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Comments

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PRODUCTION OF FERMENTABLE SUGARS AND LIPIDS BY MICROALGAE FROM SECONDARILY TREATED MUNICIPAL WASTEWATER

By

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PRODUCTION OF FERMENTABLE SUGARS AND LIPIDS BY MICROALGAE

FROM SECONDARILY TREATED MUNICIPAL WASTEWATER

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In this paper, replacing complete or partly of growth mediums with secondarily wastewater was studied. Lipid content of *Neochloris oleoabundans* grown in a 0.3 X SE medium and autoclaved secondarily treated wastewater mixture was 22.27 % (w/w). The maximum biomass concentration of *N. oleoabundans* grown in wastewater with no additional nutrients was 0.636 g/L with 33% (w/w) glucose.

Two culture lines, MA, and NA were isolated within our laboratory and could grow in secondarily treated wastewater with no additional nutrients. The maximum biomass concentration of MA in batch culture was 0.860 g/L and the sum of glucose and xylose was 40% (w/w). The maximum biomass concentration of NA was 1.562 g/l and the sum of glucose and xylose was 33.8% (w/w). The maximum specific growth rates of NA and MA were determined to be 0.0566 and 0.0337 per hour.

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CHAPTER I

INTRODUCTION

Before discovery of crude oil, biomass, such as wood, was one of the major energy sources. Standard of living was improved due to inexpensive fossil fuels; however, decreasing storage, environmental and political concerns about fossil fuels leads to search alternate fuel sources. Several alternate fuels were created in recent decades, such as biogas, bio-oil, bio-gas, ethanol and biodiesel.

Biogas is one of renewable energies which converts sunlight energy to chemical energy by photosynthesis and anaerobic digestion. Biogas composition varies among processes. For example, biogas from landfills typically comprises 30-40% carbon dioxide, 45-55% methane and 5-15% nitrogen. Biogas from sewage digesters usually contains 35-45% carbon dioxide, 55-65% methane and less than 1% nitrogen. And biogas composition from organic waste digesters usually contains 30-40% carbon dioxide, 60-70% methane and less than 1% nitrogen. [Oslaj, 2010] Trace amounts of sulfur compounds, such as hydrogen sulfide may also be present. Methane in biogas can be further processed and used to generate steam and electricity. Biogas may be produced from various sources such as corn, animal manures and corn stalks. [Oslaj, 2010; Holm-Neilsen, 2009; Chen, 2010]

Thermochemical treatment of agricultural wastes or forestry products can result in the production of bio-oil. [Li, 2008] Bio-oil can be used for power generation through

internal and external combustion by mixing with diesel or natural gas. However, significant upgrading of bio-oil is needed to make it suitable as a transportation fuel.

Another bio-based energy source is synthesis or "syn-gas". Syngas is obtained by gasifying solid or liquid carbon materials (e.g. biomass, coal or oil) with air or oxygen and steam under thermochemical treatment. [Huber, 2006] Syn-gas is primarily composed of carbon monoxide (CO) and hydrogen (H_2) with lesser amounts of carbon dioxide (CO_2), methane (CH_4) and nitrogen (N_2). It retains a significant fuel value and can also be used for synthesizing commercial products, such as methanol and alkanes. Gasification is already commercialized for producing heat and electricity.

Ethanol can be produced from biomass in the fermentation process. In this process, sugars were converted to ethanol and carbon dioxide by yeasts or other fermenting microorganisms (e.g. *Zymomonas mobilis*). There are many sugar sources for the ethanol fermentation such as corn, sugar cane and wood products. Even though cellulose from wood products can be used as the sugar source for ethanol fermentation, pretreatment is needed, such as acid or enzymatic hydrolysis.

Biodiesel is yet another renewable fuel produced from biomass. In the current biodiesel production, plant and animal oils are trans-esterified. During trans-esterification, methanol and oils (triglycerides) are mixed in a reactor at elevated temperatures (e.g. 60 ^{o}C) with a base or acid catalyst. [Huber, 2006] Methanol has been commonly used due to its low cost even though other alcohols, such as ethanol or 2-propanol can be used. In the reaction, which has two reversible reactions with diglycerides and monoglycerides as intermediates, triglycerides can be converted into alkyl easters (biodiesel) with glycerol as a byproduct. Process economics are currently

unfavorable as a result of the significant amount of glycerol produced in the reaction and its relative lack of commercial value.

In recent years, considerable research has focused on production of alternate fuels from microalgae [Gouveia, 2009a; Harun, 2009; Pan, 2010] While microalgae have faster growth rates in comparison to other biomass (e.g. trees) and require less land area for growth, production of dried microalgae is expensive. According to Seambiotic Ltd in Israel, the estimated production cost of its dried algae was 0.34 US/kg. [Schenk, 2008] To reduce production costs of dried algae mass, research avenues exist such as designing more efficient growth systems, lowering process costs and replacing costly components in a defined growth medium with residual nutrients from waste streams.

In this project, we offer the thesis that using secondarily treated wastewater as replacement nutrients for the growth medium can successfully produce microalgae biomass and offers the potential for reducing production costs. Two different strategies were investigated for achieving this goal. First potential microalgae were selected which, from growth on defined media, have been shown to produce high lipid contents (for conversion to biodiesel). Secondly, cultures were screened for biomass potential when grown on secondarily-treated wastewater. Biomass samples were then hydrolyzed and examined for sugars production (for ultimate conversion to ethanol).

Initially, our study focused on the growth of cultures such as *Botryococcus braunii.* [Órpez, 2009] In the event of minimal or no growth on secondarily-treated wastewater, a phase of work was dedicated to examining the minimum amount of key nutrients added to the secondarily-treated wastewater for sustaining algal production.

In addition to examining known microalgae cultures, a second strategy was pursued to isolate natural microalgae present in the secondarily-treated wastewater. Lipid and sugar contents of natural isolates were examined.

The ultimate goal of this project was to determine if known microalgae cultures or natural isolates could produce biomass levels and lipids or sugars in amounts sufficient to constitute a process for converting residual nutrients residing in secondarily-treated wastewater as part of a larger scheme of producing biofuels from a wastewater treatment facility.

CHAPTER II

LITERATURE REVIEW

2.1 Algal Fuels

The idea of using algae for fuel production is not new. In 1970s, algae were initially examined as potential replacement of fossil fuels. Due to high production costs and limitations, commercial algal fuels haven‘t been developed [Li, 2008a; Chisti, 2007]. With increasing petroleum prices between 2005 and 2007, and an increased concern about global warming, microalgae research shifted from discovery and production of fine chemicals such a beta carotene and health food to once again focusing on fuel production. Various fuels can be produced by microalgae, such as biodiesel, bio-oil, bioethanol, syngas and bioags.

2.1.1 Biodiesel

In biodiesel production, plant and animal oils are trans-esterified. During trans-esterification, methanol and oils (triglycerides) are mixed inside a reactor at temperatures around 60 ^{o}C with base or acid as catalysts. [Huber, 2006] Methanol has been commonly used due to its low costs even though other alcohols, such as ethanol or 2-propanol can be used. In the reaction, which has two reversible reactions with diglycerides and monoglycerides as intermediates, triglycerides can be converted into alkyl esters (biodiesel) with glycerol as a byproduct.

Competition between feed stocks for food supplies and biodiesel production have prompted researchers to focus on microalgal biodiesel production. According to estimates, 2.5% of U.S. existing crop producing area would be needed for growing microalgae with 30% (w/w) oil content microalgae capable of satisfying 50% of transport fuel needs of the United States [Chisti, 2007]. For comparison, 24% of the land area used for crops would be needed for an equivalent fuel production from palm oil. The oil content of several microalgae have been observed to be over 30% (w/w), such as *Scendesmus obliquus* (35-55%), *Chlorella vulgaris* (14-40%), *Neochloris oleoabundans* (35-65%) and *Chlorella minutissima* (57%). [Gouveia, 2009a] Clearly, microalgae can potentially replace current biodiesel feedstocks. However, current high production costs have limited the commercial feasibility of producing biodiesel from microalgae. According to Seambiotic Ltd in Israel, the estimated production cost of its dried algae was $0.34 US/kg [Schenk, 2008]. The productivity was 20 g/m^2/day and the total lipid content varied from 8 to 40%. If the average yield of total lipid was assumed to 24% and extracted at no further cost, the price of "crude oil" or "microalgal lipids" would be $209 U.S. dollar per barrel. Clearly, more research is needed for reducing production costs of microalgal biodiesel production.

2.1.2 Bioethanol

While ethanol is typically produced from sugars obtained from corn, sugar cane, or cellulosic materials such as wood or grasses, microalgae can also be used as sugar sources for ethanol fermentation, since some of microalgae have high carbohydrate contents as shown in table 2.1. Even though cellulose from wood products can be used as

the sugar source for ethanol fermentation, pretreatment, such as acid hydrolysis, is needed and production costs can be increased significantly.

Table 2.1 % (w/w) Carbohydrate, Lipid and Protein Contents of Microalgae [Becker, 1994]

Species	Protein	Carbohydrates	Lipids
Scenedesmus obliquus	50-56	10-17	12-14
Scenedesmus dimorphus	8-18	21-52	16-40
Chlamydomonas rheinhardii	48	17	21
Chlorella vulgaris	51-58	12-17	14-22
Chlorella pyrenoidosa	57	26	2
Spirogyra sp.	6-20	33-64	11-21
Dunaliella bioculata	49	4	8
Dunaliella salina	57	32	6
Euglena gracilis	39-61	14-18	14-20
Prymnesium parvum	28-45	25-33	22-38
Tetraselmis maculate	52	15	3
Porphyridium cruentum	28-39	40-57	9-14
Spirulina platensis	46-63	8-14	4-9
Spirulina maxima	60-71	13-16	6-7
Synechococcus sp.	63	15	11
Anabaena cylindrical	43-56	25-30	4-7

As shown in table 2.1, a number of microalgae species could potentially be used for ethanol fermentation after pretreatment, releasing sugars into the growth medium. In a recent study, no pretreatment was needed for ethanol fermentation, such as fermentation of *Chlorococum sp.*, by yeast, *Saccharomyces bayanus*, under different fermentation conditions. [Harun, 2009] Two types of pretreatment were conducted with microalgae

for obtaining fuel precursors. The first treatment consisted of drying the microalgae without lipid extraction. In a second pretreatment step, lipids were extracted from microalgae with supercritical carbon dioxide. Microalgae debris remaining after lipid extraction was used as a substrate for the ethanol fermentation step. According to the results, the ethanol concentration of addition of lipid extracted microalgae was 60% higher than with simple drying of the microalgae. One possible explanation noted was that polysaccharides were released from cell wall during lipid extraction by supercritical carbon dioxide. The maximal ethanol concentration of 3.83 g/L was obtained with addition of extracted lipids to the microalgae fermentation mixture.

Ethanol production from *Chlamydomonas reinhardtii*, containing 59.7% carbohydrates, hydrolyzed enzymatically was reported. [Choi, 2010] From the results, 0.235g ethanol/g dried microalgal biomass was produced.

2.1.3 Biogas

Biogas is a renewable energy source produced by conversion of sunlight energy to chemical energy by photosynthesis and anaerobic digestion. Composition of biogas varies among various processes. Biogas from landfills contains 30-40% carbon dioxide, 45-55% methane and 5-15% nitrogen. Biogas from sewage digesters usually contains 35-45% carbon dioxide, 55-65% methane and less than 1% nitrogen. Biogas from organic waste digesters usually contains 30-40% carbon dioxide, 60-70% methane and less than 1% nitrogen. [Oslaj, 2010] Biogas may also contain trace amount of sulfur compounds, such as hydrogen sulfide. Methane in biogas can be further processed, such as in steam generation and the production of electricity. Production of biogas from various sources was studied, such as from corn, animal manures and corn stalks. [Elmore,

1987; Oslaj, 2010; Holm-Neilsen, 2009; Chen, 2010] Production of biogas from microalgae was also studied and concluded to be feasible. [Golueke, 1959] The methane yield from different microalgae strains are shown in Table 2.2 Even though, microalgae can be used for biogas production and grow much faster than other crops, using microalgae as feedstock for methane production is not competitive for other feed stocks, such as corn or sunflowers since growth costs of microalgae is more expensive than other feed stocks. [Schenk, 2008] However, combinations of biogas production and wastewater treatment by microalgae may be feasible for fuels production.

Table 2.2 Biomethane Yield from Various Microalgae [Harun, 2010]

Biomass	Methane yield (m^3/kg)	Biomass	Methane yield (m^3/kg)
Laminaria sp.	0.26-0.28	*I. Digitata*	0.5
Gracitara sp.	0.28-0.4	*Ulva sp.*	0.20
Macrocytis	0.39-0.41		

2.1.4 Synthesis gas

Production of synthesis gas (or ―syngas") involves a gasification process which converts solid or liquid carbon materials, such as biomass, coal or oil into gas with air, oxygen and steam under thermochemical treatment. [Huber, 2006] The gas composition predominantly contains carbon monoxide (CO), hydrogen (H_2), carbon dioxide (CO_2), methane (CH_4) and nitrogen (N_2). This technology is not new and biomass gasification is already commercialized for producing heat and electricity. Also syngas can be used for synthesizing commercial products, such as methanol and alkanes. Gasification of microalgae species *Cladophora fracta* and *Chlorella protothecold* by steam has been studied [Demirbas, 2009]. This study showed the technical feasibility of gasification of

algae by steam gasification. Gasification of *Chlorella vulgaris* with supercritical water was also studied. [Chakinala, 2010] The feedstock for this process can be homogeneous liquid or slurry. This may give an advantage for using wet microalgae or microalgal liquid as a feedstock for reducing harvesting costs. From Chakinala's work, the syngas from *C. vulgaris* contained 7% H_2, 22% CO, 25% CH_4, 26% CO_2, and 20% C_2-C_3 organic chemicals. The gasification efficiency or carbon conversion was reported to be 53%. The author concluded microalgae can be a promising feedstock for supercritical water gasification.

2.1.5 Bio-oil

The form of products of biomass under thermochemical treatment can be varied, such as gases, bio-oil and char and depends on heating rate, temperature and reaction time. For example, in flash pyrolysis, biomass can be converted into bio-oil when the temperature is maintained at 400-650 ^{o}C. However, when the temperature is maintained at 650-900 ^{o}C, a bio-oil and gas mixture was obtained. [Huber, 2006; Zhang 2007] Bio-oil can be used for power generation through internal and external combustion by mixing with diesel or natural gas. Upgrading the bio-oil is needed for using as a transportation fuel since bio-oil has a high oxygen content, low heat value, and high viscosity at ambient temperatures typical of fuel storage. Most bio-oil studies focus on conventional biomass sources, such as agricultural wastes or forestry products. [Li, 2008] Recently, production of bio-oil from microalgae was investigated.

Pyrolysis of *Chlorella protothecoides* in absence of oxygen at 200, 300, 400, 500 and 600 ^{o}C for 5, 20, 60 and 120 minutes was studied. [Peng, 2000] The maximum yield of bio-oil from *C. protothecoides* was 52% (w/w) at 500 ^{o}C after heating 5 minutes.

The direct pyrolysis and catalytic pyrolysis of *Nannochloropsis sp*. residue after lipid extraction with HZSM-5 as a catalyst was studied. [Pan 2010] For direct pyrolysis of *Nannochloropsis sp*., the maximum yield of bio-oil was found to be 30% at 400 ^{o}C, with 24% (w/w) of biomass converted to syngas. The liquid products yield was 45.3% (w/w). From these results, authors suggest the direct pyrolysis temperature for *Nannochloropsis sp*. residues of 350-450 ^{o}C.

Liquefaction of *Chlorella vulgaris* and *Spirulina* at 300 and 350 ^{o}C using KOH, Na_2CO_3, acetic and formic acid as catalysts was reported. [Ross 2010] Acetic acid catalyst gave the best bio-crude yield at 22% (w/w). From the results presented above, the production of bio-oil from microalgae is technically feasible.

2.2 Biofuels from Wastewater

Biofuel, such as biodiesel, can be produced from microalgae. To produce biofuel, various nutrients, such as phosphate and ammonium are needed for microalgal growth. These nutrients can be found in various wastewater sources.[Park, 2009; Kong, 2010; Chinnasamy 2010a] Since wastewater can potentially be used as inexpensive medium and pollutants contained therein can be consumed by microalgae, several studies have been conducted examining the potential for using wastewater as a growth medium for microalgae production. In this section, results of microalgal production from various wastewater sources will be presented.

Mixtures of *Spirulina platensis* and *Rhodotorula glutinis* have been grown on monosodium glutamate wastewater [Xue, 2010]. Total biomass reached 1.6 g/L with 0.22 g/L lipid reported after 5 days cultivation.

Chlorella sp. grown in 10x, 15x 20x and 25x diluted digested dairy manure in a batch system for 21 days was reported. [Wang, 2010] The algal concentrations in 10x, 15x, 20x and 25x diluted digested manure at day 21 were 1.57, 1.47, 1.71, 1.48 g/L, respectively, with a corresponding lipid contents (w/w) of 9.00%, 11.00%, 13.6% and 13.7%.

An algal consortium was isolated from a pond treating municipal or winery wastewater and incubated in wastewater from the primary clarifier effluent and dairy wastewater from San Luis Obispo. [Woertz, 2009] This consortium contained species from *Actinastrum, Scenedesmus, Chlorella, Spirogyra, Nitzschia, Micractiniun, Golenkinia, Chlorococcum, Closterium, and Euglena* and two unidentified species. The lipid contents of this consortium grown in the primary effluent varied from 4.9% to 11.3% (w/w) with biomass concentrations varying from 0.412 g/L to 0.843 g/L. The maximum total production was reported when the biomass concentration reached 0.812g/L with a lipid content of 9% (w/w) during the 18 day study. Also dairy wastewater was used in this study and was diluted to 10% (v/v) and 25% (v/v) with tap water due to poor algal growth without dilution. The maximal biomass concentration was reported to be 0.5g/L for 10% (v/v) dilution and 0.9g/L for 25% (v/v) dilution. The ranges of lipid contents were from 8-14% (w/w) for 10% (v/v) dilution and 10-29% (w/w) for 25% (v/v) dilution.

Botryococcus braunii, a microalgae species noted for producing high levels of hydrocarbons intracellularly, grown in secondarily treated piggery wastewater reached its maximum biomass concentration of 8.5g/L with a hydrocarbon content of 11.18% (w/w) in a batch system.[An, 2003]

Also, *B. braunii* grown in secondarily-treated sewage was reported. [Órpez, 2009] The maximum biomass concentration was between 0.2 to 0.25 g/L after 1000 hours incubation with 17.85% (w/w) lipids and 1.91% (w/w) hydrocarbon levels. In another study, *B. braunii* was also grown in secondarily-treated sewage in a semi-batch system. [Sawayama, 1994] 1140 ml secondarily-treated sewage was added into a 2 L bioreactor after removing 1140 ml liquid from the reactor daily. The biomass concentration dropped from 0.6 to 0.4g/L after 11 days. The biomass concentration was stabilized at 0.4g/L until end of the experiment at day 31. The hydrocarbon content, which was determined by hexane extraction at day 32, was 49% (w/w)

Another study has been conducted on *Botryococcus braunii (UTEX 2629)* in livestock wastewater. [Shen, 2008] Six flasks were labeled as B1 to B6 and tested in six different conditions**.** *Botryococcus sudeticus* was grown in 50% autoclaved wastewater in B1. In B2, *B. sudeticus* was grown in 25% raw wastewater. In B3, *Chlorella sp.*, which was presented and identified in the pond, was grown in 25% raw wastewater. In B4, both species were grown in 25% raw wastewater. In B5, *B. sudeticus* was grown in the inoculation medium. In B6, *Chlorella sp.* was grown in 100% raw wastewater. Distilled water was used for dilution. From the results, lag phase of B1 was 4 days period and others were less than 2 days. The max biomass concentration in the inoculation medium was approximately 0.2 to 0.3 g/l which is the lowest by comparing other conditions. The maximum biomass concentrations of B2 - B5 were less than 0.8g/L. The maximum biomass concentration of *B. sudeticus* grown in 50% autoclaved wastewater was 2.543 g/L with oil content (w/w) of 19.8%.

Scenedesmus sp. *LX1*, was incubated in the secondary effluent in the batch system. The highest biomass concentration recorded was 0.11g/L with 31-33% oil content. [Li, 2010]

Growth and lipid content of *Chalmydomoas reinhardtii* in wastewater from a concentrate of a St. Paul Metropolitan waste treatment plant with addition of trace metal solution has been reported. [Kong, 2010] A ~~–b~~io-coil" reactor system, consisting of a 6L storage tank connected to a 9L coil, was used for growing *C. reinhardtii*. Four liter samples were taken and replaced by wastewater daily. The stationary phase was reached at day 8 and cell concentration was 7.6 $x10^7$ cells/ml. At the stationary phase, 2g/L per day biomass was produced with an oil content of 25.25% (w/w).

A microalga consortium, containing 15 species, was isolated from carpet industrial wastewater. [Chinnasamy, 2010a] This consortium was grown in wastewater containing 85-90% (v/v) carpet industry effluent with 10-15% (v/v) municipal sewage with the addition of ~250ppm nitrogen as sodium nitrate and 5-6% (v/v) carbon dioxide in four raceway ponds of 950L capacity. The maximum biomass productivity was 4.9g/m^2 per day and the average productivity during the winter was 2.64 g/m^2per day. The biochemical composition of the consortium after 10-12 days incubation was determined to be 54.5% (w/w) protein, 6.82% (w/w) lipid and 8.98 %(w/w) carbohydrates. Before growing the consortium in the raceway ponds, the consortium was grown in 250 ml flask at 25 ^oC with 6% carbon dioxide in air. The max biomass was recorded as 1.47g/L after 9 days incubation.

Another microalgal consortium isolated from carpet industrial wastewater, contained three algal strains identified as: *Chlamydomonas globosa*, *Chlorella*

minutissima and *Scenedesmus bijuga.* This consortium grown in untreated carpet industry effluent was studied. [Chinnasamy, 2010b] The highest biomass productivity was 0.07± 0.018 g/L per day within 8 days incubation. The harvested consortium biomass contained 53.8% (w/w) protein, 15.7% (w/w) carbohydrate and 5.3% (w/w) lipid.

2.3 Bioremediation

Algal biomass can be used for removing phosphate, nitrate, ammonium and dyes, such as Azo dye, Remazol Black B, Remazol Red, Remazol Golden Yellow, Acid blue 290, Acid blue 324, Acid red 274 and Malachite green. [Mohan, 2008; Aksu, 2005; Özer, 2006a; Özer, 2006b; Daneshavr, 2007; Safonova, 2004]. Furthermore, algae are capable of removal and recovery from wastewater by metal accumulation or biosorption. [Mehta, 2005] Biosorption is usually referred to metal adsorption on dead biomass; however, it can also mean metal accumulation by live biomass as well. Results from several researchers show that algae is capable of removing Al, Au, Co, Cd, Cr, Cu, Fe, Hg, Ni, Pb, Zn, Ur from the aqueous solution. [Jacinto, 2009; Chojnacka, 2005; Khani, 2006; Fraile, 2005; Gupta, 2008] Precious metals such as silver or gold can be recovered from the wastewater by the adsorption and desorption process.

Removal of heavy metal by algae involves two processes: adsorption and transportation. [Bates, 1982] During the adsorption, metals are bound into the cell wall. This process was observed to be fast and metabolism independent. However, during the transportation process, metal ions were transported through the cell membrane and bound intracellularly. Equilibrium and kinetics of this process was observed to be affected by several factors, such as temperature, pH, initial metal concentration, amount of biomass, presence of competing ions and metabolic stage of algae.

In the biosorption process, metal accumulation can occur on the cell surface or inside the cell as shown in figure. The algal wall or surface contains several functional groups, such as hydroxyl, amino, carboxyl, sulphydryl and phosphoryl. Metal ions, usually positively charged, attract and bind to those functional groups on the cell surface since those functional groups act as a negative charge on the cell surface. [Xue, 1988; Mehta, 2005] Those functional groups are parts of cell wall components, such as protein and polysaccharides. Since the cell wall composition is varied among different algal species, the adsorption capability is varied among algae. However, even though the cell wall composition varies among algae, most metals are bound with proteins and polysaccharides. [Kuyucak, 1989] . Once metals are inside the cell, they may precipitate or bind to intracellular components, such as proteins, cellular polyphosphates, polysaccharides or enzymes [Wong, 1984; Zhang, 1994] Also metals can be absorbed as nutrients for living cells. [Hammouda, 1995]

Biosorption is not the only mechanism for polishing wastewater. Pollutants, such as ammonium and phosphate can be removed by microalgae as nutrient uptake. The removal rates of both ammonium and phosphate are reported as high as 99%. Removal of phosphates and ammonium has been reported by incubating a consortium in diluted dairy wastewater or primary clarifier effluent. Initial phosphate concentrations of wastewater were changed from 1.8 mg/L to 2.6 mg/L. The ammonium concentration of primary effluent, 10% diluted and 25% diluted dairy wastewater were 39, 30.5 and 16.3 mg/L. [Woertz, 2009] *Scenedesmus sp* grown in wastewater derived from remains of fish removed 94.44% ammonia nitrogen, 77.54% phosphate and 35.59% organic matter from the wastewater. [Andrade R., 2009]. However, not all ammonium is necessarily removed

by microalgae since a portion of ammonium may be lost to the surroundings. Over 20% ammonia was released into atmosphere during the pilot plant study in Quebec, Canada. [Sérodes, 1986] Ammonia lost to the atmosphere depends on the temperature and pH. [Nunez, 2001] The photosynthetic rate can affect ammonium lost to the atmosphere due to usage of carbonate which changes the pH. Likewise, not all phosphates are consumed by microalgae since phosphate can precipitate with a range of metal ions present in wastewater, such as calcium and magnesium [Larsdotter, 2007] The amount of phosphate removed by precipitation instead of consumption by microalgae depends on temperature, pH and initial concentrations.

2.4 Nutrients and Environmental Conditions

Growth of microalgae depends on various factors, such as nutrient levels, light intensity and temperature. High light intensity may lead microalgae into photo-inhibition which causes poor growth of microalgae. However, a lack of irradiance reduces the rate of photosynthesis of microalgae, also leading to the poor growth. Similarly, temperature and nutrient levels critically impact the growth rate and product rates and composition of microalgae. Environmental factors may also significantly change the population density of individual species in a microalgal consortium. When lipid-rich microalgae fail to grow in the secondarily treated wastewater, the question becomes, "How do you 'reconstitute' or modify the secondarily-treated wastewater in order to produce targeted products and maximize the growth"? In order to answer this question, one should have some knowledge about effect of nutrients and other environmental factors on algal growth as described below

2.4.1 Phosphorus

Phosphorous is one of the major nutrients and plays an important role in any biological system. Biomolecules containing phosphorus can be used for storing energy, such as ATP, NAP and phospholipids or can be used for synthesizing other important biological components, such as coenzyme, proteins, DNA and RNA. Furthermore, the supply of phosphorus affects the composition of biomass, such as lipids content and carbohydrates. [Borowitzka, 1988] Also, the nitrogen phosphorus (N:P) ratio not only controls potential productivity but also may significantly affect the dominance of specific species in a mixed culture. [Miller, 2005] Interactive effects of N:P ratio and light with a multispecies community has been reported. [Pinto, 2010] A lower N:P ratio has been observed to promote domination of N-fixing species under high light intensity; however, dominant species shifted to green algae when the N:P ratio was high under high light intensity. In nature, there are several phosphorus sources. [Barsanti, 2006] Phosphorus can be released as phosphate ions from rock during weathering. Also decomposition of dead biological creative, such as animals, plants or microalgae can also release phosphorus compounds. Phosphorus can be introduced into the biological system from human activities, such as improperly disposed animal wastes, and agricultural or lawn fertilizers. There are three forms of phosphate: orthophosphate, metaphosphate (a benzyl ring like structure) and organically bound phosphate. Orthophosphate is a preferred form for feeding microalgae. When phosphorus is rich, algae store excess phosphorus in the form of polyphosphate. These polyphosphate disappears under phosphorus deficient conditions. [Healey, 1982; Powell, 2009] In this condition, the content of chlorophyll a is decreased, but carbohydrate is increased. Enhancement of lipid

accumulation of *Monodus subterraneus* was reported under a phosphorus deficient condition. [Khozin, 2006] As decreasing K_2HPO_4 concentration from 175 μM to 0 μM, the total lipid content was increased and its composition is changed. The proportion of triacylglycerol in the total lipid was increased from 6.5% (w/w) to 39.3% (w/w); however, the proportion of phospholipids was decreased from 8.3% (w/w) to 1.4% (w/w).

2.4.2 Nitrogen

Nitrogen is one of the abundant elements on earth and is used for synthesizing important biological components, such as NADP, ATP, DNA chlorophyll and protein. The composition of air contains around 79% nitrogen which can be converted into ammonia by nitrogen fixation under low nitrogen environment. [Barsanti, 2006] This concept is employed in bio-fertilizer research. The main idea of bio-fertilizer is to grow a nitrogen-fixer prior to planting the desired crop (for releasing fixed nitrogen for uptake by the subsequently desired crop). There are two approaches for releasing nitrogen back to the crop field. The first one is to select nitrogenase-repressed mutants which excrete ammonia into the selected field. [Latorre, 1986; Thomas, 1991] Another approach for releasing nitrogen into the selected field is by decomposition or mineralization. Bio-fertilizers have been studied intensively in rice fields [Dhar, 2007]. However, production of bio-fertilizers from secondarily treated waste water has rarely been studied. In other words, by growing a nitrogen-fixer in secondarily-treated waste water to enrich the nitrogen content of wastewater, the wastewater could then be reprocessed for biofuel production.

Even through the composition of air contains 79% nitrogen, not all microorganisms can use nitrogen directly from air. Nitrogen in other forms, such as

nitrate, nitrite, ammonia and urea can be consumed by microorganisms. The different forms of nitrogen can affect the growth or composition of microalgae. Nitrogen effects among three different kinds of nitrogen sources (i.e. nitrate, urea, and ammonium) have been studied for growth of, *Neochloris oleoabundans.* It has been reported that *N. oleoabundans* has a higher lipid content and biomass concentration in nitrate media. [Li, 2008b] However, for *Ellipsoidion sp.*, no difference in growth was observed among nitrate, ammonium and urea nitrogen sources while a higher lipid content was observed for this species when grown in ammonium media. [Xu, 2001]

A study was conducted of nitrogen source effects on four strains (i.e. UTEX 25, 31 249, and 255) of *Chlorella protothecoides*. [Shen, 2010] No significant differences in growth were observed among three nitrogen sources (urea, yeast extract and nitrate) for the microalga strain UTEX 255. However, UTEX 25 and UTEX 249 grew much better in nitrate. UTEX 31 grows marginally better in urea than the other two nitrogen sources. However, no gene information was reported in this study to indicate that UTEX 255, UTEX 25, UTEX249 and UTEX 255 were the same species even though they were each named to be *C. protothecoides.*

When microalgae grow under nitrogen limited condition, specific degradation of phycobilisomes is activated for reuse of those nutrients and bleaching is observed, (algal color is changed from green to yellow green—a phenomenon observed in our own studies). [Collier, 1992] As nitrogen concentration decreases, the rate of photosynthesis is reduced and metabolism is changed to secondary metabolism which changes from a protein synthesis pathway to a carbohydrate or lipid synthesis pathway. Accumulation of lipids or carbohydrates inside cells under nitrogen starvation conditions depends

significantly on the type of microalgae. *Neochloris oleoabundans* was reported to accumulate lipids under nitrogen-limited growth conditions. As decreasing KNO_3 concentration from 20 μM to 3 μM, total lipids (w/w) increased from 8% to 40%. [Li, 2008] Also *Botryococcus braunii* Kutz IPPAS H-252 has been reported to accumulate lipids (w/w) from 9% to 22%. [Zhila, 2005] β-carotene accumulation by *Dunaliella* cells under nitrogen limited condition has also been reported. [Ben-Amotz, 1982]

2.4.3 Carbon Dioxide

Algae are capable of consuming a variety of carbon sources including glucose, acetate, carbon dioxide and ethanol. For autotrophic microorganisms, carbon dioxide is the primary carbon source and is consumed to produce sugar during photosynthesis. This process contains two cycles: a light cycle and a dark cycle. In the dark cycle, every mole of carbon dioxide taken up by the algal cell is converted to one mole of carbohydrates with three moles ATP and two moles of $NADPH_2$. [Richmond, 2007] Carbon dioxide is considered to be a greenhouse gas, contributing significantly to global warming. At present, the carbon dioxide concentration in the atmosphere is estimated to be 0.036%. [Barsanti, 2006] Excess carbon dioxide is usually supplied into open algae ponds. A bicarbonate-carbonate buffer system *is* created as shown in the following reaction:

(2.1)

(2.2)

(2.3)

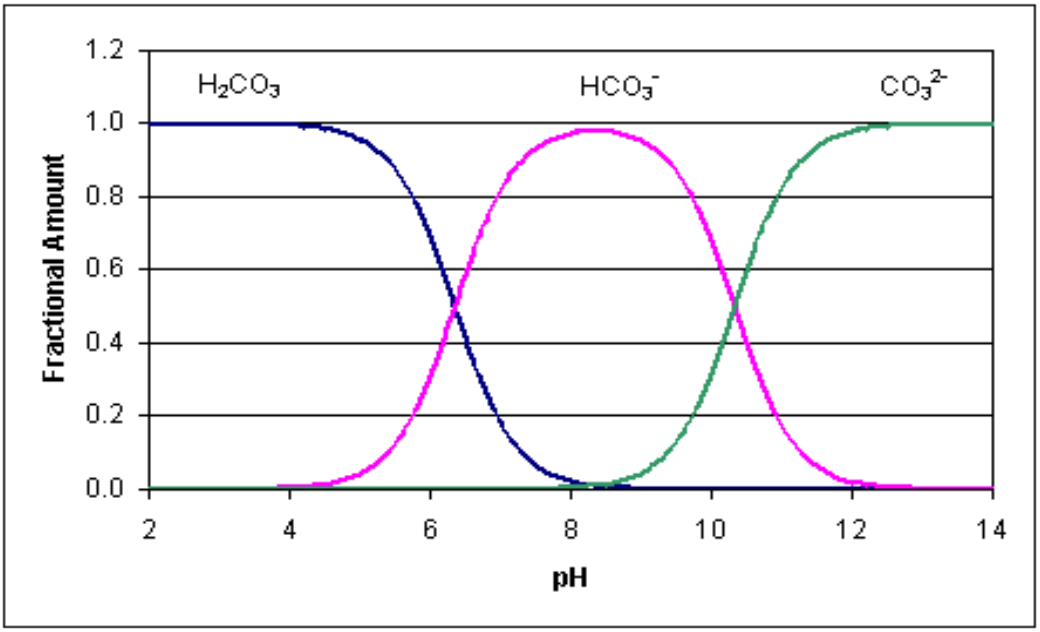

Figure 2.1 Equilibrium of Carbonate Ions in Different pH

Equilibrium constants for the above equations are dependent on temperature, pressure and salinity. [Wu, 2008] Presences of carbonate ions are dependent on pH as shown in figure 2.1. [http://www.chem.usu.edu/~sbialkow/Classes/3600/overheads /Carbonate/CO2.html] A carefully monitored supply of carbon dioxide is essential for growing algae. Oversupply of carbon dioxide (in comparison to the metabolic uptake rate) can cause a significant drop in pH and subsequent cessation of algal growth. Likewise, an inadequate supply of CO_2 will adversely affect algal growth. For example, the maximum biomass of *Chlorella vulgaris* was observed with 6 to 7 % (v/v) of carbon dioxide in air and decreased when CO_2 was increased from 7% to 12% (v/v). After increasing above 12 % (v/v) carbon dioxide, no significant growth was observed. [Chinnasamy, 2009].

Carbon dioxide concentration in the feed gas not only affects the maximum biomass concentration, but also affects the composition of products, such as lipid. Influence of carbon dioxide on four microalgae strains, *Nannochloropsis sp., Nannochloropsis oculata, Nannochloris atomus and Isochrysis sp.*, was reported [Roncarati, 2004]. A carbon dioxide addition of 2% (v/v) in air exerted a significant and more favorable influence than a lower carbon dioxide concentration (i.e. 1% v/v in air) on C18:1, C20:0 C22:6n- and C20:4n-6. Also ratio of short chain fatty acids to long chain fatty acids increased low carbon dioxide concentrations in the stationary growth phase. Desaturation of fatty acids was observed to be lower at higher CO_2 concentrations with *Chlorella vulgaris*. [Tzusuki, 1990] A similar result has been reported for *Botryococcus braunii.* [Rangarao, 2007] As carbon dioxide concentration was increased to 2.0% (v/v), the percentage of high chain carbohydrates (> 30 Carbons) increased from 34.41 % (w/w) to 57.58% (w/w). The maximum biomass concentration reached 2.0 g/L at day 25. For a carbon dioxide concentration of 1% (v/v), the biomass concentration reached ~1g/L at day 25. Increasing carbon dioxide concentration had a positive influence on total lipid composition of C16:0, C18:1 and C24:0, but have negative influence on C18:0, C18:2 and C22:1.

2.4.4 Light

Light is one of environmental factors that can affect microalgal growth. High light intensity may lead microalgae into photoinhibition which causes poor growth of microalgae. However, a lack of irradiance reduces the rate of photosynthesis of microalgae, also leading to the poor growth. This phenomenon can be represented by a light response curve. [Richmond, 2007] Three regions can be divided in this curve. As

increasing light intensity from 0 to the light saturated region, the photosynthesis rate is increased. In the light saturated region, the photosynthesis rate is maintained constant as increasing light intensity. However, once photoinhibition region is reached, photosynthesis rate is reduced. Effect of light intensity (10-200 $\mu Em^{-2}s^{-1}$) on the growth of Cryptomonas sp. was reported. [Weng, 2009] The highest specific growth was reached at 150 $\mu Em^{-2}s^{-1}$ and photoinhibition was observed at 200 $\mu Em^{-2}s^{-1}$. In the same study, a decrease tendency of Fe and P absorption with the increase in light intensity was observed. In another study, the highest specific growth rate of *Scenedesmus sp.* was reached at 6 Wm^{-2} and remained constant as increasing the light intensity from 6 to 15. [Rhee, 1981a] In the same study, the highest specific growth rate of *Fragilaria crotonensis* was reached at 12 Wm^{-2} and remained constant as increasing the light intensity from 12 to 30. No photoinhibition was observed. Authors also concludes that nutrient requirement for growth increased as irradiance decreased.

2.4.5 pH

Microalgae growth and metabolic product composition is significantly affected by pH . *Dunaliella bardawil* grown in medium at pH values of to 4, 6 7.5,9, and 10 medium was studied. [Khalil, 2010] The highest value of biomass concentration was obtained at a pH of 7.5 after 10 days incubation. The biomass concentration decreased with departures (either increases or decreases) in pH from 7.5. Cellular composition also changed with changes in pH. Both protein and carbohydrate cellular content reached a maximum at a pH of 7.5. , As expected, variances in cellular composition vary among species. For a study with *Chlorella ellipsoidea* a maximum carbohydrate concentration was observed at pH 10, while the % protein decreased with increasing pH above a maximum observed at

pH 9.0. The influence of pH on *Scendesmus obliquus* was reported [Hodaifa, 2009] with the highest maximum specific growth rate of 0.022 h^{-1} at a pH of 7.0. Not all species show a maximum growth at pH 7. The optimal growth of *Chlamydomonas acidophila* was at a range of pH from 2.6 to 3. For *Galderia sulphuraria*, a maximum growth was observed for pH values of 1-2. [Gerloff-Elias, 2005]. On application for these observations might be pH control for reducing or eliminating contamination in open pond systems. [Richmond, 2007]

2.4.6 Temperature

Temperature is one of the most important environmental factors influencing algal growth and its biological composition. It has been well documented that with changing growth temperature, the level of unsaturated fatty acids changed in the form of glycerolipids. In the most species, as temperature was increased, the degree of unsaturation of membrane lipids decreased. [Nishida, 1996]. Furthermore, accumulation of lipids depends on temperature and the optimal temperature for lipids production varies with each species. Lipids content of two microalgae strains (*Nannochloropsis oculata* and *Chlorella vulgaris*) was reported with temperature variations. [Converti, 2009]. Lipids content of *Nannochloropsis oculata* doubled from 7.90% to 14.92% (w/w) when the temperature was increased from 25 to 30 ^{o}C . In contrast, the lipids content of *Chlorella vulgaris* decreased from 14.71% to 5.90% (w/w) when the temperature was increased from 25 to 30 ^{o}C.

Temperature can also affect the starch composition and amount inside *Chlorella vulgaris* [Nakamura, 1983]. The optimal temperature of starch accumulation has been observed between 20 and 30 ^{o}C. Size of starch molecules produced is also temperature

dependent. At 20 ^{o}C, 75% of the starch produced had a molecular weight over 2,000,000 (L-starch) with 16% of remaining starch with a molecular weight less than 10000 (S-starch). At 38 ^{o}C, the ratio of L-starch to S-starch decreased.

Temperature can also affect microalgal growth and production rates. *Neochloris oleabundans* was reported to grow faster at 26 ^{o}C than at 30 ^{o}C [Guoveia, 2009b]. The specific growth rate of *Amphidinium sp*. reached a maximum at a temperature of 26 ^{o}C [Kitaya, 2008].

Temperature also affects cellular nitrogen and phosphorus composition. A study of *Scenedesmus sp.* and *A. formosa* with temperature variations and interactions with nutrient limitations was reported [Rhee, 1981b]. In this study, cell growth under non-limiting and limited nutrient conditions increased as temperature was decreased. Also, lowering the temperature caused increases in minimum cell quota which is defined as internal cell‘s nutrient contents (nutrient weight per cell), such as weight of vitamin per cell. [Droop, 1968] This shows more nutrients are needed for growth or maintenance when the temperature is below optimal temperature. Furthermore, protein synthesis rate per unit RNA is decreased as lowing temperature. Cell volume of *Scenedesmus sp.* is increased when the temperature is below optimal temperature. In the other world, more nutrients are needed to produce a cell at the same growth rate at a non-optimal temperature [Richmond, 2007] Effect of temperature on photosynthesis rate of three algal strains (*Prorocentrum minimum*, *Prymnesium parvum* and *Phaeodactylum tricornutum*) is also reported from 5 Celsius to 30 Celsius. [Hancke, 2008] The maximum photosynthesis rate is reached at 20 to 25 Celsius. As increasing temperature from 25 Celsius to 30 Celsius, the photosynthesis rate is decreased. As temperature is increasing

from 5 Celsius to 20 Celsius, the photosynthesis rate is increased. Also no photoinhibition is observed from 0-566 μmol photons m^{-2} s^{-1}.

2.5 Growth Cycle

In the batch cultures, the growth curve of algae, as with most microbial systems, can be represented by the following phases: (a) lag phase (b) exponential growth phase (c) deceleration growth phase (d) stationary phase and (e) death phase. The cellular composition and metabolic path can be varied during each phase due to nutrient levels within the batch vessel. [Shuler, 2002]

2.5.1 Lag Phase

Lag phase represents the growth phase after incubation. In this stage, the cell concentration remains essentially constant. With inoculation, cells encounter a nutrient-rich environment, but significantly different from that of the seed culture from which the cells were transferred. For growth to occur in the new system, adaption occurs by synthesizing enzymes and co-enzymes necessary for cellular reproduction. The metabolic path may be changed from secondary metabolism, such as lipid accumulation, releasing soluble polysaccharides and degradation of phycobilisomes (observed by cell bleaching or discoloration), to primary metabolism. The length of the lag phase is dependent upon the amount of cells transferred to the new growth medium, the nutrient levels present, and culture age. To ensure that the early exponential growth phase from the late lag phase is noted, it is recommended that the initial inoculum volume to be 5% to 10% of the total volume of the new batch culture. Length of lag phase is strongly

dependent upon culture age. Increasing age of a culture usually results in a longer lag phase period due to a smaller fraction of cells capable of reproduction [Monod, 1949].

2.5.2 Exponential Growth Phase

When there is sign of growth, the growth curve is moving from the lag phase into the exponential growth phase or logarithmic growth phase. After cells are adapted to the new environment and new protein/enzymes are synthesized, cells start to grow exponentially. However, exponential growth is not only happened after the lag phase but also can happen after the stationary phase. [Boiler, 1989] Also two exponential phases have been observed and separated the secondary lag phase due to the certain mixture of carbohydrates, such as glucose/sorbitol when the organic source is the limiting growing factor. [Monod, 1949] Furthermore, several successful exponential phases can occur between the lag and stationary phases. Each exponential phase has their own specific growth rate and can be separated by angular transition points.

The idea of exponential population growth was developed by Malthus. [Droop, 1983] Growth of microorganisms can be represented as cell division over time. Assume all cells in the growth phase are able to divide or majority of cells are capable of reproduce themselves. [Monod, 1949] Then the equation can be written as following after all cell divides once in a certain period time

(2.4)

where, is the number of cells per volume at time 2. is the cell number before the division at time . is the time requirement for all cells divide once. After n division at t_3, it becomes as

(2.5)

Assume the cells divide at the constant rate [Ferenci, 1999] which can be represented as

(2.6)

Then

(2.7)

Rearranged, the equation becomes

(2.8)

where r is the number of divisions per unit time. As approaches to 0 and replace to , the model can be represented as

(2.9)

where, N is cell number per unit volume, t is time and u_{net} is net specific growth rate which is characteristic of the cell population and depends on the growing conditions, such as nutrient level and space required for cell reproduction. In other words, this model is developed based on unlimited spaces and resources. [Droop, 1983] Assuming the average cell density (weight per cell over volume per cell) doesn‘t change during the growth, the equation can be written as

(2.10)

where, X is the biomass concentration which is cell weights per volume. Several assumptions are applied to simplify the model and to determine the maximum specific growth rate under one set of conditions. while in the batch system, the space (i.e. culture volume) is limited, the effects of volume per cells on the cell growth rate is assumed to be

negligible. In other words, available space for cell reproduction is assumed to greatly exceed needed space. It also assumes that a change in nutrient level doesn‘t affect the specific growth rate. Accumulation of waste has a negligible effect on the cell growth in this stage. Furthermore, variations of environmental factors, such as light insensitivity and temperature, are very small and have neglectable effect on growth. Also, cells‘ properties are assumed to be constant or steady state, such as size, transport of nutrient and affinity [Ferenci, 1999] Basing on all assumption and integrals, the specific growth rate can be determined in the following from:

(2.11)

(2.12)

(2.13)

2.5.3 Deceleration Growth Phase

In this growth phase, nutrients are consumed and toxins are released into the environment. Space or volume required for cellular reproduction may become limited. Assumptions in the exponential phase are no longer valid. In other words, increasing toxicity and decreasing nutrient level reduce the growth rate of cell. Also the cellular morphology and physiology may change (i.e. size, cell composition and metabolic pathways). This phase is called deceleration growth phase before the net growth rate reaches zero. The model developed on the exponential growth phase cannot be used to predict accurate biomass concentration without remodeling the equation.

2.5.4 Stationary Phase and Death Phase

Once the net growth rate reaches zero, the growth curve enters the stationary phase from deceleration growth phase. The metabolic pathway is changed from primary metabolites to secondary metabolites. Several products can be formed in this phase such as accumulation of lipid. [Guoveia, 2009] In this phase, the total cell concentration may stay constant but total viable cells may decrease. This leads to the continuous decrease of the net specific growth rate into the death phase. Another exponential phase may be observed after the stationary phase with a lower net specific growth rate (in comparison to that observed during the initial exponential growth phase). [Boiler, 1989] This may due to cell lysis with products of lysed cells can be used for growth. After the stationary phase, the death phase occurs. However, the demarcation between late stationary and early death phase may be difficult to define with precision. In the death phase, the net specific phase is negative due to lowering numbers of viable cells, nutrient depletion and toxic stress. Apparent or ”cryptic” growth may occur, but the death rate is higher than the growth rate.

CHAPTER III

RESEARCH OBJECTIVES AND STRATEGIES

The goal of this project is to determine if known microalgae cultures or natural isolates could produce biomass levels and lipids or sugars in amounts sufficient to constitute a process for converting residual nutrients residing in secondarily-treated wastewater as part of a larger scheme of producing biofuels from a wastewater treatment facility. To achieve this goal, two different strategies were used. The first strategy was to select identified microalgae cultures known to produce high levels of biomass (from which sugar may be obtained) or lipids when grown on defined medium. Performances of these cultures were examined when grown on limited nutrients in defined medium or in combination with secondarily-treated wastewater.

The second strategy was to screen or isolate from natural sources potential microalgae that can grow in secondarily treated wastewater with (and ultimately without) nutrient addition.

Rates and yields of biomass, lipids and sugars resulting from an acid hydrolysis treatment are reported herein for a variety of culture growth conditions.

At the beginning of this project, the first strategy was employed to using known high lipids-producing microalgae, *Botryococcus sudeticus UTEX2629* and *Neochloris oleoabundans UTEX1185*. A baseline of experimental data was established for defined medium proven to support growth of these cultures, followed by studies with synthetic

wastewater. From these initial studies, poor growth of *B. sudeticus* and *N. oleoabundans* were observed. The reason of poor growth appeared to be a lack of key nutrients.

Growth of *N. oleoabundans* in actual secondarily-treated wastewater was studied. From results, additions of nutrients into secondarily wastewater appeared to be needed for supporting both culture growth and lipids production.

In the processing of secondarily-treated wastewater from local wastewater treatment facilities, natural isolates were observed and transferred to defined medium for enrichment. Through the enrichment of two isolates, designated "MA" and "NA", the second research strategy was initiated to investigate the potential of these isolates when grown (after enrichment in defined medium) in secondarily-treated wastewater without nutrient addition. The two natural isolates and *N. oleoabundans* gave favorable results when grown on secondarily-treated wastewater. Their sugar contents and maximum specific growth rates were determined. Results were used for a rough estimation of ethanol production from sugars (based upon a model facility presented in the literature). A study of NA growth was performed in secondarily treated wastewater with the addition of a commercial liquid fertilizer. From these results, biomass concentrations were not increased, but the glucose:xylose ratio of culture NA was observed to be influenced by the addition of supplemental nutrients via addition of the liquid fertilizer into secondarily-treated wastewater.

CHAPTER VI

METHODS AND PROCEDURES

4.1 Microalgae Cultures

Six different cultures, pure and mixed consortia, were used in this study. *Botryococcus sudeticus* (*UTEX 2629)* and *Neochloris oleoabundans* (*UTEX 1185)* were maintained in growth media identified from the literature for culture maintenance. Additionally, natural isolates were obtained from various sources and examined for growth on secondarily-treated wastewater (hereafter designated STW). Table 4.1 shows the culture designation and sources for natural isolates showing significant growth and/or lipids productivity when grown on STW.

Table 4.1 Natural Isolates Chosen for Study

Culture Designation	Source Material	Location
E	Winogradsky column	Swalm lab 185
NA	STW	Water treatment plant, Tuscaloosa, AL
MA	STW	Water treatment plant, Tuscaloosa, AL

Culture ~~"E~~" was isolated from a Winogradsky column—one of several maintained in the Swalm School of Chemical Engineering microbiology laboratory, each of which indicated a significant presence of algae and other microbial cultures emerging from inocula obtained from various soil, water and manure samples. At the time of sampling,

cultures in these columns had aged 3-4 years. Two isolates designated –NA", and –MA" were isolated from different sources of STW and maintained with autoclaved STW throughout the study. Culture –NA" was isolated from STW obtained from a wastewater treatment plant in Tuscaloosa, Alabama. Isolate –MA" consisted of a mixture of both NA and *N. oleoabundans*, isolated from an attempt to grow *N. oleoabundans* in non-autoclaved STW. Earlier experiments with *N. oleoabundans* in non-autoclaved-STW had shown little to no significant growth. While no growth was observed in all flasks but one, a small bright green floc appeared at the end of one experiment (described in detail in later sections). This floc was transferred to new, autoclaved STW with subsequent strong growth. Determination of the culture identity is beyond the capability of our labs. Thus, while the possibility certainly exists that this culture may likely be *N. oleoabundans*, it is also distinctly possible that it may be a mixed consortium or an entirely different organism. For the purposes of this project, it was designated –MA" to distinguish its performance from studies with pure *N. oleaabundans*. Additional culture isolation details are presented in later sections describing specific experimental work.

Procedurally, STW was obtained from local wastewater treatment plants in five gallon polyethylene carboys. Once returned to the laboratory, STW was autoclaved in 2-3 liter Erlenmeyer flasks prior to batch microalgae studies. After autoclaving, STW was returned to sanitized carboys (washed with a bleach/soap solution and thoroughly rinsed).

4.2 Culture Maintenance and Batch Systems Used

In this project, cultures were maintained in stationary 150 ml serum bottles and on agar slants with defined medium nutrients (or STW in the case of –NA" and –MA" isolates) in an incubator and in agitated batch flasks. Batch systems consisted of 0.5 L

and 1.0 L Erlenmeyer flasks fitted with rubber stoppers and stainless tubing for aeration/carbon dioxide transfer and for maintain aseptic conditions were used for studying culture performance.

4.2.1 Incubator

The incubator consisted of an insulated chamber, 65 cm in width X 125 cm in length X 61 cm in height. Temperature control was maintained at 25 ^{o}C by a fan connected to thermostatic control. Fluorescent "daylight" lamps were used as a light sources and the dark/light period was controlled at 12:12 hours by a timer. No carbon dioxide (CO_2) was supplied to stored cultures other than diffusion of CO_2 present in the ambient atmosphere.

4.2.2 Batch Systems

The batch system, shown in Figure 4.1, was used for studying culture performance. In this system, 0.5 and 1.0 L Erlenmeyer flasks were used as batch reactors and sealed with rubber stoppers with two stainless tubes through which air and CO_2 was fed and exhausted. Pipettes filled with glass wool were used as filters and connected between stainless steel tubes and gas sources. Stir bars and stir plates were used for maintaining culture suspension. Sixteen fluorescent lamps were employed as the light source for growth with an average light intensity of 15.09 ± 0.81 klux (determined by a Sper Scientific light meter 840010). The volumetric flow rate of carbon dioxide and air mixture was maintained from 0.250-0.542 ml/s by adjusting valves from a gas manifold (shown in Figure 4.2). The CO_2 concentration was maintained between 3 to 5 % (v/v) by mixing air through two rotameters from an air compressor and a CO_2 high pressure

cylinder to the gas manifold (Fig. 4.2). Bioreactor temperature was monitored at ambient laboratory conditions (approximately 20 ^{o}C).

Figure 4.1 The Front View of Batch Systems

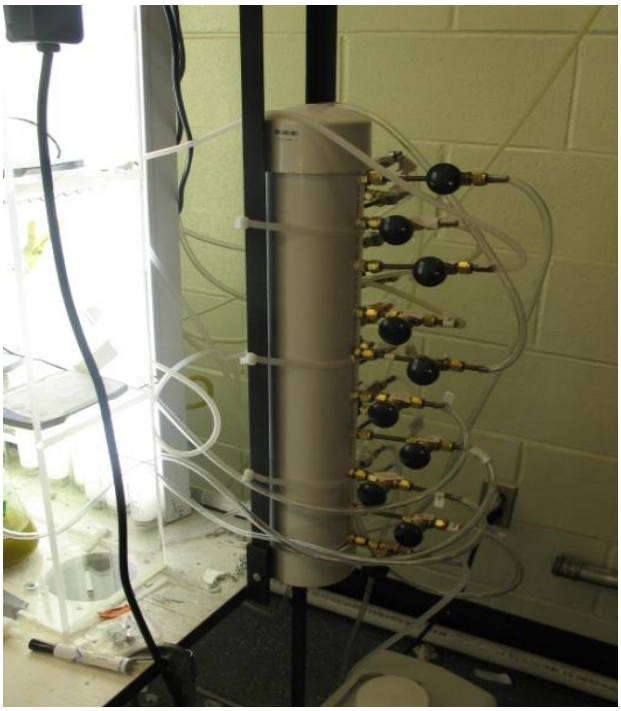

Figure 4.2 Gas Manifold for Batch Systems

Figure 4.3 Carbon Dioxide Feed to Batch Systems

4.3 Culture Growth Conditions and Media

Seed cultures were grown in 0.5 L Erlenmeyer flasks using various media (defined, STW or in combination). Different growth media were used for the incubation of UTEX microalgae cultures as suggested by literature or culture repository sources. For *B. sudeticus*, *N. oleoabundans* and the ―E" isolate, CHU13 and SE medium were employed. For ―NA" and ―MA" isolates, autoclaved STW was used for culture maintenance. In the 0.5L batch flasks, CO_2 was supplied (3-4% v/v) in air. Media formulations used in this project are listed in Tables 4.2 to 4.4. A synthetic wastewater formulation [Ghosh, 2004] was used in some studies with *B. sudeticus*, *N. oleoabundans* and ―E" cultures for examining the effect on growth of various nutrients.

Table 4.2 SE Medium [Li, 2008b]

SE Chemicals	Concentration (mg/L)	SE Trace Solution chemicals	Concentration (mg/L)
KNO_3	505.5	H_3BO_3	286
K_2HPO_4	114.5	$MnCl*4H_2O$	181
KH_2PO_4	350	$ZnSO_4*7H_2O$	22
$MgSO_4*7H_2O$	150	$CuSO_4*5H_2O$	7.9
NaCl	50	$Na_2MoO_4*2H_2O$	5.345
$CaCl_2*2H_2O$	50		
SE solution	10 ml		

Table 4.3 CHU13 Medium [Chu, 1942]

CHU 13 Chemicals	Concentration (mg/L)	PIV Solution Chemicals	Concentration (mg/L)
KNO_3	400	Na_2EDTA	750
K_2HPO_4	80	$FeCl_3*6H_2O$	97
$MgSO_4*2H_2O$	200	$MnCl_2*4H_2O$	41
Ferric citrate	20	$ZnCl_2$	5
Citric acid monohydrate	100	$CoCl_2*6H_2O$	2
$CaCl_2*2H_2O$	107	$Na_2Mo_4*2H_2O$	4
PIV solution	12 ml		
pH	7.5		

Table 4.4 Synthetic Wastewater [Ghosh, 2004]

Synthetic Wastewater Chemicals	Concentration (mg/L)	SL7 Solution Chemicals	Concentration (mg/L)
$(NH_4)_2SO_4$	50	$Cr(NO_3)_3*9H_2O$	0.77
K_2HPO_4	25	$CuCl_2*2H_2O$	0.536
Na_2HPO_4	400	$MnSO_4*H_2O$	0.108
Gelatin	150	$NiSO_4*6H_2O$	0.336
Casamino Acid	10	$PbCl_2$	0.1
$CaCl_2$	0.06	$ZnCl_2$	0.208
Starch	70		
Yeast extract	10		
SL 7 solution	0.1 ml		
pH	7.5		

4.4 Analytical Methods and Cell Preparation

Several analytical methods were employed for setting experimental operating parameters and for determining sugar and lipid contents of selected microalgae.

4.4.1 Bubble Count

To determine the gas flow rate to each batch reactor, flow through ¼" stainless steel tubing into each reactor was calibrated with a bubble count method (described below). Two rotameters (one for air and one for carbon dioxide) were used to fix the percentage (v/v) of CO_2 mixed with air. The volumetric flow of carbon dioxide in batch systems was controlled by bubble count and was calculated by an empirical equation. The setup of this experiment was shown in figure 4.4. Bubbles from a submerged 24.2 cm steel tube with 3.15 mm inside diameter and 6.25 mm outer diameter were counted within a period of time and the gas volume was measured inside a 2L cylinder. The empirical equation was constructed correlating bubble counts and a corresponding accumulated gas volume.

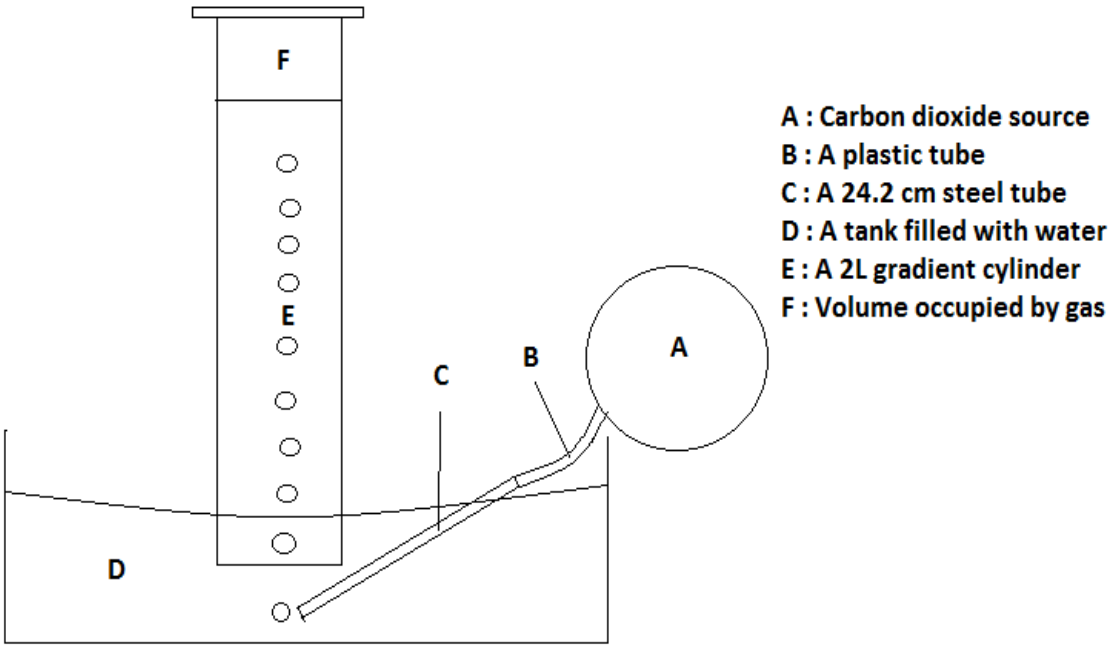

Figure 4.4 Bubble Count System for Constructing an Empirical Equation.

A flow diagram, shown below in Figure 4.5 depicts the progress of analyses from cell harvesting through determination of lipids and/or sugar content.

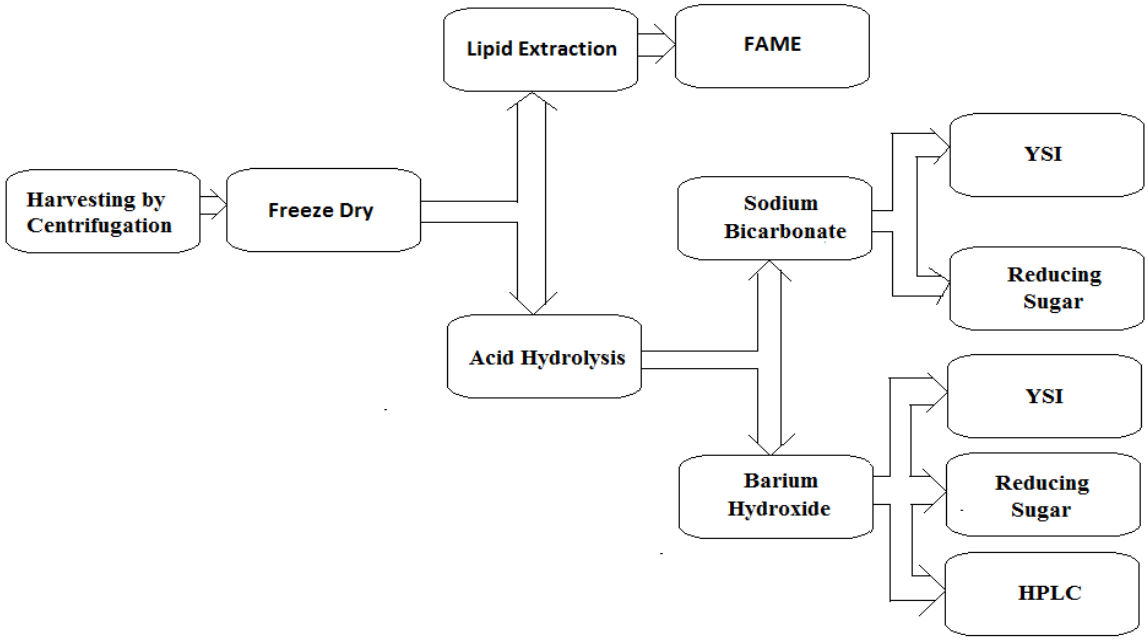

Figure 4.5 The Flow Diagram of Analytical Procedures

4.4.2 Harvesting/Centrifugation/Freeze Drying

Cultures were harvesting by transferring 50-200 ml samples into pre- weighed centrifuge tubes and centrifuged at 6000 rpm for 15-20 minutes. The supernatant was decanted. Remaining solids or concentrated microalgal solutions were stored inside the freezer for 1 to 2 days for ice formation. After becoming solidified, samples were placed in the freeze dryer until completely dessicated—as indicated by a constant dry weight over time. Depending on culture type, the period of drying ranged from 24 to 72 hr. After freeze drying, biomass weight was determined by a final weight from which the initial centrifuge tube weight was subtracted. The dry algal biomass was used for determining lipid and sugar contents.

4.4.3 Lipid Extraction

The lipid content of microalgae was determined by a modified Bligh and Dyer method [Bligh & Dyer, 1959]. For our analyses, 6.25 ml of chloroform and 12.5 ml of methanol were added to 5ml of aqueous mixture of water and microalgae forming a single phase. After vigorously mixing the solution for 10 minutes, 6.25 ml of chloroform and 6.25 ml of water with 0.5% (w/w) sodium chloride were added to the solution to obtain a two phase mixture. This mixture was shaken for 10 minutes and then centrifuged at 3000 rpm for 10-15 minutes to form three phases: and aqueous, solid cellular material and organic phase. The organic phase at the bottom of the test tube was removed with pipettes and filtered through a glass pipette filled with glass wool. Organic layers of three extractions were combined and evaporated using a ~~t~~urbovap"—an evaporative device in which tubes of sample are partially immersed in a constant temperature bath and with a nitrogen stream blown into each tube to speed up solvent evaporation. The weight of non-volatile, extracted lipids was measured and recorded as total lipids.

4.4.4 Fatty Acid Methyl Ester (FAME) Analysis

The total lipids from Bligh and Dyer method were trans-esterified to fatty acid methyl esters by reacting with methanol and 2% H_2SO_4 (v/v) as a catalyst at 60 ˚C for 2 hours. After neutralization with 2% (w/w) $KHCO_3$ and a 5% (w/w) sodium chloride solution, FAMEs were extracted by toluene. Toluene containing FAME was analyzed by using an Agilent 6890 series Gas Chromatograph with Supelco SP 2380 capillary column (100 m x 0.25 mm x 0.2 micron, model number Supelco 24317) and a flame

ionization detector (FID). The concentrations of FAME peaks were determined and identified by comparing samples‘ responses with known standards.

4.4.5 Sugar Analysis

For sugars analysis, three different methods were employed. First, dried microalgae samples were treated with acid hydrolysis for releasing sugars into the solution. Then HPLC/MS was employed for determine potential sugars in solutions. A YSI® Biochemistry Analyzer 2700 Select was used to determine the xylose and glucose concentration in solutions. [Choy, 2007; Kastner, 2001] For an estimate of total sugars, reducing sugars were measured colorimetrically by a di-nitro-salicylic (DNS) method [Miller, 1959]

4.4.5.1 Acid Hydrolysis

To prepare dried cellular material for sugars analysis, it must first be hydrolyzed. Dried biomass samples (0.5 to 1g) were weighed into dried 20 ml test tube. Controls were run in parallel by performing all procedural steps with two dried test tubes containing no cellular material. To each tube 8.3 ml of ~ 0.6 M sulfuric acid and 1.7 ml water were added to give a final sulfuric acid concentration inside test tubes of ~ 0.5M. Diluted sulfuric acid concentrations were determined to be ~ 0.6 M by titration with a prepared concentration of NaOH. Test tubes were sealed with caps and placed into boiling water bath for 4 hours. [Brown, 1992] The water temperature was maintained between 90 and 100 ^{o}C with water addition every 10-20 minutes to compensate for evaporation over time. After cooking 4 hours, samples were cooled overnight at room temperature. Prior to analysis, two different neutralization methods were employed. For

HPLC analysis, barium hydroxide octa-hydrate powders were added to neutralize sample pH to a range of 6 to 8. After neutralization, samples were filtered through glass pipettes filled with glass wool. A second filtration was performed through 0.45 μm leur-lock filters fitted to 10 ml syringes. Filtrates were stored in the freezer for HPLC, YSI and reducing sugar analysis.

Due to a long period required for neutralization by the previous method (5 to 20 minutes per sample), a second method of neutralization was attempted using sodium bicarbonate. After neutralization, samples were centrifuged at 6000 rpm for 10 minutes. Cell debris floating on the top of the sample was removed by pipette and the remaining sample liquid was filtered through a 0.45 μm filter. Filtrates were stored in the freezer for YSI and reducing sugar analysis.

4.4.5.2 Reducing Sugar Analysis

For reducing sugar analysis, the DNS method was employed [Miller, 1959]. In this method, a DNS solution was prepared by dissolving 1 gram of 3, 5 - dinitrosalicylic acid and 30 grams of potassium tartarate tetrahydrate into 20ml of 2M sodium hydroxide and 80 ml water. For DNS analysis, 4ml DNS solution was added to 2 ml acid hydrolyzate. Then the mixtures were cooked for 10-15 minutes in a boiling water bath for color development. Depending on the sugar concentration, the solution color may vary from yellow to orange to dark red. A calibration curve was constructed for every DNS analysis by preparing known glucose concentration solutions in parallel to sample preparation. After cooking, all solutions (i.e. samples and glucose) were allowed to cool overnight and an optical density (OD) reading was recorded at 570nm using a GEAE SYS 20 Thermo Spectronic UV spectrophometer The reading was converted into

concentrations by employing a calibration curve prepared from the OD readings of the known glucose concentrations.

4.4.5.3 YSI/HPLC

Filtered, neutralized samples were analyzed by HPLC/MS in the Mississippi State Chemistry lab. Two sugar components were observed and determined to be glucose and xylose. Since two major sugar components were xylose and glucose, the YSI 2700 was employed for determining xylose and glucose concentrations by comparing sample responses and known standard responses from immobilized enzyme biosensors.

4.4.6 Ion Chromatography

Phosphate, nitrate, and nitrite concentrations of STW were identified and determined by comparing samples‘ responses and standard responses from a DIONEX ICS-3000s ion chromatograph.

4. 5 Experimental Procedures of the Each Study

Several experiments were conducted in this research. Between each experiment, procedures could be varied due to different experimental designs.

Experiments were designed for several purposes:

1) to establish a baseline of growth on various defined media and known cultures for comparison to the scientific literature and to studies with natural inocula
2) to examine culture performance when grown on a defined synthetic wastewater
3) to determine capabilities for growth on secondarily-treated wastewater (STW) alone and with nutrient additions of various formulations

The ultimate goal of this project was to determine if known microalgae cultures or natural isolates could produce biomass levels and lipids or sugars in amounts sufficient to constitute a process for converting residual nutrients residing in secondarily-treated wastewater as part of a larger scheme of producing biofuels from a wastewater treatment facility. For the experimental work, unless otherwise noted, the following experimental conditions were used: carbon dioxide supply was maintained between 3 to 5% (v/v) in air. The volumetric flow rate of carbon dioxide and air mixture was maintained between 0.250-0.542 ml/s and the light intensity was measured to be 15.09 ± 0.81 klux.

4.5.1 Growth of *Botryococcus sudeticus* in CHU13 Medium

Initially, to establish a baseline of culture growth and performance for comparison with the literature and with studies of natural isolates, *B. sudeticus (UTEX 2629)* was grown in 0.5 L flasks containing CHU13 supplied with carbon dioxide mixing with air. Algal biomass was harvested by centrifugation at 6000 rpm and freeze dried at different culture ages. The growth and calibration curves were constructed correlating the mass of freeze and oven dried algal samples with optical density readings. The dried biomass was used in FAME analysis, and Bligh and Dyer methods for determining lipid contents and composition.

4.5.2 Effect on Growth by Replacing $(NH_4)_2SO_4$ with KNO_3

From observation of limited growth in the synthetic wastewater, nitrogen was assumed to be a potential limiting nutrient. This assumption was tested in this experiment. As highlighted in the literature review, nitrogen sources have been cited as playing an important role in culture performance. This experiment was conducted in two

successive generations of *B. sudeticus* (to lessen the likelihood of nutrient –carryover" from CHU13 medium—since it has been shown to readily sustain algae growth in comparison to wastewater). In the first generation of incubation, *B. sudeticus* grown in CH13 was transferred into 3 different medium formulations: CHU13 (as a control), synthetic wastewater and synthetic wastewater replacing potassium nitrate in the medium formulation with ammonium sulfate. The cultures were grown in 0.5 L flasks, each containing 0.4 L medium, fed with carbon dioxide in air under a constant light source. No forced air/carbon dioxide flow was supplied until day 3. Optical density readings (OD) were taken daily and converted into biomass concentrations. The calibration curve was constructed by measuring optical density of *B. sudeticus* samples at a wavelength of 550 nm correlated to dry weight of samples obtained via filtration and oven drying.

For the second generation of *B. sudeticus*, the first generation culture was transferred into the same medium. The growth conditions were the same as the first generation. In this experiment, for comparison using a natural isolate, –E" culture (isolated from a Winogradsky column in our labs) was also used. –E" culture was first grown in synthetic wastewater for avoiding nutrient —carryover" effects from CHU 13 medium and then was incubated into three different media: CHU13, synthetic wastewater and synthetic wastewater with addition of potassium nitrate. Optical Density (OD) was measured daily.

4.5.3 Effects on Growth by Removal of Nutrients from the Medium

N. oleoabundans was grown in the synthetic wastewater then incubated into CHU13, CHU13/synthetic wastewater mixtures, CHU13/synthetic wastewater deficient in of one nutrient, and synthetic wastewater. For example, in one flask, K_2HPO_4 was

reduced (by the amount normally present in CHU13) and mixed with synthetic wastewater containing its normal formulation of K_2HPO_4. Optical density readings were taken daily at wavelength of 600 nm and was converted to biomass concentration base on the calibration curve and resulting empirical equation established for this culture. [Li, 2008b] To increase the total amount of biomass recoverable, cultures were grown in 1L flasks containing 700 mL medium under constant light without supplement of carbon dioxide until day 3. After 3 days incubation, carbon dioxide and air mixture was pumped into batch systems.

4.5.4 Growth of *N. oleoabundans* in STW

N. oleoabundans, first grown in SE medium, was transferred to four different medium formulations: (1) SE medium as the control; (2) a mixture containing 50% (v/v) SE medium with 2X nutrient levels and 50% (v/v) non-autoclaved STW from Tuscaloosa Alabama (to give a total initial nutrient concentration equivalent to SE medium but with the added change of the 50% STW); (3) a mixture containing 50% (v/v) SE medium with 2X nutrient concentrations and 50% autoclaved STW; and (4) autoclaved STW alone. Cultures were grown in 1L flasks containing 0.7 L liquid supplemented with carbon dioxide under constant and a gas flow rate was maintained from 8.33 ml/s to 3.94 ml/s with a bubble flow meter. OD readings were taken daily. The mixtures of SE medium with STW were chosen to investigate the possibility that STW might be deficient in key nutrients needed to sustain growth.

4.5.5 Growth of *N. oleoabundans* in STW and SE Medium Mixtures

In this study, *N. oleoabundans* was first grown in SE medium then transferred to four different mixed solutions: (1) SE medium as a control, (2) 1X SE medium constituents in autoclaved STW, (3) 0.6X SE nutrients in autoclaved STW, and (4) 0.3X SE nutrients in autoclaved STW. The cultures were grown in 1L flasks containing 0.7 L liquid supplemented with carbon dioxide under constant light sources. Gas flow rates were maintained between 2.08-4.39 ml/s. OD readings were taken daily. Lipid contents of cultures were determined by Bligh and Dyer method.

4.5.6 Cultures Used for Kinetic Studies

The isolate designated –MA" was discovered while studying *Neochloris oleoabundans* in the non-autoclaved secondarily wastewater from Tuscaloosa Alabama (STW-TA). Throughout much of the duration of the experiment, no visible culture growth was observed. At the experiment's end, two flasks still showed no growth but one flask had a small floc of distinctly green algae growth. This algae clump continued to grow in size and was eventually transferred to new autoclaved wastewater and continued to grow well without any additional nutrients. While determining culture identity is beyond the capabilities of our laboratory, in all likelihood, this culture could be *N. oleoabundans*, a natural isolate, or a consortium. The generic –MA" designation was thus carried throughout the duration of this project, rather than assuming its identity.

Culture –NA" was isolated by transferring a small portion of non-autoclaved secondarily-treated wastewater from Tuscaloosa Alabama (STW-TA) into 0.5 L flasks containing 0.4L CHU13 or SE media separately with supplemental carbon dioxide under constant light sources. After flasks showed visible growth (as indicated by the

appearance of bright green culture), the cultures were transferred back to both autoclaved and non-autoclaved STW-TA for continued growth. After transferring, only one out of three flasks containing autoclaved STW-TA showed growth. Algae from this flask was transferred into new autoclaved STW-TA and continued to grow without added nutrients. This culture was designated ―NA".

Another ―line" of microalgae culture studied originated by transferring *N. oleoabundans* into three flasks containing autoclaved secondarily treated wastewater from Starkville Mississippi (STW-SM). Only one of flasks displayed any growth. This culture was transferred into new autoclaved STW-SM and continued to grow. In all likelihood, this culture is *N. oleoabundans*. All *N. oleoabundans*, ―NA" and ―MA" cultures were subsequently maintained in autoclaved STW-TA or STW-MS (as indicated in the experimental results and discussion chapter).

4.5.7 NA and MA Growth in Autoclaved and Non-autoclaved STW

Cultures ―NA" and ―MA" were grown in autoclaved and non-autoclaved STW-TA. Cultures were grown with supplemental carbon dioxide in air under a constant light with intensity. OD was measured daily. The purpose of this experiment was to investigate any effects autoclaving might have on biomass production. Recognizing that, in the non-autoclaved flasks, the potential exists for a consortium of microbial cultures to proliferate. With a project objective of maximizing biomass production on STW, this was determined to be an acceptable experimental approach capturing the potential of either our isolates or another resident microbial culture (or both) exhibiting significant growth under the experimental conditions.

4.5.8 Kinetic and Growth Study of NA, MA, *N. oleoabundans*

Cultures ―NA", ―MA" and ―*N. oleoabundans*" were grown in 1 L flasks containing 0.7 L autoclaved STW-SM with supplemental carbon dioxide in air under a constant light source. For a kinetic study of ―NA" and ―MA" growth, OD readings were taken at a frequency between 2.5 and 4 hours from the point of inoculation for 40 hours incubation. The specific growth rate was determined based on these data from the early exponential growth phase. Replicates of eight flasks were prepared for final biomass determinations at different elapsed periods.

To establish growth characteristics of ―NA" and ―MA", these same flasks were allowed to continue growing to establish a significant biomass accumulation for harvest. For each sampling period, the entire contents of two duplicate flasks each of ―NA" and ―MA" were harvested after 120, 167, 218 and 264 hours incubation, respectively. OD readings were correlated with and dry weights of harvested biomass. Sugar contents of dried samples were determined by YSI and DNS reducing sugars methods. For isolate ―*N. oleoabundans*", procedures were identical. The only difference was that flasks of ―*N. oleoabundans*" were harvested after 120 and 144 hours incubation. No kinetic study was performed on isolate *N. oleoabundans* due to heavy flocculation in the early growth stage which prevents homogeneity of biomass sampling. Inaccurate O.D readings with this type of growth would lead to false kinetic data.

4.5.9 Growth of *B. sudeticus* in Autoclaved STW

For comparison with natural isolates, *Botryoccus sudeticus* grown in autoclaved STW-SM was studied. Only second generation data was recorded to reduce nutrient ―carryover" effects (from the maintenance medium, CHU13 which readily sustained

growth). The culture was incubated in 1L flasks containing 0.7 L autoclaved wastewater with supplemental carbon dioxide in air under a constant fluorescent light. OD readings were taken daily for 10 days. The biomass was harvested at day 10 and cell concentration was determined by the centrifugation and freeze dry method. The sugar contents of *B. sudeticus* at day 10 was determined by reducing sugar and YSI method.

4.5.10 NA Growth and Sugar Contents with Addition of Fertilizer

To evaluate the addition of a commercial fertilizer to STW-MS, a study of isolate ―NA" was made. The composition of the commercial fertilizer (w/w) contained 1.2% ammonia-based nitrogen, 1.2% nitrate nitrogen, 5.6% urea nitrogen, 7% phosphate, 6% soluble potash and 0.1 % iron. The following concentrations (in g/L commercial fertilizer:STW-SM) were used for this study, 0± 0, 0.0433± 0.0055, 0.0665± 0.0020, 0.0952± 0.0095, 0.1332± 0.0025, 0.2020± 0.0054, 0.2709± 0.0031. Standard deviations were obtained from averaging three individual measurements of each amount fertilizer added to three individual flasks for a given level of fertilizer addition. Isolate ―NA" was incubated in 1L flasks containing 0.7 L medium with supplemental carbon dioxide in air under a constant fluorescent light. OD readings, at a wavelength of 600nm, were taken daily and biomass was harvested after 240 hours. The calibration curve was constructed based on OD readings correlated with dry weights. YSI and reducing sugar method were used for determined the sugar contents of NA.

4.5.11 Semi-batch Study

For the small semi-batch system, isolates ―MA" and ―NA" were incubated in 1L flasks containing 0.7 L autoclaved STW-SM with supplement carbon dioxide in air under

a constant fluorescent light. Sample volumes of 300 ml were taken at days 10, 23, 31 with flasks refilled back to 0.7 L with fresh, autoclaved STW from Starkville. The experiment was ended at day 43 and all cultures were harvested. Harvested samples were freeze dried and dried biomass concentrations were determined. Lipid and sugar contents of dried biomass were determined by Bligh and Dyer and the DNS reducing sugar method.

CHAPTER V

RESULTS AND DISCUSSION

5.1 Construction of Empirical Equation for Bubble Counts

To determine the amount of carbon dioxide being supplied to each culture flask, a calibration was performed. In this experiment, CO_2 was flown through a 24.2 cm steel tube, which the inside diameter was 3.15 mm and the outer diameter was 6.25 nm, from the gas mixer into a cylinder which is filled with water. The bubbles were counted within a period of time and the gas volume was measured. An empirical model as shown in the figure 5.1 was developed basing on the linear relationship between bubble sizes and the volumetric flow rate. As increasing the volumetric flow rate, the size of bubble was increased.

Bubble counts were converted into volumetric flow rates by using the empirical equation from the calibration curve and the fact between bubbles counts and volumetric flow rates as shown below.

(5.1)

where:

Vf = Volumetric flow rate (ml/second)

Bc = Bubble count (bubbles/second)

Bs = Bubble size (ml/bubble)

From the empirical model

(5.2)

Combine both equations and rearrange

(5.3)

This equation was employed for determining and controlling the carbon dioxide volumetric flow rate into flasks.

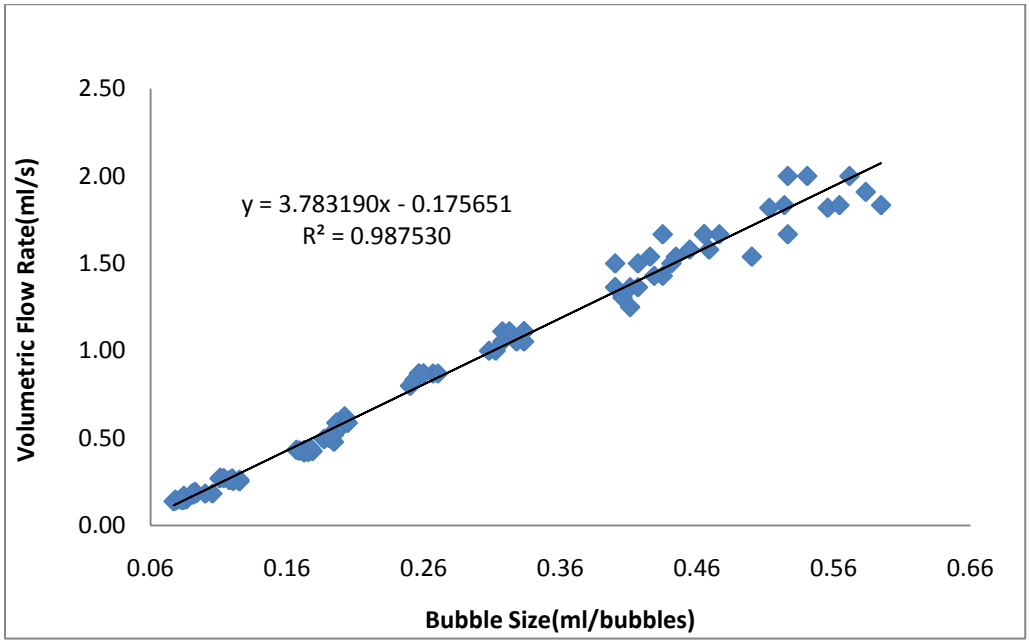

Figure 5.1 Calibration Curve of CO_2 Volumetric Flow Rate

5.2 Calibration Curve of *Botryococcus sudeticus* Grown in CHU13

The calibration curve of *Botryococcus Sudeticus* was constructed from cultures incubated 5-22 days. Optical densities and dried weight of samples were determined and used for constructing the calibration curve as shown in figure 5.2. The empirical equation for the calibration showed a linear relationship between optical densities and biomass

concentrations. This empirical equation was used for determining *B. sudeticus* concentrations in later studies.

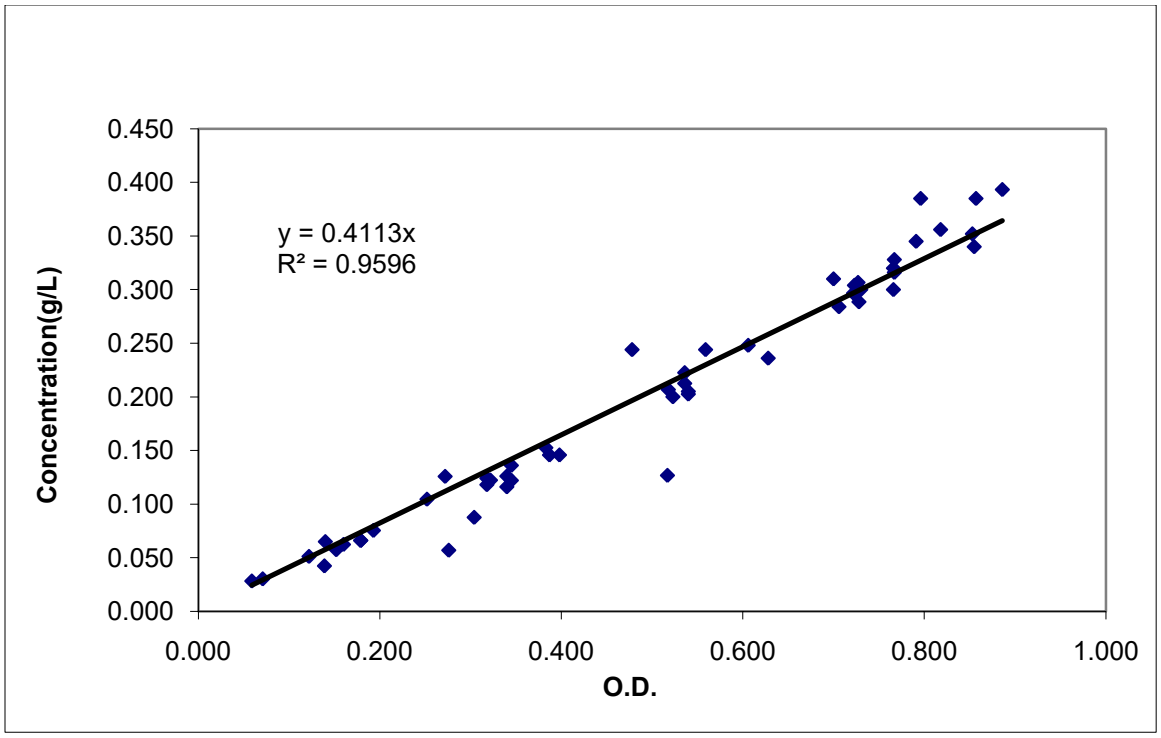

Figure 5.2 The Calibration Curve of *Botryococcus sudeticus*

5.3 Growth and Lipid Contents of *Botryococcus sudeticus* in CHU13

B. sudeticus was grown in CHU13 under constant light with supplement of carbon dioxide. The maximum accumulated biomass was approximately 1.6 g/L at day 27 as shown in figure 5.3. The exponential growth phase of this culture lasted 22 days. The maximum average lipid content was 21% of the dry biomass at day 15 even though the total lipid was varied from 12% to 21% as shown in figure 5.4. From the literature, maximum biomass concentrations of *B. sudeticus* was 1.4 g/L and lipid contents were varied from 9.39% (w/w) to 23.19 % (w/w). [Rafael, 1987] Our results agreed well with literature values. From the FAME analysis, the maximum value was recorded to be 9.00

% of the dry biomass weight. The average FAME values varied over the course of the experiment from 6.19% to 9.00% as shown in figure 5.4. The weight ratio of FAME over total lipid was 44.78% ± 7.29%. This showed that not all of total lipids can be converted to biodiesel and indicated that the total lipid does not only contain glycerides, but also contain other extractable organic chemicals.

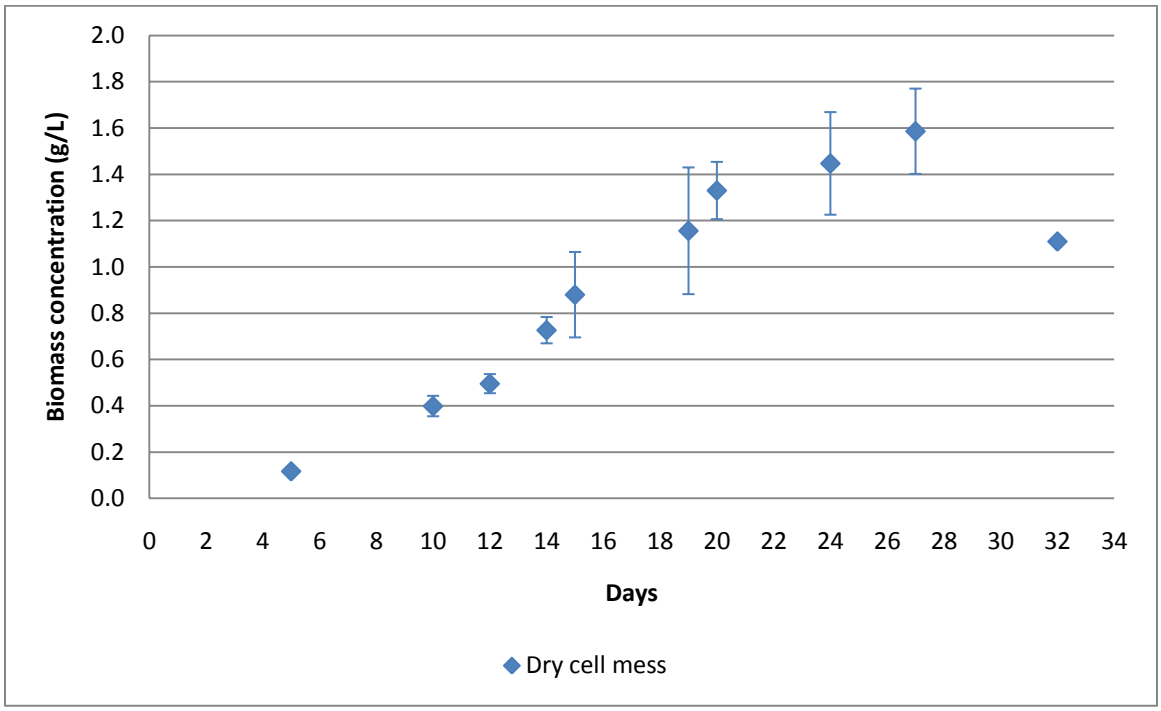

Figure 5.3 The Growth Curve for *Botryococcus sudeticus* in CHU13

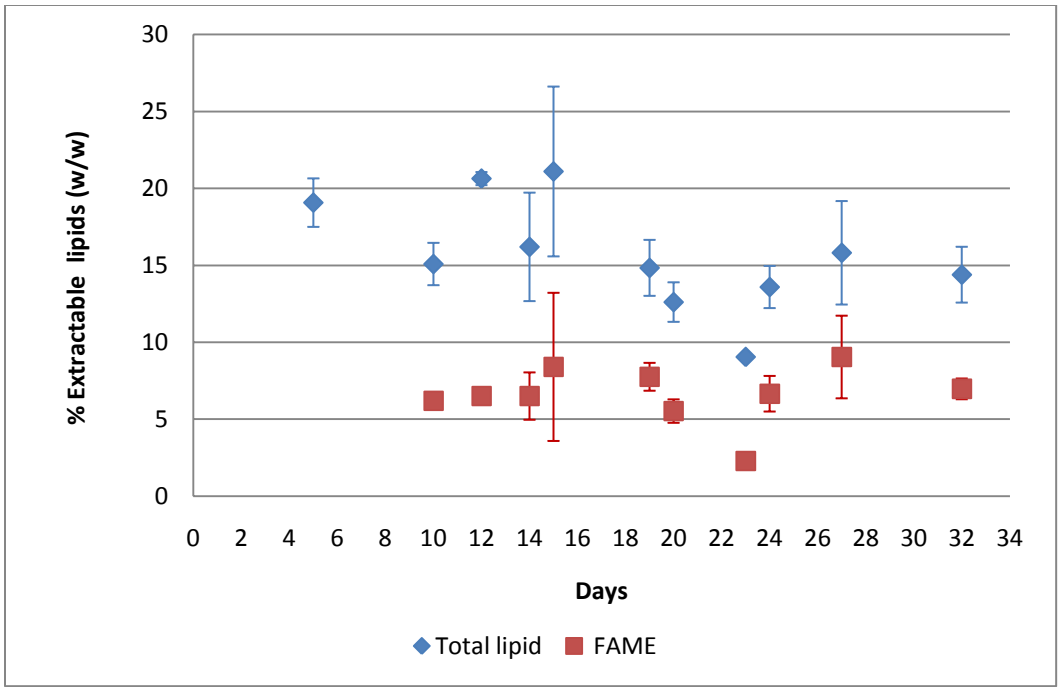

Figure 5.4 % Lipid and FAME Analysis of *B. sudeticus* in CHU13 Medium

5.4 Microalgal Growth in Synthetic Wastewater

As an initial strategy for assessing microalgae growth on wastewater, two microalgae strains, proven from the literature to produce high amounts of lipids, were selected and tested for growth in synthetic wastewater. For comparison, one of our early laboratory isolates, ―E" culture was selected. Synthetic wastewater was selected for the initial study because its chemical composition was defined. After growing for 4 days, average values of optical densities of cultures increased from 0.161 to 0.813 (approximately 0.334 g/L) and a stationary growth phase was reached. A bleaching effect, indicated by microalgae turning from green to a pale-yellow coloration then to white, was observed from days 8 to days 20. Culture pH was measured at day 20 and ranged from 4.33 to 5.79 (from an initial pH of approximately 8.0), indicating the

possibility of cumulative toxic effects of carbon dioxide accumulation during the stationary phase.

In cultures without supplemental carbon dioxide, optical densities increased from 0.152 to 0.438 over 4 days when the stationary phase was reached. However, the culture didn‘t turn to white over the 20 day incubation period. The pH of these flasks at day 20 was measured to be 9.16. To examine the possibility of reinitiating culture growth, carbon dioxide was supplied from day 15 to day 33 (data in Appendix A). No significant growth was observed. From day 33 to 38, supplemental carbon dioxide was discontinued and no additional growth was observed.

For comparison, another batch of culture was grown without supplement of carbon oxide from day 0 to day 33. Here, too, the bleaching effect was observed beginning at day 15. However, the culture turned back to green at day 23. Also pH rose from 7.54 at day 20 to 9.32 at day 23. A second exponential phase was observed from day 21 to day 24. When carbon oxide was supplied from day 32 to day 38, a third exponential growth was observed and O.D was increased from 0.159 to 0.608 (0.250g/L). Qualitatively, clearly pH bears an important role (in conjunction with carbon dioxide uptake) for culture viability and growth. With the poor growth observed in culture E, neither sugars nor lipids were measured and no further study was pursued with this culture.

5.5 Effect on Growth by Replacing $(NH_4)_2SO_4$ with KNO_3

From previous experiments, two facts were noticed. The maximum biomass concentration of *B. sudeticus* grown in CHU13 medium was 1.6 g/L, but the maximum biomass concentration of *B. sudeticus* grown in synthetic waste water was less than 0.334

g/L Comparative compositions between CHU13 and synthetic wastewater (shown in Table 5.1 below) were different. Among possible influencing factors, one is that key nutrients are missing in the synthetic wastewater. Another possibility is that the synthetic wastewater contains some chemicals inhibiting growth of *B. sudeticus.* In subsequent experimentation, the problem was approached by assuming that poor growth of *B. sudeticus* in synthetic wastewater was due to missing one or more key nutrients.

Table 5.1 Comparisons of CHU13 and Synthetic Wastewater Compositions

CHU13 medium constituents	Concentration (mg/L)	Synthetic wastewater constituents	Concentration (mg/L)
KNO_3	400	$(NH_4)_2SO4$	50
K_2HPO_4	80	K_2HPO_4	25
$MgSO_4*2H_2O$	200	Na_2HPO_4	400
Ferric citrate	20	Gelatin	150
Citric acid monohydrate	100	Casamino Acid	10
$CaCl_2*2H_2O$	107	$CaCl_2$	0.06
PIV solution	12 ml	Starch	70
pH	7.5	pH	7.5
		Yeast extract	10
		SL 7 solution	0.1 ml
PIV solution (trace elements)	Concentration (mg/L)	SL 7 solution (trace elements)	Concentration (mg/L)
Na_2EDTA	750	$Cr(NO_3)_3*9H_2O$	0.77
$FeCl_3*6H_2O$	97	$CuCl_2*2H_2O$	0.536
$MnCl_2*4H_2O$	41	$MnSO_4*H_2O$	0.108
$ZnCl_2$	5	$NiSO_4*6H_2O$	0.336
$CoCl_2*6H_2O$	2	$PbCl_2$	0.1
$Na_2Mo_4*2H_2O$	4	$ZnCl_2$	0.208

Another experiment was designed to determine evaluate nutrient effects on growth. Comparing both media, nitrogen sources were clearly different between CHU13 and synthetic wastewater. The nitrogen source in CHU13 is KNO_3. In synthetic

wastewater, nitrogen sources could be $(NH_4)_2SO_4$, gelatin, yeast extract and casamino acid. The capability of *B. sudeticus* to use gelatin, casamino acid, yeast extract and $(NH_4)_2SO_4$ as nitrogen sources was not known. Also it was noted that the trace elemental solutions were different between two mediums. Also, synthetic wastewater contained a higher phosphorous concentration than CHU13. The cause of low *B. sudeticus* growth in synthetic wastewater may be due to a lack or excess of these nutrient sources.

The synthetic wastewater was modified by replacing ammonium sulfate with potassium nitrate for testing nitrogen source effects. *B. sudeticus* grown in CHU13 was transferred into three different media: CHU13, synthetic wastewater and synthetic wastewater with the ammonium sulfate replaced by potassium nitrate (to give an equivalent nitrogen supply). No supplemental carbon dioxide was supplied until day 3. To dampen carryover effects from the CHU13 medium used for the seed culture, two successive generations of *B. sudeticus* were studied (each transferred successively into its same experimental medium).

As described above, to promote strong growth in the seed culture, *B. sudeticus* was grown in CHU13. Once strong growth was established, *B. sudeticus* was transferred into the three medium compositions described above AND transferred, after 14 days, into fresh medium (again, each in their own medium composition). For example, *B. sudeticus* grown in synthetic wastewater for 14 days was transferred into fresh synthetic wastewater medium. Both growth curves are shown in Figures 5.5 and 5.6 below.

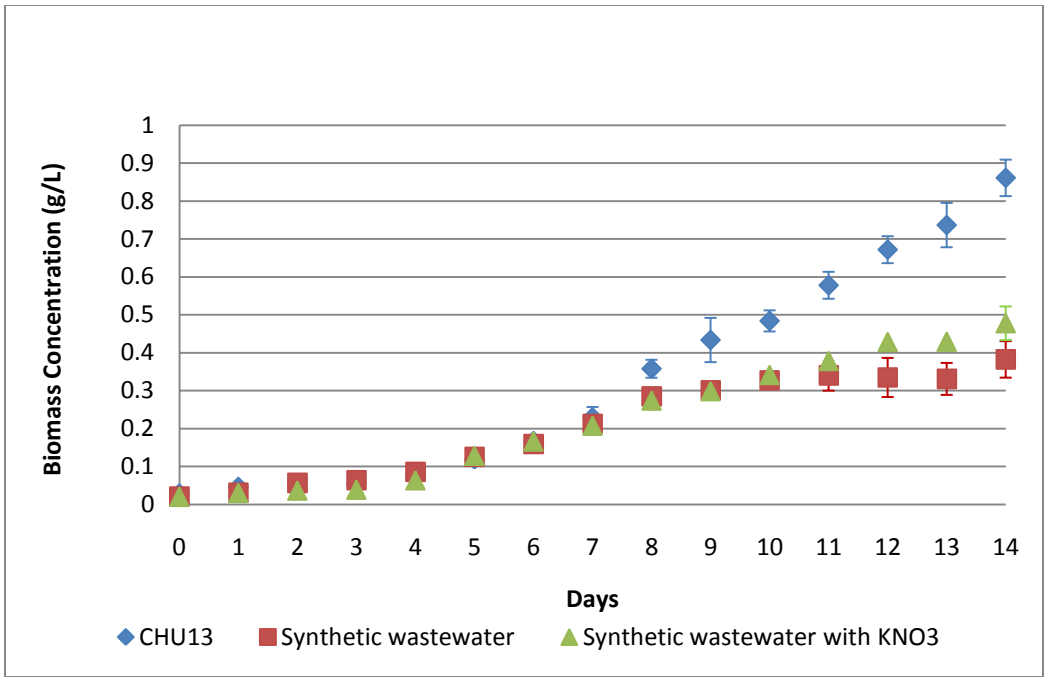

Figure 5.5 First Generation Growth Curves of *Botryococcus sudeticus*

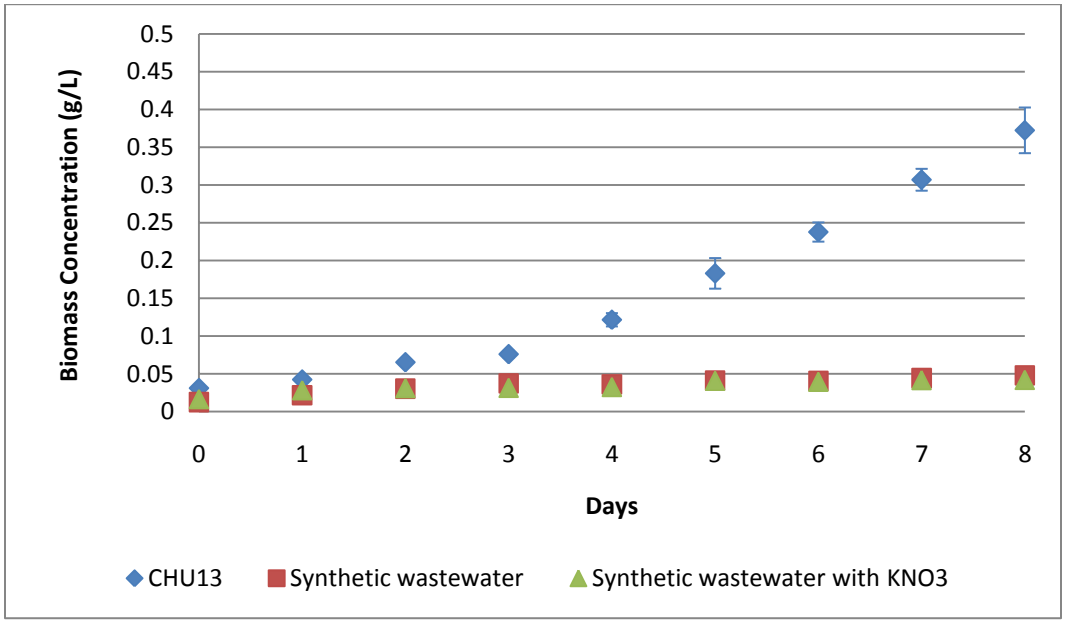

Figure 5.6 Second Generation Growth Curves of *Botryococcus sudeticus*

In Figure 5.5, a lag phase from day 0 to day 3 (during which no supplemental carbon dioxide was supplied) for all medium compositions was observed. After

supplying carbon dioxide in day 3, all cultures began growing at similar rates. However, in the synthetic wastewater and synthetic wastewater with potassium nitrate a stationary growth phase was observed before 12 days of incubation. Replicate flasks containing *B. sudeticus* grown in CHU13 were still in exponential phase at day 14. Also bleaching effects were observed in the cultures containing synthetic wastewater (denoted by cultures turning white in coloration).

A similar experiment was performed with isolate ―E". The result is included in Appendix A.1. Flasks containing ―E" isolate were first incubated in synthetic wastewater before beginning the experiment (again, to dampen nutrient ―carryover" effects). Then E culture grown in synthetic wastewater was transferred into three different mediums: CHU13, synthetic wastewater, and synthetic wastewater with potassium nitrate. The growth conditions were identical for all flasks containing *B. sudeticus* grown in those three mediums. A similar result (to that described above in Figures 5.5 and 5.6) was observed. Flasks containing ―E" isolate grew well in CHU13; however, poor growth was observed for other mediums. Furthermore, daily pH data (Appendix A.2) showed the pH was stayed between 6 and 8.

From these experimental results, replacing ammonium sulfate with potassium nitrate in synthetic wastewater did not improve the growth.

5.6 Effects on Growth by Removing One Nutrient From the Mixture

A new experiment was designed to grow *Neochloris oleoabundans* in modified media. The modified medium contained both chemical compositions from CHU13 and synthetic wastewater (varying individual medium constituents among sets of replicate flasks). In one set of flasks, magnesium sulfate ($MgSO_4$) was deleted from the medium

(i.e. the medium was prepared by adding all chemicals from CHU13 into synthetic wastewater except $MgSO_4$). In another set, dipotassium phosphate (K_2HPO_4) was absent. In other sets of flasks, *N. oleoabundans* was grown in a mixture of CHU13 and synthetic wastewater, a set of flasks with no potassium phosphate and CHU13 alone and synthetic wastewater alone as controls. Before *N. oleoabundans* were transferred into each of these different media combinations, the culture was incubated in synthetic wastewater. The results are shown in Figures 5.7 and 5.8. Kinetic profiles for this experiment are reported in Figures 5.9 and 5.10 as natural log (Biomass at time, t^+/biomass at t=0) and in Table 5.2

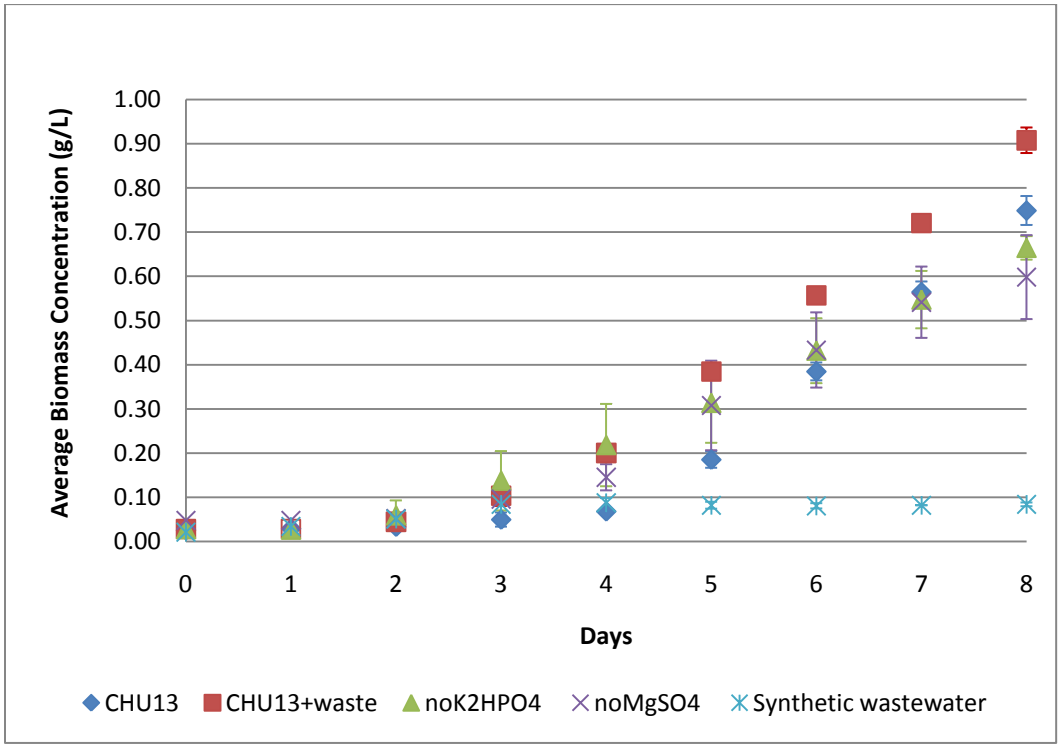

Figure 5.7 Growth Curves of *N. oleoabundans* in Nutrient Deficient Media

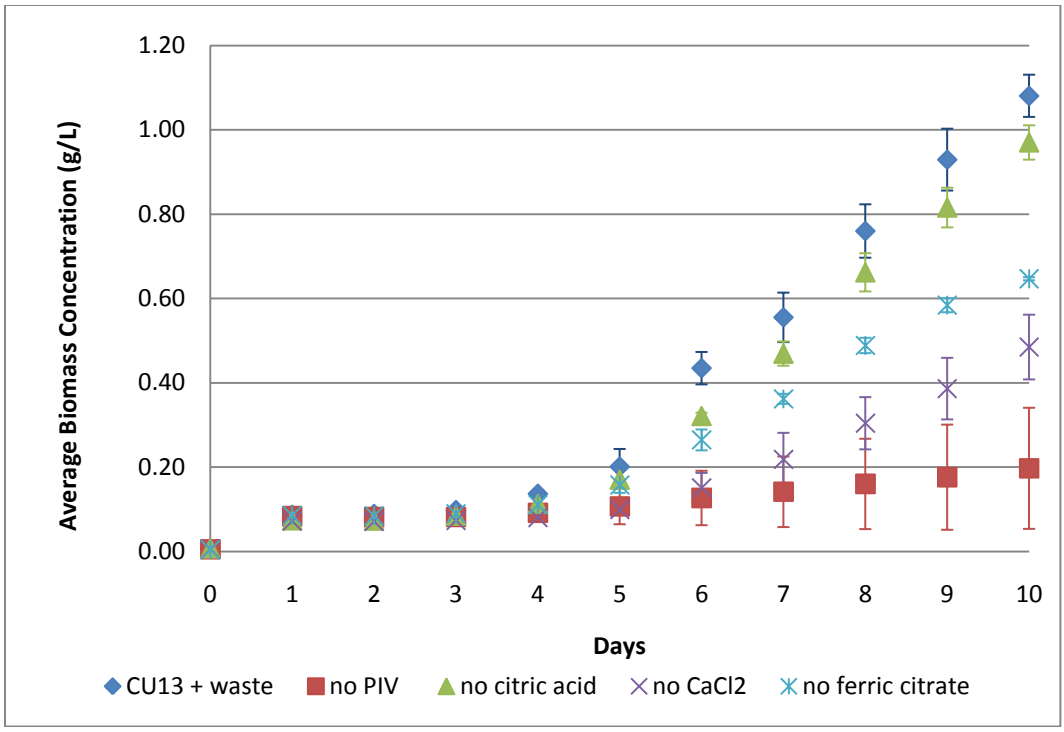

Figure 5.8 Growth Curves of *N. oleoabundans* in Nutrient Deficient Media

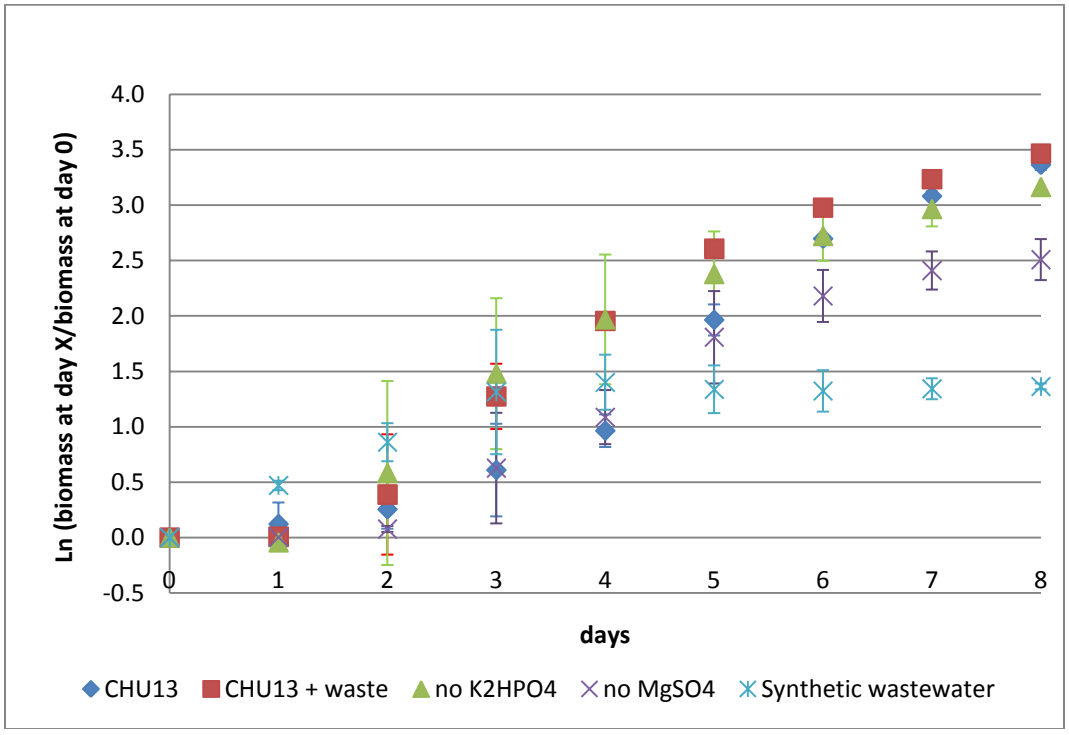

Figure 5.9 Kinetic Profiles of *N. oleoabundans* in Nutrient Deficient Media

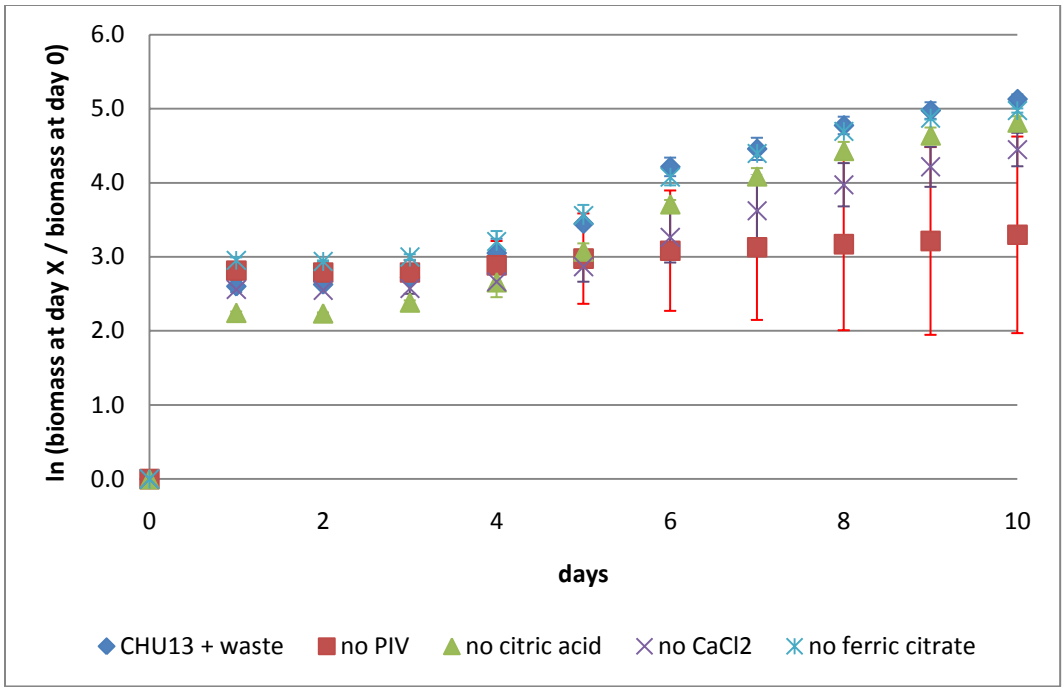

Figure 5.10 Kinetic Profiles of *N. oleoabundans* in Nutrient Deficient Media

Table 5.2 Maximum Specific Growth Rates of *Neochloris oleoabundans*

Growth Condition	Maximum specific growth rates (day^{-1})	Growth Condition	Maximum specific growth rates (day^{-1})
CHU13	0.7260 ± 0.1370	No PIV	0.0935 ±0.2034
CHU13/Synthetic wastewater mixtures	0.5230 ± 0.0983	No Citric acid	0.4591 ±0.0820
No K_2HPO_4	0.5656 ± 0.0580	No $CaCl_2$	0.3376 ±0.0079
No $MgSO_4$	0.6155 ± 0.0419	No ferric citrate	0.4315 ±0.0340
Synthetic wastewater	0.3918 ± 0.1400		

In Figure 5.7, poor growth of *N. oleoabundans* in synthetic wastewater was observed. Flasks of *N. oleoabundans* incubated in a mixture of CHU13/synthetic wastewater medium had higher biomass concentrations at day 8 than the culture grown in CHU13. However, no difference in maximum specific growth rates was observed as

shown in table 5.2. This indicated the growth rates of both cultures were the similar and synthetic wastewater contained no chemical constituent significantly inhibiting growth in comparison to the CHU13 medium. Replicate flasks of *N. oleoabundans* grown in media deficient in K_2HPO_4 or $MgSO_4$ had lower biomass concentrations than *N. oleoabundans* grown in a mixture of CHU13 and synthetic wastewater, but, again, there was no large difference in maximum specific growth rates. This indicated that the nutrient levels of these constituents supplied by the synthetic wastewater were sufficient to sustain growth. However, as shown in Figure 5.9, the specific growth rate of *N. oleoabundans* grown in media without $MgSO_4$ decreased after day 6, indicating that, in this set of flasks, the culture entered the deceleration growth phase and stationary phase. Also from Figure 5.9, the specific growth rates approached 0 and this leaded to poor growth as shown in Figure 5.7. From Figure 5.8 and Table 5.2, no difference in growth between citric acid depleted mediums and mixtures of CHU13 and synthetic wastewater medium was observed. However, the biomass concentration at day 10 and the growth rate of *N. oleoabundans* incubated in media without ferric citrate, $CaCl_2$ or PIV solution was lower than the biomass concentration at day 10 and the growth rate of *N. oleoabundans* incubated in the mixture of CHU13 and synthetic wastewater. By comparing maximum specific growths of *N. oleoabundans* incubated in ferric citrate depleted mediums with *N. oleoabundans* grown in CHU13/synthetic wastewater mixtures, there was no difference. However, By comparing maximum specific growths of *N. oleoabundans* incubated in media without $CaCl_2$ or PIV solution mediums with *N. oleoabundans* grown in CHU13/synthetic wastewater mixtures, maximum specific growth rates were lower. Furthermore, as shown in Figures 5.8 and 5.10, the lag phase of *N. oleoabundans* grown in CHU13/synthetic

wastewater mixtures without addition of $CaCl_2$ was increased. From above results, $CaCl_2$ and PIV solutions appear necessary for growth of *N. oleoabundans* in synthetic wastewater.

5.7 Growth of *N. oleoabundans* in Secondarily Treated Wastewater

In this experiment, *N. oleoabundans* was grown in secondarily treated wastewater from Tuscaloosa Alabama (STW-TA) with supplemental carbon dioxide under a constant light source. Analysis of this STW-TA by ion chromatography analysis showed only trace amounts of nitrite (less than 0.53 ppm), nitrate (less than 0.24 ppm) and phosphate (less than 0.53 ppm). It was anticipated that these low nutrient rates might inhibit microalgae growth on STW-TA alone (which proved not to be the case in later experimentation). In this experiment, *N. oleoabundans* was grown in SE medium (described in the Methods chapter and chosen from the literature as supporting strong growth of *N. oleoabundans*). Seed cultures grown first in SE medium were transferred into four different media and grown under constant light with supplemental carbon dioxide. The four media were as follows: (1) SE medium as a control; (2) a mixture of SE and autoclaved secondarily treated wastewater; (3) a mixture of SE medium and non-autoclaved secondarily treated wastewater; and, (4) non-autoclaved secondarily treated wastewater. Culture performance is presented in Figures 5.11 and Table 5.3. Maximum specific growth rates were determined by using data from day 1 to day 3.

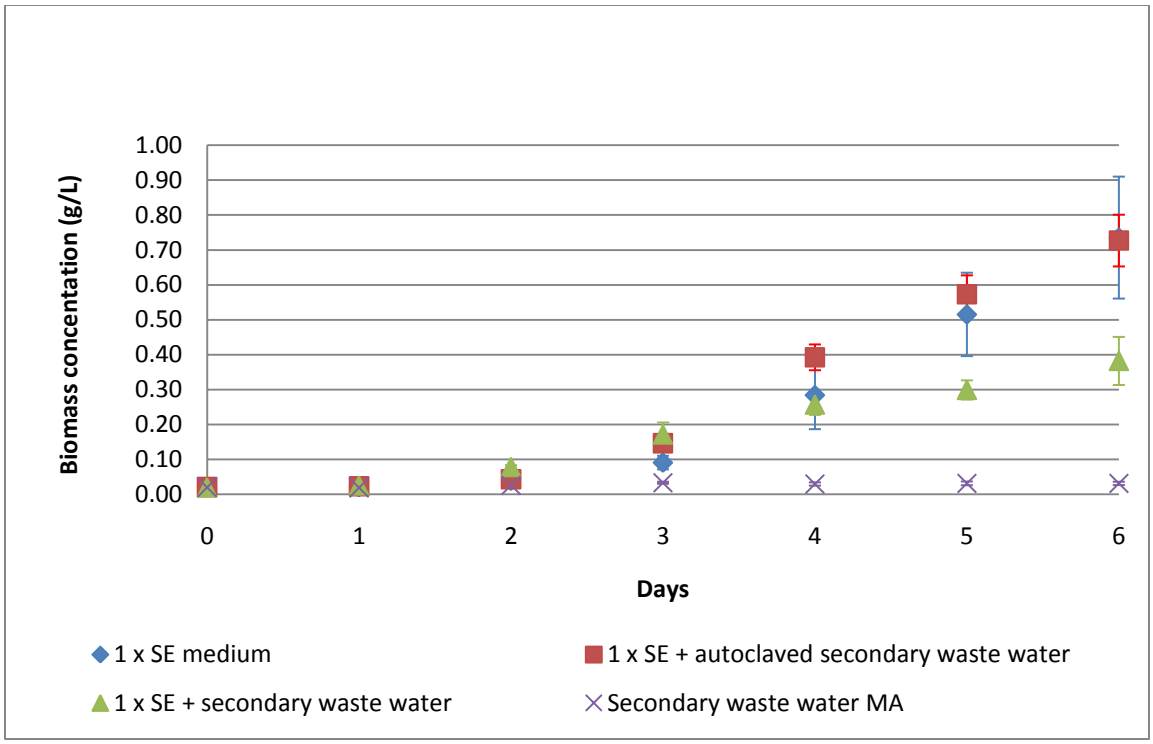

Figure 5.11 Growth of *Neochloris oleoabundans* in Secondarily-treated Wastewater

Table 5.3 Maximum Specific Growth Rates of *N. oleoabundans* in Wastewater

Growth Condition	Maximum specific growth rates (day^{-1})
1 X SE medium	0.879 ± 0.090
1 X SE medium + autoclaved wastewater	0.969 ± 0.043
1 X SE medium + non-autoclaved wastewater	0.940 ± 0.117
Non-autoclaved wastewater	0.281 ± 0.051

As shown in Figure 5.11, the lag phase lasted approximately 1 day and cultures were still in exponential growth phases at day 6 for all media compositions other than flasks containing non-autoclaved wastewater. No difference in biomass concentrations was observed between SE medium and mixture of SE/autoclaved secondarily wastewater medium. However, the biomass concentrations of *N. oleoabundans* in a mixture of SE and non-autoclaved secondarily treated wastewater medium was lower than one in a

mixture of SE and non-autoclaved secondarily treated wastewater medium. This difference in biomass concentrations does not have a ready explanation. As shown in Table 5.3, there were no differences in maximum specific growth rates for *N. oleoabundans* grown in SE medium, SE/autoclaved wastewater and SE/non-autoclaved wastewater mixtures. From Table 5.3, the maximum specific growth rate of *N. oleoabundans* grown in SE/non-autoclaved wastewater mixtures was determined to be 0.940 ± 0.117 day^{-1}. The maximum specific growth rate of *N. oleoabundans* grown in non-autoclaved wastewater was 0.281 ± 0.051 day^{-1}. By comparing those two results, this showed that the poor growth of *N. oleoabundans* in non-autoclaved wastewater as shown in figure 5.8 was likely due to lack of nutrients.

5.8 *N. oleoabundans* Growth in The STW and SE Medium Mixture

From the above experiment, it was concluded that addition of SE medium appeared necessary for growing *N. oleoabundans* in the autoclaved STW-TA. To investigate the amount of nutrients in SE medium needed to sustain culture growth, this experiment was structured to examine culture growth with reduced levels of SE medium. Reduced strength SE medium was added to autoclaved STW-TA and the effect on culture growth was studied. The results are shown below in Figure 5.12.

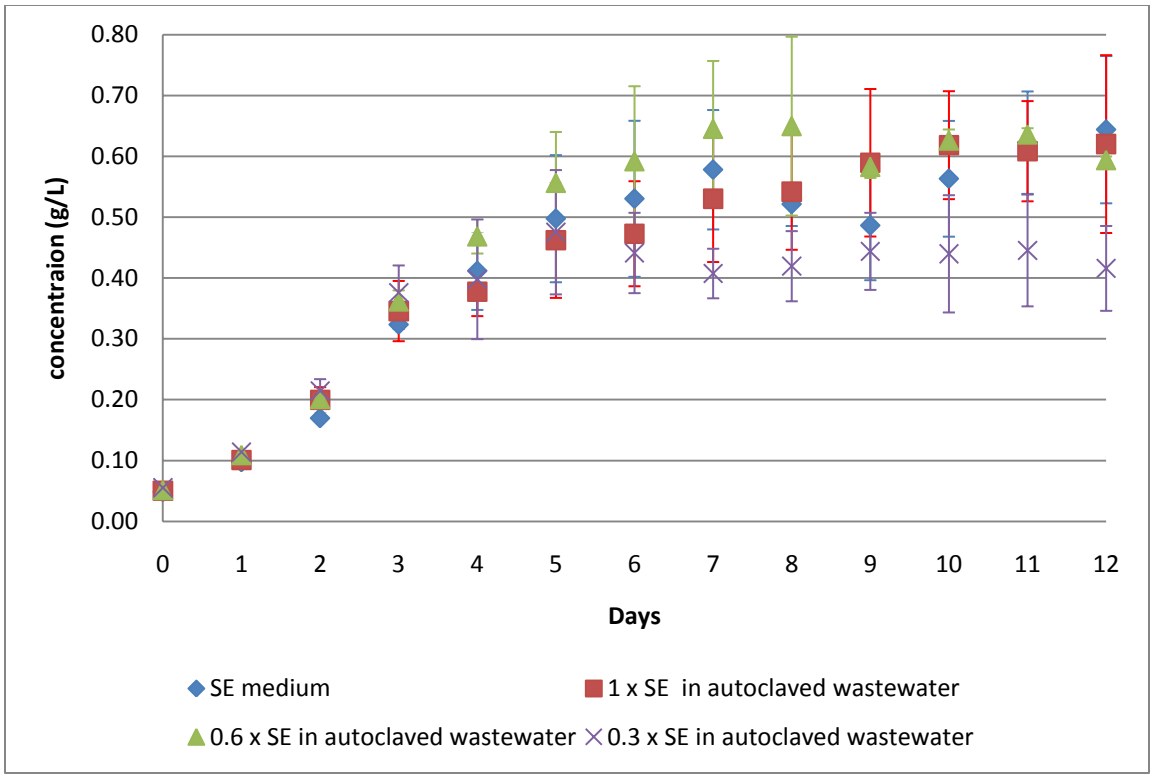

Figure 5.12 Growth Curves of *Neochloris oleoabundans* in SE/Wastewater Mixtures

N. oleoabundans was first grown in SE medium (to establish a strong seed culture) then transferred into four different media compositions: (1) SE medium as control; (2) equal volumes of a mixture of full strength SE and autoclaved STW-TA (a mixture contains 50%(v/v) 2 X strength of SE and 50%(v/v) autoclaved wastewater); (3) equal volumes of a mixture of 0.6 strength SE and autoclaved secondarily treated wastewater (a mixture contains 30%(v/v) 2 X strength of SE and 70%(v/v) autoclaved wastewater); and, (4) equal volumes of a mixture of 0.3 strength SE and autoclaved STW-TA (a mixture contains 15%(v/v) 2 X strength of SE and 85%(v/v) autoclaved wastewater). The cultures were grown in 1L flasks containing 700 ml solutions with supplemental carbon dioxide under constant light source. From Figure 5.12 (above), no significant lag phase was observed and *N. oleoabundans* in all media reached stationary

phase after seven days incubation. There was no significant difference in growths and biomass concentrations between all media, even though, the average biomass concentration of *N. oleoabundans* grown in the mixture of 0.3 strength SE medium and autoclaved wastewater was much lower than the average biomass concentration of *N. oleoabundans* grown in other three mediums. Also cultures of *N. oleoabundans* grown in the mixture of 0.3 strength SE medium and autoclaved wastewater were light yellowish green and others are light green. This would indicate an approach to some nutrient limitations required to maintain strong growth.

Lipid contents of *N. oleoabundans* grown in each of the four media were determined by Bligh & Dyer method for cells harvested at day 12. Lipid contents of *N. oleoabundans* were determined to be 15.4 ± 1.14%, 14.51 ± 4.25%, 15.16 ± 1.08%, and 22.27 ± 6.89% for (1) SE medium as control, (2) the mixture of full strength SE and autoclaved STW-TA, (3) the mixture of 0.6 strength SE with STW-TA, and (4) the mixture of 0.3 strength SE and autoclaved STW-TA, respectively. The culture showing visible signs of nutrient limitations (i.e. discoloration of cells) clearly had the highest lipids composition at day 12. This agrees with general observations from the literature that lipids accumulation is often dissociated from strong culture growth. Also, from the results, it is apparent that full strength of SE medium was not needed for growing *N. oleoabundans* in an STW-TA/SE medium mixture. The optimal strength of the mixture appeared to be between 0 and 0.6. This ended the first experimental strategy for attempting to grow an identified microalgae culture proven to produce significant lipids amounts on secondarily treated wastewater with or without addition of nutrients.

5.9 Enrichment procedures for culture lines "MA" and "NA"

A second strategy was begun to investigate the potential for lipids and sugars production through enrichment techniques of natural isolates. Choosing the culture with the best observed growth to date (i.e. *N. oleoabundans*), an enrichment process was initiated to possibly grow a microalgae consortium with *N. oleaabundans* as a foundational organism. To accomplish this, flasks of *N. oleoabundans* (maintained in defined medium using aceptic techniques to ensure culture purity) were transferred to flasks of non-autoclaved secondarily-treated wastewater (STW) in hopes of finding a consortium which could grow only on STW. With *N.* oleoabundans inoculated into a series of flasks of non-autoclaved STW, only one flask showed significant growth. Culture from this flask was transferred back to autoclaved STW for maintenance. And, while no visible variation between this culture growth and that of *N. oleoabundans* was obvious, our inability to definitively state that this culture was *N. oleabundans* in pure form prompted a designation of ~~"M~~MA" as a separate culture line. Additionally, the lack of growth observed in the transfer of *N. oleoabundans* to all flasks but one of non-autoclaved STW supports the view that the cell growth observed in the one flask may be a mixed consortium. Clearly, future work will need to consult suitable expertise in microbiology to identify cultures isolated and studied in this research. To ensure that the culture line ~~"M~~MA" could grow in the secondarily treated wastewater, it was transferred and incubated in both autoclaved and non-autoclaved STW-TA with supplemental of carbon dioxide under constant light. Results for this transfer are shown below in Figure 5.13. Significant growth was observed in both autoclaved and non-autoclaved STW-TA (with slightly higher optical densities observed for flasks containing MA grown in

autoclaved STW-TA). This experiment showed that MA could clearly grow in STW with no additional nutrients—an observation not seen with earlier culture studies using identified microalgae species.

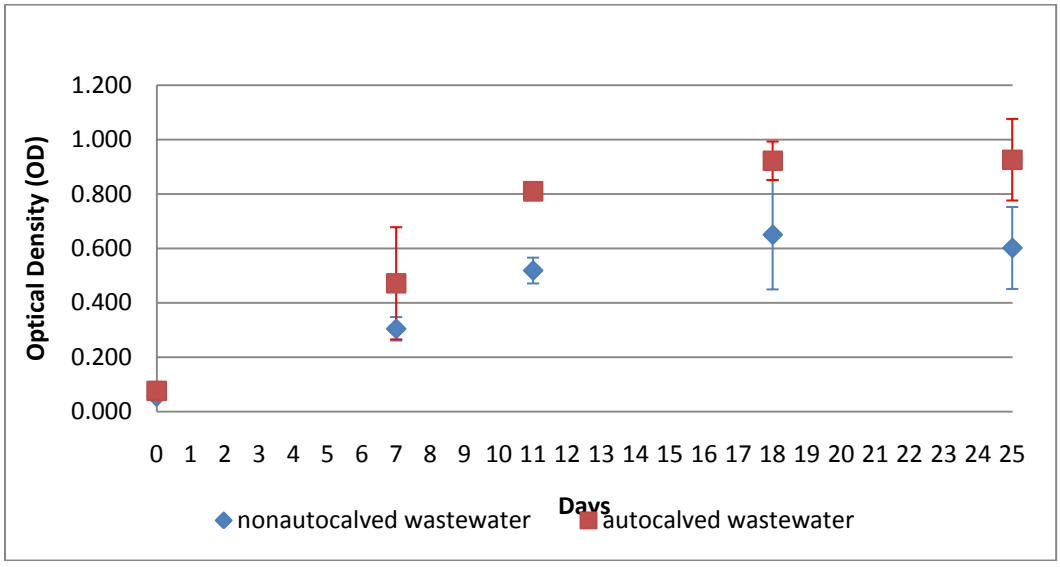

Figure 5.13 Growth Curves of MA in Non-autoclave and Autoclaved Wastewater

In an attempt to enhance the possibility of finding a culture that could grow strongly on STW as its sole source of nutrients, a small portion of non-autoclaved wastewater (~ 5ml STW-TA) was transferred to defined media (i.e. CHU 13 and SE). After several days‘ incubation, both media exhibited visible growth; however, growth was visibly much stronger in CH13 (as indicated by a bold dark green color in comparison to the pale, yellowish coloration in SE medium). Therefore, this culture in CHU13 was transferred back to autoclaved and non-autoclaved secondarily treated wastewater sources. These results are shown below in Figures 5.14 and 5.15. As shown in figure 5.14, poor growth was observed before day 18. However, the data masks the fact that, for individual flasks (data shown in Figure 5.15), only one of three flasks was

able to grow in the autoclaved secondarily treated wastewater. This experiment was designated –NA" and the flask labeled NA2, showing significantly higher growth, was chosen to further study and designated as a new culture line –NA". Again, the reasoning behind this process was to try and discover additional culture lines that exhibited strong microbial growth in STW alone with accompanying significant production of lipids or sugars.

An interesting observation for these –NA" cultures it that, as they aged for a number of weeks, a clear, highly viscous substance was excreted into the medium. Later attempts to grow the culture in a spiral tubular reactor resulted in complete clogging of the reactor tubing. This indicated possible presence of an extra-cellular polysaccharide. Also, after autoclaving old cultures and left to be cool overnight, flasks showed almost complete solidification giving an agar-like appearance.

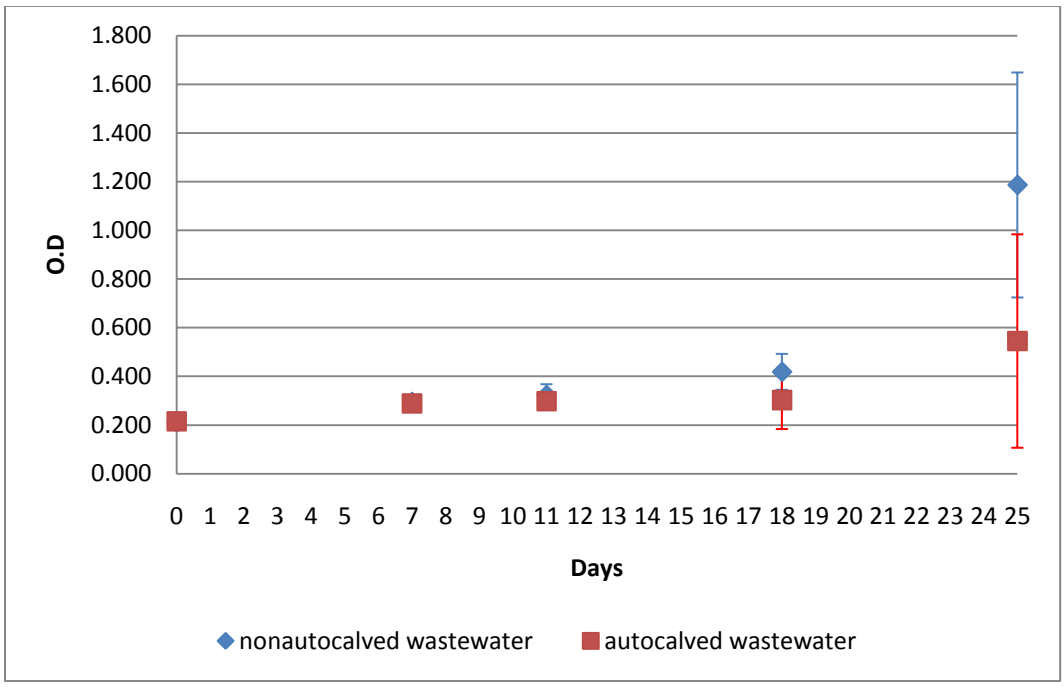

Figure 5.14 Growth Curves of NA in Non-autoclaved and Autoclaved Wastewater

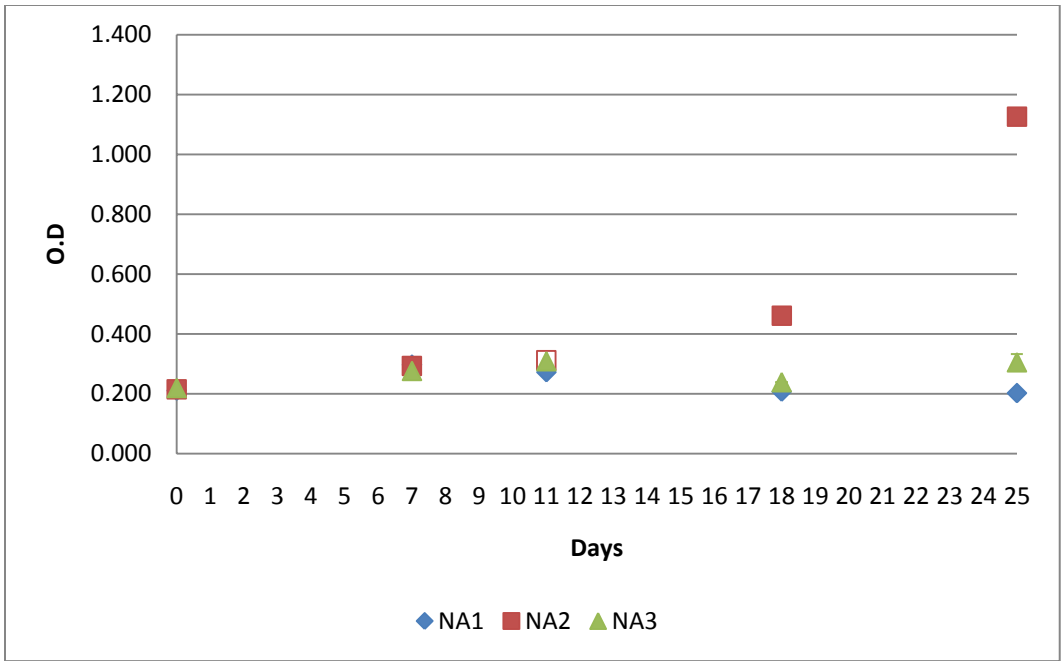

Figure 5.15 Growth Curves of Individual Flasks of ~~"~~NA"

Similarly to the procedure described above for culture line ~~"~~MA", *N. oleoabundans* were transferred into three flasks containing autoclaved secondarily treated wastewater from Starkville Mississippi (STW-SM). Only one of flasks displayed any growth. This culture was transferred into new autoclaved STW-SM and continued to grow. In all likelihood, this culture is *N. oleoabundans*. This culture was used for later studies.

Images of SEM and light microscopes are shown in Figures 5.16-5.27. While general cell morphology appears similar among the different culture lines, the enrichment process used for obtaining significant microbial growth in STW precludes definitively stating that these cultures are the same.

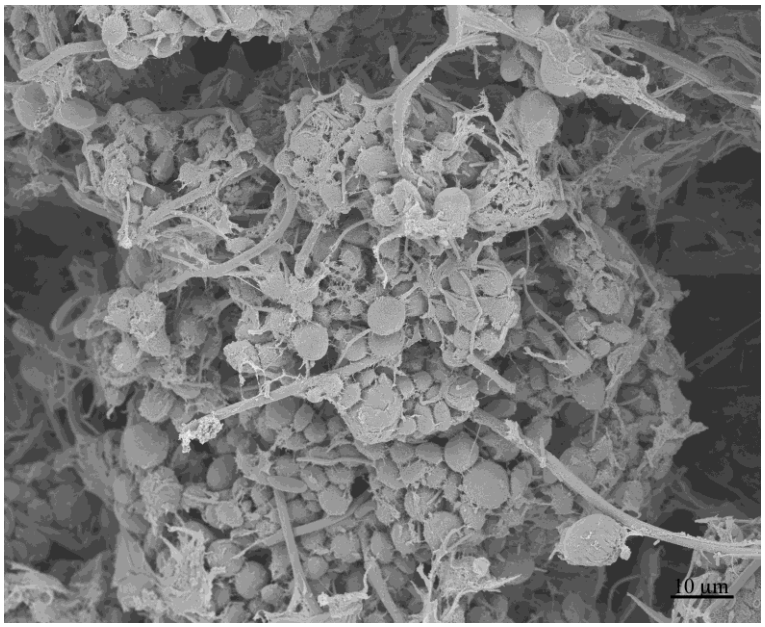

Figure 5.16 SEM Micrograph of MA

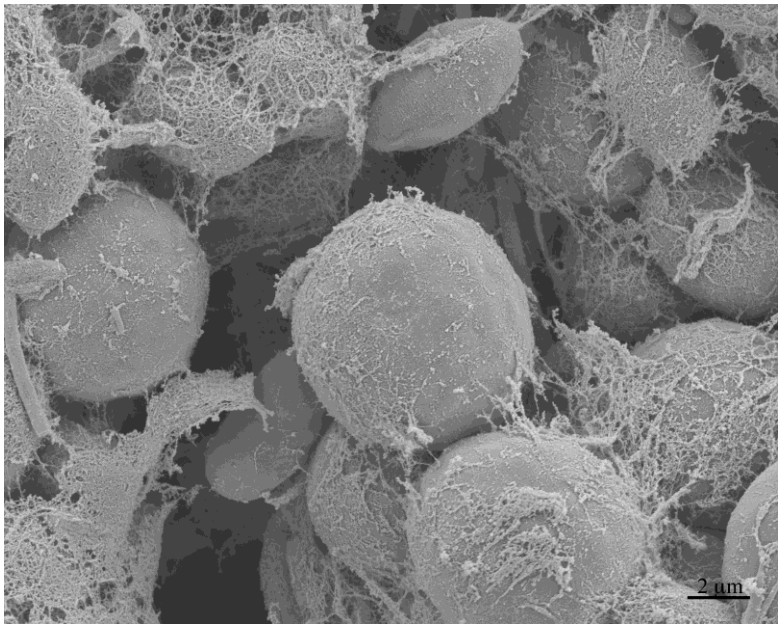

Figure 5.17 SEM Micrograph of MA

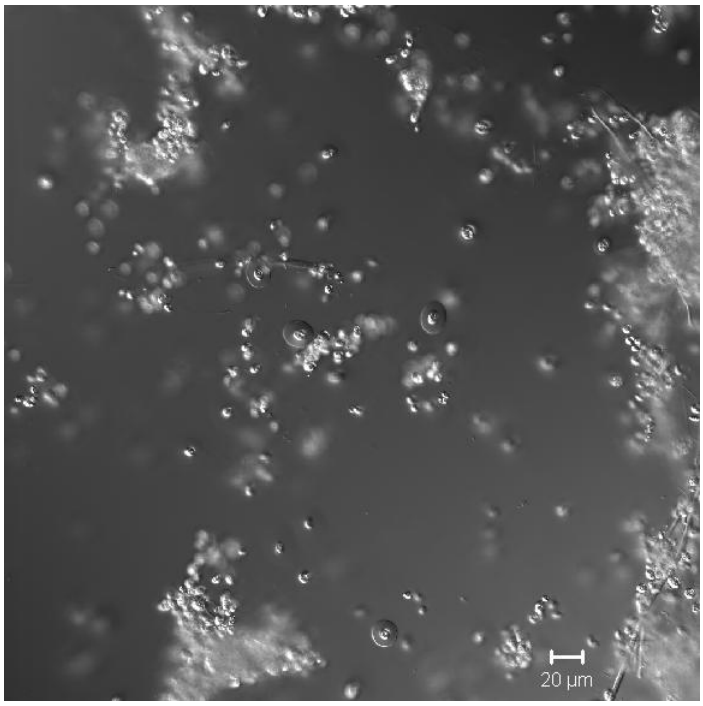

Figure 5.18 Light Micrograph of MA

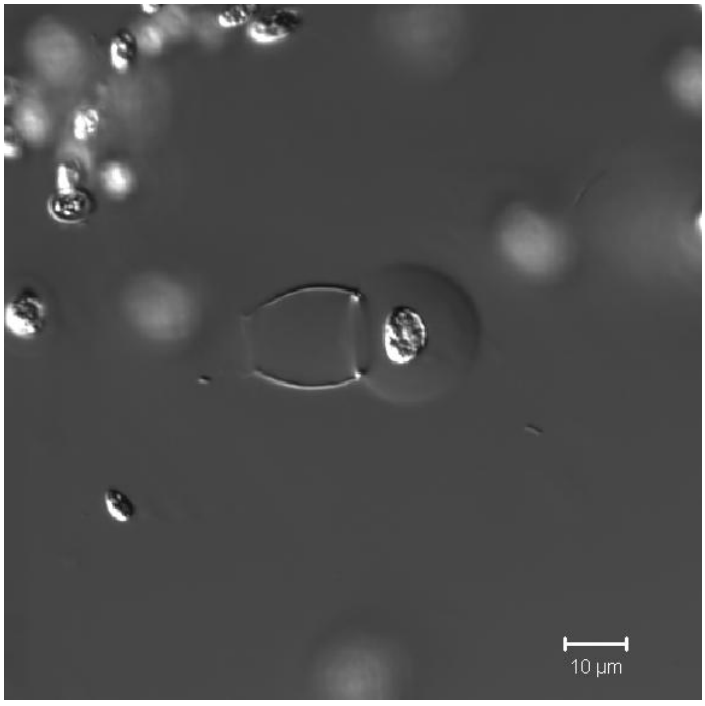

Figure 5.19 Light Micrograph of MA

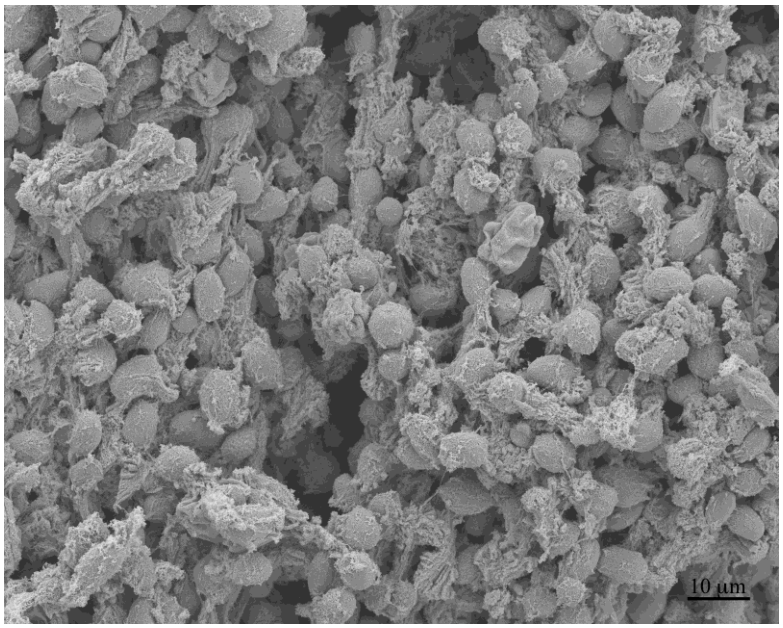

Figure 5.20 SEM Micrograph of NA

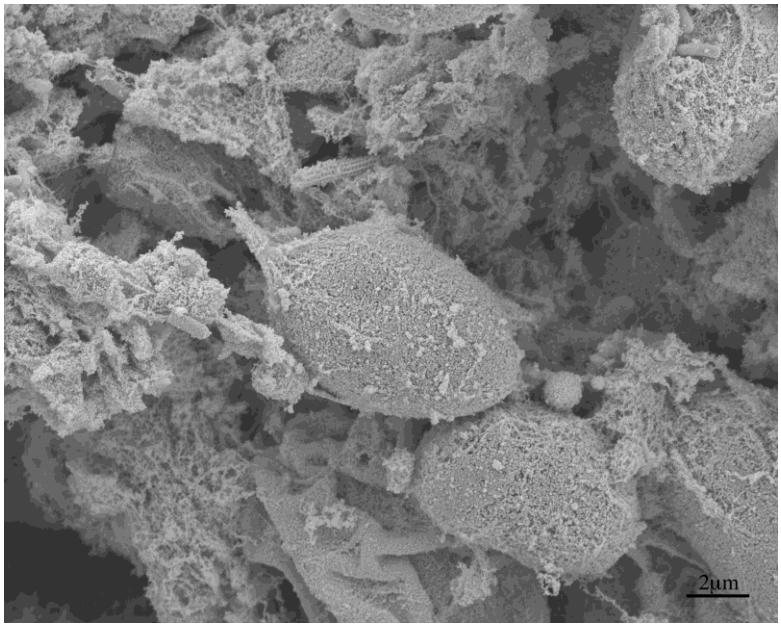

Figure 5.21 SEM Micrograph of NA

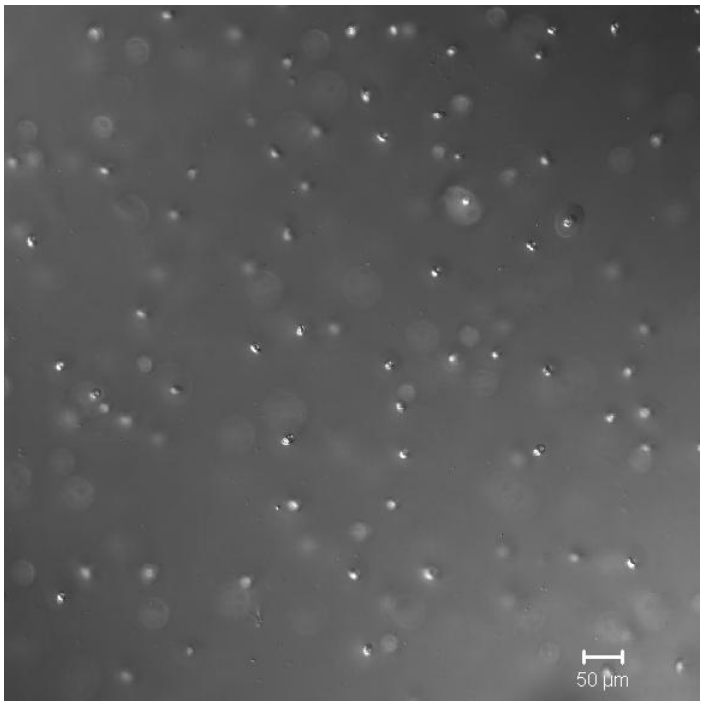

Figure 5.22 Light Micrograph of NA

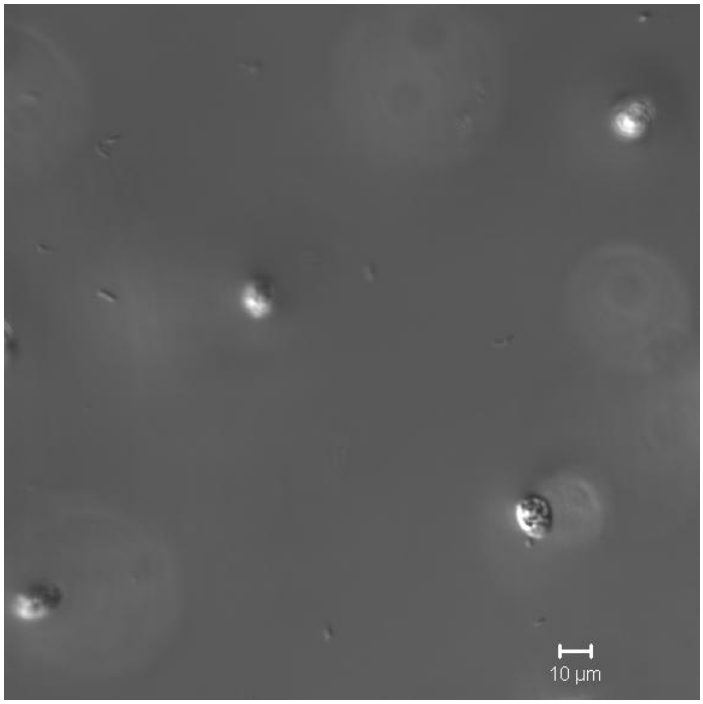

Figure 5.23 Light Micrograph of NA

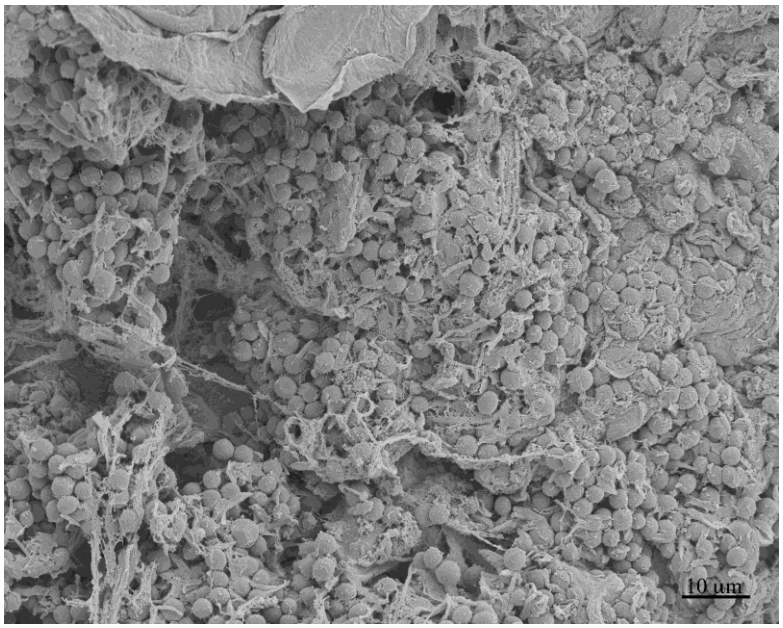

Figure 5.24 SEM Micrograph of *N. oleoabundans*

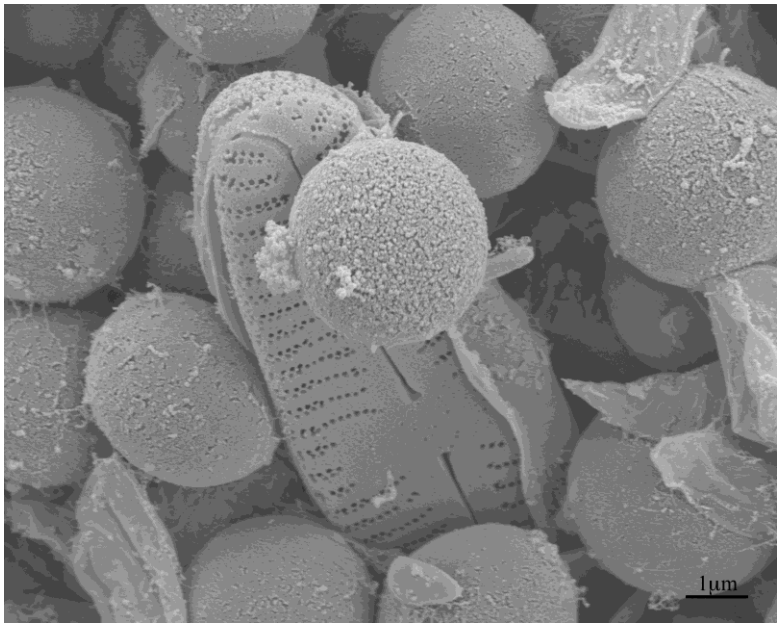

Figure 5.25 SEM Micrograph of *N. oleoabundans*

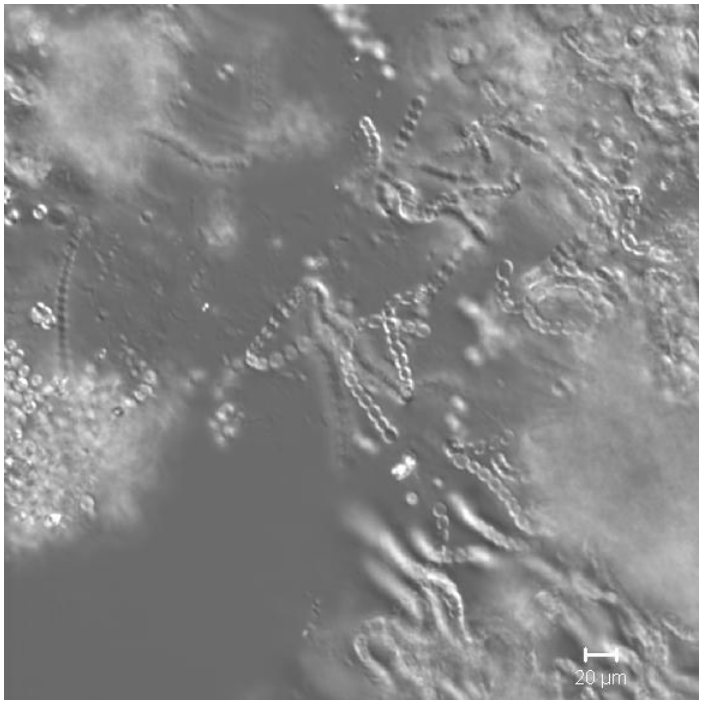

Figure 5.26 Light Micrograph of *N. oleoabundans*

Figure 5.27 Light Micrograph of *Neochloris oleoabundans*

SEM and light micrographs of MA were shown in Figures 5.16 to 5.19. Cell morphology for all culture lines was spherical or ovoid in appearance. From SEM

micrographs as shown in Figure 5.16 and 5.17, the cell diameters were between 5.5 and 8.5 micrometers. Based on shape and size, these cultures are possibily microalgae, since most of coccid bacteria with spherical symmetry have a diameter between 1 and 2.5 micrometers. [Piondexter, 1986]

Also, the tubular structures appear connecting cells, having a diameter of approximately 1.8 micrometer. The length of one particular tube was over 33.2 micrometers. In Figure 5.17, several small disconnected tubes are observed. On the upper right of the image, a fiber-like network is seen. One possibility is that this could indicate presence of extracellular polymeric substances which might be potential polysaccharides sources (which were observed macroscopically in several experiments). Under light micrograph images shown in figure 5.18 and 5.19, different structures are observed: ellipsoidal and circular cells and a transparent layer surrounding some cells. The diameters of circular cells were between 5 and 7 micrometers. Lengths of ellipsoidal cells were from 5 to 8 micrometers and their widths were between 3.5 and 6 micrometers. Lengths of ellipsoidal cells with transparent layers were between 18 and 20 micrometer and their widths were from 14 to 20 micrometers. Lengths of cells inside transparent layers were between 8 and 10 micrometers and their widths were from 5.5 to 6.5 micrometers. On the bottom right of figure 5.18, a long tube was connected through several aggregate colonies and its length was over 140 micrometers. Microbial cells appeared to be aggregated around the tube rather than dispersed homogeneously throughout the image. Though not definitely linked to macroscopic observations, the presence of distinct, strong flocculation in some experiments could be supported by the appearance of these microscopic structures.

SEM and light micrographs of NA are shown in Figures 5.20-5.23. As shown in Figure 5.20, NA contained similarly contained the two shapes of cells: ovoid or ellipsoidal and spherical. The majority of cells were ellipsoidal with lengths between 7 to 9 micrometers and their widths are from 5 to 7 micrometers. The diameter of spherical-shaped cells was determined to be between 4.5 to 7.5 micrometers. Again, the cell sizes and shapes are in agreement with cell morphology of microalgae. On both Figures 5.20 and 5.21, the extracellular fiber-like network was observed. As shown on the top right side of Figure 5.21, trace amounts of bacteria are observed (i.e. small, rod-like structures). This supports the view that culture line NA is a consortium. From light micrographs (Figures 5.22 and 5.23), cells appeared to be mixed homogenously throughout the image. The shapes of cells were again ellipsoid or spherical in form. One particular cell size was determined to be 10 micrometers in the width and 15 micrometers in the length as shown in Figure 5.23.

SEM and light micrographs of culture line *N. oleoabundans* are shown in Figures 5.24-5.26. The images here show spherical-shaped cells 3 to 4 micrometers in diameter. Small disconnected tubes with diameters from 0.83 to 0.72 micrometers and the extracellular fiber-like network are observed in Figure 5.24. As seen in Figure 5.26, two different morphological characteristics, filamentous and coccid, are seen. The length of filaments in some instances are over 60 micrometer.

Comparing images of MA, NA and *N. oleoabundans* with the light micrographs of *N. oleoabundans* from the University of Texas microalgae repository (UTEX) (Figure 5.27), clearly, the appearance of various structures (including bacteria) indicate a likelihood of a microbial consortium emerging through our enrichment process.

However, there was no solid evidence for proving or disproving that these cultures do not have *N. oleoabundans* present. In all likelihood, *N. oleoabundans* remained as one member of the consortium as the culture enrichment procedures progressed.

A distinct advantage of such an enrichment is that such a mixed consortium is much more likely to survive and thrive in an open-pond system reducing the risks of a competitive disadvantage introduced by microbial contaminants in a pure culture and greatly improving process economics through deployment of such an open-pond system (as compared to the stringent requirements and high operational costs of maintaining a closed, pure-culture system). [Schenk, 2008]

5.10 Kinetic and Growth Study of NA, MA, *N. oleoabundans* in Batch Systems

Growth Kinetic and carbohydrate of three culture lines: MA, NA, and *N. oleoabundans* were studied. Cultures were transferred and incubated in autoclaved STW-SM with supplemental carbon dioxide and continuous light supply. The carbohydrate contents were determined by YSI and DNS after acid hydrolysis. Autoclaved wastewater was used for eliminating contamination. However, this process would contribute significantly to operational costs in scale-up. Also, autoclaving those microorganisms may release potential nutrients for microalgal growth which is known as cryptic growth. One major assumption made for the kinetic study was that compositions of microorganism species within each culture line were assumed to have a negligible effect on growth kinetics. Though not demonstrated, this was assumed after numerous transfers of each culture line. Future work should investigate the validity of this assumption. The culture growth, specific growth rates, sugar contents and sugar yield are shown in Figures 5.28-5.35.

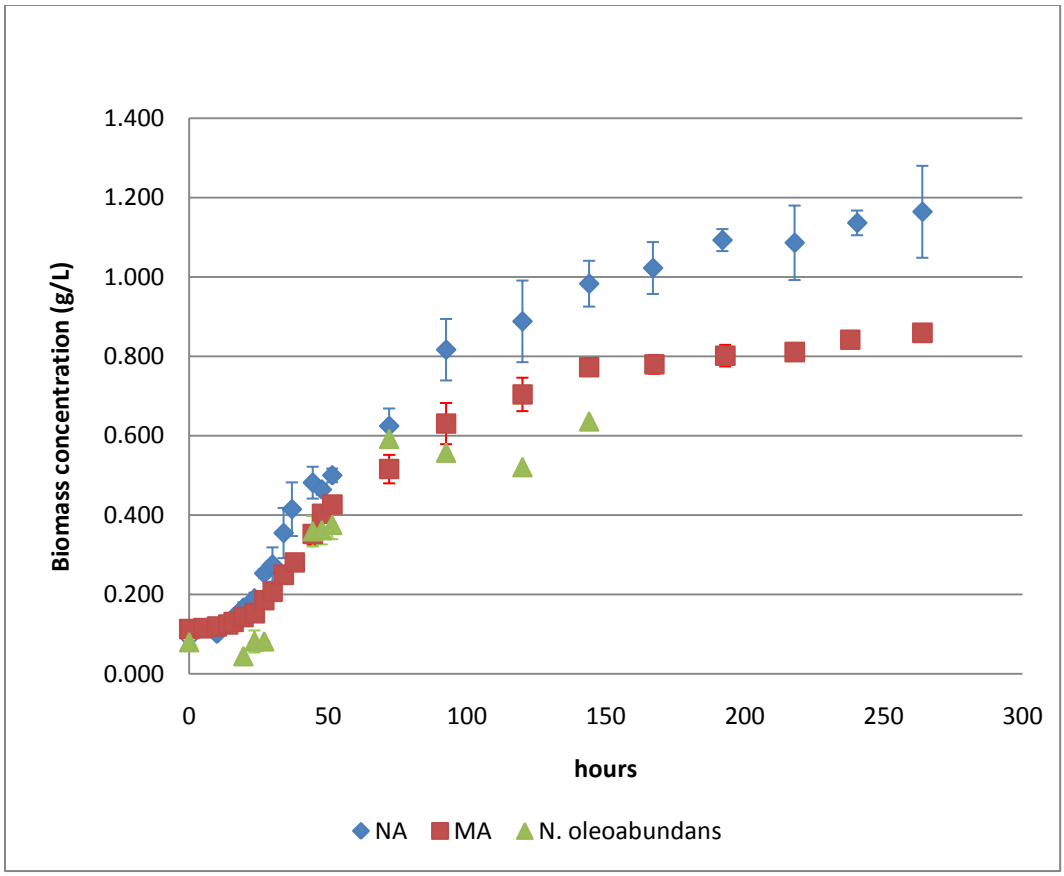

Figure 5.28 Growth Curves of NA, MA and *N. oleoabundans*

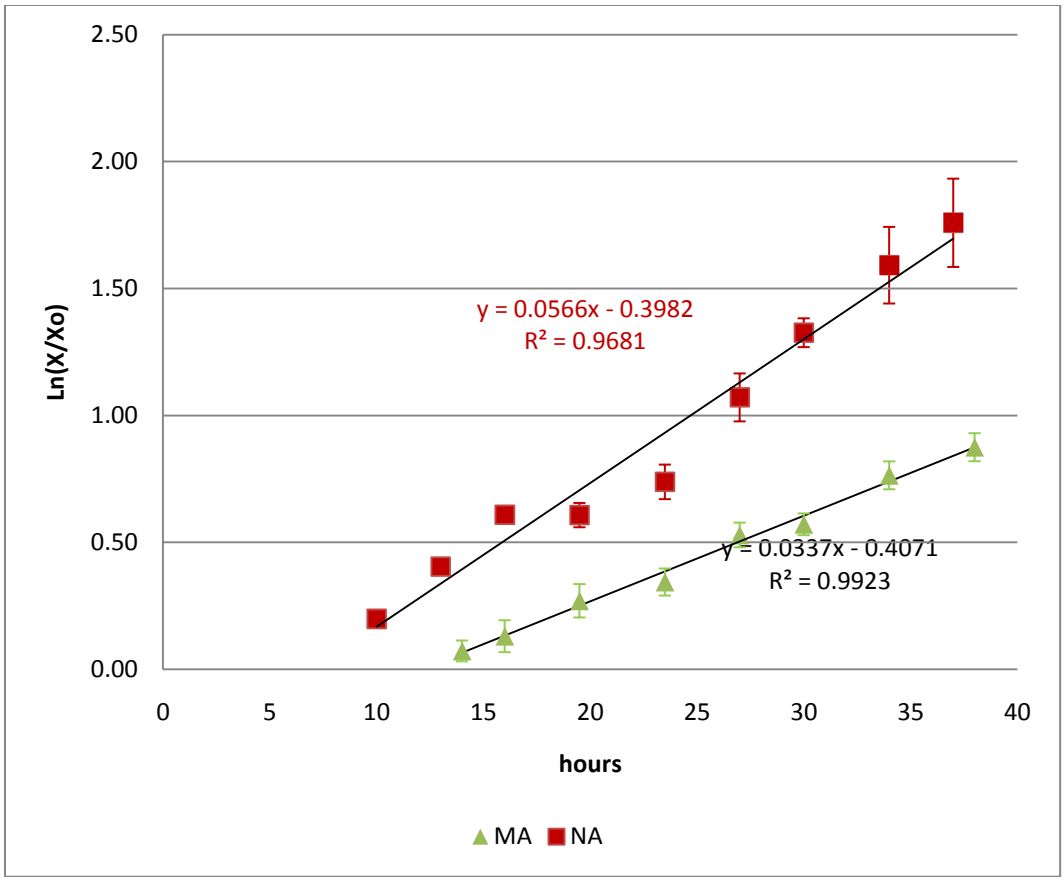

Figure 5.29 Determination of Specific Growth Rate of NA and MA

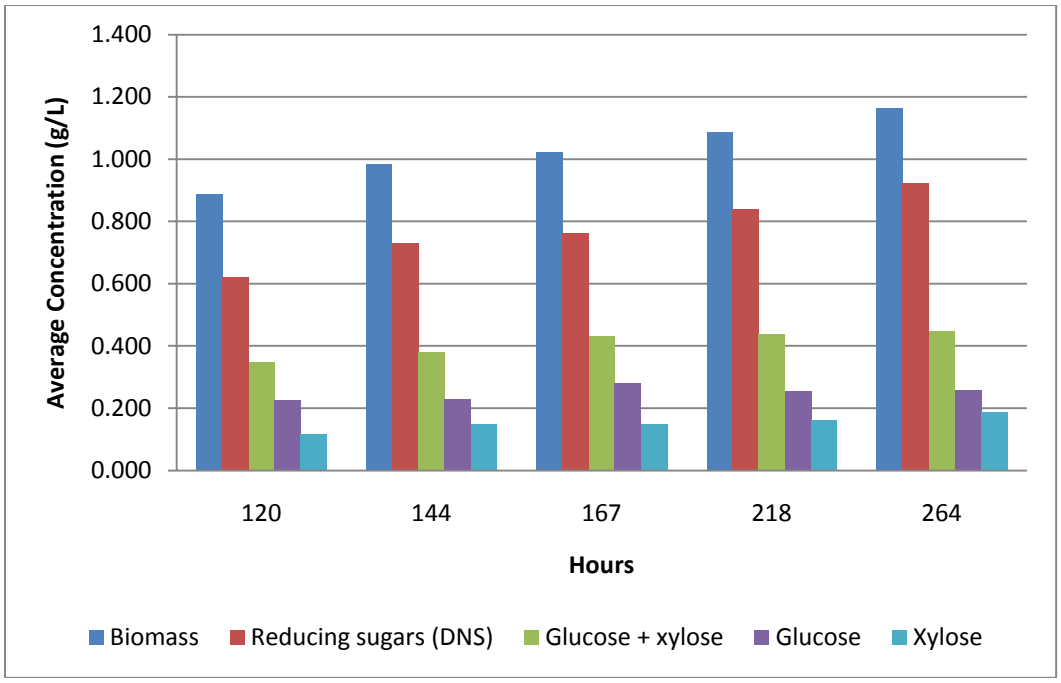

Figure 5.30 Sugar Production of NA from 120 to 264 Hours

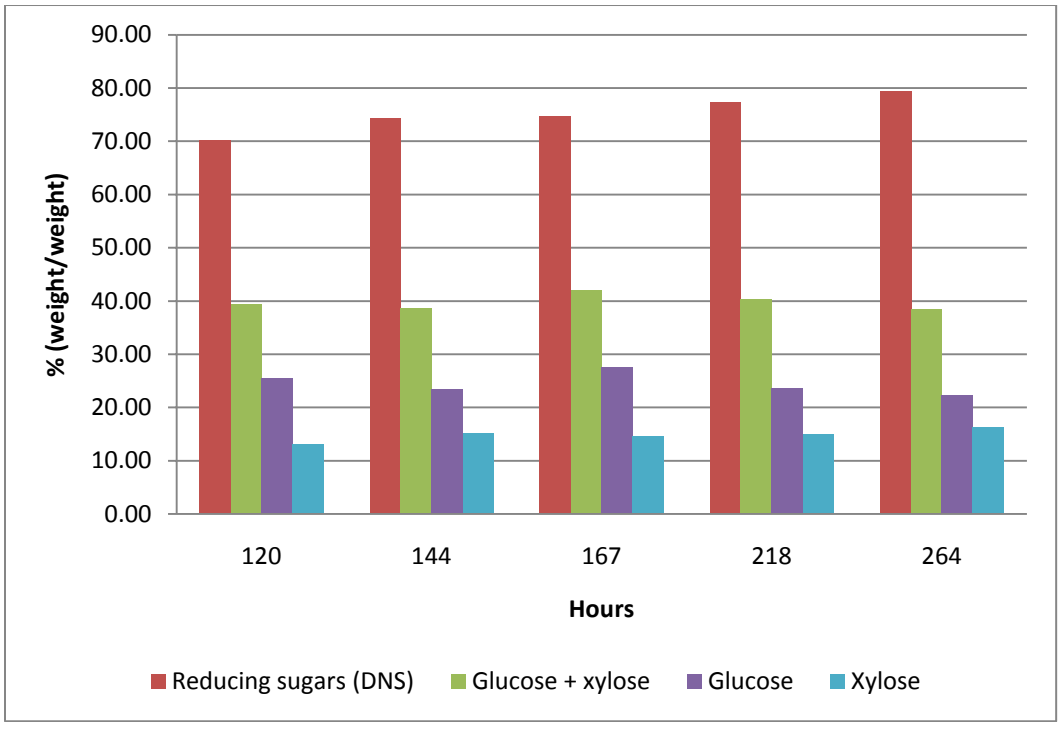

Figure 5.31 Sugar Contents of NA from 120 to 264 Hours

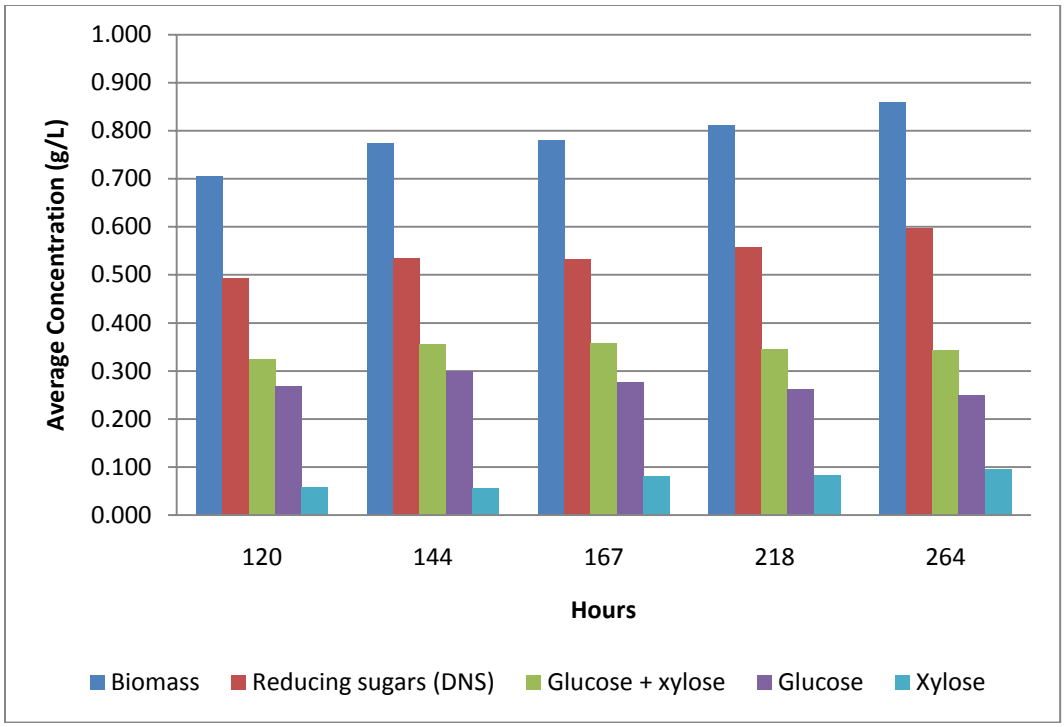

Figure 5.32 Sugar Production of MA from 120 to 264 Hours

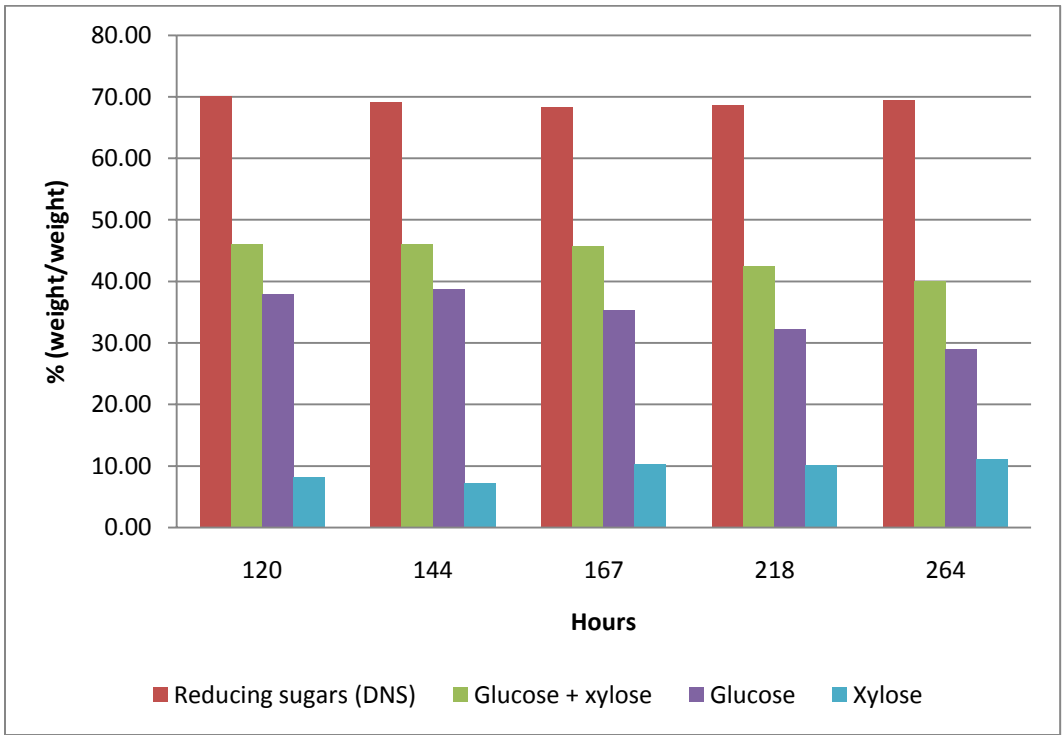

Figure 5.33 Sugar Contents of MA from 120 to 264 Hours

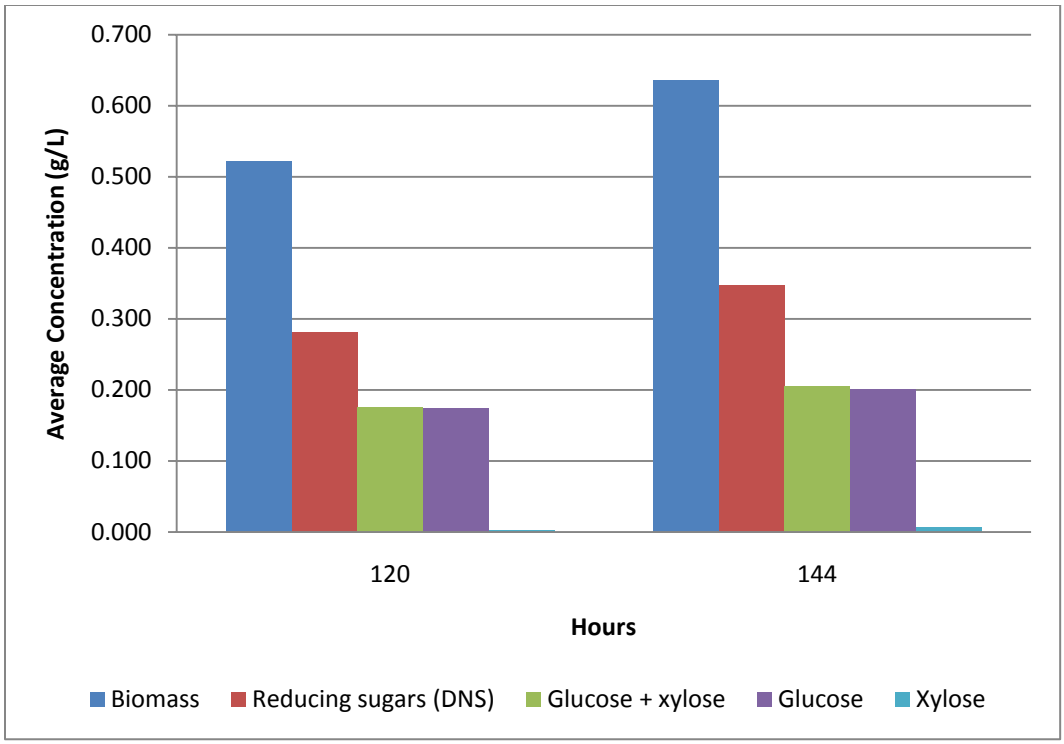

Figure 5.34 Sugar Productions of *N. oleoabundans* from 120 to 264 Hours

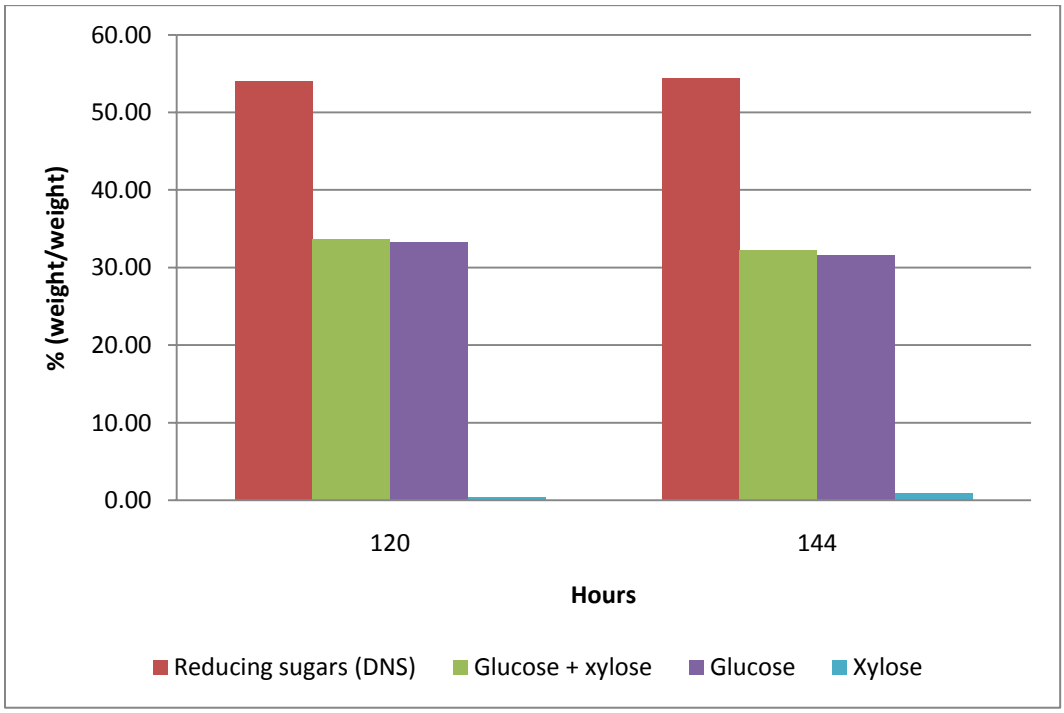

Figure 5.35 Sugar Contents of *N. oleoabundans* from 120 to 264 Hours

As shown in Figure 5.28, growth of NA exhibited four phases: lag, exponential, deceleration and stationary phases during 264 hours incubation. The lag phase length of NA was determined to be around 7 hours from calculation of the maximum specific rate. The maximum specific rate of NA was found to be 0.0556 per hour as shown in Figure 5.29. The exponential phase was observed from around 7 hours to 37. After 37 hours incubation, the culture entered the deceleration phase. The stationary phase was reached after 167 hours incubation. The maximum average biomass concentration reached 1.165 g/L. According to DNS analysis, the NA consortium contained 70-79 % (w/w) reducing sugars as shown in Figure 5.31. HPLC was employed for determining the sugar compositions of the NA acid hydrolyte. Xylose and glucose were two major carbohydrates presented in the NA acid hydrolytes. As shown in Figure 5.31, the average glucose content of NA was between 22 and 27.5 % (w/w) and the average xylose content was between 13 and 16.2% (w/w). The total YSI carbohydrate content, which combined xylose and glucose contents, was between 38 and 42 % (w/ w). By comparing total reducing sugars and the combination of xylose and glucose, the difference was between 41 and 31 %. Other work has shown that such variations might be due to 5-hyhroxymethyl furfural, interference of xylose, presence of other sugars, such as pentose, and other chemicals, such as low molecular-weight aldehyde or pigments. [Marsden, 1982; River, 1984] From the sugar productions of NA as shown in Figure 4.30, the maximum glucose production of 0.282 g/l was reached after 167 hours incubation and xylose production increased slowly from 0.116 g/l to 0.189g/L. The overall xylose and glucose production were increased from 0.349 g/L to 0.448 g/l from 120 to 264 hours incubation.

As shown in Figure 5.28, growth phases of MA contained four phases, similar to NA: lag, exponential, deceleration and stationary phases during 264 hours incubation. The lag phase length of MA was determined to be around 12 hours from calculation of the maximum specific growth rate. The maximum specific growth rate of MA was found to be 0.0337 per hour as shown in Figure 5.29. The exponential phase was observed from around 12 hours to 48. After 48 hours incubation, the culture entered the deceleration phase. The stationary phase was reached after 120 hours incubation. The maximum average biomass concentration reached 0.860 g/L. According to DNS analysis, the MA consortium contained around 69 % (w/w) reducing sugars as shown in Figure 5.33. The average glucose content of MA varied from 29-38% (w/w) and the average xylose content was between 8-11% (w/w). The total YSI carbohydrate content (i.e. combined xylose and glucose) was 40-46% (w/ w). Sugar production of MA is shown in Figure 5.32. The maximum glucose production from MA was recorded as 0.300 g/L after 144 hours incubation, and its xylose production increased slowly from 0.058 to 0.095 g/l during the experiment. The overall xylose and glucose production was 0.356 g/l at 144 and 167 hours.

The growth curve of *N. oleoabundans* is shown in Figure 5.28. The lag phase was estimated to be 27 hours and the stationary phase was reached after 72 hours incubation. The specific growth rate was not determined due to strong flocculation which leaded to inaccurate O.D readings. In contrast to cultures of NA and MA which were homogeneously dispersed throughout the medium giving a consistent coloration throughout (thus giving consistent optical density readings), flasks of *N. oleoabundans* in this experiment showed such a strong tendency to flocculate that the growth medium

appeared completely clear with flocs approximately 1-3 mm in diameter dispersed throughout the medium. The maximum biomass concentration (determined through direct measurement of freeze-dried cells) was 0.636 g/L. By comparing results between Figures 5.28 and 5.11, *N. oleoabundans*, which were maintained in autoclaved wastewater, had higher growth rates than *N. oleoabundans* that was maintained in SE medium. The reducing sugar content of *N. oleoabundans* was approximately 54% (w/w) as shown in Figure 5.34. Trace amounts of xylose were present in *N. oleoabundans* and glucose content was between 31-33% (w/w). The sugar production of *N. oleoabundans* is shown in Figure 5.34. Glucose production from *N. oleoabundans* was estimated to be 0.201 g/l after 144 hours incubation.

For comparison, *Botryococcus sudeticus* was also incubated in autoclaved STW-SM. The stationary phase was reached after 72 hours incubation as shown below in Figure 5.36.

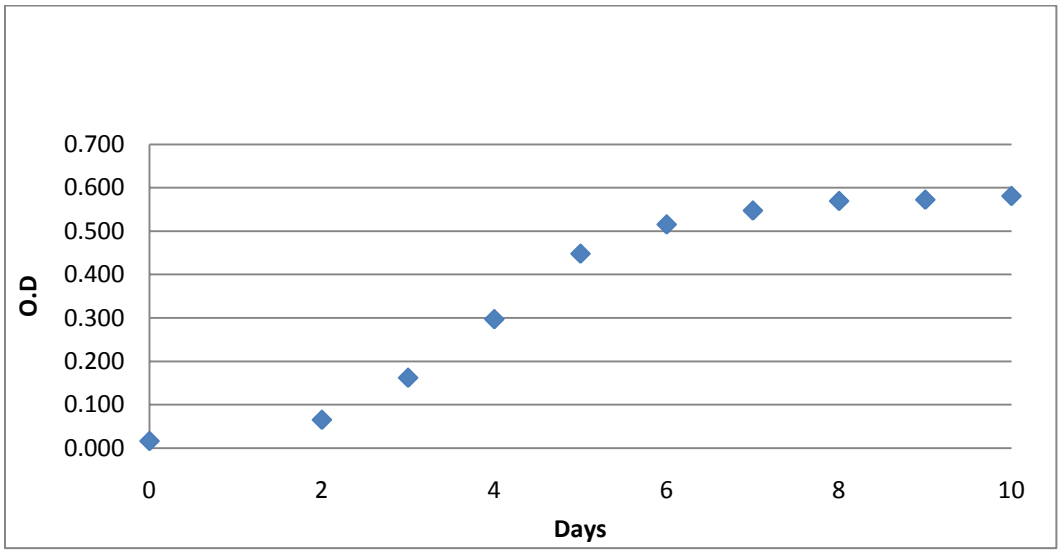

Figure 5.36 The Growth Curve of *Botryococcus sudeticus* in Autoclaved Wastewater

The biomass was harvested at day 10 and the average biomass concentration was determined to be 0.443 g/L. The reducing sugar content of *B. sudeticus* at 240 hours was 56.12% (w/w) and its glucose content was 38.05% (w/w). The xylose content was less than 1.4% (w/w). Comparing results of all cultures, NA, MA, *N. oleoabundans* and *B. sudeticus*, NA had the highest overall xylose and glucose production (0.448 g/l) even through MA had higher glucose + xylose contents (46.11 %w/w). Also, NA had the highest biomass concentrations (1.165g/l) of all four cultures, but MA had the highest overall glucose production (0.300g/l).

However, it is important to note one technical challenge related to culture NA. The production of the extracellular, highly viscous material (reported earlier) made for significant difficulty in harvesting NA. During centrifugation, this highly viscous fluid prevented the microalgae cells from forming a solid pellet on the centrifuge tube bottom. Such challenges would have to be addressed for scale-up technical and cost considerations.

5.11 NA Growth and Sugar Contents with Addition of Fertilizer

To assess the impact of the addition of nutrients for improving NA growth in autoclaved STW-SM, an experiment was conducted using a commercial liquid fertilizer. The composition of the commercial fertilizer contained 1.2% ammoniacal nitrogen, 1.2% nitrate nitrogen, 5.6% urea nitrogen, 7% phosphate, 6% soluble potash and 0.1 % iron. Different amounts of this fertilizer were added into STW-SM as shown in Table 5.4. Experimental observations are summarized below.

Table 5.4 Growth & Sugar Contents of NA with Addition of Commercial Fertilizer

Nutrient Addition (g/L)	Max biomass concentration (g/L)	Glucose Content (w/w)	Xylose Content (w/w)	Glucose + Xylose content (w/w)	Reducing sugar content (w/w)
0	1.562±0.100	20.17±3.92	14.10±2.18	33.82±5.45	71.04±6.74
0.0433 ± 0.0055	1.279±0.052	21.45±3.59	11.61±2.01	33.05±5.46	59.85±6.20
0.0665 ± 0.0020	1.440±0.128	22.53±1.75	13.9±1.64	36.44±1.77	68.69±2.60
0.0952± 0.0095	1.641±0.170	24.72±2.12	11.78±1.76	36.50±2.88	72.85±6.01
0.1332± 0.0025	0.883±0.064	34.08±1.82	5.57±0.94	39.65±2.24	55.05±3.26
0.2020± 0.0054	1.708±0.068	29.31±2.91	9.66±0.69	38.96±2.72	65.11±4.90
0.2709± 0.0031	1.199±0.072	34.08±2.19	4.09±0.69	38.16±1.90	56.07±2.13

After 216.5 hours incubation, cultures containing the highest addition of commercial fertilizer (i.e. 0.2020 and 0.2709 g/L) reached the stationary phase. Other flasks of NA with lesser amounts of the added fertilizer reached the stationary phase after approximately 144 hours incubation. As shown in table 5.4, no difference in reducing sugars and glucose + xylose contents was observed among all levels of fertilizer addition. While there was no difference in glucose + xylose contents, the ratio of glucose to xylose increased with increasing amounts of fertilizer addition. This indicates that glucose contents of NA can be controlled by adding nutrients. However, no improvement maximum biomass concentration was observed by adding the commercial fertilizer in any amount.

CHAPTER VI

ENGINEERING SIGNIFICANCE

Using the results from this research, a bio-ethanol plant was designed based on a model reported in a National Renewable Energy Laboratory (NREL) report [Aden, 2002]. This model was assumed to be appropriate for estimating ethanol/biomass ratios (gallon/ton) and ethanol/wastewater ratios (gallon/ton). Also, ethanol/land ratios were estimated by assuming that "~~N~~", "~~M~~A", and "~~*N*~~*eochloris oleoabundans*" were grown in indoor raceway bioreactors. The depth of the bioreactor was assumed to be maintained at 20 cm and the days of operation were assumed to be 350 days per year. The overall process flow diagram is shown below in Figure 6.1

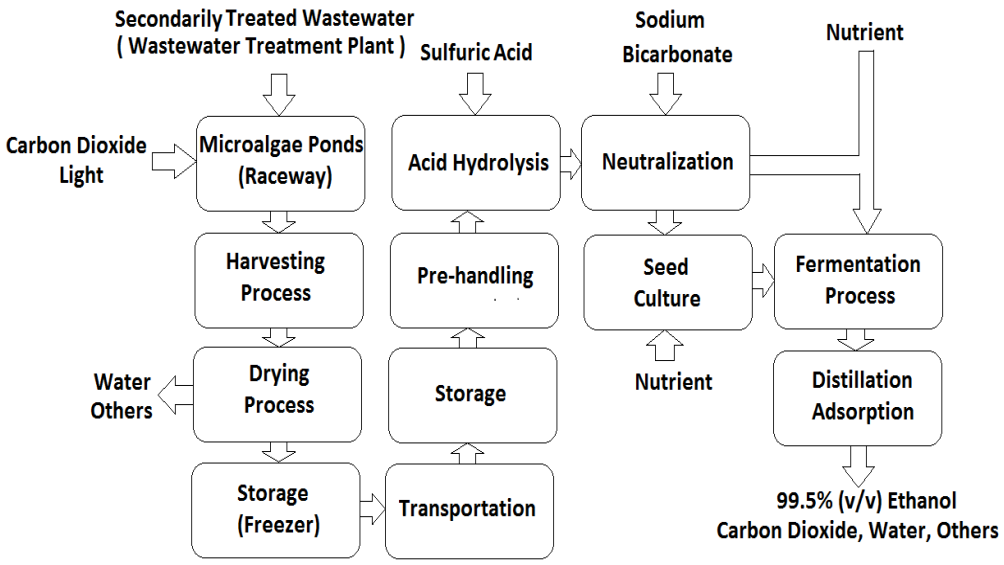

Figure 6.1 The Flow Diagram for Bioethanol Production from Microalgae

From our research, we have shown that NA, MA and *N. oleoabundans* are able to be grown in secondarily treated wastewater without addition of nutrients. Observed glucose compositions ranged between 22% and 38% (w/w). Xylose composition ranged between 0 and 16.2% (w/w). This indicates the feasibility for bio-ethanol production plant from secondarily treated wastewater by growing microalgae. This lead to questions about required resources for ethanol production from microalgae grown in secondarily treated wastewater. For our purposes, we assumed that 10,000,000 gallons of 99.5% ethanol was to be produced annually. From this assumption, the question of how much wastewater was needed must be addressed. From another perspective, how much ethanol could be produced from one ton of NA, MA or *N. oleoabundans?* Alternately, how much ethanol could be produced from one gallon secondarily treated wastewater? Several assumptions made for designing a system to answer the above questions are presented.

Firstly, an annual production of 10,000,000 gallon 99.5% (v/v) ethanol was converted into annual mole production of ethanol (i.e. 648,294,007 moles per year). Assuming no ethanol loss during distillation and adsorption processes, the molar flow rate of ethanol out of fermentation must then be 648,294,007 moles per year. Fractional conversions of glucose or xylose as shown in Table 6.1 were adapted from the NREL report [Aden, 2002].

Table 6.1 Fractional Conversion of Xylose or Glucose to Ethanol [Aden, 2002]

Reaction (seed culture)	Reactant	Fraction converted to ethanol
Glucose → 2 Ethanol + 2 CO2	Glucose	0.90
3 Xylose → 5 Ethanol + 5 CO2	Xylose	0.8
Reaction (Fermentation)	Reactant	Fraction converted to ethanol
Glucose → 2 Ethanol + 2 CO2	Glucose	0.95
3 Xylose → 5 Ethanol + 5 CO2	Xylose	0.85

For calculation purposes, we assume "NA", "MA" and "*N. oleoabundans*" are harvested every five days. Based on this assumption, the biomass concentrations and their sugar compositions are known from our research data, summarized in Table 6.2 below. After calculating the biomass needed for producing 10,000,000 gallon 99.5% (v/v) ethanol, wastewater needed for producing 10,000,000 gallon 99.5% (v/v) ethanol per year can be calculated by using biomass concentrations from our research data.

Then the ethanol/wastewater ratio and the ethanol/biomass ratio can be calculated. Before calculating daily production rate (g/m^2 per day), two assumptions were made. The first assumption was that the total number of operating days were 350 days per year. The second assumption was that "NA", "MA" and "*N. oleoabundans*" were grown in indoor raceway bioreactor which area was one hectare and the depth was 20 cm. The biomass concentrations after incubating 120 hours (five days) can be used. Based on these assumptions and our experimental results, daily production rates (g/m^2 per day) and land needed for 10,000,000 gallon annual production of 99.5% (v/v) ethanol can be estimated. Since land needed for 10,000,000 gallon annual production of 99.5% (v/v) ethanol was known, the ethanol/land ratios (gal/ha) can be calculated. Results of these calculations are shown in Table 6.2 and details of calculation are included in appendix B.

An additional calculation was made, assuming that xylose would be unusable (results shown in Table 6.3). The same approach was used, but conversion of xylose to ethanol was assumed to be 0. The conversion of glucose was assumed to be the same shown in Table 6.1.

Table 6.2 Estimation of Ethanol Production

Culture	NA	MA	Neochloris oleoabundans
Harvested Concentration (g/L)	0.888	0.704	0.521
% Conversion of Biomass to Glucose (w/w) from acid hydrolysis with 0.5 M sulfuric acid	25.52	37.94	33.28
% Conversion of Biomass to Xylose (w/w) from acid hydrolysis with 0.5 M sulfuric acid	13.06	8.17	0
Mass needed for producing 10,000,000 gallon 99.5% (v/v) ethanol per year (ton)	166,129.24	136,581.57	185,688.61
Wastewater needed for producing 10,000,000 gallon 99.5% (v/v) ethanol per year (gallon)	49,421,952,666	51,251,459,153	94,153,033,934
Daily production rate (g/m^2 per day)	35.52	28.04	20.84
Land needed for 10,000,000 gallon annual production of 99.5% (v/v) ethanol	1,336	1,392	2,546
Ethanol/biomass ratio (gallon/ton)	60.19	73.22	53.85
Ethanol/wastewater ratio (%v/v)	0.02023	0.01951	0.01062
Ethanol/Land ratio (gal/ha per year)	7,483	7,185	3,928

Table 6.3 Estimation of Ethanol Production from Glucose only (not Xylose)

Culture	NA	MA	Neochloris oleoabundans
Harvested Concentration (g/L)	0.888	0.704	0.521
% Conversion of Biomass to Glucose (w/w) from acid hydrolysis with 0.5 M sulfuric acid	25.52	37.94	33.28
% Conversion of Biomass to Xylose (w/w) from acid hydrolysis with 0.5 M sulfuric acid	13.06	8.17	0
Mass needed for producing 10,000,000 gallon 99.5% (v/v) ethanol per year (ton)	242,151.91	162.881.31	185,688.61
Wastewater needed for producing 10,000,000 gallon 99.5% (v/v) ethanol per year (gallon)	72,038,012,083	61,120,285,507	94,153,033,934
Daily production rate (g/m^2 per day)	35.52	28.04	20.84
Land needed for 10,000,000 gallon annual production of 99.5% (v/v) ethanol	1,948	1,660	2,546
Ethanol/biomass ratio (gallon/ton)	41.30	61.39	53.85
Ethanol/wastewater ratio (%v/v)	0.01388	0.01636	0.01062
Ethanol/Land ratio (gal/ha per year)	5,134	6,025	3,928

As shown in Table 6.2, the estimated ethanol production of ―MA" was 73.22 gallon per ton based the fraction conversion of glucose or xylose to ethanol from the NREL report. [Aden, 2002] The estimated ethanol production for ―NA" and *N. oleoabundans* were 60.19 and 53.85 gallons per ton of biomass, respectively. Even though ―MA" had the highest estimated ethanol production per ton biomass, lesser amount of wastewater and land were needed for growing ―NA" to achieve 10,000,000

gallon 99.5% (v/v) ethanol. It‘s because –NA” has higher growth rates than –MA”. Annual ethanol production of NA grown in one hectare raceway ponds was estimated to be 7,483 gallon/hectare per year. For –MA” and *N. oleoabundans*, annual ethanol production rates for one hectare raceway ponds were 7,185 and 3,928 gallon/hectare, respectively.

If other yeasts were used and weren‘t able to convert xylose to ethanol, ethanol production per ton biomass of –NA” and –MA” were decreased. Estimated ethanol productions of –MA” and –NA” dropped from 73.22 gallon to 61.39 gallon per ton biomass and from 60.19 gallon to 41.30 gallon per ton biomass, respectively. The estimated ethanol production of *N. oleoabundans* remained constant since no xylose was detected in our experiments. The ethanol/land ratio for –NA” and –MA” decreased from 7,483 gallon/ha-yr to 5,134 gallon/ha-yr and 7,185 gallon/ha-yr to 6025 gallon/ha-yr, respectively. The ethanol/wastewater ratio (%v/v) of –NA” and –MA” dropped from 0.02023 to 0.01388 and 0.01951 to 0.01639, respectively. This demonstrates that more wastewater and land are needed for 10,000,000 gallon ethanol production. Also, in this case, –MA” required less land and wastewater than –NA” for producing 10,000,000 gallon ethanol.

Table 6.4 Estimated Production Costs of NA, MA and *Neochloris oleoabundans*

	Seambiotic (model data)	NA	MA	*Neochloris oleoabundans*
Manpower (8 men)	120,000	120,000	120,000	120,000
Electricity & residual energy	30,000	30,000	30,000	30,000
Fertilizers and other chemicals	36,000	0	0	0
Domestic Land Taxes	10,000	10,000	10,000	10,000
Carbon dioxide	5,000	5,000	5,000	5,000
Sea Water	5,000	0	0	0
Fresh Water	10,000	0	0	0
Other supplies and Miscellaneous	20, 000	20, 000	20, 000	20, 000
Total	236,000	185,000	185,000	185,000
Yearly production of dry algae biomass (kg)	700,000	1,243,200	981,400	729,400
Cost per 1KG dry microalgae	0.34	0.15	0.19	0.25

Table 6.5 Profitability of NA, MA and *Neochloris oleoabundans*

	NA	MA	*Neochloris oleoabundans*
Biomass Cost (dollar/kg)	0.14881	0.18851	0.25363
The total cost of one ton feedstock	148.81	188.51	253.63
Ethanol/biomass ratio at the best condition (gallon/ton)	60.19	73.22	53.85
Income from ethanol sale (1.8392 dollar per gallon)	110.70	134.67	99.04
Ethanol sale – feedstock costs (US dollar per ton biomass)	-38.11	-53.84	-154.59

Production costs of ―NA", ―MA" and *N. oleoabundans* could be estimated as shown in table 6.4 with four assumptions. The first assumption was that production costs of dried microalgae from Seambitoic/ICE plant studies [Ben-Amotz, 2009] was the

appropriate model for estimating production costs of ―NA", ―MA" and *N. oleoabundans.* The second assumption was that all chemicals and fertilizers in the model can be replaced by secondarily treated wastewater; therefore, costs of other chemicals and fertilizers were 0. The third assumption was that the sea water cost only contained the cost of the sea water and no other costs, such as the pumping cost, was included. The fourth assumption was that the fresh water cost only contained the cost of the sea water and no other costs, such as the pumping cost, was included. Since secondarily treated wastewater can be used as water sources; therefore, the total water cost was 0. Also profitability of ―NA", ―MA" and *N. oleoabundans* was estimated and only feedstock costs were considered. The price of ethanol was 1.8392 dollar per gallon and the data was from http://www.ethanolmarket.com/fuelethanol.html. From table 6.4, NA has the lowest production cost of all three cultures. However, as shown in table 6.5, ethanol productions from all cultures have negative profits. This indicated this process was not profitable and further studies were required to reduce biomass production costs or to other alternate fuels productions from ―NA","MA" and *N. oleoabundans*, such as syngas.

CHAPTER VII

CONCLUSIONS

Results of this thesis demonstrated that some microalgae can grow in secondarily treated wastewater form Starkville M.S without nutrient addition. However, for some microalgae, addition of nutrients into secondarily treated wastewater was needed for the growth.

In the first phase of this work, *Botryococcus sudeticus* and *Neochloris oleoabundans* were selected as known species due their ability to accumulate lipids which can be used for biodiesel production. [Shen, 2008; Gouveia, 2009a]

Poor growth in synthetic wastewater was observed for both cultures and the contributing causes were found to be lack of PIV solutions and $CaCl_2$ from nutrient depletion studies of *N. oleoabundans*. However, *N. oleoabundans* was selected for incubation in actual secondarily treated wastewater. In early studies, poor growth was observed. Based on results, poor growth appeared to be a result of low nutrient levels present in the secondarily treated wastewater.

Therefore, additional studies were conducted with added nutrients for improving *N. oleoabundans* growth. In the study of *N. oleoabundans* in the mixture of defined medium (i.e. SE) and autoclaved secondarily treated wastewater (STW), the highest lipid contents were observed at a mixture of 0.3 strength SE medium in secondarily treated wastewater. No difference in biomass concentrations was observed between *N.*

oleoabundans grown in higher strength SE/wastewater mixtures. This suggested the optimum nutrient addition through mixing SE medium and secondarily wastewater mixtures could be lower than the 30% (v/v) addition of 2 X strength SE with STW.

Through an enrichment process for discovering a viable consortium in non-autoclaved STW, culture line MA was found. A second research strategy was developed for attempting to grow these enriched cultures on STW for studying lipids and sugars production. Through a series of enrichment steps, culture lines MA, NA and *N. oleoabundans* (i.e. a line of *N. oleoabundans* that appeared to adapt to growing only on STW) were isolated. NA was isolated from natural species presented in secondarily treated wastewater from Tuscaloosa Alabama. Through a series of enrichment steps, a flask of *N. oleoabundans* demonstrated significant growth in autoclaved secondarily treated wastewater from Starkville Mississippi. This line was isolated from *N. oleoabundans* grown in SE mediums. Each of these three culture lines showed significant growth in autoclaved secondarily treated wastewater from Starkville Mississippi without additional nutrients.

In batch studies, the maximum biomass concentration of *N. oleoabundans* was recorded to be 0.636 g/L and contained 33% (w/w) glucose. For MA, the max biomass concentration was recorded to be 0.860 g/l with over 40% (w/w) xylose and glucose. The maximum biomass concentration of NA was recorded to be 1.562 g/L and contained 33.82% (w/w) glucose and xylose. The maximum specific growth rates of NA and MA were determined to be 0.0566 and 0.0337 per hour. Those results showed that all three culture lines were able to grow under autoclaved secondarily treated wastewater and also could serve as potential feed stocks for ethanol production.

Different amounts of commercial fertilizers were added into autoclaved secondarily wastewater for growing NA. No improvement in growth has been observed; however, sugar compositions were shifted from xylose to glucose production. As increasing nutrient levels, the glucose content (w/w) inside cells was increased and xylose content (w/w) was decreased. However, there was no difference observed between sum of glucose and xylose contents between all microalagal samples grown in different level of nutrient addition. This showed sugar compositions of NA can be controlled by addition of nutrients.

Ethanol productions from NA, MA and *N. oleoabundans* were estimated from experimental results with several assumptions which were discussed in Chapter 5. Ethanol productions of ―MA", ―NA" and *N. oleoabundans* were estimated to be 73.22, 60.19 and 53.85 gallon per ton, respectively. Annual ethanol production of ―MA", ―NA" and *N. oleoabundans* grown in one hectare raceway ponds with a 20 cm depth were estimated to be 7,185, 7,483 and 3,928 gallon/hectare per year, respectively. If xylose is not converted to ethanol, ethanol productions per ton biomass of ―NA" and ―MA" were decreased from 73.22 gallon to 61.39 gallon per ton biomass and from 60.19 gallon to 41.30 gallon per ton biomass, respectively. The estimated ethanol production of *N. oleoabundans* remained constant since no xylose was detected in our experiments. Ethanol/land ratio for ―NA" and ―MA" were decreased from 7,483 gallon/ha per year to 5,134 gallon/ha per year and 7,185 gallon/ha per year to 6025 gallon/ha per year. This indicated ―MA" may be better feed stocks for ethanol production than NA; however, ethanol production from ―NA" and ―MA" was not profitable as shown in table 6.5.

Therefore, further studies were needed for reducing biomass production costs or other alternate fuel productions from MA and NA, such as syngas.

In summary, biodiesel can be produced from *N. oleoabundans* grown in autoclaved secondarily with addition of nutrients. *N. oleoabundans*, NA and MA can grow in autoclaved secondarily wastewater with high xylose and glucose contents. They could be used for ethanol production and other alternate energy sources. From estimation of ethanol productions, "MA" may be better feed stocks for ethanol productions; however more studies were required for reducing biomass production costs since so far there was no profit for ethanol production from "NA" and "MA" as shown in table 6.5

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http://www.ethanolmarket.com/fuelethanol.html Ethanol Market (Ethanolmarket.com, LLC)

http://www.chem.usu.edu/~sbialkow/Classes/3600/overheads/Carbonate/CO2.html Equilibrium of carbonate ions

APPENDIX A

ADDITIONAL GRAPH AND DATA

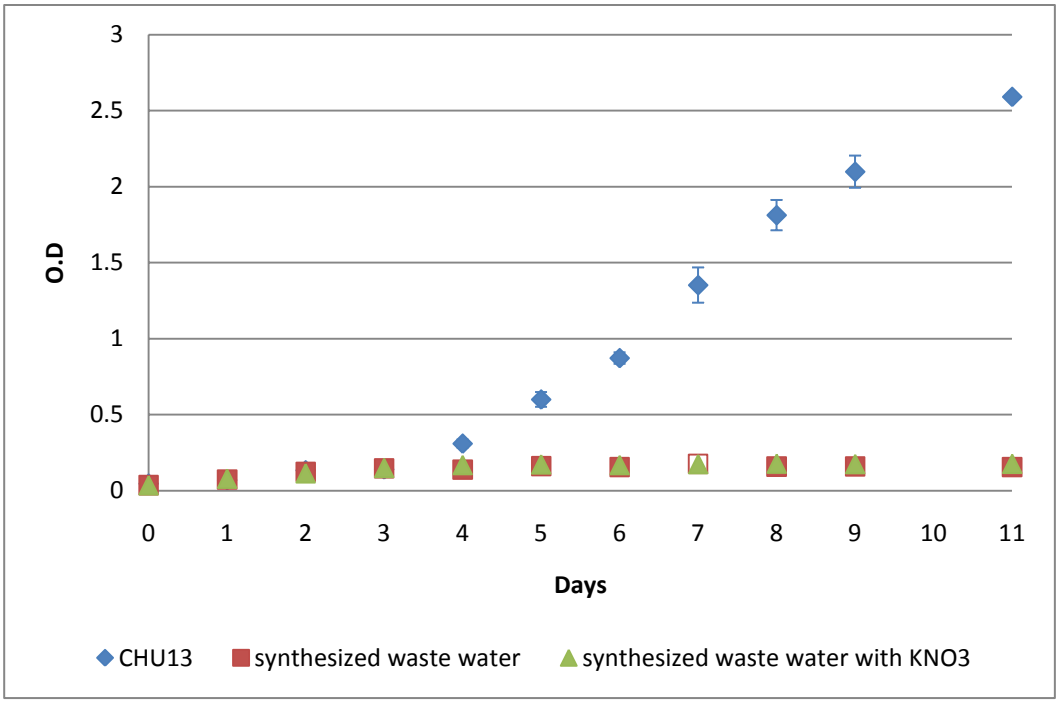

Figure A.1 The Second Generation Growth of *E* Culture

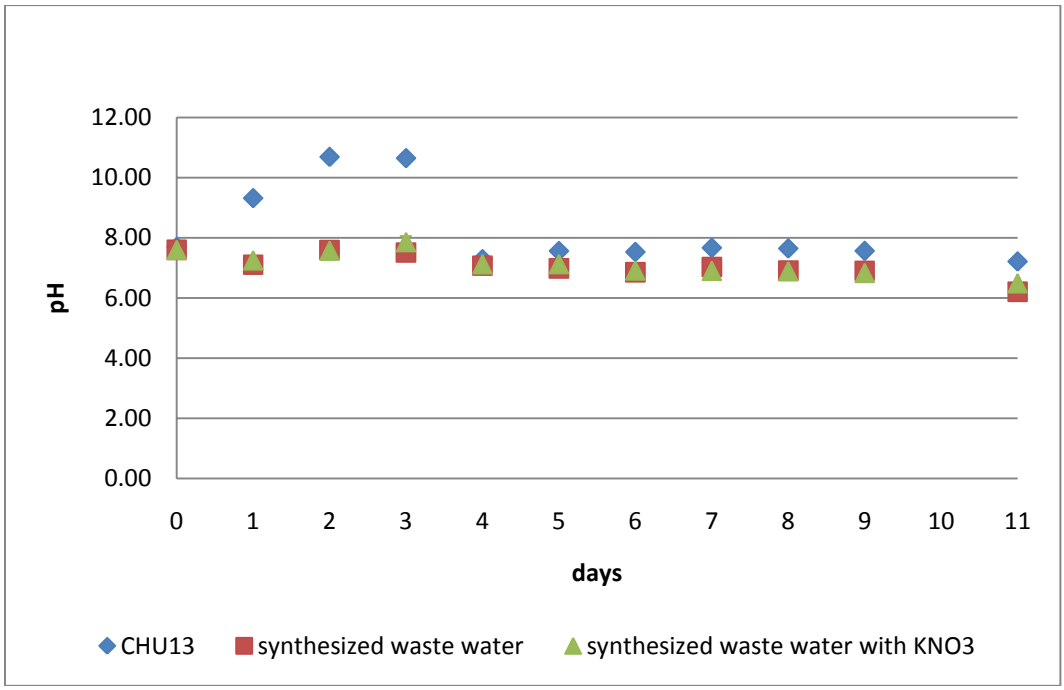

Figure A.2 pH Curve of E Culture

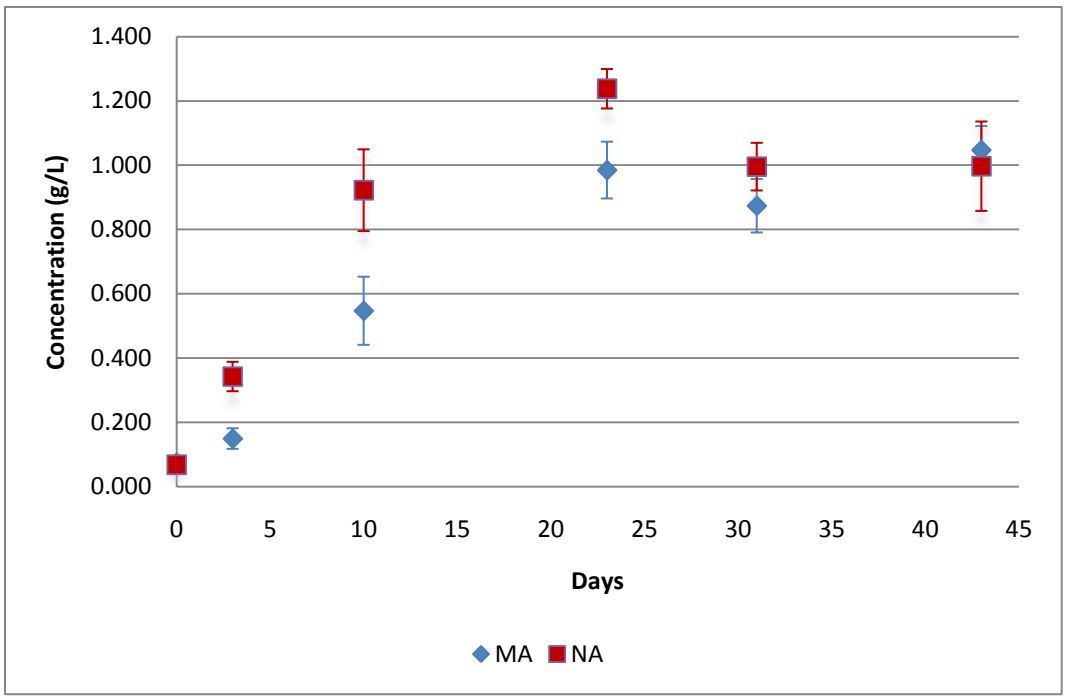

Figure A.3 Growth Curves of MA and NA in the Semi-batch System

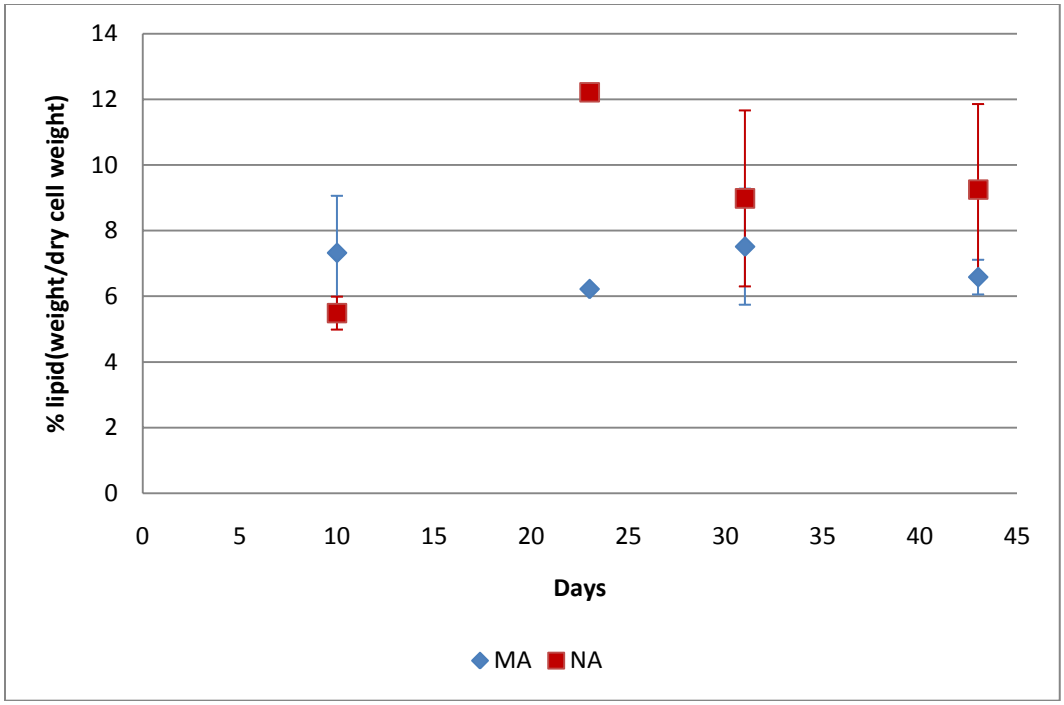

Figure A.4 Lipid Contents of NA and MA in the Semi-batch System

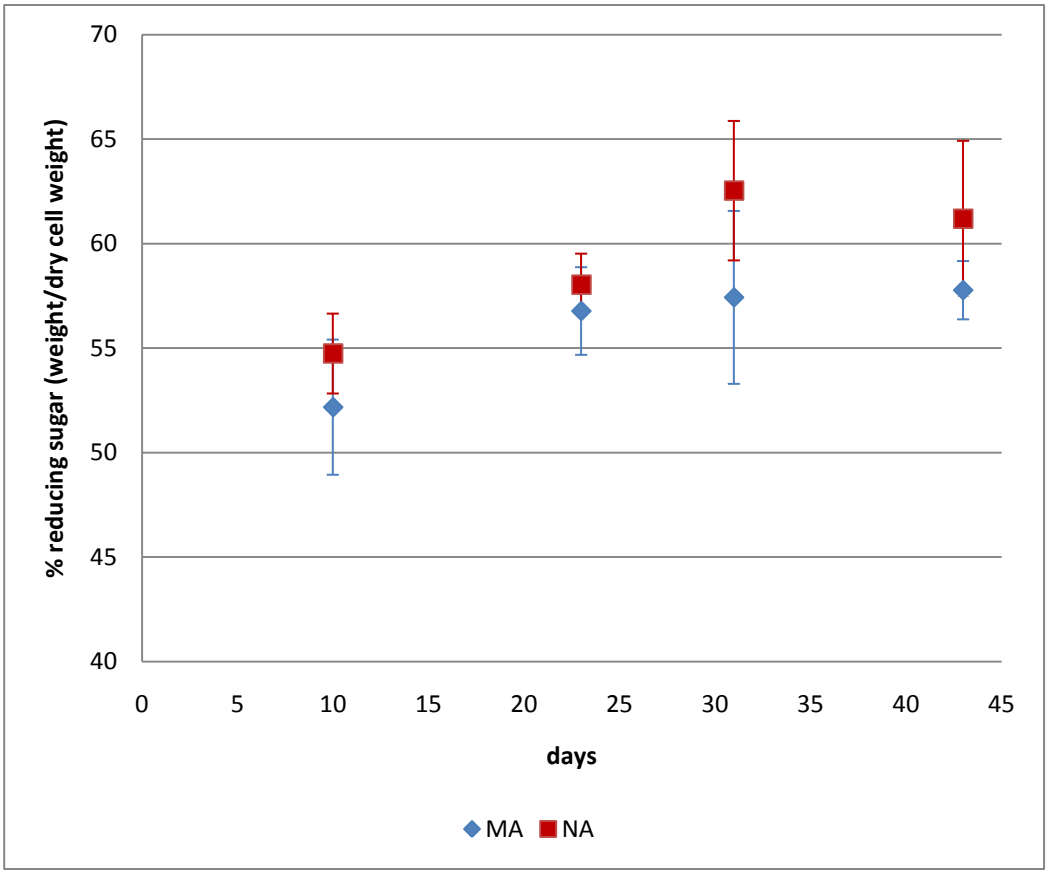

Figure A.5 Reducing Sugar Contents of NA and MA in the Semi-batch System

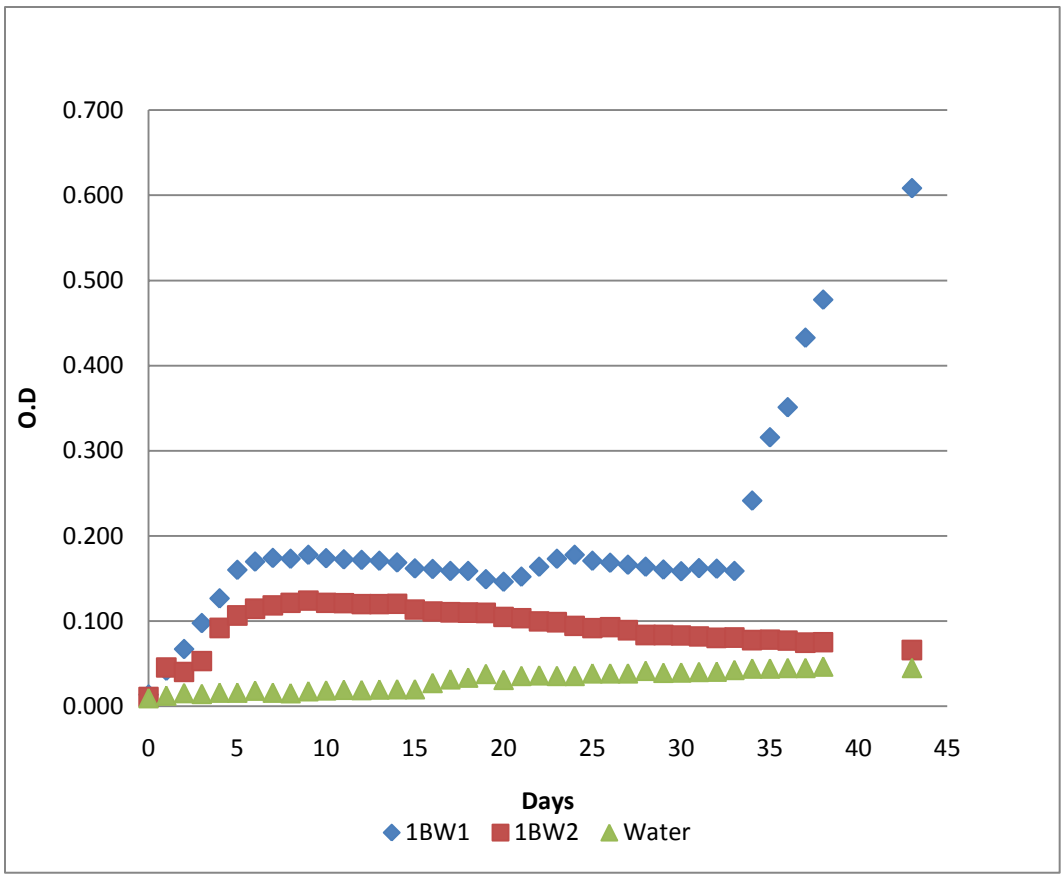

Figure A.6 Growth Curve of *Botryococcus sudeticus* in synthetic wastewater

As shown in figure A.6, no carbon dioxide is supplied for 1BW1 until day 33. For 1BW2, carbon dioxide was supplied from day 15 to day 33. Water is used as the control in this experiment. Optical density is shown in figure A.6 and pH data is shown in figure A.7.

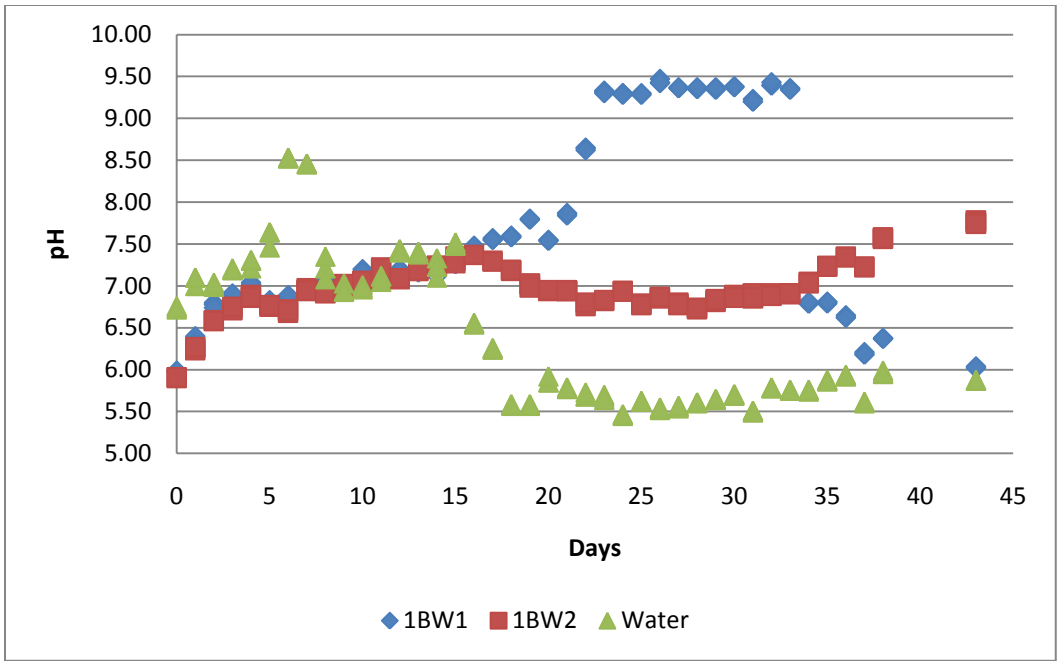

Figure A.7 pH Curve During Growth of *B. sudeticus* in Synthetic Wastewater

APPENDIX B

CALCULATION FOR ESTIMATING BIOETHANOL PRODUCTION

Annual of 10,000,000 gallon 99.5 % (v/v) ethanol productions were estimated for –NA" , –MA" and Neochloris oleoabundans. Fraction conversions of glucose and xylose to ethanol were shown in table B.1 which was adopted from the national renewable energy laboratory report [Aden, 2002] In the design, Zymomonas mobilis strain was used for the fermentation and 10% of acid hydrolysate was fed to the seed culture reactor and 90% was fed into the fermentation reactors. This process was assumed to be appropriate for estimating annual ethanol production by using –NA", –MA", Neochloris oleoabundans A as feed stocks. Details of calculation were shown after table B.1.

Table B.1 Fraction Conversion of Xylose or Glucose to Ethanol

Reaction (seed)	Reactant	Fraction converted to ethanol
Glucose → 2 Ethanol + 2 CO2	Glucose	0.90
3 Xylose → 5 Ethanol + 5 CO2	Xylose	0.8
Reaction (Fermentation)	Reactant	Fraction converted to ethanol
Glucose → 2 Ethanol + 2 CO2	Glucose	0.95
3 Xylose → 5 Ethanol + 5 CO2	Xylose	0.85

Conversion of annual 99.5 % (v/v) ethanol production from gallon into mole

Calculation of Annual biomass mass needed for 10,000,000 gallon 99.5% (v/v) ethanol production

Assume no ethanol was lost during purification processes, such as distillation processes

Simplify the equation

Rearrange the equation

Rearrange the equation

Biomass of NA needed for producing 10,000,000 gallon 99.5% (v/v) ethanol annually

166,129,241,433 g/year = 166,129.24 ton/year

Biomass of MA needed for producing 10,000,000 gallon 99.5% (v/v) ethanol annually

136,581,572,778 g/year = 136,581.57 ton/year

Biomass of Neochloris oleoabundans needed for producing 10,000,000 gallon 99.5% (v/v) ethanol annually

185,688,606,966 g/year = 185,688.61 ton/year

Ethanol/biomass ratio (gallon/ton)

Wastewater needed for producing 10,000,000 gallon 99.5% (v/v) ethanol per year (gallon)

Ethanol/Wastewater ratio (%v/v)

Daily production rate of dry microalgae biomass (g/m^2 per day)

Assume the depth of the raceway pond to be 0.20 cm and biomass were harvested every 5 days

---

Land needed for 10,000,000 gallon annual production of 99.5% (v/v) ethanol

Assume operating days to be 350 days per year.

Ethanol/Land ratio

Calculation of Annual biomass mass needed for 10,000,000 gallon 99.5% (v/v) ethanol production when xylose can't be converted to ethanol (Calculation of table 6.3)

Since no xylose was detected by YSI for Neochloris oleoabundans, the calculated results of Neochloris oleoabundans in table 5.3 would be the same as table 5.2. Therefore, no calculation was preformed. Fraction conversion of glucose to ethanol was assumed to be the same as shown in table B.1

Simplify and rearrange above equations

Biomass of NA needed for producing 10,000,000 gallon 99.5% (v/v) ethanol annually

242,151,913,787 g/year = 242,151.91 ton/year

Biomass of MA needed for producing 10,000,000 gallon 99.5% (v/v) ethanol annually

162,881,308,377 g/year = 162,881.31 ton/year

Ethanol/biomass ratio (gallon/ton)

Wastewater needed for producing 10,000,000 gallon 99.5% (v/v) ethanol per year (gallon)

Ethanol/wastewater ratio (%v/v)

Land needed for 10,000,000 gallon annual production of 99.5% (v/v) ethanol

Assume operating days to be 350 days per year. Daily biomass production in table 5.3 was the same as table 5.2.

Ethanol/Land ratio