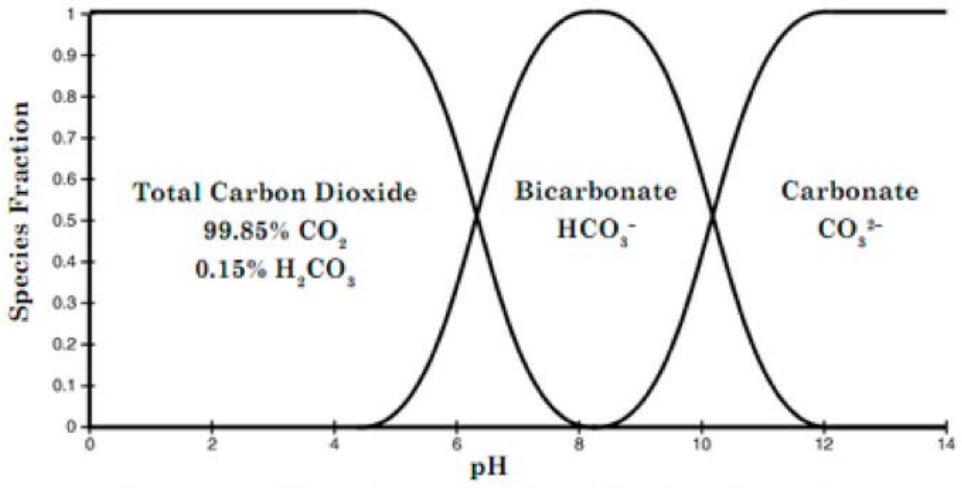

Figure 2-2 Distribution of total carbon dioxide, bicarbonate and carbonate as a function of pH in and aqueous solution (Wojtowicz, 2001).

2.3 Photosynthesis

Photosynthesis is the process of solar energy conversion that converts inorganic compounds and light energy, normally from sun, into organic products or chemical energy by photoautotrophs (plants, algae, and cyanobacteria). During photosynthesis, carbon dioxide and water are converted to oxygen and carbohydrates by using solar energy to power the redox reaction from oxygenic photosynthesis. The overall redox reaction is:

$$6\ CO_2 + 6\ H_2O + \text{light energy} \rightarrow C_6H_{12}O_6 + 6O_2 \qquad (2\text{–}2)$$

Photosynthesis is generally recognized as occurring in two general steps; the so-called light reactions and dark reactions (Richmond, 2004). For light reactions, once one photon is captured by the pigment molecule (usually chlorophyll), one electron will be released at the same time. This electron goes into a pheophytin (modified form of chlorophyll), which is an electron carrier and then pushes the electron to a quinone molecule, making a reduction of NADP to NADPH through a flow of electron transport chain. On the other hand, during a photolysis process, the pigment molecule grabs the lost electron from a water molecule, which releases a dioxygen (O_2) molecule. The overall equation for the light reactions under the conditions of non-cyclic electron flow is (Raven, 2005):

$$2\ H_2O + 2\ NADP^+ + 3\ ADP + 3\ P_i + \text{light} \rightarrow 2\ NADPH + 3\ ATP + O_2 \qquad (2\text{-}3)$$

In the dark or light-independent reactions, the enzyme (RuBisCO) captures CO_2 from the air and releases three-carbon sugars (C3) through Calvin-Benson cycle that requires the newly formed NADPH. C3s are later gathered to form sucrose and starch. Not all plants only have the C3 pathway. Before CO_2 goes into Calvin-Benson or PCR cycle, it is changed into bicarbonate and fixed by phosphoenolpyruvate carboxylase (PEPC) in the mesophyll cells of C4 plants. The product of this step is a 4-carbon molecule (C4 acids) presented in carbon fixation. The stable C4 acids are rapidly converted and diffuse to the bundle sheath cells, where CO_2 and C3 are released by decarboxylating enzymes. The released CO_2 is fixed by RuBisCO again, which as a material of Calvin-Benson cycle (Gowik & Westhoff, 2011).

Generally, C3 plants, including rice, wheat, and forage grasses, exhibit higher efficiency of photosynthesis at cool temperatures, whereas sugarcane or maize that fix CO_2 by the C4 pathway have higher rate of photosynthesis at warm temperatures (Klass, 1998). Green algae have higher solar energy capture efficiency than C4 plants (Table 2-1). Several studies have reported that aquatic microalgae have 10 to 50 times higher photosynthetic efficiency than that of terrestrial plants in terms of fixing CO_2 using solar energy (Maroto-Valer, 2010).

Table 2-1 Examples estimated solar energy capture efficiency for various biomass types (Klass, 1998).

Biomass type	Solar energy capture efficiency (%)
Maize	0.79
Rice	1.04
Sugarcane	2.24
Green algae	4.9

2.4 Nutrients

2.4.1 Nitrogen

Nitrogen is a primary constituent of all functional and structural proteins in microalgal cells, and accounts for typically about 7-10% of cell dry weight. Under nitrogen-sufficient conditions, microalgae do not generally produce nitrogen storage materials (Richmond, 2004).

Many studies have been conducted to determine the optimal nitrogen source and concentration for algae. Nitrate, ammonium, urea, and complex nitrogen sources such as those found in wastewater have been used successfully to culture microalgae. Some microalgae strains may have different cell growth rates depending the form of N utilized, even when the initial nitrogen concentrations provided were identical (Lourenco et al., 2002).

Oxidized N species, particularly nitrate (NO_3^-), are more abundant than NH_4^+. Collectively, these dissolved inorganic forms of N are known as DIN. In all cases, NO_3^- assimilation begins with the two-electron reduction to NO_2^- (Glass et al., 2009):

$$NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O \quad (2\text{-}4)$$

Following NO_3^- reduction, a six-electron reduction of NO_2^- to NH_4^+ is essential for N incorporation. Six electrons reduce NO_2^- to NH_4^+ as following the overall reaction (Glass et al., 2009):

$$NO_2^- + 8H^+ + 6e^- \rightarrow NH_4^+ + 2H_2O \quad (2\text{-}5)$$

Microalgae can assimilate ammonium easily since reduction is not required of ammonium before it is incorporated into amino acids (Wang et al., 2008). Thus, electrons must be donated to NO_3^- and NO_2^- to reduce to NH_4^+. The highest cell growth of algae was achieved within a restricted certain range of ammonia concentrations. Like for *Chlorella vulgaris*, the optimal molar concentration of ammonia was 10 mM/L. Low concentration of ammonia did not meet the requirement of cell growth while high concentrations of ammonia inhibited the growth as well (1 M/L was too high for *C. vulgaris*) (Jeong et al., 2003; Lourenco et al., 2002). However there are some adverse environmental and economic issues owing to release of ammonia into atmosphere (Wang et al., 2008).

As another N source, urea (CH_4N_2O) is an uncharged chemical, which does not have an effect on either pH or alkalinity (Goldman et al., 1982). *Isochrysis galbana* and *Synechococcus subsalsus* showed larger cell volumes with ammonia while *Hillea sp.* and *Nannochloropsis oculata* produced larger cell volumes with urea (Lourenco et al., 2002). Also, urea is one of the dissolved organic nitrogen sources and can be taken up by microalgae easily. Urease as an activator in the pathway begins with a reduction action (Goldman et al., 1982):

$$CO(NH_2)_2 + H_2O + 2H^+ \rightarrow 2\ NH_4^+ + CO_2 \quad (2\text{-}6)$$

Ammonium will join cell metabolism after being assimilated through the glutamine synthetase-glutamate synthase (GS-GltS) pathway.

2.4.2 Phosphorus

Phosphorus is another essential element for microalgae growth since phosphorus is required for both cell growth and metabolism. Phosphorus is used for the formation both of organic and inorganic compounds, DNA, RNA, and adenosine triphosphate (ATP). As a component of ATP, phosphorus essential for cellular energy transfer including cellular processes like photophosphorylation and phosphorylation. Unlike nitrogenous compounds, microalgae cannot use both inorganic and organic phosphates equally. Inorganic phosphates in the forms of $H_2PO_4^-$ and HPO_4^{2-} are assimilated preferentially from the culture medium rather than organic P (Martinez et al., 1999).

2.4.3 Potassium

As a bio-component, potassium is the primary component that related with cytoplasm in the cytosol. Especially for aquatic plants, potassium element can occupy up to 2-4% of the dry weight. Besides, a lot of enzymatic reactions are involved with potassium as a co-factor, which is exchanged with the outer medium and affect biological rhythms (Talling, 2010).

2.5 The Effects of pH

Given the importance of ions in the reactions discussed in this literature review, it is not surprising that the pH of the culture has a marked effect on the growth of microalgae. Sometimes it is not easy to maintain the ideal pH of certain microalgae strains. The freshwater *Scenedesmus obliquus* demonstrated a range of pH from 6.5 to 7 to obtain maximum biomass productivity. However, it is typically necessary to control pH in algal cultures. This is exemplified by freshwater algal *Chlorella vulgaris*, and the marine species *Dunaliella tertiolecta* whose growth is severely inhibited at non-optimal pHs (Figure 2-3, Goldman et al., 1982). These two species of algae have an optimal pH around 8 and when pH changes, the steady-state concentration of algal carbons are obviously dropped, which indicates that the final concentration or biomass of algae are much less than at optimal pH.

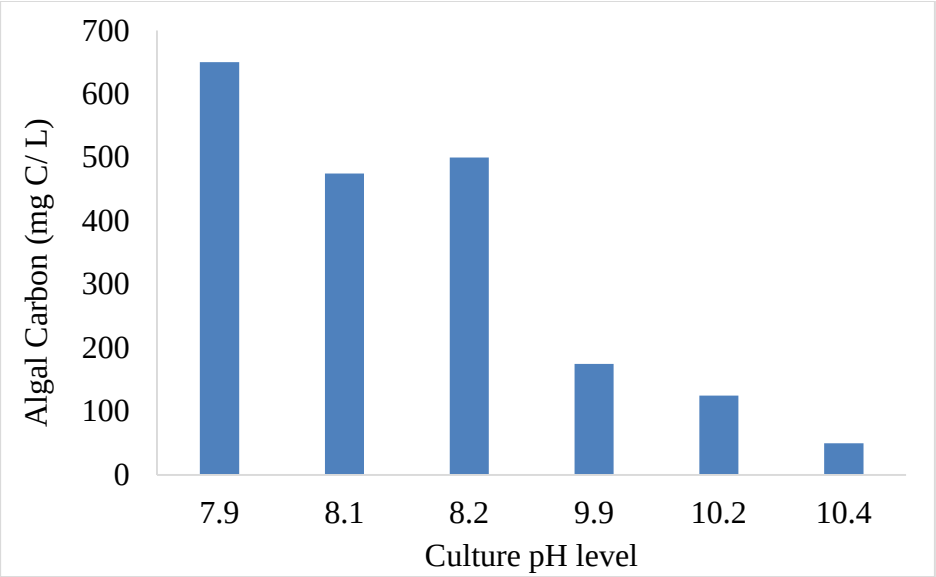

Figure 2-3 Effect of culture pH on steady-state concentration of algal carbon for continuous cultures maintained at a dilution rate of 0.5/day for *Chlorella vulgaris* (Goldman et al., 1982).

Photosynthesis and respiration have influences on day and night pH variation through the environment they build. During the light period, CO_2 is absorbed through photosynthesis that increases the pH levels, while CO_2 is released through respiration that lowers pH levels during the dark period (Bartley et al., 2014). For example, to culture *Chlorella sp.*, pH was raised up to pH 9 during the daytime and then decreased to the pH 7.6 by the end of following dark period (Moheimani, 2013).

2.6 Temperature

The growth of all microorganisms is influenced by environmental temperatures. As processing factors, temperature and pH both have a considerable effect on microalgal growth for *Scenedesmus obliquus* strain M2-1, while temperature more intensely impacts the maximum growth rate rather than pH (Guedes et al., 2011).

Not only does temperature directly affect cellular metabolism, but it also indirectly affects the algae by impacting the solubility of CO_2 and O_2 in the culture medium (Martinez et al., 1999). Each microalgae strain must be tested to determine its

temperature range for optimal growth. For example, *S. obliquus* reaches maximum biomass productivity at 30 °C when the culture medium composition is controlled (Martinez et al., 1999). In previous studies, *C. rosae* had been tested at 4, 10 and 15 °C in BBM, and it grew well at 4 °C (*unpublished, Dawson, 2012*).

The rate of various metabolic reactions is limited by ambient temperature in a nonlinear manner. It is well known that enzyme kinetics are extremely dependent on temperature, and the majority of metabolic reactions are mediated by enzymes. Thus enzyme-mediated reactions will constrained non-linearly depending on the sensitivity of a particular enzyme to temperature (Guedes et al., 2011).

2.7 Bioreactors

The choice of microalgal bioreactor if of crucial importance to designing a satisfactory microalgal-CO_2 fixation (Ho et al., 2011) system. Since most microalgae are photoautotrophs and absorb light energy, microalgal bioreactor systems are usually designed differently than traditional bioreactors used other microorganisms (Eriksen, 2008). Microalgal bioreactor systems basically can be divided into open or closed systems.

Most open systems are simpler to install and operate than closed systems because they typically use simpler technology. Open systems use both natural and artificial freshwater to cultivate microalgae (Ho et al., 2011). Many commercial operations are attracted to the open pond systems which use solar energy directly. There is evidence that reasonable amounts of algal biomass can be achieved at relatively low expense in these systems (Chen et al., 2011; Ho et al., 2011).

However, these outdoor open pond bioreactors are usually limited in performance due the difficulty in controlling the culture conditions, variations in light intensity, varying temperatures, pollution, as well as the requirement of a large land area (Chen et al., 2008; Chen et al., 2011). A large problem when using natural sunlight are the diurnal variations in light intensity, which results in low productivity and low energy conversion efficiencies (Chen et al., 2011).

Although open systems have such drawbacks, they are still widely used in commercial microalgal production, and have been modified to overcome some of the open-pond system limitations. *Chlorella sp.*, for example, has been cultured in outdoor open thin-layer photobioreactor s with biomass productivity up to 4.3 g/L/d (Doucha & Livansky, 2015) .

Closed bioreactor systems are typically designed to have a larger surface area (high surface-to-volume ratio) than open systems, in order to reduce the shadow effect and improve the photosynthetic efficiency (Carvalho et al., 2006; Ho et al., 2011). However, closed systems are difficult to scale up. The systems quickly lose their efficiency because light utilization decreases, poor biomass circulation, and the difficulty in controlling growth parameters (Ho et al., 2011).

There are three main configurations used for closed microalgae cultivation systems including 1) tubular systems, 2) flat plate systems, and 3) continuously-stirred tank reactor (CSTR) systems.

Column photobioreactors are relatively inexpensive compared to other tubular systems since they are manufactured with relatively common materials (Carvalho et al., 2006). Bubble column photobioreactors have well-defined flow patterns and circulation times when using risers and downcomers. Although tubular systems are effective in their ability to produce microalgae from CO_2 fixation, requirement for the removal of O_2, consumption of CO_2, and control of other parameters restrain their diameter and length, tubular systems are difficult to scale up.

Unlike tubular systems, it is relatively easy to scale up flat plate systems (Ho et al., 2011). Flat plate systems have high productivity and an even distribution of light, and the reactors can be built outside facing the sun, which improves the efficiency of light absorption (Carvalho et al., 2006). Furthermore, flat plate systems achieve both high cell density and good CO_2 mitigation. They achieve this through their large illuminated surface area and relatively low O_2 accumulation. Flat plat systems are not as easily controlled as open systems and the algae may experience high hydrodynamic stress (Ho

et al., 2011). Nevertheless, flat plate systems seem to be the best closed systems for cultivating microalgae.

CSTRs are the least useful closed system because they typically have a low surface-to-volume ratio; therefore the efficiency of sunlight utilization is poor. Moreover, this problem worsens when the reactors are scaled up. However, these systems can control processing parameters like temperature and pH accurately, and many companies have experience using CSTRs in the food and pharmaceutical industries. If the microalgae productivity can be sufficiently increased, fermenter-type systems would be promising for industrial microalgal cultivation systems.

Regardless of the high operation cost and difficult scale-up for closed systems, there is evidence that a closed system has advantages compared to an open system. These advantages include: a higher rate of CO_2 fixation, greater biomass productivity, higher photosynthetic efficiency, and easier process control. However, at present, large-scale cultivations in closed systems are limited by the high capital costs especially for the bioreactor system (Ho et al., 2011). Thus it is essential to the successful scale up CO_2 fixation by microalgae that a low-cost bioreactor suitable for microalgae be used.

When microalgae are cultivated outdoors, the most common environmental stresses encountered are high light and low temperature, since the original sunlight density and outside temperature will change by weather and climate.

2.8 Applications for Microalgae

Microalgae have several components (e.g. proteins, biomass, or lipid content), from which bioproducts such as biofuels, animal feed, and nutritional supplements can be produced.

2.8.1 Biofuels

Producing biodiesel from algae fuel has a shorter development history than other biofuels. Though algal fuel has only been proposed for 50 years, it is a promising biofuel. Many believe that the sustainability and economic viability of this fuel will improve within next 10-15 years (Wijffels & Barbosa, 2010).

Microalgae biomass can be converted into various biofuels through different downstream processing paths (Figure 2-4). The simplest way is directly burning biomass as a replacement of coal to get electricity, which saves the producing cost. Another choice is either having a biochemical or thermochemical conversion, or taking a chemical reaction to convert biomass into various types of biofuels, depending on conversion technologies. Those products can be ethanol, methane, bio-oil, and biodiesel. One main component used to make biofuel is the lipids extracted from algae. Green algae have been shown: 1) to contain relatively higher concentration of lipids than other algae species (REN21); 2) to exist widely within the natural world; 3) to be easy to isolate; and 4) to grow rapidly in the laboratory (Hu et al., 2008). For these reasons, it is most common to see green algae used in studies when looking at the conversion of microalgae to biodiesel.

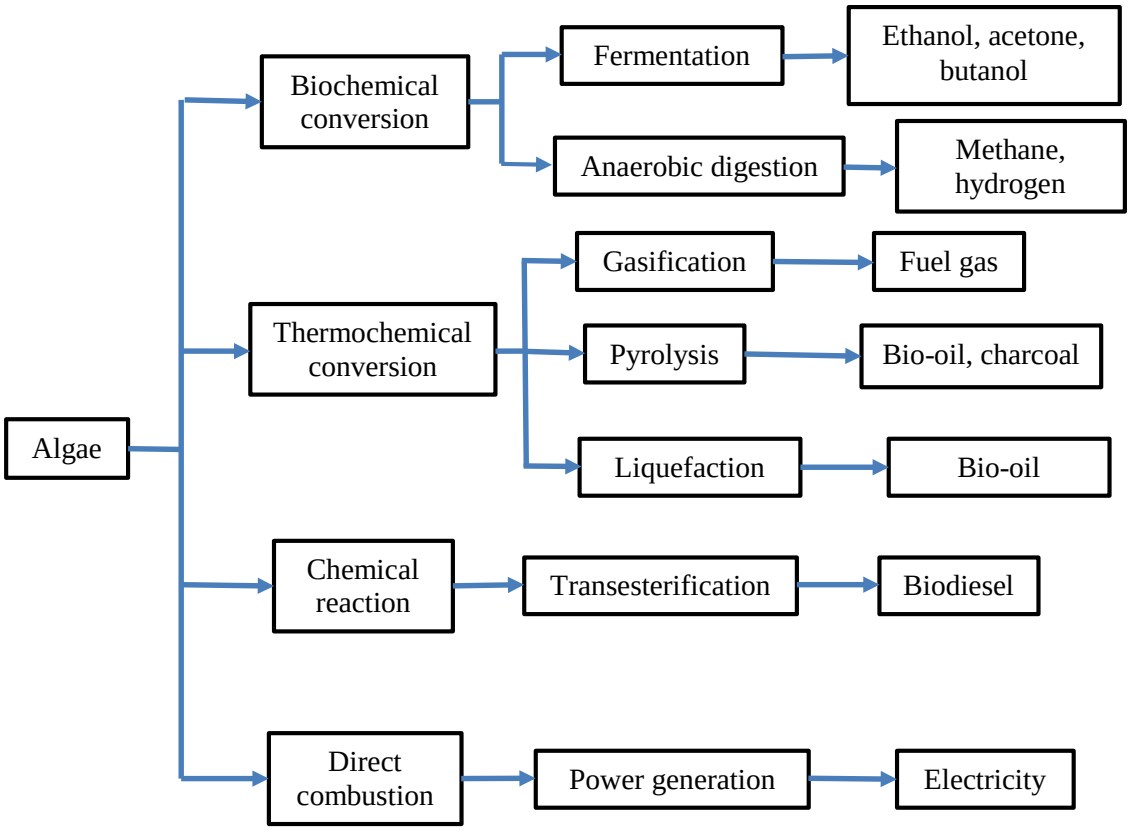

Figure 2-4 Microalgae biomass conversion processes (Medipally et al., 2015).

Carbohydrates from microalgae (50% w/w of carbohydrates) also can be converted to bioethanol through fermentation. It has been estimated that up to 38% of the dry biomass could be converted to ethanol through fermentation. Unlike other ethanol production systems, it is possible to use wet microalgal biomass directly in the fermentation, which may be more energy efficient and economical because the energy required to dry the biomass would not be needed (Milledge & Heaven, 2014).

There are drawbacks to biofuel production from microalgae that limit the use of microalgae in the biofuels industry. These disadvantages can be organized into two main categories, namely the upstream process results in low concentrations of biomass in the culture and therefore low lipid content while requiring a costly photobioreactor in the process(Callander et al., 1992); and the costly downstream processing and the harvesting and drying of the algal biomass is complicated (Medipally et al., 2015). These difficulties must be overcome before commercial production can become a reality.

2.8.2 Food products

In addition, microalgae can be used in animal feed due to its high crude protein and carotenoid contents. Compared to soybean, corn and wheat, microalgae have relatively high protein or lipid contents (may vary by various species). In animal feed, replacing soybean meal by natural alternatives like microalgae is attractive since protein is the most expensive nutrient (Lum et al., 2013). Assuming the microalgae have 50% protein and 20% lipid content and replace soybean meal in animal feeds, this switch would use almost 30 times less land area than what is needed to produce soybean protein (Brune et al., 2009). Moreover, it is possible to improve daily body weight gain by improving animal growth performance through feeding microalgae. Even the de-fatted biomass that is a by-product from the biofuel industry can replace corn and soybean meal for pigs, cows and chickens (Lum et al., 2013).

Spiralina and *Chlorella* microalgae are dried and sold in several nutritional products without any further processing. *Spirulina* is produced in both Asia and the United States, typically for its Phycocyanin (colorant), while *Chlorella* is produced only in Asia. Also, EPA/DHA (omega-3 fatty acids) and Algalin Flour (Lipid Additive) are microalgal

products developed to be used in food, although they are not currently marketed. (Enzing et al., 2014). Even though most of these nutritional supplements still need further development, they may represent promising new products, which may improve the viability of both biofuel production and food production from microalgae.

CHAPTER 3: INFLUENCE OF MEDIA INGREDIENTS ON THE GROWTH OF *CHLOROMONAS ROSAE*

3.1 Background

The key to optimal microalgae cultivation and CO_2 fixation is developing balanced medium, which contains adequate amounts of nutrients required for growth (Mandalam & Palsson, 1998). There is an optimal concentration of most nutrients, and more is not always better. Nitrogen can affect the balance between algal oil production and high biomass productivity, so it is essential that the correct amount be provided to the cultures (Wang et al., 2008). As no more information about *Chloromonas rosae var. psychrophila* can be provided, it has been cultivated using Bold's Basal Medium (BBM, Table 3-1) which is a "defined" medium that is good for a lot green algal classes in freshwater by experiments (Gualtieri & Barsanti, 2006). In BBM, the nitrogen source is $NaNO_3$ that is most widely used as laboratory grade nitrogen source but not well for commercial use due to its cost. Thus, a replacement of $NaNO_3$ needs to be developed to extend from lab to industry.

Table 3-1 Recipe for Bold's Basal Medium (BBM) (Bold, 1949; Bischoff & Bold, 1963).

Component	Concentration
Macronutrients (g/L)	
$NaNO_3$	0.25
$CaCl_2.2H_2O$	0.025
$MgSO_4.7H_2O$	0.075
K_2HPO_4	0.075
KH_2PO_4	0.175
NaCl	0.025
Micronutrients (mg/L)	
EDTA anhydrous	50
KOH	31
$FeSO_4.7H_2O$	4.98
H_2SO_4	1 mL (100% solution)
H_3BO_3	11.42
$ZnSO_4.7H_2O$	8.82
$MnCl_2.4H_2O$	1.44
MoO_3	0.71
$CuSO_4.5H_2O$	1.57
$Co(NO_3)_2.6H_2O$	0.49

Urea is a common commercial nitrogen source and it has been shown to work well in algae cultivation systems (Crofcheck et al., 2012). Though urea can decrease the lipid content, it is still the main factor that impacts the growth rate of algae.

The final recipe uses commercial fertilizers with urea, triple superphosphate, potash and Sprint 330. As a nitrogen source, urea ($CO(NH_2)_2$) can dissolve in solution easily with high N content (46%) and involves almost no hazards, which makes it a widely used nitrogen fertilizer in the agricultural industry. Triple superphosphate, containing phosphorus pentoxide (P_2O_5), is a high phosphorus content (over 40%) fertilizer (EPA, 1996). Potash is also soluble in water, which may contain multiple potassium chemicals, e.g., potassium chloride (KCl) or potassium nitrate (KNO_3), to make potassium fertilizers. Sprint 330 used as a commercial form of EDTA. Table 3-2 shows the price for each major chemical.

Table 3-2 Prices for lab grade chemicals and commercial fertilizers with supplier information.

Chemicals	Price $/Kg	Supplier
$NaNO_3$	100	Fisher Scientific
Urea(Lab)	97	Fisher Scientific
Urea (Commercial)	0.79	Southern States Cooperative
KH_2PO_4	194	Fisher Scientific
K_2HPO_4	295	Fisher Scientific
$MgSO_4.7H_2O$	241	Fisher Scientific
Potash	0.86	Southern States Cooperative
Triple superphosphate	1.03	Southern States Cooperative
Na.EDTA.Fe	148	Sigma Aldrich
Sprint /330	14.52	BFG Supply Co.
$CaCl_2$	268	Fisher Scientific
NaCl	128	Fisher Scientific
EDTA anhydrous	15.13	BFG Supply
KOH	157	Fisher Scientific

3.2 Materials and Methods

3.2.1 Algae culture maintenance

C. rosae (UTEX# SNO11) was purchased from the Culture Collection of Algae at the University of Texas at Austin. Mediums were prepared with deionized (DI) water or city water sterilized by UV light for two hours. Since *C. rosae* has a slow growth rate due to low temperature influence, experiments last an average of 9 days (216 hr) to achieve maximum culture density. Microalgal cells were cultured in Bold's Basal Medium (BBM) prior to experiments and also used as a control medium. Algae cells were collected by centrifuging at a speed of 1300 rpm for 13 minutes and the supernatant was discarded. Harvested algal cells were re-suspended in DI water (liquid stock culture) and used as inoculums for experiments. Flasks (500 mL) were inoculated in a laminar flow hood, using 2 mL of the initial algae culture added to 450 mL fresh medium. Cool white

(Philips F32T8/TL735 Alto, 32 Watts) fluorescent lights (70 µmol/m2 per second) in a 16:8 hours light: dark illumination period was used as light source to culture newly transferred algae. Flasks were bubbled with 3% cylinder CO_2 (approximately 0.15 L/min CO_2 and 4.4 L/min air for the total system of flasks), this percentage of CO_2 being sufficient to ensure the saturation of the flask with CO_2. The algae cultivation setup is shown in Figure 3-1. Flasks were placed on a shelf and cultured in an environmental chamber at 4 °C.

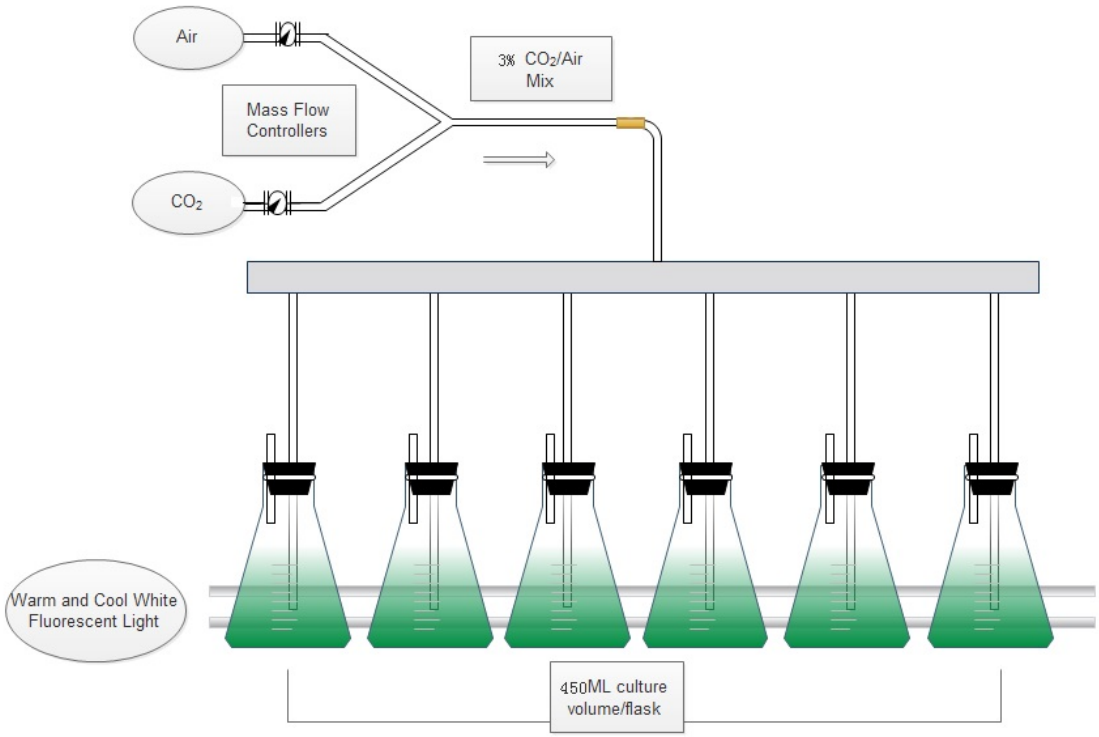

Figure 3-1 Schematic of the lab-scale algae cultivation system.

3.2.2 Dry weight and growth rate

Triplicate 8 mL algae samples per flask were collected for dry weight measurement every 24 hr (± 1 hr). Each sample was filtered through a binder-free glass microfiber filter (Whatman type 934-AH, 24 mm diameter) and dried at 105 °C in the oven for 24 hr. Algae dry weight was obtained from the difference between the weight of the dried algae plus filter versus the clean filter and quantified as dry mass per unit volume (mg/L). Growth rate was calculated using the slope of the exponential phase of the algae growth curve (Shuler & Kargi, 1992).

3.2.3 Medium formula development for lab grade

C. rosae was maintained in Bold's Basal Medium (Bischoff & Bold, 1963), which is a widely used artificial freshwater medium. Though it's a general medium, which can culture mostly green algae, it might be not the best recipe for some special species. The numerous ingredients in the BBM recipe are complicated, and can be costly and time consuming to prepare, meanwhile the main nitrogen source is sodium nitrate which is very expensive compared to other nitrogen source.

In order to simplify the BBM into a better recipe for *C. rosae*, the first step is to keep all macronutrients and remove all micronutrients except EDTA from original medium. EDTA, Ethylenediaminetetraacetic acid, is a widely used chemical that helps to keep metal ions remain in solution without reducing reactivity. In this study, Na.EDTA.Fe is used to keep Fe^{3+} in suspension. Sodium chloride (NaCl) is also eliminated from the recipe since sodium and chloride are not necessary in large amount and they are present in other ingredients. Thus, the Modified I recipe contains $NaNO_3$, KH_2PO_4, K_2HPO_4, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, and Na.EDTA.Fe.

Secondly, urea (lab grade) was substituted as the nitrogen source instead of sodium nitrate ($NaNO_3$) while the rest of the macronutrients stayed the same to study the algae adaptability to urea. Next, water source was changed by replacing DI water with sterilized city water. The final goal was to culture *C. rosae* is in a large scale, which will require a large amount of water. Therefore, city water was a much better water source since it was easily assessable and inexpensive. As the city water in the Lexington contains a lot of calcium (0.5 g/L, Ward labs report on Lexington KY tap water from July 2013), it is reasonable to eliminate calcium chloride ($CaCl_2$) from the recipe. As for potassium and phosphorus sources, KH_2PO_4 and K_2HPO_4 are used. The various modified recipes are summarized in Table 3-3.

Table 3-3 Recipes for the various modified mediums (MI, MII, MIII, and MIV) tested to determine possible simplifications to the typically used BBM media.

Ingredient (g/L)		BBM	MI	MII	MIII	MIV
Water		DI	DI	DI	Tap*	Tap*
P, K Source	KH_2PO_4	0.175	0.168	0.168	0.168	0.1184
	K_2HPO_4	0.075	0.085	0.085	0.085	
	KOH	0.031				
N source	Urea			0.1375	0.0882	0.0882
	$NaNO_3$	0.25	0.25			
EDTA, Fe Source	$FeSO_4.7H_2O$	0.00498				
	Na.EDTA.Fe		0.06	0.06	0.06	0.02
	EDTA anhydrous	0.05				
Micro-nutrients	$MgSO_4.7H_2O$	0.075	0.075	0.075	0.075	0.075
	$CaCl_2.2H_2O$	0.025	0.025	0.025		
	NaCl	0.025				
	H_2SO_4	0.00176				
	H_3BO_3	0.01142				
	$ZnSO_4.7H_2O$	0.00882				
	$MnCl_2.4H_2O$	0.00144				
	MoO_3	0.00071				
	$CuSO_4.5H_2O$	0.00157				
	$Co(NO_3)_2.6H_2O$	0.00049				

*City UV-sterilized water

3.2.4 Commercial grade medium formula

It was assumed that the nutrient sources utilized for scale up microalgae cultivation were commercial fertilizers, generally using urea, potash, triple super phosphate (TSP) and Sprint330. All fertilizers were purchased from Southern States Cooperative. Those fertilizers were tested and supported healthy algae growth in previous work with

Scenedesmus (Graham, 2013). Based on the modified laboratory grade medium, the amount of all lab nutrients was converted into the proper amount of fertilizer nutrients (Table 3-4). Since the fertilizers did not contain $MgSO_4$, absence of magnesium also needed to be tested. Also, EDTA is relatively expensive compared to other nutrients, so the importance of EDTA was investigated. Basically two experiments were run, MIV was used as the control and compared to the same recipe made with commercial grade components without $MgSO_4$ (UI), without EDTA and MgSO4 (UII), and with EDTA and $MgSO_4$ (UIII). The four medium formulations are summarized in Table 3-4.

Table 3-4 Nutrient medium formulations for the lab and commercial recipes.

	Ingredient (g/L)	M IV	U I	U II	U III
N source	Urea, lab	0.0882			
	Urea, commercial		0.882	0.882	0.882
P, K Source	KH_2PO_4	0.1184			
	Triple superphosphate (P source)		0.14	0.14	0.14
	Potash (K source)		0.068	0.068	0.068
EDTA, Fe Source	Na.EDTA.Fe	0.02			
	Sprint /300 Na.EDTA.Fe(III)		0.0044		0.0044
	$MgSO_4.7H_2O$	0.075			0.075

3.2.5 Experimental procedure

Prior to the experiment, the *C. rosae* stock culture (cultivated in 400 mL BBM medium with 3% CO_2 for 216 hr) was centrifuged at 1300 rpm for 13 min and the supernatant (BBM medium) was discarded. 450 mL of each medium and 2 mL of suspended algae were combined in a 500 mL flask and bubbled with 3% CO_2. Algae was cultured in different mediums at 4 °C for 216 hr. The mediums were prepared with DI water and sterilized city water. For all of the cultures, the gas flow rates were kept at 0.15 L/min CO_2 and 4.40 L/min air by being regulated with a mass-flow controller (model 5850E, Brooks Instrument, Hatfield, PA) and a flow meter (model VA20439, Dwyer Instrument, Inc., Michigan City, IN). Growth system was in continuous illumination in a 16:8 hours light: dark illumination period (70 µmol/m^2 per second) was used as light source to

culture newly transferred algae. For each flask 8 mL samples were taken at times 0, 24, 48, 72, 96, 120,144,168,192, and 216 hr (± 1 hr) to measure the dry weight and pH level.

3.2.6 Initial concentrations of BBM

During this study, each individual experiment used BBM as the control. While steps were taken to keep the initial concentration of algae constant, the actual initial concentration varied for each experiment. As a result, the growth curves for the BBM control were used to show the effect of varying initial concentration, while recognizing that the experiments were also done on different weeks.

3.3 Results and Discussion

3.3.1 Different initial concentrations with BBM

Comparing all of the runs using Bold's Basal Medium (BBM) from the various experiments (done on different weeks), it was obvious that even though all other conditions stayed the same, changing the initial concentration of each experiment could have different algae growth curves (Figure 3-2) and even growth rates (Table 3-5). In Figure 3-3, the increasing initial algae concentration of microalgal stock had a generally positive effect on algae growth. This illustrates why extra effort was made to attempt to have the same concentrations for each treatment and its corresponding control.

Table 3-5 Growth rate ± standard error for growth with different initial concentrations of algae (n=3).

Average initial algae conc. (mg/L)	Average growth (mg/L/hr)
24	2.00±0.08
43	2.07±0.04
87	2.76±0.10
121	2.54±0.15
215	3.85±0.07

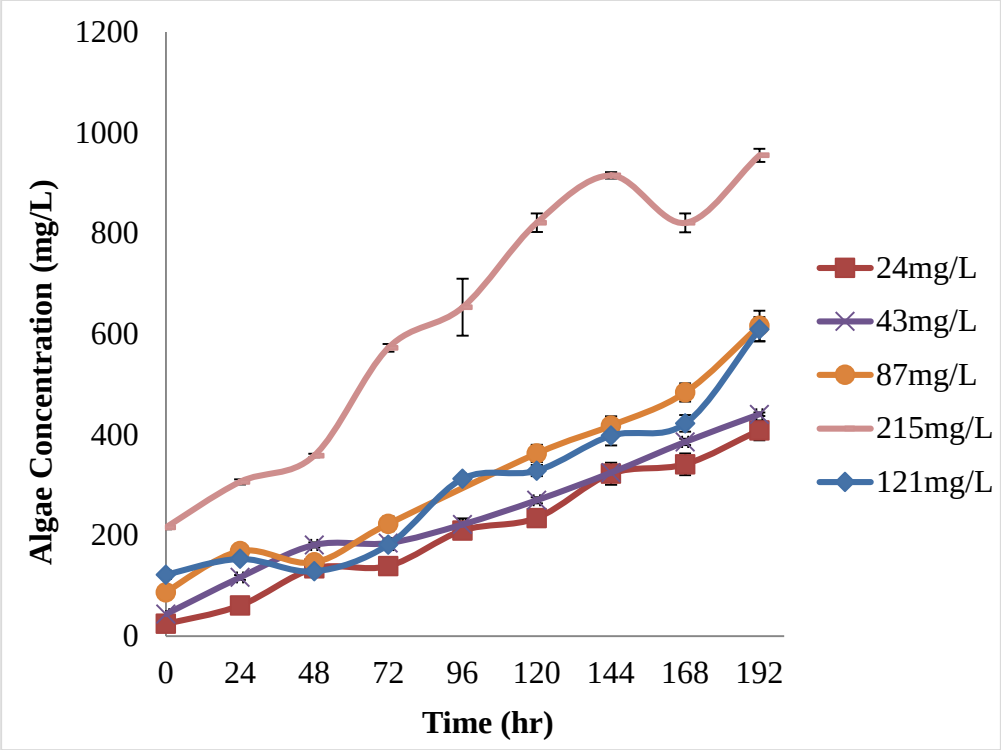

Figure 3-2 Growth curves during a 192 hr cultivation using BBM formula with different initial algae concentrations. Error bars represent standard error (n=3).

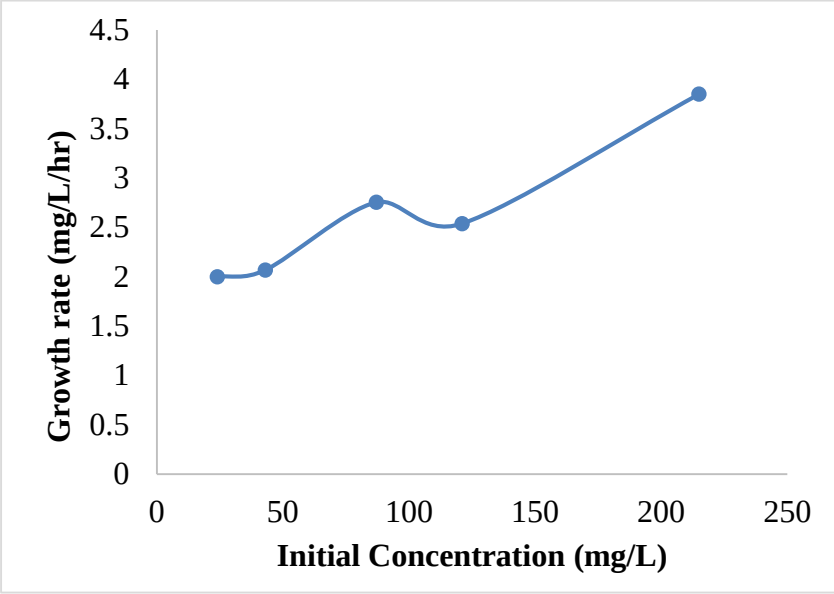

Figure 3-3 Growth rate for increasing initial algae concentrations during a 192 hr cultivation using BBM formula.

3.3.2 Modified Mediums vs BBM

Four modified versions of BBM were tested in four different trials, each with a BBM control. The growth curves for each trial are shown in Figure 3-4 and the resulting growth rates are shown in Table 3-6.

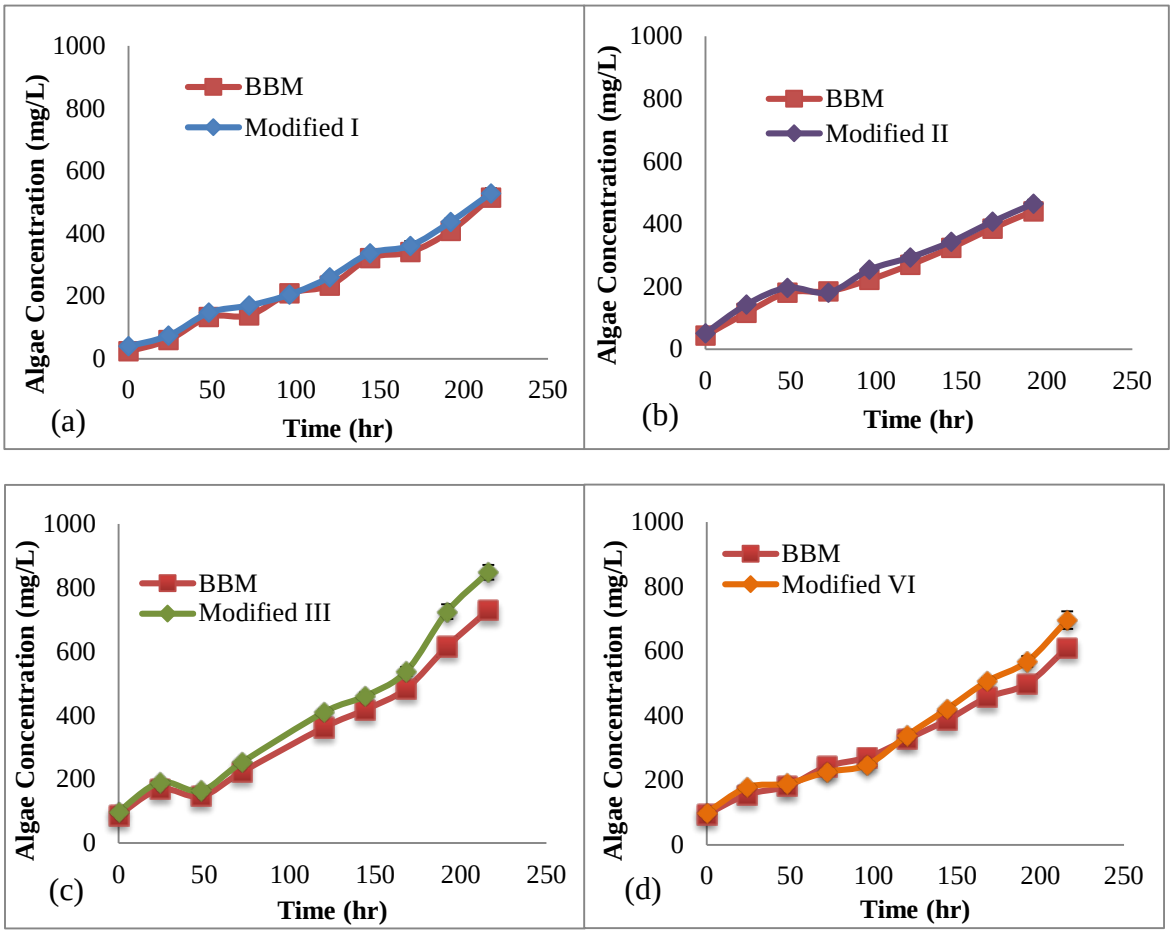

Figure 3-4 Growth curves for the four trials with different modified media [(a) MI, (b) MII, (c) MIII, and (d) MIV] each shown with the growth curve for the BBM control. Error bars show standard errors (n=3).

Table 3-6 Average growth rates for each modified treatment and control (BBM) with p-values.

Average growth rate over 192 hr (mg/L/hr)			
Medium Type	Treatment	Control (BBM)	P-value
Modified I	2.26±0.051	2.28±0.089	0.8928
Modified II	2.154±0.056	2.069±0.036	0.2686
Modified III	3.473±0.109	2.975±0.104	0.0717
Modified IV	2.762±0.096	2.389±0.051	0.0297

In the MI experiment, *C. rosae* grew well in both the modified recipe and the control (BBM). Though the initial concentration of the treatment was a little bit higher than the control, the growth curves for the two mediums had the same slope (even with the difference in the y-intercept, Figure 3-4a). Both curves had three flat stages at 72, 120, and 168 hr of cultivation. The growth rates of treatment and control were 2.26 and 2.28 mg/L/hr (Table 3-6), respectively, and there was not a significant difference between the two total growth rates with a p-value of p=0.8928. This experiment showed that the addition of micronutrients did not affected the growth of *C. rosae*.

The growth curves of *C. rosae* grown in the Modified II recipe and BBM are shown in Figure 3-4b. The treatment curve was slightly above the control growth curve. Only one flat stage was seen at 72 hr after cultivation. In this experiment, the initial concentrations were almost the same, while the treatment (Modified II recipe) had a slightly greater growth rate (Table 3-6). There was only 0.1 mg/L/hr difference between treatment and control and the total growth rate was not significantly different (p=0.2686). This result shows that the lab grade urea could replace $NaNO_3$ as a good nitrogen source to grow *C. rosae*.

The result turned out to be that treatment (Modified III) in tap water worked better than control (BBM) in DI water, and the difference of total growth rate was almost 0.5 mg/L/hr (Table 3-6), which was 17% higher than the control in DI water. It might be because tap water contains slightly more nutrients than DI water. Unlike the previous two experiments, late in this experiment the slopes of growth curves increases (Figure

3-4c), especially for the treatment curve. However, there was one dip at 48 hr after cultivation in both curves. The total growth rates were much greater than Modified II experiments, since the initials were also higher. In the statistical analysis, there was still no significant difference in total growth rate ($p=0.0717>0.05$). The total chemical nutrient cost for MIII was \$0.064/L, which was about 56% of the BBM cost (\$0.114/L excluding micronutrients).

In the Modified IV experiment, the growth curves were pretty close except for the last few days of cultivation (Figure 3-4d). Compared with Modified III, even the total growth rate was not very high and the total growth rate differences between treatment and BBM (Modified –BBM) was 0.4 mg/L/hr (Table 3-6). There was significant difference between treatment and control ($p=0.0297<0.05$). The total chemical cost for Modified IV was \$0.053/L that was only 46% of the cost of BBM. Both of the modified recipes could be good cultivation media, however, Modified IV is less expensive. Thus, the Modified IV media was selected to culture *C. rosae* in subsequent experiments, since these results confirm that lab grade urea can be used in place of $NaNO_3$.

3.3.3 Commercial grade recipes vs BBM

Unlike the experiments done in the lab, the cost of media chemicals will be a significant contribution to the overall costs of the full-scale commercial system. Thus, it is important to make sure every nutrient component is necessary and cost effective. To evaluate the effect of using lab vs. commercial grade, using the best lab based recipe (determined in the previous section) an equivalent recipe using only commercial grade nutrients, urea (N), triple superphosphate (P), potash (K), and Sprint 300 (EDTA) was formulated (UI). In a large scale using fertilizers, Sprint 330 acted as commercial EDTA, but might be not necessary to use, so the second commercial recipe tested was the same as UI without the EDTA (UII). Finally, the importance of MgO_4, which is in the lab grade recipe, was tested, by adding MgO_4 into a final recipe (UIII), even though there isn't a commercial grade equivalent. The UI and UII recipes were tested at the same time with the same BBM control. The growth rates for each of the recipes are shown in Table 3-7, along with the growth rates for the BBM control.

Table 3-7 Average growth rates for using the control (BBM) and the three commercial grade recipes (UI, UII, and UIII) with p-values.

Average growth rate over 240 hr (mg/L/hr)			
Medium Type	Treatment	Control (BBM)	P-value
UI	4.346± 0.127	3.851± 0.067	0.0262
UII	4.520± 0.086	3.851± 0.067	0.0035
UIII	3.641±0.257	3.265±0.150	0.2740

The initial concentrations of UI treatment and BBM control were the same, while the UII treatment had a slightly higher initial concentration (lower than 10%). UI and UII had very similar growth curves; they were fast growing and had one downward trend at the end. For BBM, there was one flat stage, but similar to UI and UII, one downward curve at the end. Since they all had a decrease in growth at the same time, there might have been external issues (Figure 3-5).

The culture medium without Sprint 330 had a greater growth rate than UI and BBM (Table 3-7), which indicated that UII worked a little bit better than UI, though not significantly different (p=0.3190). The growth rates for both commercial grade mediums were significantly different from BBM, with p-values of 0.0262 and 0.0035, respectively. Thus, for a large-scale cultivation of *C. rosae*, Sprint 330 could be removed which would save more money.

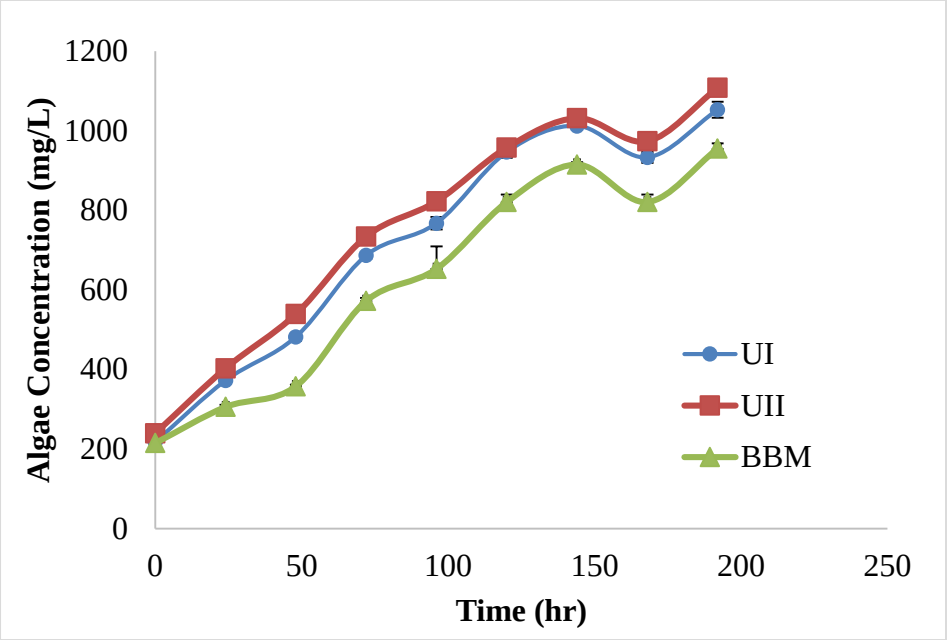

Figure 3-5 Growth curves during a 192 hr cultivation using commercial grade with and without Sprint 330. Error bars represent standard error (n=3).

However, none of these nutrients contains magnesium, so $MgSO_4$ needed to be added to the first commercial grade recipe to be able to compare the replacement of the remaining N, P. and K nutrients. In order to see if *C. rosae* adapted fertilizers or not without nutrients' effect, making all nutrients stay at the same level, which means keeping the same $MgSO_4$ level in commercial urea recipe (UI) and (UIII) to compare with BBM. Also, compared to later experiment, it tested magnesium sulfate would affect microalgal growth or not in a commercial grade media. The growth curves in Figure 3-6 showed that commercial fertilizers with $MgSO_4$ worked a little better than BBM in lab scale. Both started at very close initial concentration of algae, and the growth curves had almost same trend with two flat stages during the beginning and in the middle of cultivation, then ended up this a quite fast growing. From Table 3-7, it is easy to see that the growth rates difference between UIII and BBM is 0.4mg/L/hr, while there is no significant difference ($p=0.274>0.05$). It proved that *C. rosae* could grow as well as BBM in fertilizers with $MgSO_4$.

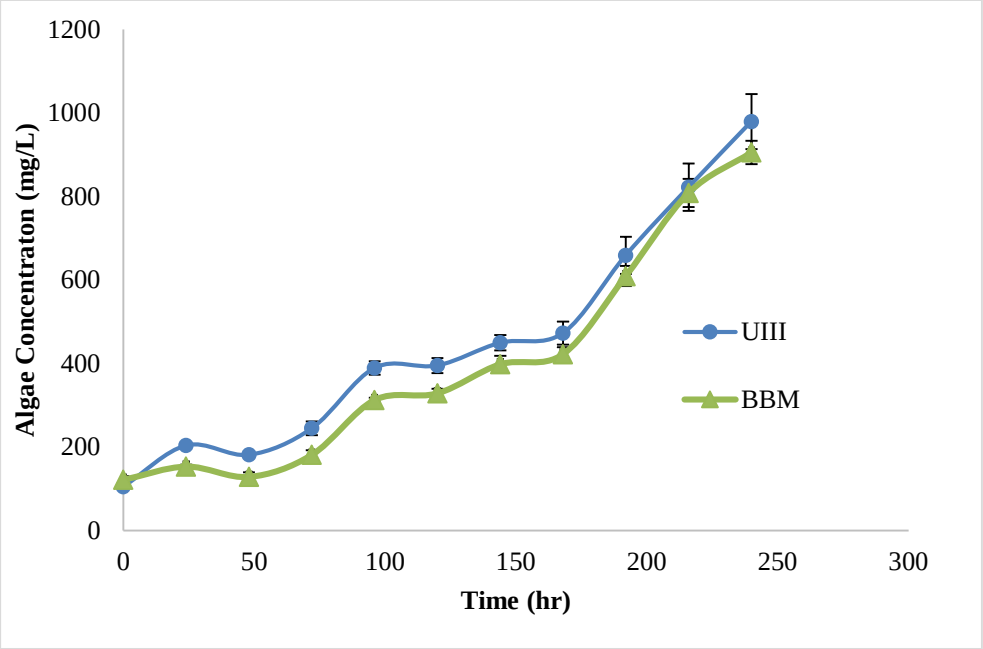

Figure 3-6 Growth curves during a 240 hr cultivation using the commercial grade media with $MgSO_4$ and the BBM media. Error bars represent standard error (n=3).

CHAPTER 4: INFLUENCE OF MEDIA PH ON THE GROWTH OF *CHLOROMONAS ROSAE*

4.1 Background

Most microalgae favor a neutral pH, while some strains can live in higher or lower pH or even have a wider tolerance of pH. The relationship between CO_2 concentration and pH in microalgal bioreactor systems is complex, due to chemical equilibrium among CO_2, H_2CO_3, HCO_3^-, and CO_3^-. Microalgae productivity will be greater with increasing CO_2 concentrations, while the pH will decrease, which may have an adverse effect on microalgal physiology. Conversely, the consumption of CO_2 by microalgae leads to a rise in pH to 10-11 in open ponds, which inhibits microalgal growth (Kumar et al., 2010).

4.2 Materials and Methods

4.2.1 Mediums preparation

Two mediums, lab and commercial grade, were tested at different pH levels. The lab grade media was made using the MIV recipe and the commercial grade media was using UI recipe (Table 2-1). The N, P, K, and EDTA levels were the same in UI and MIV, however UI did not include $MgSO_4$. Both mediums were prepared with sterilized city water.

Table 4-1 Recipes for the lab grade recipe (MIV) and the commercial grade recipe (UI).

	MIV g/L
Urea	0.0882
KH_2PO_4	0.1184
Na.EDTA.Fe	0.02
$MgSO_4.7H_2O$	0.075

	UI g/L
Urea	0.0882
TSP	0.14
Potash	0.068
Sprint330	0.0044

4.2.2 Algae culture maintenance

Mediums were prepared with city water sterilized by UV light for two hours. Microalgal cells were cultured in Bold's Basal Medium (BBM) and centrifuged at a speed of 1300 rpm for 13 minutes, then the supernatant was discarded. Harvested algal cells were re-suspended in DI water (liquid stock culture) and used as inoculums for an experiment. Working in a laminar flow hood, 2 mL of liquid stock culture was transferred to 400 mL fresh medium in a 500 mL Erlenmeyer flask. Cool white (Philips F32T8/TL735 Alto, 32 Watts) fluorescent lights (70 µmol/m^2 per second) in a 16:8 hours light: dark illumination period was used as light source to culture newly transferred algae. Flasks were bubbled with 3% cylinder CO_2 (approximately 0.15 L/min CO_2 and 4.4 L/min air for the total system of flasks), this percentage of CO_2 being sufficient to ensure the saturation of the flask with CO_2. The algae cultivation setup is shown in Figure 3-1. Flasks were placed on a shelf and cultured in an environmental chamber at 4 °C.

4.2.3 pH buffer preparation

The pH for the original mediums for lab grade and commercial grade without adjustment was 6.8 and 7.6, respectively. The commercial grade media pH was higher due to the additional compounds (e.g. fillers and binders) found in the commercial fertilizers. For the pH experiments, mediums were buffered before adding algal cells. The pH buffers were selected such that there wouldn't be additional side reactions and the algae would be unaffected by the buffer. For the lab grade recipe, the mediums were buffered to pH values of 5, 6, 7, and 8. MES buffer was used to adjust medium pH to pH 5 and 6. Similarly, HEPES-NaOH buffer was used to adjust medium pH to 7 and 8. For the commercial grade recipe, when the pH was adjusted from 7.6 to 8, it would either create sediment or decrease to a pH of 6.7 during experiment, which was too far from pH 8. Thus, mediums were buffered to pH 5 and 6 using MES buffer, and adjusted from 7.6 to 7 using HEPES-NaOH buffer. All buffers were purchased from Sigma Aldrich and Fisher.

4.2.4 Experimental procedure

Prior to experiment, the *Chloromonas rosae var. psychrophila* stock culture (cultivated in 400 mL BBM medium with 3% CO_2 for 216 hr) was centrifuged at 1300 rpm for 13 min

and the supernatant (BBM medium) was discarded. 400 mL of each media and 2 mL of suspended algae were combined in a 500 mL flask and bubbled with 3% CO_2. Algae were cultured in different mediums at 4 °C for 168 hr. For all of the cultures, the gas flow rates were kept at 0.15 L/min CO_2 and 4.40 L/min air by being regulated with a mass-flow controller (model 5850E, Brooks Instrument, Hatfield, PA) and a flow meter (model VA20439, Dwyer Instrument, Inc., Michigan City, IN). The growth system was in continuous illumination with a 16:8 hours light: dark period (70 µmol/m^2 per second) through warm and cool white fluorescent light. For each flask 8 mL samples were taken at times 0, 24, 48, 72, 96, 120,144, and168 hr (± 1 hr) to measure the dry weight and pH level.

4.3 Results and Discussion

4.3.1 Lab grade

Even though the pH was adjusted to high levels which were 7.5 and 8.4 at beginning, it did not stay constant during experiment periods. Several pH buffers were used to change culture mediums to high pH value, unfortunately none of them worked well. They all caused mediums to create white sediment at pH higher than 8.5. The pH level of high pH mediums would drop fast after 48 hr and then stay almost constant around 6.7 and 7, respectively (Figure 4-1). The reason might be when CO_2 was bubbled into the culture solutions, making carbonic acid and decreasing the pH until reaching equilibrium.

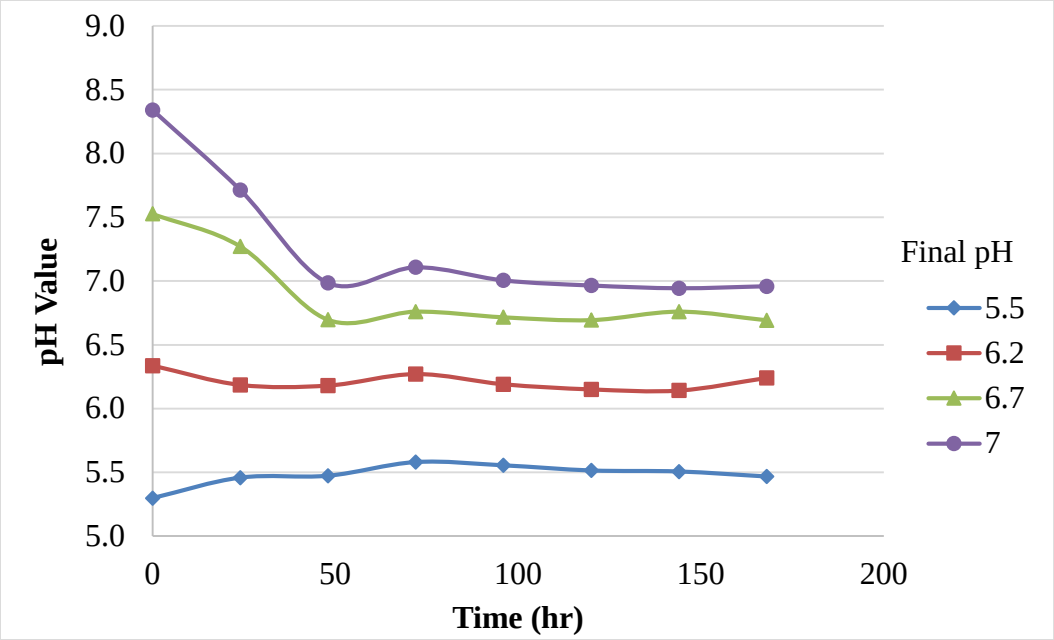

Figure 4-1 pH value during 168 hr cultivation in lab based media, MIV, for four levels of cultivation pH.

During the 168 hr cultivation, the pH levels stayed at 5.5 to 7, and after 72 hr there was little change in the pH value. The average initial algae concentration was 210 mg/L. The best performing pH was pH 6.5 with a slightly higher growth rate at 4.7mg/L/hr (Figure 4-2 and Figure 4-3). In general, the four growth curves for different pH values had the same trend. At a pH of 5.5, there was no significant difference between the other three pH levels, while pH 6.2 were significantly different from pH 6.7 and 7. The lowest growth rate was 3.7 mg/L/hr at pH 7 (Figure 4-3). As a result, pH 6.2 could be the best pH value for *C. rosae* cultivation in a lab grade recipe.

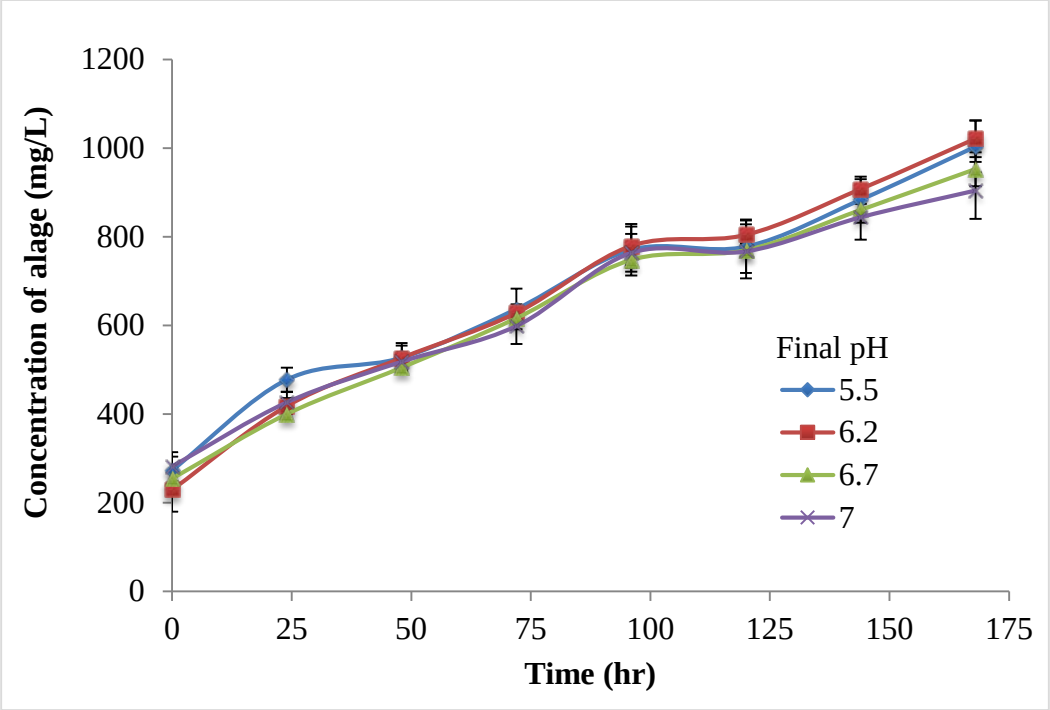

Figure 4-2 Growth curves during a 168 hr cultivation using the laboratory grade media at four different pH levels. Error bars represent standard error (n=3).

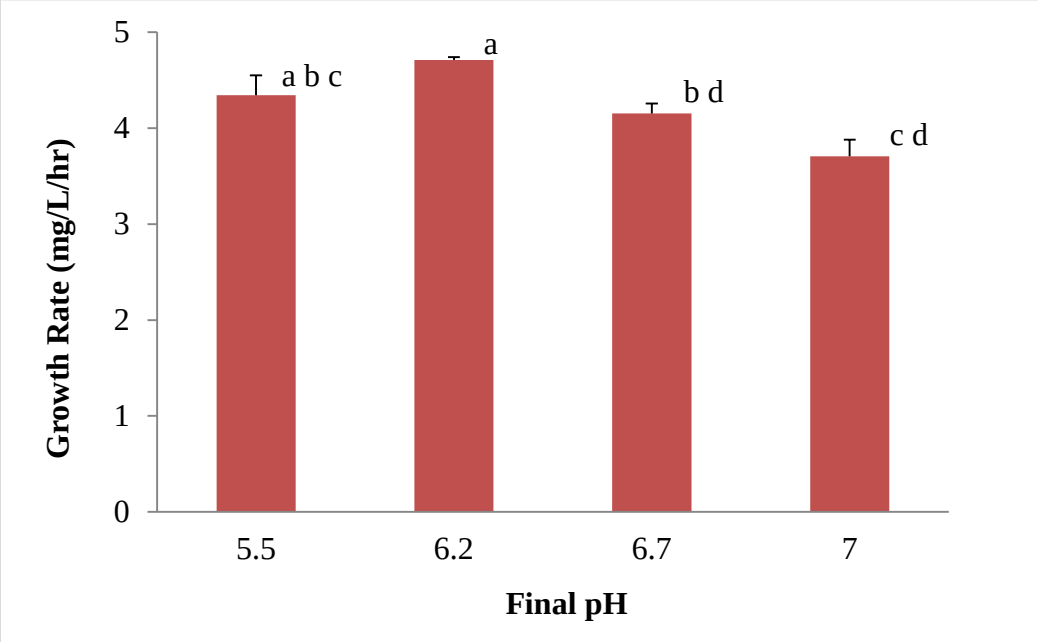

Figure 4-3 Growth rate during a 168 hr cultivation using lab grade media at four different pH levels. Treatments with the same letter are statically the same (α=0.05) Error bars represent standard error (n=3).

4.3.2 Commercial grade

The most constant pH value during 168 hr cultivation occurred after 24 hours (once the CO_2 had reached equilibrium with the solution), so the cultivation pH value was assumed to be the average pH after 24 hours: 4.8, 5.6, and 6.7 instead of the target pH values of 5, 6, and 7, respectively (Figure 4-4).

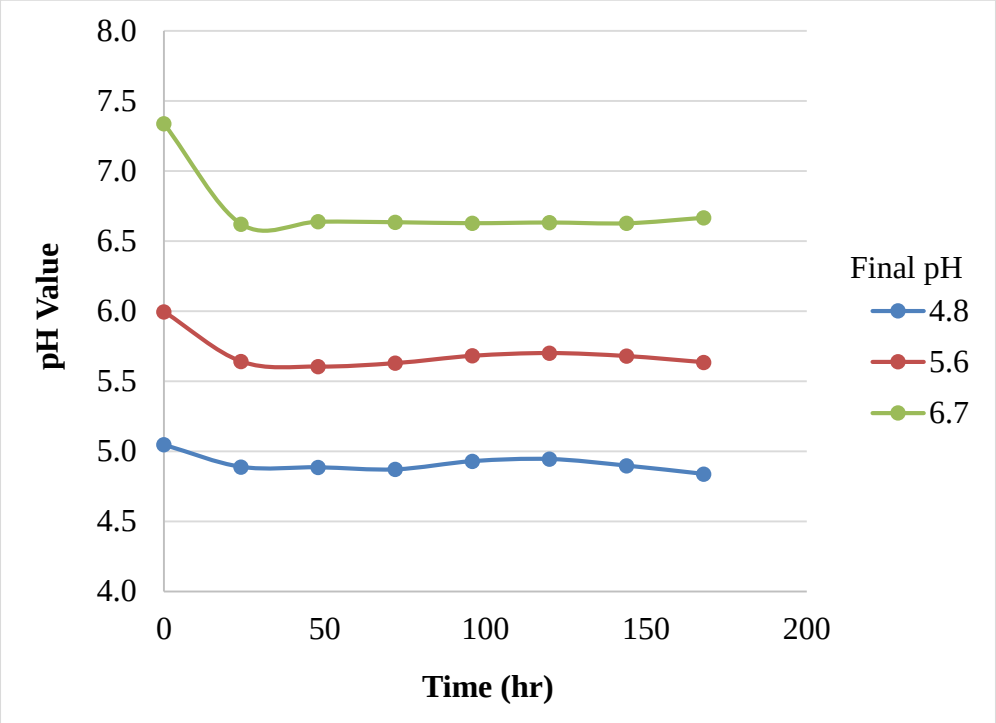

Figure 4-4 pH value during 168 hr cultivation with UI media (made with commercial ingredients).

During a 168-hr cultivation, there were barely any differences between pH levels from 4.8 to 6.7 that were considered valid pH values at the end of experiment. The best performing pH was pH of 6.7 with a slightly higher growth rate at 4.6 mg/L/hr, while there was no significant difference between other two pH values (Figure 4-5, Figure 4-6). The growth curves for these three treatments were very close, pH 4.8 and 5.6 could be regarded as the same, while pH 6.7 just had a higher curve at the end of experiment. Thus, *C. rosae* grows well at a pH range of 4.8 to 6.7 using fertilizers.

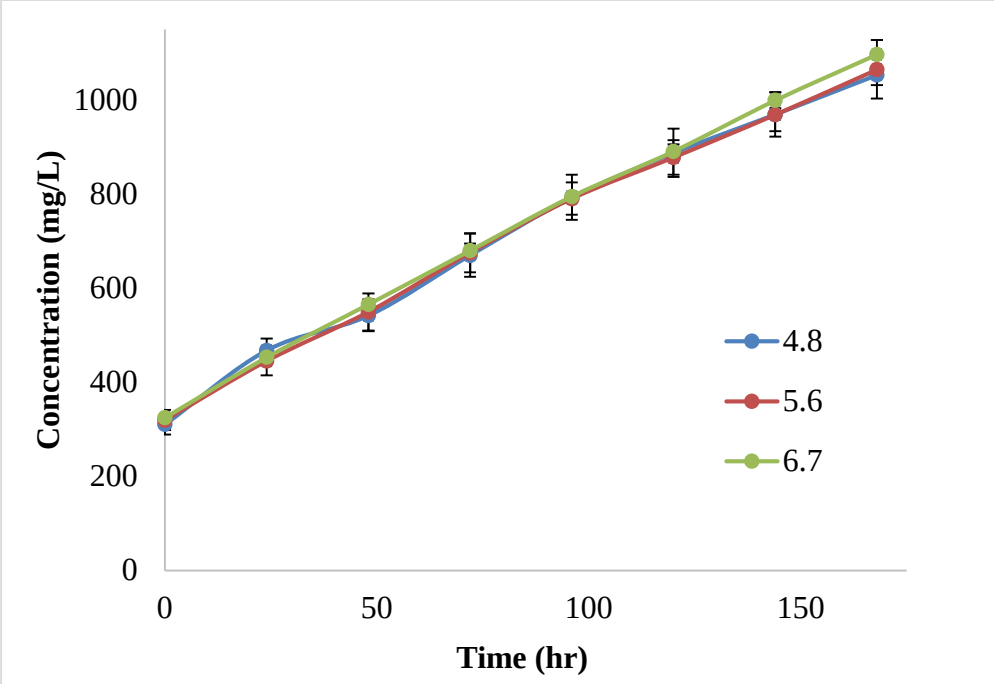

Figure 4-5 Growth curves during a 168 hr cultivation using UI recipe (commercial ingredients) in three different pH levels. Error bars represent standard error (n=3).

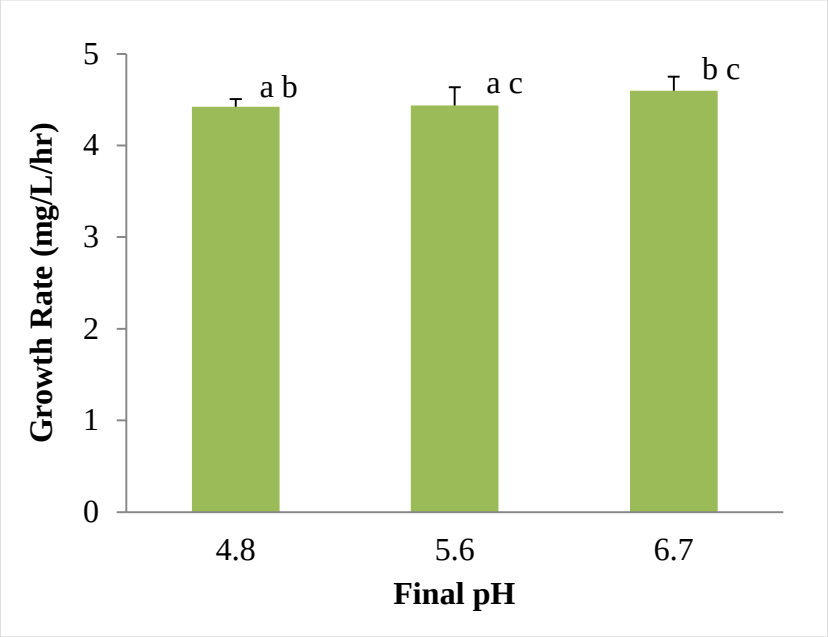

Figure 4-6 Growth rate during a 168 hr cultivation using lab grade media at four different pH levels. Treatments with the same letter are statically the same (α=0.05) Error bars represent standard error (n=3).

CHAPTER 5: CONCLUSIONS AND FUTURE WORK

This research focused on microalgae cultivation conditions, specifically the nutrient sources and pH levels. The objective of investigating media ingredients was to save money; and an optimal pH could improve the microalgae growth rate. These two improvements might also protect the algae from the influence of the external environment.

The nutrients used for lab and commercial grade are: nitrogen, phosphate, potassium, EDTA, and iron sources. The urea medium worked as well as the BBM according to the result from Chapter 3. When changing the nitrogen source and keeping other ingredients (potassium, phosphate, EDTA, and Fe) at the same level, it did not decrease the microalgae growth rate. Urea and $NaNO_3$ both are suitable to culture *Chloromonas rosae var. psychrophila.* In addition, when Sprint 330, which acted as EDTA in commercial grade medium, was removed and only iron was added the growth rate slightly increased. In conclusion, the results indicate that commercial urea medium using fertilizers without Sprint330 performed the best.

Even though the pH levels were adjusted from 5 to 8 for the lab grade and from 5 to 7 for the commercial grade, the actual pH levels were around 5.5 to 7 for the lab grade and 4.8 to 6.7 for the commercial grade. In retrospect, the testing pH range might have been too narrow, and therefore it was difficult to determine the optimal pH. The best among the pH values tested was 6.2, which was very similar to the original pH level without buffering.

The next step relevant to these experiments is to actually scale up the cultivations to large scale. Even the commercial recipe using urea did well in growing *C. rosae* at lab scale, the recipe still needs to be tested on large scale because large reactors have different growth conditions than the lab flasks.

For example, in large scale the CO_2 is from coal-fired plants, which may not be as pure as cylinder CO_2. Also, on a large scale the sunlight is the major light source and the outdoor temperature oscillates rather than staying at a consistent 4 °C. The bioreactor used to culture other microalgae at Center for Applied Energy Research at the University of

Kentucky is a column system with a total volume of up to 5,500 L. Thus, the performance of *C. rosae* using commercial urea recipe in large scale is not obvious and must be verified in the field.

Another issue is with the culture time. The laboratory scale of the study limited the hours of microalgal growing period. The majority of the growth seen in this study was exponential growth and even with a 9 day experiment it wasn't clear whether the exponential growth phase was complete. For this reason, it was hard to tell if the maximum concentration of microalgae using final modified urea recipe was achieved. Additionally, the longest cultivation period is unknown, which means for a large-scale bioreactor, the exact time to dilute the microalgae solution with fresh medium is not clear. As a result, it will affect the efficiency to absorb CO_2.

Once the components of *C. rosae* are obtained, then it will be clear to see which type of bioproducts can be developed from it. Observing from the experiments during the culture period, there were bubbles floating in the top of microalgae stock, which indicates this strain of microalgae may contain considerable lipids. The bioproducts from this algae strain could be numerous.

APPENDICES

Appendix A. **Converting the lab recipe to a fertilizer recipe**

Medium calculations were applied to get the amount of fertilizers required to make the nutrient levels equivalent to the laboratory urea recipe. Though experiment supports that BBM has been used on algae growth feasibly in lab grade, but its cost makes large scale unreasonable. The lab grade urea recipe was modified and then is converted into commercial grade using fertilizers.

Table A-1: Lab grade Modified IV nutrients (without $MgSO_4$) amounts used with tap water.

MIV	Amount (g/L)
Urea, lab	0.0882
KH_2PO_4	0.1184
Na.EDTA.Fe	0.02

Converted g/L to moles of K, P and Fe:

$$0.1184\frac{g}{L}KH2PO4 \times \frac{1mol\ KH2PO4}{136g\ KH2PO4} \times \frac{1\ mol\ K}{1\ mol\ KH2PO4} = 0.00087\frac{mol}{L} of\ K$$

$$0.1184\frac{g}{L}\text{KH2PO4} \times \frac{1mol\ KH2PO4}{136g\ KH2PO4} \times \frac{1\ mol\ P}{1mol\ KH2PO4} = 0.00087\frac{mol}{L} of\ P$$

$$0.02\frac{g}{L}Na.EDTA.Fe \times \frac{1mol\ Na.EDTA.Fe}{367.08g\ Na.EDTA.Fe} \times \frac{1molFe}{1mol\ Na.EDTA.Fe}$$
$$= \frac{0.000054mol}{L} of\ Fe$$

Using Potash (KCL) fertilizer to supply element K, while fertilizer is measured with valid Potassium given by K_2O. Calculate the amount of Potash needed the mol/L of K were converted to g/L of Potash:

$$0.00087\frac{mol}{L} of\ K \times \frac{1mol\ K2O}{2mol\ K} \times \frac{94.15g\ K2O}{1mol\ K2O} = 0.041g/L\ K2O$$

Only 60% of the theoretical amount can dissolve or used from fertilizer instruction.

$$\frac{0.041g/L\ K2O}{60\%} = 0.068\frac{g}{L} of\ Potash$$

Triple Super Phosphate fertilizer ($CaH_4P_2O_8$) was used to offer P element, while fertilizer is measured with valid Phosphorous given by P_2O_5. Calculate the amount of Triple Super Phosphate needed the mol/L of P were converted to g/L of TSP:

$$0.00087\frac{mol}{L} of\ P\frac{1mol\ P2O5}{2mol\ P}\times\frac{141.94\ g\ P2O5}{1mol\ P2O5}=0.062\ g/L\ P2O5$$

Only 44% of the theoretical amount can dissolve or used from fertilizer instruction.

$$\frac{0.062\ g/L\ P2O5}{44\%}=\frac{0.14g}{L}TSP$$

Table A-2 Commercial grade UI nutrient amounts used with tap water.

UI recipe	g/L
Urea, commercial	0.0882
TSP	0.14
Potash	0.068
Sprint330	0.0044

Appendix B. **Bold's Basal Medium (BBM) recipe**

The recipe for Bold's Basal Medium (BBM) was taken from (Bold, 1949) and (Bischoff & Bold, 1963).

Macronutrients	
Component	**Concentration (g/L)**
$NaNO_3$	0.25
$CaCl_2.2H_2O$	0.025
$MgSO_4.7H_2O$	0.075
K_2HPO_4	0.075
KH_2PO_4	0.175
NaCl	0.025
Micronutrients	
Stock Solution	**Concentration (mg/L)**
Alkaline EDTA Stock Solution	
EDTA anhydrous	50
KOH	31
Acidified Iron Solution	
$FeSO_4.7H_2O$	4.98
H_2SO_4	1 mL (100% solution)
Boron Stock Solution	
H_3BO_3	11.42
Trace Metal Solution	
$ZnSO_4.7H_2O$	8.82
$MnCl_2.4H_2O$	1.44
MoO_3	0.71
$CuSO_4.5H_2O$	1.57
$Co(NO_3)_2.6H_2O$	0.49

1. Measure and add all 6 macronutrients to 996 mL of deionized water in a 1 L bottle.
2. Mix medium thoroughly and label it as BBM.
3. To prepare a 1 L alkaline EDTA stock solution, add 50 g of EDTA anhydrous and 31 g of KOH to 1000 mL deionized water.
4. In a similar manner, individually prepare the rest of the stock solutions according to the recipe.
5. Add 1 mL each of the four stock solutions to BBM.
6. Mix and autoclave media.
7. Store unused media at 4 °C.

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