Cultivation of *Nannochloropsis salina* and *Synechocystis* sp. PCC6803 in Anaerobic Digestion Effluent for Nutrient Removal and Lipid Production

**THESIS**

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

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2012

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Abstract

Energy demands and climate change are among the major issues that challenge the development and sustainability of our society. Biofuels are one of the most promising substitute energy resources for fossil fuels because they are renewable and help reduce greenhouse gas emissions. Microalgae are a group of organisms living in a wide range of environments and have the potential to fix carbon dioxide and produce biomass more efficiently than other plants. Thus, microalgal biomass is believed to be one of the most promising feedstocks for biofuel production. The water and nutrients required for the growth of microalgae could be supplied from wastewater. Open ponds and photobioreactors are the two main types of algae cultivation systems, with the former often being more suitable for large scale biomass production due to low capital costs. However, contaminants and water loss through evaporation are two major concerns for open pond systems.

Anaerobic digestion (AD) is used worldwide to treat organic waste and produce biogas as a renewable energy. The digester liquor (AD effluent) is rich in nitrogen and phosphorus and is usually land applied as fertilizer. When there is not enough land within an economic transportation range, AD effluent needs specific treatment before being discharged. The abundant nutrients in AD effluent make it a good nutrient supplement for
algae cultivation. Most of previous research about using AD effluent to grow algae has focused on freshwater algal species in digested wastewater from livestock production systems. However, there are no reports on the study of seawater algae *Nannochloropsis salina* and *Synechocystis* sp. PCC6803 in digested municipal wastewater.

The approach of cultivating marine algae *N. salina* in AD effluent has several advantages. Firstly, the high salinity growth conditions for *N. salina* could help reduce the invasion of other species. Secondly, the lipid content of *N. salina* is in the range of 21-36%, making it a good candidate for lipid production. In addition, the use of AD effluent could not only provide nutrients required by *N. salina*, but also make up the water loss due to daily evaporation. Cyanobacteria *Synechocystis* sp. PCC6803 is a robust strain which could survive in a wide range of salinities. Its potential for producing large amounts of biomass for bioenergy production is also attractive because of its high growth rate.

In this study, the growth rate, nutrient removal efficiency, and biomass and lipid productivity of *N. salina* and *Synechocystis* sp. PCC6803 were evaluated in both batch and semi-continuous cultivation systems using digested municipal wastewater as nutrient supplement. Batch results showed that *N. salina* grew well at AD effluent loadings of 3, 6, 12, and 18%. In contrast, the growth of *Synechocystis* sp. PCC6803 was gradually inhibited as the effluent loading increased from 3% to 24%, with a highest growth rate of 0.717 d⁻¹ obtained at 3% loading. For both *N. salina* and *Synechocystis* sp. PCC6803, the nutrient removal efficiency decreased as the effluent loading increased, while *N. salina* was more efficient than *Synechocystis* sp. PCC6803 for nutrient removal. Although the
highest biomass yield of *Synechocystis* sp. PCC6803 was 1.6 times that of *N. salina*, its lower lipid content resulted in lower lipid production. The optimal growth rate of *N. salina* and *Synechocystis* sp. PCC6803 was obtained at 6% and 3% effluent loading, respectively.

The semi-continuous studies were carried out for 18 days using these optimal loading rates at different harvesting frequencies (1-, 2-, 3-day intervals) and harvesting ratios (25% and 50%). Compared to the batch study, both biomass and lipid productivity of *N. salina* were improved in the semi-continuous study at the same effluent loading. The highest lipid productivity of *N. salina* was 38.7 mg L^{-1} d^{-1} with 35.3 mg L^{-1} d^{-1} nitrogen removal and 3.8 mg L^{-1} d^{-1} phosphorus removal, when harvested every two days at a 50% harvesting ratio. The biomass productivity of *Synechocystis* sp. PCC6803 was 40% higher than that of *N. salina*, but its maximum lipid productivity (29.1 mg L^{-1} d^{-1}), obtained at a 50% harvesting ratio and daily harvesting, was lower than that of *N. salina*.

This study demonstrated the feasibility of cultivating *N. salina* and *Synechocystis* sp. PCC6803 in AD effluent for nutrient removal and for biomass and lipid production. The results obtained from this study will be scaled up in pilot-scale and commercial scale raceway ponds to evaluate the commercial potential of algae culture with AD effluent.
Dedication

This document is dedicated to my family.
Acknowledgments

I want to give my deepest gratitude and appreciation to my advisor, Dr. Yebo Li, for his constant encouragement, tremendous support and guidance throughout my graduate study. I would like to thank professors serving on my committee: Dr. Jay Martin and Dr. Jiyoung Lee for their time, suggestions and encouragement. Thank Dr. Jiyoung Lee for her precious advice and help with my research project. I also want to thank OSU/OARDC for providing me with two honorable scholarships for my graduate study at OSU.

I would like to thank the staff members of Food, Agricultural and Biological Engineering Department: Mr. Mike Klingmans for his great technical support for my experiment setup, Mrs. Mary Wicks for her time and extreme patience in correcting my writing and improving my thesis, Mrs. Peggy Christman and Mrs. Candy McBride for their kind help and administrative support.

I feel lucky to have been working with these excellent members in our lab. Special thanks go to Mr. Stephen Y. Park for his generous help and enthusiastic support for my study. I want to thank Dr. Zhongjiang Wang, Dr. Xiaolan Luo, Ms. Fuqing Xu, Ms. Lo Niece Liew, Mr. Shengjun Hu, Mr. Dan Brown, Mr. Phil Cherosky, and Mr. Jia Zhao for their help and encouragement during my stay in Wooster.
Especially, I want to thank my dearest parents, my brother and my family for their deep understanding and infinite support for my study. Finally, I would like to thank my husband, Ye Yuan, for always standing by my side through the good and bad times. I would not have been able to finish this thesis without his love and support.
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Fields of Study

Major Field: Food, Agricultural and Biological Engineering
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Chapter 1 Introduction

With increasing population and rapid developing industries, the world primary energy consumption, which was latest reported 12 billion tonnes of oil equivalent per year, continues to increase (BP p.l.c., 2011). Fossil fuel accounted for more than 85% of the primary energy consumption, with oil (34%), coal (30%), and natural gas (24%) as the major energy sources, while nuclear energy, hydroelectricity and renewable energy accounted for 5%, 6% and 1.3% of the total primary energy consumption, respectively (BP, 2011). Though still widely used, the conventional fossil fuels such as coal, petroleum and natural gas have caused serious environmental problems and at the same time they are depleted. It is one of the major challenges for current society to find sustainable and renewable energy resources for the future because the potential energy crisis may lead to global instability, economic recession, and may also affect people’s quality of life. Biofuel is among the top choices in renewable energy list.

Microalgae, which are environmentally friendly biofuel feedstock, have attracted increasing interest for commercial production. The cultivation of microalgae is favored because of the following advantages: (1) microalgae do not compete with crops for arable land and freshwater because they can be cultivated in brackish water and non-arable land; (2) microalgae can grow rapidly and have high oil contents of 20–50% on a dry weight basis (Chisti, 2007); (3) microalgae have the ability to fix carbon dioxide, thus reducing
greenhouse gas emissions; (4) microalgae can utilize nutrients from wastewaters, providing an alternative method for wastewater treatment; and (5) byproducts of microalgae cultivation after lipid extraction, namely algae biomass residue, can be used as a nitrogen source, such as a protein-rich animal feed or fertilizer for crops (Spolaore et al., 2006). In summary, microalgae cultivation combines biofuel production, carbon dioxide mitigation, and wastewater treatment, consequently displaying great application potential. However, mass cultivation of microalgae requires large amounts of freshwater/seawater and nutrients, which are among the major parts of costs for algal biofuel production.

Besides microalgae, cyanobacteria (blue green algae) are another group of efficient photosynthetic microorganisms and have been the subjected to studying for bioenergy production in recent years. Cyanobacteria could produce diverse types of bioenergy including hydrogen, ethanol, diesel, and methane (Hallenbeck, 2012; Stal and Moezelaar, 1997; Varel et al., 1988). Compared with microalgae or crops, less attention has been placed on cyanobacteria for production of lipid-based biofuels because their lipid content is usually lower than most microalgae (Parmar et al., 2011). However, the fast growth rate of cyanobacteria during exponential phase could also lead to high lipid productivity (Sheehan et al., 1998). Genetic modification of cyanobacteria has been attempted to increase their lipid productivity (Liu et al., 2010). Nutrient removal from wastewater by cyanobacteria was investigated due to their robustness and simple growth needs and some strains of cyanobacteria were shown to be efficient for nitrogen and phosphorus removal.
Large amount of water used for agricultural, municipal, and industrial purposes result in issues due to superfluous wastewater generation. Excessive nutrients, such as nitrogen and phosphorus, in the wastewater may cause eutrophication and upset the balance of the ecosystem. Eutrophication as a serious environmental pollution problem has become more widespread since the mid-20th century (Smith and Schindler, 2009; Le et al., 2010; Dodds et al., 2009; Smith, 2003; Rodhe, 1969). The adverse impacts of eutrophication have brought severe damages to the environment, threatened human health, and added extra financial burden to the government and society (Dodds et al., 2009). These facts reflect the urgent need of an economical approach for reducing nutrient runoff and recycling nutrient in wastewaters. An integrated microalgae cultivation and wastewater treatment system will help remove the nutrients in wastewater while producing useful products. Algal biofuel is to be economically viable if combined with wastewater treatment. Microalgae have been proven to be efficient in removing nitrogen, phosphorus, and toxic metals from a wide variety of wastewaters (Ahluwalia and Goyal, 2007; Mallick, 2002).

Anaerobic digestion (AD) is a mature technology used to decompose organic matter under oxygen-free conditions and produce biogas (Abbasi and Abbasi, 2010). It is applied worldwide for the treatment of agricultural wastes, industrial wastewater, and domestic wastewater, etc (Verstraete et al., 2005). Issues arise in the treatment of digester effluent. The digester liquor, which contains high amounts of nutrients such as ammonium, phosphate, etc., is typically disposed in agricultural fields and used as a fertilizer (Salminen et al., 2001). However, it is concerned that the excessive application of digested effluent in agricultural lands will become a potential source of nitrogen
pollution in farming areas (Salminen et al., 2001; Woli et al., 2004; Lei et al., 2007). Therefore, an effective process for nutrients removal is necessary for the post-treatment of the anaerobic digester effluent. AD effluent biological treatment by algae cultivation is an appealing option, but the potential of using AD effluent as algae culture medium is not yet demonstrated as an economic approach to recycle the nutrients and produce biomass.

A majority of algae strains that have been studied for the removal of nutrients from wastewater were freshwater algae (Hongyang et al., 2011; Min et al., 2011; Zhen-Feng et al., 2011; Chaiklahan et al., 2010; Kong et al., 2010). However, the supply of freshwater for algae cultivation is a big issue where freshwater is lacking. Marine microalgae *Nannochloropsis salina* is a promising candidate for biofuel production due to high lipid content and they can grow in brackish water or seawater (Hoffmann et al., 2010; Emdadi and Berland, 1989; Boussiba et al., 1987). Limited information about removal of nutrients by *N. salina* from AD effluent is available. Blue-green algae *Synechocystis* sp. PCC6803 is found to be a very robust strain that lives in a wide range of environment and is able endure high salt stress. Its simple structure and well-known genome information make it a popular subject for bioenergy production via genetic modification and regulation (Huang et al., 2002; Schubert et al., 1993). The broad existence of *Synechocystis* sp. PCC6803 in natural environment such as lakes and reservoirs could also provide large amount of biomass feedstock for bioenergy production. So far, no research on growing *Synechocystis* sp. PCC6803 in AD effluent has been reported.

The first purpose of this study is to investigate the feasibility of cultivating
microalgae *N. salina* for nutrients removal and lipid production in AD effluent from a commercial scale anaerobic digester fed with municipal wastewater. The digested municipal wastewater had high concentrations of carbon, nitrogen and phosphorus, which could support the growth of microalgae. However, high solids content and large bacteria population in the original effluent may inhibit algal growth. Pre-treated by centrifugation to removal large particles and bacteria, the AD effluent was then diluted at different ratios before fed to algae. Batch cultivation was conducted to find the optimal effluent loading with which higher growth rate could reach. Semi-continuous experiment was aimed at finding the proper harvesting frequency and ratio in order to provide information for operation of four large algae ponds (one acre area) on a local farm to facilitate the commercialization of algae cultivation in AD effluent.

This study also compared the nutrients removal efficiency and lipid productivity of *N. salina* and *Synechocystis* sp. PCC6803 for bioenergy production and treatment of AD effluent. Although the lipid content of *Synechocystis* sp. PCC6803 is lower than *N. salina* as reported, its high biomass productivity is attractive and may lead to high lipid production. The robustness of *Synechocystis* sp. PCC6803 makes it less susceptible to invasive species and reduces the risk of contamination in large ponds, thus easy to maintain. The results from this study could also provide base-line data for growing *Synechocystis* sp. PCC6803 in polluted water bodies in a controlled manner to help clean the water and produce biomass at the same time.
Chapter 2 Literature Review

The increasing scarcity of water, rapid population growth, and increasing urbanization calls for efficient wastewater treatment and recycle in the world. Anaerobic digestion as a mature waste treatment process generates large amounts of nutrient-rich effluent. Excessive nutrients discharge to the environment will cause eutrophication, which promotes the growth of harmful algae. Algal fuel as a promising biofuel is to be economically viable if combined with wastewater bioremediation. Interests have been raised in the use of blue-green algae (cyanobacteria) for bioenergy production due to their high growth rate. Green algae are commonly studied for biofuel production and have been proved to be effective in removing nitrogen, phosphorus, and even heavy metals from various streams of wastewater.

2.1 Wastewater Characteristics and Eutrophication due to Nutrients Runoff

2.1.1 Characteristics of different wastewater streams

Wastewater refers to liquid waste discharged by domestic residences, commercial properties, industry, and agriculture and has a wide range of potential contaminants and concentrations. According to the 2008 Clean Watersheds Needs Survey, the amount of wastewater generated in the United States is 12 million t d$^{-1}$ (U.S. Environmental Protection Agency, 2002). The composition of wastewaters varies with sources: municipal wastewater, agricultural wastewater, industrial wastewater.

The increasing urbanization and expansion of urban populations results in
increased amount of municipal wastewater. A city with a population of 500,000 and water consumption of 0.2 t d$^{-1}$ per capita would produce approximately 85,000 t d$^{-1}$ of wastewater (Pescod, 1992). Municipal wastewater contains small concentrations of suspended and dissolved organic and inorganic solids. Table 1 shows the levels of the nitrogen and phosphorus in different sources of wastewater. Compared with animal wastewater, municipal wastewater has less nitrogen and phosphorus. However, there are often considerable amounts of heavy metals such as lead, zinc, and copper in raw municipal sewage. Municipal wastewater treatment processes include three stages: primary treatment, secondary treatment, and advanced treatment. Buoyant and non-buoyant materials are separated in the first stage using physical or chemical methods. Dissolved organics and colloidal materials are removed during the second stage by biological or chemical treatments. The removal of dissolved inorganic components, including nitrogen and phosphorus, takes place in the advanced treatment process through a number of different unit operations including ponds, post-aeration, filtration, carbon adsorption, and membrane separation. Microalgae cultivation for nitrogen and phosphorus removal from municipal wastewater has been most extensively studied (Bhatnagar et al., 2010; Ruiz-Marin et al., 2010).
<table>
<thead>
<tr>
<th>Wastewater category</th>
<th>Description</th>
<th>TN (mg L(^{-1}))</th>
<th>TP (mg L(^{-1}))</th>
<th>N/P</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Municipal wastewater</td>
<td>Sewage</td>
<td>15-90</td>
<td>5-20</td>
<td>3.3</td>
<td>(Bennette D. Burks and Minnis, 1994)</td>
</tr>
<tr>
<td>Animal wastewater</td>
<td>Dairy</td>
<td>185-2636</td>
<td>30-727</td>
<td>3.6-7.2</td>
<td>(Bradford et al., 2008; Barker et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>Poultry</td>
<td>802-1825</td>
<td>50-446</td>
<td>4-16</td>
<td>(Bradford et al., 2008; Yetilmezsoy and Sakar, 2008a)</td>
</tr>
<tr>
<td></td>
<td>Swine</td>
<td>1110(^{-1})-3213</td>
<td>310-987</td>
<td>3.0-7.8</td>
<td>(Barker et al., 2001; Millmier et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>Beef feedlot</td>
<td>63-4165</td>
<td>14-1195</td>
<td>2.0-4.5</td>
<td>(Bradford et al., 2008; Barker et al., 2001)</td>
</tr>
<tr>
<td>Industrial wastewater</td>
<td>Textile</td>
<td>21-57(^{*})</td>
<td>1.0-9.7(\dagger)</td>
<td>2.0-4.1</td>
<td>(Sen and Demirer, 2003), (Chinnasamy et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>Winery</td>
<td>110(^{*})</td>
<td>52</td>
<td>2.1</td>
<td>(Mosse et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>Tannery</td>
<td>273(^{*})</td>
<td>21(\dagger)</td>
<td>13.0</td>
<td>(Durai and Rajasimman, 2011)</td>
</tr>
<tr>
<td></td>
<td>Paper mill</td>
<td>1.1-10.9</td>
<td>0.6-5.8</td>
<td>3.0-4.3</td>
<td>(Slade et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>Olive mill</td>
<td>532</td>
<td>182</td>
<td>2.9</td>
<td>(Ammary, 2004)</td>
</tr>
<tr>
<td>Anaerobic digestion effluent</td>
<td>Dairy manure</td>
<td>125-3456</td>
<td>18-250</td>
<td>7.0-13.8</td>
<td>(Wang et al., 2010a; Cho et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>Poultry manure</td>
<td>1380-1580</td>
<td>370-382</td>
<td>3.6-4.3</td>
<td>(Yetilmezsoy and Sapei-Zengin, 2009; Yetilmezsoy and Sakar, 2008b)</td>
</tr>
<tr>
<td></td>
<td>Sewage sludge</td>
<td>427-467</td>
<td>134-321</td>
<td>-</td>
<td>(Montusiewicz et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>Food waste and dairy manure</td>
<td>1640-1885(^{*})</td>
<td>296-302</td>
<td>-</td>
<td>(El-Mashad and Zhang, 2007)</td>
</tr>
</tbody>
</table>

\(^{*}\) Total Kjeldahl nitrogen (TKN), \(^{\dagger}\) Total orthophosphates (PO\(_4\)\(^{3-}\)-P)
It was reported that an annual average of 665 billion ton of water was used by industries around the world between 1987 and 2003. The total water usage of the U.S. was reported to be approximately a third of the world total (Newman, 2011). Although it varies depending on the source, most industrial wastewater normally contains more heavy metal pollutants and less nitrogen or phosphorus than other types of wastewater (Ahluwalia and Goyal, 2007). Due to the limited amount of nitrogen and phosphorus, it is hard to achieve large-scale microalgal cultivation in industrial wastewater. Most research of microalgal cultivation in industrial wastewater is focused on the bioremediation of heavy metals (Ahluwalia and Goyal, 2007). Selection of strains with high metal sorption capacity is crucial to the achievement of high metal removal efficiency. So far, only a few algal species have been studied for metal sorption ability. There are several reports evaluating the nitrogen, phosphorus and heavy metal removal from industrial wastewater by algae, such as those from carpet industries (Chinnasamy et al., 2010).

Agricultural wastewater, which is mainly produced from livestock production, is another major source of wastewater. Current animal feeding operations in the U.S. generate more than 450 million ton of manure and manure-contaminated runoff water annually (Kellogg et al., 2000). Livestock operations have shifted from small and medium scale to large scale during the past decade. As a result, nutrients have become spatially concentrated in high livestock production areas (Kellogg et al., 2000). The wastewater produced from animal farms is often rich in nitrogen and phosphorus as shown in Table 1. Approximately half of the nitrogen in animal waste is in the form of ammonium, and half is in the form of organic nitrogen. Factors such as animal diet, age, usage, productivity, management, and location will significantly affect the actual nutrient
content in animal wastewater. The nitrogen-to-phosphorus (N/P) ratio is typically 2–8 for dairy, swine and beef feedlot wastewater. The traditional treatment of animal manure is land application as fertilizer. However, the nutrients in manure cannot be totally taken up by crops due to different N/P ratio requirements of the plants and nutrient availability. Therefore the excess nutrients accumulate in the soil, which can increase nutrient loss through runoff, resulting in eutrophication in receiving waters. Other than manure, agricultural runoff can also contain herbicides, fungicides, insecticides, and nitrate and phosphate components from agricultural operations. Agricultural runoff may bring particles, dissolved ions and molecules, and living microorganisms into receiving waters and reduces the water quality.

Anaerobic digestion (AD) is a mature technology which uses microorganisms to decompose organic waste and produce biogas. Many AD systems have been built all over the world for municipal, industrial, and agricultural waste treatment (Verstraete et al., 2005). Efforts have been focused on the optimization of biogas yield and degradation of the volatile solids (Mayer et al., 2009); one neglected aspect is the post-treatment of the AD effluent. Most of the AD effluent is separated by a dewatering system into liquid and solid fractions. The solid portion is usually composted then marketed as potting media or soil amendment, while the liquid portion is traditionally used as fertilizer for land application (Mayer et al., 2009). Excessive land application of AD effluent can result in nitrogen and phosphorus runoff, and may contribute to eutrophication. Efficient and cost-effective nutrient recovery methods should be considered in order to reduce the risk of nitrogen and phosphorus pollution from AD. Compared with typical agricultural, municipal, and industrial wastewater, AD effluent contains relatively more nitrogen and
phosphorus and less carbon because the microbial activity during the digestion converts the carbon to methane (Wang et al., 2010a). The nitrogen in AD effluent is mainly in the form of ammonium (Singh et al., 2011).

2.1.2 Eutrophication due to nutrients runoff

Eutrophication, which can be human-caused or natural, is defined as an increase in the rate of supply of organic matter in an ecosystem (Nixon, 1995). Humans now have significant influence on almost every major aquatic ecosystem, and their activities have greatly changed the nutrients fluxes from the landscape to receiving waters. Agricultural run-off carrying fertilizers and untreated sewage effluent will cause human-induced eutrophication. Rapid agricultural intensification leads to application of vast quantities of fertilizer each year (Matson et al., 1997). It was estimated that the global production of nitrogen fertilizer will increase from current 80 million metric tonnes per year to 134 million metric tonnes per year by 2020 (Levy and Kashibhatla, 1994).

The application of animal manures as nitrogen fertilizer for croplands is another big source of nitrogen input to the ecosystem (Carpenter et al., 1998). Phosphorus (P) is an essential nutrient element for crop production and livestock agriculture. The balance in P inputs in feed and fertilizer and P output in farm produce has been disrupted due to the rapid growth and intensification of crop and livestock farming in many areas. Surface runoff with P was accelerated by soil P building up due to excessive use of P fertilizer (Sharpley et al., 2003). With the rapid development of AD technology, large amount of AD effluent are being produced and the traditional disposal of AD effluent as agricultural fertilizer will also cause human-induced eutrophication.

Eutrophication as a serious environmental pollution problem has become more
widespread since the mid-20th century (Rodhe, 1969). According to the survey conducted by the International Lake Environment Committee, 48% of lakes and reservoirs in North America are eutrophic; in Asia & the Pacific, 54%; in Europe, 53%; in South America, 41%; and in Africa, 28% (ILEC and Lake Biwa Research Institute, 1993). The adverse ecological impacts caused by eutrophication can be categorized based on three aspects: (1) reducing the biodiversity and replacing the dominant species, (2) increasing the water’s toxicity, and (3) increasing the turbidity of the water and decreasing the lifespan of the lakes. The economic loss due to eutrophication in U.S. freshwaters was estimated to be $2.2 billion per year (Dodds et al., 2009). One specific example of freshwater eutrophication is Ohio’s largest inland lake, Grand Lake St. Marys (GLSM). Animal manure applied to the land in the lake’s watershed is one of the primary causes for nutrient overloading, while wastewater treatment plants surrounding the lake contribute 5–10% of the phosphorus load to the lake (the Ohio Departments of Natural Resources, 2010). The algal blooms in GLSM generate health concerns for the surrounding residents and directly affect the quality of drinking water, fishing and tourism industries which play a significant role in the local economy.

Eutrophication generally promotes the rapid growth of algae and plankton, which is known as algae blooms. Algae blooms are problematic in natural waters because they cause a severe reduction in water quality. Dissolved oxygen content and pH of the water are two of the major influences of algal blooms. The mass growth of algae during the day produces excessive oxygen and the water becomes supersaturated. While, at night, the algae consume most of the oxygen in the water and cause low dissolved oxygen levels, which may kill fish and other organisms in the water (Reid et al., 1990). Large algal
blooms can raise the pH of the water as high as 9.5 and many natural processes occurring in the water are affected as a result (Moore et al., 1997). Algal bloom takes place when one or more species of phytoplankton reproduces at a rapid rate, multiplying quickly in a short time, which leads to visible discoloration (most often yellow, green, blue-green, red or brown) of the water. Phytoplanktons are photosynthetic microscopic organisms that live beneath the surface of almost all oceans and bodies of fresh water. Phytoplanktons obtain energy from sunlight through the process of photosynthesis. Blue-green bacteria (called cyanobacteria) is one group of organisms that belong to the phytoplankton community and it is commonly found in algal blooms (Diersing, 2009). Many species of the bloom-forming cyanobacteria produce toxic secondary metabolites (cyanotoxins) and pose a potential risk to public health (Paerl et al., 2001). Therefore, research has been focused on the study of cyanotoxins. With the world-wide occurrence of harmful cyanobacterial blooms, there are more and more studied of cyanotoxins going on in universities or research institute (Jaiswal et al., 2008). Cyanotoxins can be categorized into three groups: hepatotoxin, neurotoxin and dermatoxin. Microcystins and nodularins, which belong to hepatotoxin, are two most common cyanotoxins and they have many structural variants (Rinehart et al., 1994).

There are about 2,000 species of cyanobacteria known and they are divided into 150 genera (Pulz and Gross, 2004). Not all cyanobacteria are toxic. Some species of cyanobacteria can produce very high value bio-products and have been used in food supplements, pharmaceuticals and cosmetics (Gantar and Svircev, 2008). *Spirulina*, also known as *Arthrospira*, contains a high amount of essential amino acids, vitamins, and fatty acids, and thus can be used as nutritional products or animal feed.
Immunomodulator produced by *Lyngbya majuscule* has potential use in pharmaceuticals (Skulberg, 2000). Many bioactive compounds isolated from cyanobacteria have been used to substitute synthetic chemicals in cosmetics (Singh et al., 2005). Another unique feature of cyanobacteria is their simple growth needs, which makes it a viable option for biofuel production. Biotechnologists have been working on the culture, screening, and genetic engineering techniques to exploit cyanobacteria for the cost-effective production of high-value products (Balasubramanian et al., 2012; Hallenbeck, 2012; Quintana et al., 2011; Brooks, 2010).

### 2.2 Cultivation of Cyanobacteria for Nutrient Removal and Biofuel Production

#### 2.2.1 Use of cyanobacteria for nutrients removal from wastewater

Previous experiments showed that cyanobacteria tend to grow better with high concentrations of phosphorus and low N/P ratio (Havens et al., 2003). According to data collected from lakes with various N/P ratios, Smith et al. drawn a conclusion that N: P mass ratio lower than 22: 1 will more probably stimulate cyanobacteria dominance (Smith, 1983; Smith et al., 1995). The reason for this phenomenon is that cyanobacteria are better at competing for nitrogen than other phytoplankton when N is scarce. Thus, when the supply of P increases, the concentration of N is relatively lower and N the limiting factor, which will make cyanobacteria the dominant species (Smith and Bennett, 1999). When grown in controlled environment such as ponds and reactors, many strains of cyanobacteria are shown ability to remove nitrogen and phosphorus from wastewater. Extensive research has been done on cyanobacteria due to their robustness and simple growth needs.

* Spirulina (Arthrospira) was cultivated in outdoor raceways under tropical
conditions to evaluate its ability to remove nutrients from anaerobic effluents of pig wastewater (Olguin et al., 2003). The results of semi-continuous test showed that the average annual biomass productivity of *Spirulina* is 11.8 g m\(^{-2}\) d\(^{-1}\), the ammonium nitrogen removal rate is 13.6 mg L\(^{-1}\) d\(^{-1}\), with removal efficiency ranging from 84% to 93%, and total phosphate removal rate were 72%-85%. Since the culture system is outdoor raceways, temperature and the depth of raceway ponds are two factors that have major influences on the productivity. Average productivity in semi-continuous cultures was higher in summer than in autumn or winter. The productivity was 14.4 g m\(^{-2}\) d\(^{-1}\) and 15.1 g m\(^{-2}\) d\(^{-1}\) when the depth was 0.15 m and 0.20 m, respectively. The protein content of biomass was not affect by season in batch test, but was affected by the time when the biomass was harvested during each season in semi-continuous test. The average protein content of the semi-continuous cultures was 48.9% ash-free dry weight. The ability of nutrients removal of cyanobacteria *Sprirulina* was demonstrated in another study in olive oil mill wastewater (OMWW). The OMWW was pretreated with sodium hypochlorite to decrease the phenol concentration and turbidity. Maximum biomass production (1.7 g L\(^{-1}\)) was obtained when the OMWW is loaded at a rate of 10% of total volume with the supplementation of 1 g L\(^{-1}\)NaNO\(_3\) and 5 g L\(^{-1}\) NaHCO\(_3\). It was observed that phenols, phosphorus and nitrogen were totally removed in 18-day batch test.
Table 2 Nutrient removal, biomass productivity and lipid content of various genera of cyanobacteria and microalgae in different waste streams

<table>
<thead>
<tr>
<th>Category</th>
<th>Genus and species</th>
<th>Waste stream</th>
<th>Reactor type</th>
<th>Retention time (d)</th>
<th>TN Initial conc. (mg L(^{-1}))</th>
<th>Removal efficiency (%)</th>
<th>TP Initial conc. (mg L(^{-1}))</th>
<th>Removal efficiency (%)</th>
<th>Biomass productivity (g L(^{-1}) d(^{-1}))</th>
<th>Lipid content (% dry cell weight)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanobacteria</td>
<td>Oscillatoria sp. c</td>
<td>Raceway</td>
<td>14</td>
<td>498</td>
<td>100</td>
<td>76</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Cragg et al., 1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c Photobioreactor</td>
<td>2-3</td>
<td>12-17</td>
<td>53-62(^*)</td>
<td>3-18</td>
<td>100</td>
<td>0.023-0.057</td>
<td>-</td>
<td>-</td>
<td>(Laliberte et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>Phormidium bohneri a</td>
<td>Photobioreactor</td>
<td>30</td>
<td>0.9-1.1</td>
<td>82(^*)</td>
<td>0.08-0.15</td>
<td>85</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Dumas et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>P. bohneri c</td>
<td>Photobioreactor</td>
<td>9</td>
<td>43-59</td>
<td>26-40(^*)</td>
<td>7.5</td>
<td>100</td>
<td>0.027-0.05</td>
<td>-</td>
<td>-</td>
<td>(Margarita Silva-Benavides and Torzillo, 2012)</td>
</tr>
<tr>
<td></td>
<td>Planktothrix isothrix c</td>
<td>Photobioreactor</td>
<td>14</td>
<td>43-59</td>
<td>26-40(^*)</td>
<td>7.5</td>
<td>100</td>
<td>0.027-0.05</td>
<td>-</td>
<td>-</td>
<td>(Laliberte et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>Spirulina sp. d</td>
<td>Raceway</td>
<td>-</td>
<td>-</td>
<td>84-96(^*)</td>
<td>-</td>
<td>72-87</td>
<td>1.44-1.51</td>
<td>-</td>
<td>-</td>
<td>(Olguin et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>Spirulina sp. b</td>
<td>Photobioreactor</td>
<td>28</td>
<td>167</td>
<td>100</td>
<td>20</td>
<td>100</td>
<td>0.1</td>
<td>7.1-16.9</td>
<td>-</td>
<td>(Markou et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>Spirulina platensis c</td>
<td>Raceway</td>
<td>15</td>
<td>2-3</td>
<td>96-100(^*)</td>
<td>18-21</td>
<td>87-99</td>
<td>5.32-7.42</td>
<td>8.1-11.2</td>
<td>-</td>
<td>(Phang et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>Synechococcus elongatus e</td>
<td>Photobioreactor</td>
<td>8</td>
<td>25.5</td>
<td>29-54(^*)</td>
<td>6.7</td>
<td>77-88</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Aguilar-May and del Pilar Sanchez-Saavedra, 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.48-7.67</td>
<td>100(^{**})</td>
<td>0.04-0.39</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Sawaya et al., 2012)</td>
</tr>
<tr>
<td>Chlorophyte</td>
<td>Botryococcus braunii, c</td>
<td>Flask</td>
<td>10</td>
<td>4.48-7.67</td>
<td>100(^{**})</td>
<td>0.04-0.39</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Sawaya et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>B. braunii, c</td>
<td>Photobioreactor</td>
<td>35</td>
<td>5.5</td>
<td>27.3(^{**})</td>
<td>0.08</td>
<td>62.5</td>
<td>0.4</td>
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<td>-</td>
<td>(Sawaya et al., 2012)</td>
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<tr>
<td></td>
<td>Chlorella sp. c</td>
<td>Photobioreactor</td>
<td>13-21</td>
<td>290</td>
<td>61(^{\dagger})</td>
<td>530</td>
<td>61</td>
<td>3.46</td>
<td>4.7-6.3</td>
<td>-</td>
<td>(Min et al., 2011)</td>
</tr>
<tr>
<td></td>
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<td>Photobioreactor</td>
<td>21</td>
<td>100-240</td>
<td>76-83</td>
<td>15-30</td>
<td>63-75</td>
<td>-</td>
<td>9-13.7</td>
<td>-</td>
<td>(Wang et al., 2010b)</td>
</tr>
<tr>
<td></td>
<td>Chlorella sp. d</td>
<td>Flask</td>
<td>15</td>
<td>70</td>
<td>90.5</td>
<td>180</td>
<td>92</td>
<td>1.38</td>
<td>-</td>
<td>-</td>
<td>(Wang et al., 2010a)</td>
</tr>
<tr>
<td></td>
<td>C. vulgaris a</td>
<td>Flask</td>
<td>30</td>
<td>1074</td>
<td>89.5</td>
<td>180</td>
<td>92</td>
<td>1.38</td>
<td>-</td>
<td>-</td>
<td>(Wang et al., 2010c)</td>
</tr>
<tr>
<td></td>
<td>C. vulgaris d</td>
<td>Flask</td>
<td>8</td>
<td>1722</td>
<td>93.6</td>
<td>111.6</td>
<td>89.2</td>
<td>0.4-0.76</td>
<td>-</td>
<td>-</td>
<td>(Heredia-Arroyo et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>C. vulgaris a</td>
<td>Photobioreactor</td>
<td>9</td>
<td>36.3</td>
<td>90-95(^*)</td>
<td>111.8</td>
<td>10-60</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Gonzalez et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>C. vulgaris b</td>
<td>Raceway</td>
<td>12</td>
<td>0.47-50.83</td>
<td>4.4-45.1(^*)</td>
<td>0.07-4.01</td>
<td>33.1-33.3</td>
<td>0.1-0.2</td>
<td>40</td>
<td>-</td>
<td>(Lim et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>C. kessleri e</td>
<td>Flask</td>
<td>12</td>
<td>129.6</td>
<td>81.1(^{\dagger})</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Lee and Lee, 2002)</td>
</tr>
<tr>
<td></td>
<td>C. kessleri e</td>
<td>Flask</td>
<td>3</td>
<td>168</td>
<td>8-19(^{**})</td>
<td>10-12</td>
<td>8-20</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Lee and Lee, 2001)</td>
</tr>
<tr>
<td></td>
<td>Chlamydomonas reinhardtii</td>
<td>Photobioreactor</td>
<td>31</td>
<td>128.6</td>
<td>55.8(^{\dagger})</td>
<td>120.6</td>
<td>17.4</td>
<td>2.0</td>
<td>25.25</td>
<td>-</td>
<td>(Kong et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>Scenedesmus sp. e</td>
<td>Photobioreactor</td>
<td>0.2-4.5</td>
<td>14-44</td>
<td>30-100</td>
<td>1.4-6</td>
<td>30-100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Zhang et al., 2008)</td>
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<tr>
<td></td>
<td>Scenedesmus sp. d</td>
<td>Photobioreactor</td>
<td>5</td>
<td>100</td>
<td>90(^{\dagger})</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
<td>(Park et al., 2010)</td>
</tr>
<tr>
<td></td>
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<td>Photobioreactor</td>
<td>15</td>
<td>15-15</td>
<td>83-99</td>
<td>0.2-1</td>
<td>99</td>
<td>0.15-0.65</td>
<td>30-53</td>
<td>-</td>
<td>(Xin et al., 2010b)</td>
</tr>
<tr>
<td></td>
<td>Scenedesmus sp. c</td>
<td>Flask</td>
<td>15</td>
<td>15.5</td>
<td>98.5</td>
<td>0.5</td>
<td>98</td>
<td>0.11</td>
<td>31-33</td>
<td>-</td>
<td>(Xin et al., 2010a)</td>
</tr>
<tr>
<td></td>
<td>S. obliquus c</td>
<td>Photobioreactor</td>
<td>8</td>
<td>2.7</td>
<td>79-100(^{\dagger})</td>
<td>12</td>
<td>47-98</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Ruiz-Marin et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>S. obliquus c</td>
<td>Photobioreactor</td>
<td>8</td>
<td>2.7</td>
<td>79-100(^{\dagger})</td>
<td>11.8</td>
<td>55-98</td>
<td>0.024</td>
<td>27-34</td>
<td>-</td>
<td>(Martinez et al., 2000)</td>
</tr>
</tbody>
</table>

* Ammonia nitrogen (NH\(_4^{+}\)-N), ** Nitrate (NO\(_3^{-}\)-N), Nitrite (NO\(_2^{-}\)-N), \(^{\dagger}\) Total Kjeldahl nitrogen

a. Animal wastewater; b. Industrial wastewater; c. Municipal wastewater; d. AD effluent; e. Artificial wastewater
The nitrogen and phosphorus removal ability of *Planktothrix isothrix* was studied in municipal wastewater (Margarita Silva-Benavides and Torzillo, 2012). The effects of mixing condition (shaking or not) and irradiance (20 and 60 μmol photons m$^{-2}$ s$^{-1}$) on growth of *Planktothrix isothrix* were tested. *Planktothrix isothrix* co-cultured with *Chlorella* had the highest growth under unshaken condition, which corresponded to 80% nitrogen removal and 100% phosphorus removal. Due to the small cell size of the microalgae, harvesting technique is one of the bottlenecks for large scale production of algal biofuel (Singh and Dhar, 2011). Filamentous cyanobacteria with the ability of self-aggregation and sedimentation could help reduce the cost of harvesting process and offer an attractive option for large-scale cultivation of cyanobacteria for biomass production and wastewater bioremediation (Noüe and Proulx, 1988). Co-cultivation of the *Planktothrix* and the unicellular *Chlorella* under unshaken condition could take advantage of the self-aggregation ability of filamentous *Planktothrix* and also make full use of the space in wastewater column because *Planktothrix* and *Chlorella* inhabit in different depth of wastewater.

*Oscillatoria* is a genus of marine filamentous cyanobacterium that inhabiting near-surface waters of tropical and subtropical seas and it is capable of fixing atmospheric nitrogen (Carpenter and Price, 1976). Cultivation of *Oscillatoria* in municipal wastewater diluted with seawater was performed in corrugated raceway ponds by Craggs et al.(1997) to evaluate its ability to removal ammonium nitrogen and orthophosphate in both batch and continuous experiments. Complete removal of ammonium and orthophosphate from wastewater diluted by seawater with 1:1 ratio was obtained in continuous running. No extra mixing was needed in corrugated raceway
system. The adherent property of *Oscillatoria* also simplified the biomass harvesting mechanism.

*Synechococcus* is a widespread unicellular cyanobacterium in the marine environment and makes important contributions to global carbon fixation (Scanlan, 2003). *Synechococcus* has been extensively studied since its discovery and identification because of its beneficial features: 1) it is non-toxic; 2) it is able to removal nitrogen, phosphorus and even heavy metals very efficiently; 3) it has high tolerance to a wide range of temperature; and 4) it produces high-value products such as glutamate and hydrogen (Sasikala and Ramana, 1994; Matsunaga et al., 1988; Ikeya et al., 1997). Aguilar-May et al compared the growth rate and nutrients removal rate of chitosan-immobilized and free cells of *Synechococcus elongatus* in artificial wastewater (Aguilar-May and del Pilar Sanchez-Saavedra, 2009). It appeared that immobilized cell cultures had a lag phase of growth due to the immobilization method, but their growth rate was similar to that of free-living cell cultures. Free cells were more efficient in ammonia and phosphorus removal than immobilized cells, but immobilized cells has higher protein content.

### 2.2.2 Bioenergy products from cyanobacteria

Cyanobacteria are a special group of photosynthetic bacteria that are able to convert sunlight, H₂O and CO₂ into carbohydrates, lipids and proteins, all of which are potential feedstock for bioenergy production. The lipids can be converted into fuels by chemical, biochemical, and thermochemical processes, or a combination of these approaches. Carbohydrates may be fermented under dark and anaerobic conditions to produce ethanol, with carbon dioxide as byproducts (Stal and Moezelaar, 1997).
Cyanobacteria may possess nitrogenase or hydrogenase that enables them to generate hydrogen gas, which is a potential alternative to limited fossil fuels (Hallenbeck, 2012). Methane is another bioenergy source that can be produced by anaerobic digestion of cyanobacterial cellular biomass (Varel et al., 1988). Four types of bioenergy (hydrogen, ethanol, diesel, and methane) that can be produced from cyanobacteria biomass are discussed in the following paragraphs.

Hydrogen (H₂) is a promising candidate as an ideal fuel because: first of all, it is clean, the combustion of hydrogen only produces water, no any carbon dioxide; secondly, it has high energy yield (122kJ g⁻¹) (Lay et al., 1999). Besides energy carrier, hydrogen also plays various roles in chemical synthesis and electrical storage. H₂ is used as a hydrogenating agent to increase the level of saturation of unsaturated fats and oils, and also used in the production of ammonia and methanol (Das and Veziroglu, 2001).

Currently, nearly 90% of hydrogen is produced from natural gas or light oil fractions with steam at high temperatures. Biological hydrogen production by photosynthetic microorganisms has several advantages over hydrogen production by water electrolysis, thermochemical processes and radiolytic processes. Biological hydrogen production can utilize renewable energy resources, such as the organic fraction of solid waste (Lay et al., 1999), and it is normally operated at ambient temperature and atmosphere pressure. A wide variety of species and strains have been examined for hydrogen production. Bandyopadhyay et al. (2010) proved that *Cyanothece* sp. ATCC 51142, a unicellular, diazotrophic cyanobacterium with the capacity to generate high levels of hydrogen under aerobic conditions, can produce hydrogen at rates as high as 465 μmol per mg of chlorophyll per hour in the presence of glycerol. Further research showed that
### Table 3 Bioenergy productivity of various species of cyanobacteria

<table>
<thead>
<tr>
<th>Fuel</th>
<th>Species</th>
<th>Productivity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td><em>Oscillatoria</em> sp.</td>
<td>400 μmol L⁻¹ d⁻¹</td>
<td>(Heyer and Krumbein, 1991)</td>
</tr>
<tr>
<td></td>
<td><em>Synechococcus</em> sp.</td>
<td>54 nmol L⁻¹ d⁻¹</td>
<td>(Deng and Coleman, 1999)</td>
</tr>
<tr>
<td></td>
<td><em>Synechocystis</em> PCC6803</td>
<td>5.2 mmol L⁻¹ d⁻¹</td>
<td>(Dexter and Fu, 2009)</td>
</tr>
<tr>
<td>Hydrogen</td>
<td><em>Anabaena variabilis</em></td>
<td>6.91 nmol μg⁻¹ of protein h⁻¹</td>
<td>(Sveshnikov et al., 1997)</td>
</tr>
<tr>
<td></td>
<td><em>Cyanothece</em> sp.</td>
<td>465 μmol mg⁻¹ of chlorophyll h⁻¹</td>
<td>(Bandyopadhyay et al., 2010)</td>
</tr>
<tr>
<td></td>
<td><em>Spirulina maxima</em></td>
<td>400 μmol L⁻¹ h⁻¹</td>
<td>(Ananyev et al., 2008)</td>
</tr>
<tr>
<td>Lipids</td>
<td><em>Synechococcus</em> sp.</td>
<td>75 mg L⁻¹ d⁻¹</td>
<td>(Griffiths and Harrison, 2009)</td>
</tr>
<tr>
<td></td>
<td><em>Nostoc paludosum</em></td>
<td>1.9 g m⁻² d⁻¹</td>
<td>(Vargas et al., 1998)</td>
</tr>
<tr>
<td></td>
<td><em>Synechocystis</em> PCC6803</td>
<td>0.133 g L⁻¹ d⁻¹</td>
<td>(Liu et al., 2010)</td>
</tr>
<tr>
<td></td>
<td><em>Synechocystis</em> PCC6803</td>
<td>6.4 nmol L⁻¹ d⁻¹</td>
<td>(Kaczmarzyk and Fulda, 2010)</td>
</tr>
<tr>
<td></td>
<td><em>Synechococcus</em> sp.</td>
<td>8.4 nmol L⁻¹ d⁻¹</td>
<td>(Kaczmarzyk and Fulda, 2010)</td>
</tr>
<tr>
<td>Methane</td>
<td><em>Spirulina</em> maxima</td>
<td>0.33 L CH₄/g VS</td>
<td>(Varel et al., 1988)</td>
</tr>
<tr>
<td></td>
<td><em>S. maxima</em></td>
<td>0.09 L CH₄/g VS</td>
<td>(Inglesby and Fisher, 2012)</td>
</tr>
<tr>
<td></td>
<td><em>S. maxima</em></td>
<td>0.63–0.74 L CH₄/g VS</td>
<td>(Sialve et al., 2009)</td>
</tr>
<tr>
<td></td>
<td><em>S. platensis</em></td>
<td>0.47–0.69 L CH₄/g VS</td>
<td>(Sialve et al., 2009)</td>
</tr>
</tbody>
</table>
Cyanothece 51142 is able to use high concentrations of CO$_2$ or glycerol for enhanced H$_2$ production, making biohydrogen production by *Cyanothece* 51142 an attractive option because both of these carbon sources are abundantly available as industrial waste products. *Anabaena* species and strains also have high rate of hydrogen production according to Sveshnikov et al. (1997). Two mutants of *Anabaena variabilis* ATCC 19413 were studied and the results showed that mutants deficient in uptake and reversible hydrogenases have higher rates of H$_2$ production compared with wild-type strains. Mutant *A. variabilis* PK84 can produce H$_2$ by rate of 6.91 nmol nmol/μg of protein/h in 350 ml cultures, which is more than 4 times of that of the wild type. Nitrogen and CO$_2$ starvation could improve the H$_2$ production by 1.8-1.9 and 1.4-1.5 times, respectively.

Bioethanol produced from renewable resources is another promising biofuel and it has been used as vehicle fuel to reduce the use of fossil fuels in many countries. The amount of ethanol fuel produced from sugarcane in Brazil and corn in U.S. accounts for 87.1% of the world’s total ethanol fuel production in 2011 (Cuellar, 2012). However, the use of agricultural crops such as sugarcane and corn for ethanol production remains controversial as it competes with world’s food supply (Rittmann, 2008). Cyanobacteria can secrete glucose and sucrose to the media and the anaerobic fermentation of these sugars under dark conditions produces ethanol (Parmar et al., 2011). The advantage of using cyanobacteria for ethanol production is that the fermentation process can be performed by cyanobacteria themselves without addition of yeast as that in the fermentation of traditional agricultural crops. Various strains have been screened and evaluated for ethanol production. Heyer and Krumbein (1991) examined 37
cyanobacterial strains to test their ability of fermentation and secretion of fermentation products. Each strain was able produce at least one fermentation product, but only 16 strains could produce ethanol and only two *Oscillatoria* strains could secrete remarkable amounts of ethanol (>10 μmol/sample). Normally, cyanobacteria only produce a small amount of energy through fermentation to maintain cell activities. Genetic modification has been applied in order to increase the ethanol production of cyanobacteria.

*Synechococcus* sp. strain PCC 7942, a unicellular freshwater cyanobacterium, is one of the few cyanobacterial strains that have been relatively well-characterized in terms of physiology, biochemistry, and genetics. Deng and Coleman (1999) successfully transformed *Synechococcus* sp. strain PCC7942 with bacterial genes in order to create a novel pathway for ethanol production in this cyanobacterium. Ethanol is usually excreted to the medium by algae and cyanobacteria under dark and anaerobic conditions (Heyer and Krumbein, 1991). The new pathway created for ethanol synthesis in *Synechococcus* sp. strain PCC7942 functions during oxygenic photosynthesis and does not require anaerobic environment. It was anticipated that ethanol production at industrial scale could be achieved with the optimization of growth conditions and the development of ethanol retrieval or sequestering technologies. Besides sugars, cellulosic material is another substrate for ethanol production. Some cyanobacteria can also excrete cellulose to the media and provide feedstock for ethanol production. For example, *Crinalium epipsammum*, a filamentous cyanobacteria isolated from the surface layer of sandy soil of coastal dunes in the Netherlands was observed to produce cellulose of more than 25% of its dry cell weight, which is unusual for cyanobacteria (De Winder et al., 1990). Genetic
modification with the cellulose synthase genes from *Gluconobacter xylinus* enabled *Synchococcus* sp. PCC 7942 to produce extracellular non-crystalline cellulose, which is an ideal feedstock for ethanol production (Nobles and Brown, 2008).

Compared with microalgae or crops, less attention has been placed on cyanobacteria for production of lipid-based biofuels because their lipid content is usually lower than most microalgae (Parmar et al., 2011). However, the fast growth rate of cyanobacteria during exponential phase could also lead to high lipid productivity (Sheehan et al., 1998). Among the 2000 species of cyanobacteria identified, only a few have been examined for biodiesel production and limited information regarding the lipid content and production, or lipids profile is available (Miao and Wu, 2006). The lipid content of cyanobacteria is usually between 5%-15% (Quintana et al., 2011). Griffiths and Harrison (2009) reviewed the information about growth rates, lipid content and lipid productivities for 55 species of microalgae, including 17 chlorophyta and 5 cyanobacteria as well as other taxa and found that although the lipid content of most microalgae can be increased under nitrogen starvation, the lipid content of cyanobacteria was hardly increased, with *Synechococcus* showing a relatively high lipid content (11% of dry cell weight) and lipid productivity (75 mg L$^{-1}$ d$^{-1}$). Vargas et al. (1998) analysed the biochemical composition and fatty acid content of twelve strains of filamentous, heterocystous, nitrogen-fixing cyanobacteria, which were all isolated from natural environments. The data showed that the lipid content of the 12 strains varied between 8% to 11% of the dry weight, and the protein and lipid level reached the highest during stationary phase. According to lipid profile analysis by gas chromatography–mass
spectrometry (GC-MS), total fatty acid levels in the strains assayed ranged between 3 and 5.7% of the dry weight. All strains showed high levels of polyunsaturated fatty acids (PUFAs) and saturated fatty acids (SAFAs), with values of 24–45% and 31–52% of total fatty acids, respectively.

_Synechocystis_ sp. PCC6803, a freshwater cyanobacterium, is one of the most extensively studied model organisms for the analysis of photosynthetic processes. Its genomic, biochemical and physiological date have been thoughtfully reported, offering valuable information for genetic modification for this strain (Huang et al., 2002). Liu et al. (2010) successfully applied a fatty acid secretion strategy in _Synechocystis_ sp. PCC6803 by introduced acyl-acyl carrier protein thioesterases into the cell. High levels of fatty acids could be produced and secreted into the medium by the mutant strains at an efficiency of up to 133 mg L⁻¹ of culture per day at a cell density of 1.5 × 10⁸ cells ml⁻¹ (0.23 g of dry weight/liter). The effects of growth temperature on fatty acid composition _Synechocystis_ PCC6803 were studied by Wada and Murata (1990). They found that the composition of the fatty acid did not change when the temperature kept constant. However, a shift from higher temperature to lower temperature will stimulate the desaturase activities of C₁₈ and C₁₆ acids, which is related to the photosynthetic electron transport. Many biotechnology companies are working on the gene transformation of cyanobacteria to increase their lipid content. It was reported that biologists from a Seattle-based bioscience firm Target Growth Inc. (TGI) increased the lipid content of cyanobacteria by about 400 percent (Roberts et al., 2011). However, they did not disclose the lipid content of the genetic modified cyanobacterium, which made it hard to compare
this cyanobacterium with other lipid-producing algae. ExxonMobil invested $600 million in Synthetic Geonomics for cyanobacteria based fuel development. BP Oil, the world’s third-largest energy company, also tries to explore biofuel from cyanobacteria with $500 million investment to support the cyanobacteria research in the Arizona State University's BioDesign Institute (Brooks, 2010). Joule Unlimited, a Massachusetts biotechnology company, also holds a key patent about genetically-engineered cyanobacteria that can simply secrete diesel fuel or ethanol when it is fed with sunlight, water and carbon dioxide (Woods et al., 2001). It is said that the cyanobacteria will produce ethanol in recoverable quantities of at least 1.7 mole ethanol per mg of chlorophyll per hour. The company claimed that they are able to produce 75,708 liters of fuel per acre per year and the product will be cost competitive with crude oil at $50 a barrel (Totty, 2012).

Methane, as a major component of biogas from anaerobic digestion (AD), is produced by the biological breakdown of organic matter. The biomass or biomass residue after lipids extraction from microalgae and cyanobacteria is another source of feedstock for anaerobic digestion. The conversion of microalgal and cyanobacterial biomass into methane also increases the energy recovery from the cellular biomass, enhancing their economic value. The methane yield and production rates of anaerobic digestion are affected by the feedstock characteristics, reactor design and operation conditions (Zhang et al., 2007). The biochemical composition (lipids, carbohydrates, protein) of the feedstock is a major factor that affects the microbial populations (hydrolytic, acetogenic, and methanogenic bacteria) in the AD system. However, it was confirmed that cyanobacterial biomass alone is not favorable for supporting methanogenic activity.
Varel et al. (1988) evaluated the methane production from fermentation of the cyanobacterium, *Spirulina maxima*, as the sole substrate. The ultimate methane yield of 0.33 L CH$_4$/g VS fed was obtained after 105 days of batch fermentation, which is significantly lower than that of cattle or swine wastes. The biomass of *Spirulina maxima* as the sole feedstock for an advanced flow-through anaerobic reactor was investigated for production of methane and the maximum methane yield is 90±19 ml CH$_4$ per g VS with a hydraulic retention time (HRT) of 10 days and an organic loading rate (OLR) of 0.5 g L$^{-1}$d$^{-1}$ (Inglesby and Fisher, 2012). There are mainly two factors that influence the methane yield of the anaerobic digestion of cyanobacterial biomass. First of all, the high protein content of algal and cyanobacterial biomass often results in the release of high amount of ammonia into the reactor, which negatively inhibits the microbial activities. The other factor is the high sodium concentrations brought by the marine species may have negative impact on the AD reactors (Sialve et al., 2009). Maintaining a proper C/N ratio by combining different substrates will increase the performance of the digester and improve the biogas productivity (Mata-Alvarez et al., 2000). Co-digestion of microalgal and cyanobacteria biomass with other feedstock is necessary to improve the health of the AD reactor and improve the methane yield consequently, making the microalgal biodiesel economically sustainable. When the C/N ratio of the substrate is lower than 20, Excessive nitrogen released in the form of ammonia will cause an accumulation of volatile fatty acids, inhibiting the microbial activities (Sialve et al., 2009). Park and Li (2012) evaluated the methane yield of the co-digestion of algae biomass residue (ABR) with lipid-rich fat, oil, and grease waste.
(FOG). The optimal methane yield of 0.54 L CH₄/g VS d was observed when the algae biomass residue was loaded at 50% loading rate, which also resulted in greater lipid degradation than that on the digestion of 100% FOG or 100% ABR. Yen and Brune (2007) studied the co-digestion of waste paper and algal sludge. The maximum methane yield (1.6 L CH₄/L d) was obtained when the algal sludge was mixed with waste paper at a ratio of 2:3. The optimal C/N ratio for co-digestion of algal sludge and waste water appeared to be 20-25 based on the results. Co-digestion of sewage sludge and *Spirulina maxima* was reported to increase the methane yield of digester by 2.1 fold (0.36 L CH₄/g VS) when the mixing ratio is 1:1 (Samson and Leduy, 1983). It was proved that the addition of sewage sludge to algal biomass helped balanced the C/N ratio and significantly increased the activity of the methanogenic bacteria, leading to the improvement of methane production.

### 2.3 Cultivation of Microalgae in Wastewater for Nutrient Removal and Biofuel Production

Compared with cyanobacteria, microalgae have gained more interests in the past few decades because of the dual benefits of cultivating microalgae for both nutrients removal and biofuel production. From 1978 to 1996, the Aquatic Species Program (ASP) funded by the U.S. Department of Energy (DOE) studies about 300 species, mostly green algae and diatoms, focusing on their ability to produce oils under normal and severe conditions such as extreme temperature, salinity or pH, in the meanwhile, utilizing waste CO₂ from coal fired power plants (Sheehan et al., 1998). The feasibility of large-scale algae production in open ponds was also tested in this program. Later, research focus
shifted to both biodiesel production and wastewater bioremediation by microalgae to enhance the sustainability of microalgal cultivation system (Figure 1). Various species of microalgae have been studies in different sources of wastewater and their nutrients removal efficiency was also reviewed by numerous researchers. So far, the bottlenecks of large-scale algae cultivation system lie in the downstream processing such as harvesting, lipids extraction process and biodiesel conversion process. The energy cost for those processes need to be balanced with the energy output of algae biofuel to make it economically viable. A complete life cycle analysis of the algae cultivation system is still needed to evaluate the sustainability of algal biofuel production.

Figure 1 Algae cultivation in wastewater for bioremediation and biofuel production
2.3.1 Nutrients requirement of microalgae growth

The growth of microalgae needs a variety of micro-/macro- elements which not only constitute algae cells, but also help maintain their metabolism system. Except the four basic elements, i.e., carbon, nitrogen, phosphorus and sulfur, ionic components such as sodium, potassium, iron, magnesium, calcium, and trace elements must be provided in algae cultivation (Richmond, 1986). Silicon is required for the cultivation of certain algae strains (Andersen, 2005). The concentration of any nutrient element could be a limiting factor for algae growth. Carbon is always supplied to the cells during cultivation by pumping air or pure CO₂, or flue gas into the cultures. Nitrogen to phosphorus ratio is a mainly concerned factor when analyzing the nutrients compositions of the culture medium. It has been pointed out that the optimal nitrogen to phosphorus ratio for algae is 16:1 (Stumm and Morgan, 1970). Therefore, adjustment of nutrients concentrations may be needed in order to match the proper ratio. Most of the wastewaters contain the major nutrient components for algae growth. According to different sources, the composition of wastewater will vary significantly.

According to their ability of using organic or inorganic carbon source, microalgae are divided into three categories: autotrophic, heterotrophic, and mixotrophic. Currently, all of the three types of microalgae are being studied for biofuel production. Mixotrophic microalgae are preferred because they can utilize both organic carbons in the wastewater and CO₂ from different resources. Microalgae are able to fix CO₂ from atmosphere, industrial exhaust gases, and soluble carbonates (HCO₃⁻, CO₃²⁻) (Wang et al., 2008).
Mitigation of CO₂ from flue gas is another research focus of algae which would bring dual benefits to the environment and biofuel production (Zeiler et al., 1995).

Nitrogen is the second most important nutrient after carbon for microalgae and a number of nitrogen compounds are available for algae to assimilate: nitrate, nitrite, ammonium, and organic nitrogen including amino acid, peptides, and proteins. Among them, ammonium is the most preferable chemical form because it takes less energy for algae to assimilate into amino acid (Y. Collos, 1986). Ammonium tolerances of different algae species varies from 25 µmol N/L to 1000 µmol N/L (Y. Collos, 1986). Studies show that nitrogen concentration in the culture medium affects the speed of lipids accumulation by microalgae. Nitrogen starvation will increase lipids content of microalgae. However, the total lipids yield is usually offset by the low biomass yield under nitrogen starvation condition (Sheehan et al., 1998). Therefore, the nitrogen concentration in the medium is critical for optimal lipids production. Ammonia stripping is an undesirable phenomenon in algae cultivation ponds using wastewater. Studies showed that significant amount of ammonia will escape to the atmosphere when the pH is higher than 9 (Konig et al., 1987). Temperature is also a key factor affecting the speed of ammonia stripping. Huge surfaces of algae ponds exposing to warm weather will accelerate ammonia release even when the pH is below 9 (Green et al., 1996).

Inorganic phosphates play a significant role in algae cell growth and metabolism (Theodorou et al., 1991). P in the forms of H₂PO₄⁻ and HPO₄²⁻ are preferred inorganic phosphates for algae growth. The consumption rate of P is affected by not only the concentration of P in the medium and the growth phase of algae, but also the pH, the
concentrations of other ions such as Ca$^{2+}$, Mg$^{2+}$, and temperature (Martínez et al., 1999). In most cases, addition of P is needed for algae growth because of the precipitation of phosphates in culture medium. Excessive phosphates added that were not utilized may cause eutrophication if not processed properly before discharged, which is a big issue for microalgae cultivation in wastewater (Yun et al., 1997).

Excessive P is the most common cause of eutrophication in freshwaters (Correll, 1998). The amount of P is always more limiting than other essential elements because C, N can be obtained from the atmosphere. However, human activities contribute a lot to the increase of P concentration in receiving water streams. The concentrations of P higher than 20µg/L will be big problem for most waters (Correll, 1998).

Silicon is generally considered as one kind of macronutrients needed for growth of diatoms, silicoflagellates, and some chrysophytes (Andersen, 2005). Silicate is added as Na$_2$SiO$_3$·9H$_2$O. However, it is better to omit it from the medium if the species do not require silicate because silicic acid will enhance precipitation of some other essential elements. Microalgae growth depends not only on an adequate supply of essential macronutrients (C, N, P, Si) and major ions (Na$^+$, K$^+$, Mg$^{2+}$, Ca$^{2+}$, Cl$^-$, SO$_4^{2-}$) but also on a number of micronutrients (iron, manganese, zinc, cobalt, copper, molybdenum, selenium, etc.). But, many of the micronutrients are toxic to most algae species at high concentrations. Some of them also form precipitations with other essential elements and reduce their availability. However, some algae strains are particularly tolerant to heavy metals and their potential to absorb metals was demonstrated in many studies (Mehta and Gaur, 2005).
2.3.2 Cultivation of microalgae in wastewater for nutrients removal and lipid production

As reviewed in the first section, most wastewater contains carbon, nitrogen, phosphorus and other essential elements that support the growth of microalgae. Tremendous efforts have been put on research of microalgae cultivation in wastewaters in the past decades. Studies showed positive results regarding the potential of utilizing microalgae to remove nitrogen, phosphorus as well as heavy metals from wastewaters. However, different species varied in the ability of nutrients removal and lipid production. *Chlorella* is a genus of single-cell green algae which belongs to the phylum Chlorophyta and it is one of the most extensively studied species for wastewater bioremediation and lipid production (Kessler, 1976). Wang et al. (2010b) studied the growth of a wild-type *Chlorella* sp. in municipal wastewaters collected from different points of the treatment process flow of a municipal wastewater treatment plant. It was found that this algae strain grew best in the centrate because of its much higher levels of nitrogen, phosphorus, and COD than the other three wastewaters. A specific growth rate of 0.948 d\(^{-1}\), total nitrogen removal rate of 82.8\% and total phosphorus removal rate of 85.6\% were observed in centrate during 10-day batch cultivation. Min et al. (2011) further investigated the growth of this wild strain *Chlorella* sp. in centrate in a pilot-scale photobioreactor (PBR). The effects of different harvesting ratio and exogenous CO\(_2\) levels on the biomass productivity of *Chlorella* sp. were tested in the pilot-scale test. The system was able to produce algal biomass at a rate of 34.6 g m\(^{-2}\) d\(^{-1}\) under low light conditions (25 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) and a one fourth harvesting rate, which corresponded to 70\% chemical oxygen demand, 61\% total Kjeldahl nitrogen, and 61\% soluble total phosphorus.
reduction. However, the addition of CO₂ to the system did not significantly increase the biomass productivity or nutrient removal in centrate because its organic carbon concentration was already enough for the growth of *Chlorella* sp.. Wang et al. (2010a) also tested anaerobic digested dairy manure as culture medium for microalgae *Chlorella* sp. The growth rate of *Chlorella* sp. was greatly affected by the dilution times of digested manure, with the highest growth rate of 0.409 d⁻¹ at 25 times dilution. Ammonia was completely removed in all of the four diluted samples during the 21-day batch test. However, the removal rate of total nitrogen, total phosphorus and COD varied with different diluted manures. The total fatty acid content of the dry biomass increased from 9% to 13.7% as the dilution multiples increased from 10 to 25. Based on the results of these batch tests, Wang et al. (2010c) moved on to the lab-scale semi-continuous cultivation of *Chlorella vulgaris* in undigested and digested dairy manure to further exploit the potential of *Chlorella vulgaris* for nutrient reduction and biomass production. It was shown that in order to achieve the same removal rate of ammonium nitrogen, *Chlorella* sp. grown in undigested dairy manure required a shorter hydraulic retention time (5 day) than that grown in digested dairy manure due to the fact that the undigested dairy manure had much higher organic carbon content which could support the growth of mixotrophic *Chlorella* sp.. Algae grown in digested dairy manure performed better in terms of CO₂ sequestration per milligram of harvested dried biomass (1.68 mg CO₂/mg dry weight (DW)) than that grown in undigested dairy manure (0.99 mg CO₂/mg DW). However, the higher biomass productivity was obtained with the undigested one. The advantage of mixotrophic cultivation of *Chlorella vulgaris* was also demonstrated by
other researchers. Heredia-Arroyo et al. (2011) studied the biomass and lipid production of *Chlorella vulgaris* under autotrophic, heterotrophic, and mixotrophic conditions with different substrates. The results showed that although the lipid content of *Chlorella vulgaris* grown under autotrophic and heterotrophic conditions are higher than that grown under mixotrophic condition, the higher biomass productivity of mixotrophic cultivation resulted in higher total lipid production. This distinct feature of *Chlorella vulgaris* makes it favorable for the treatment of carbon-rich wastewater for nutrients recovery and lipid production.

*Scenedesmus* is another green alga that has been widely used in wastewater bio-treatment. *Scenedesmus sp* is often single celled or colonial, forming 2- to 32-celled, usually 4- or 8-celled colonies. A parallel comparison study of the efficiency of ammonia and phosphorus removal by *Chlorella vulgaris* and *Scenedesmus dimorphus* was conducted using the secondary effluent from an agroindustrial wastewater of a dairy industry and pig farming (Gonzalez et al., 1997). *S. dimorphus* was shown to be more efficient in removing ammonia than *C. vulgaris*. However, they both were able to remove all ammonia from the wastewater and showed similar phosphorus removal efficiency by the end of 9-day batch experiment. The growth rate and nutrients removal efficiency of *Scenedesmus* sp. LX1 in liquor prepared from the effluent of an electronic device factory in batch and continuous mode was investigated by Zhen-Feng et al. (2011). The nitrogen concentration reduced from 35 mg L$^{-1}$ to 10 mg L$^{-1}$ and the phosphorus concentration reduced from 2.0 mg L$^{-1}$ to 0.25 mg L$^{-1}$, with a specific algal growth rate of 0.09 d$^{-1}$. However, the algal growth rate decreased to 0.02 d$^{-1}$ under continuous inflow of 60 L h$^{-1}$,
which also led to lower nitrogen and phosphorus removal efficiency. It was noticed that the accumulated metal ions in the effluent inhibited the algal growth, thus reducing the bio-treatment efficiency of wastewater.

Although N to P atomic ratio (N: P) of natural phytoplankton is about 15, it was reported that *Scenedesmus* sp. requires an N/P ratio of approximately 30 to grow without limitation by either nutrient (Rhee, 1978). In the study of effects of nitrogen and phosphorus concentrations on growth, nutrient uptake, and lipid accumulation of a *Scenedesmus* sp. LX1 by Li et al. (2010b), it was also proved that the nutrient uptake ratios (N/P) is not consistent with the N/P ratio in empirical elementary composition in microalgal cells. The authors inferred that *Scenedesmus* sp. LX1 has the ability to over-uptake nitrogen or phosphorus, when the N/P ratio in growth medium is the atomic ratio of algae cell. For example, at N/P ratio of 2:1 and 4:1 in growth medium, the nutrient uptake ratios (N/P) were around 2:1 and 4:1, respectively, after 13 days of cultivation, indicating an over-uptake of phosphorus in microalgal cells. At N/P ratio of 12:1 and 20:1, the nutrient uptake ratios (N/P) were about 10:1 and 9:1, respectively, indicating the over-uptake of nitrogen. The results showed that when *Scenedesmus* sp. LX1 was grown in an environment with N/P ratio of 5:1–12:1, 83–99% nitrogen and 99% phosphorus could be removed. Although unbalanced N/P ratio will cause insufficient nutrient removal from medium, the lipid content of algal biomass could increase under nutrient limitation, which has been testified for many algae species, including *Scenedesmus* sp.. The lipid content of *Scenedesmus* sp. LX1 could reach 53% of dry cell weight under phosphorus limitation (0.1 mg L⁻¹) (Xin et al., 2010b).
There are many other microalgae species which are able to remove nutrients from wastewater effectively. *Chlamydomonas* is a unicellular green alga that is typically spherical to subspherical with two flagella and it is widespread in freshwater (Algaebase,). Kong et al. (2010) cultivated *Chlamydomonas reinhardtii* in municipal wastewater for nutrients removal and lipid production in biocoil photobioreactor. The maximum algal biomass yield was 2.0 g L$^{-1}$ d$^{-1}$ in the 10-day batch cultivation, corresponding to reduction of 55.8 mg nitrogen and 17.4 mg phosphorus per liter per day. The optimal growth pH for *C. reinhardtii* is found to be around 7.5 and the lipid content of dry cell weight is about 25%. It was demonstrated that a proper amount of external inorganic carbon supply through injection of CO$_2$ could boost algae biomass production. Sawayama et al. (1992) found that *Botryococcus braunii*, a hydrocarbon-rich green microalga, was able to remove phosphorus, nitrate and nitrite effectively, but not ammonium, when grown in secondarily treated sewage from domestic waste-water in batch experiment. Ammonium was reported to be inhibitory to cell growth in this particular culture. Later in the continuous cultivation system, the growth rate of *Botryococcus braunii* was maintained at 196 mg dry weight/L per week for one month. However, the nitrate removal rate (27%) and phosphorus removal rate (62.5%) was lower compared with that in batch system (both are close to 100%).

*Nannochloropsis* is a genus of yellow green algae of the family Monodopsidaceae that is less than 5 μm in its maximum dimension (Hibberd, 1981). Members of this genus include 6 species: *N. gaditana, N. granulate, N. limnetica, N. oceanica, N. oculata, N. salina*. (Hibberd, 1981; Andersen et al., 1998). Among these various species,
Nannochloropsis salina is attractive to us because it is marine algae and has a relatively high biomass and lipid productivity (Emdadi and Berland, 1989; Boussiba et al., 1987). Therefore, it is a promising candidate for biofuel production. Additionally, Nannochloropsis salina is mixotrophic which means it can not only uptake the organic carbon in the ADE, but also perform photosynthesis to sequence carbon oxide in the air or flue gas (Huerlimann et al., 2010; GonenZurgil et al., 1996; Zittelli et al., 1999), which is helpful to reduce greenhouse gas in the air. Most of previous studies on Nannochloropsis focused on lipid content and compositions to evaluate its nutritional components such as EPA, DHA and GLA (Emdadi and Berland, 1989; Zou et al., 2000; Zou and Richmond, 1999; Hu et al., 1997; Christian et al., 2009). For N. salina, studies showed that there is no lipid accumulation during the exponential growth phase. Instead, the lipid content could be as high as 50% of dry weight during the stationary phase (Emdadi and Berland, 1989). The biomass productivity of N. salina could reach 24.5 g m⁻² d⁻¹ in outdoor ponds (Boussiba et al., 1987) and its lipid content could be enhanced up to 70% of dry weight by nitrogen starvation (Shifrin and Chisholm, 1981). However, high lipid content is often offset by low growth rate, which leads to low overall oil productivity (Huerlimann et al., 2010; Rodolfi et al., 2009). Therefore, for mass continuous algae cultivation, it is important to determine an efficient feeding frequency and harvesting ratio according to the growth rate and lipid content of algae in order to achieve maximum overall lipids production.
Chapter 3 Cultivation of *Nannochloropsis salina* in AD Effluent for Nutrient Removal and Lipid Production

The growth, lipid productivity, and nutrient removal of *Nannochloropsis salina* in anaerobic digested municipal wastewater were evaluated in this study. Results from batch reactors showed that *N. salina* was able to grow well under 3, 6, 12, and 18% AD effluent loading with the highest growth rate being 0.645 d\(^{-1}\). The growth of *N. salina* was inhibited when the effluent loading increased to 24%. The nutrient removal efficiency and lipid content of *N. salina* decreased as the effluent loading increased. The highest biomass yield (0.92 g L\(^{-1}\)) was obtained with 6% effluent loading, while the highest lipid productivity (29.2 mg L\(^{-1}\) d\(^{-1}\)) occurred in 3% effluent loading. Three harvesting frequencies (1-, 2-, and 3-day interval) and two harvesting ratios (25%, 50%) were tested in semi-continuous reactors and the highest lipid productivity (38.7 mg L\(^{-1}\) d\(^{-1}\)) was achieved with a 2-day harvesting interval and 50% harvesting ratio, where nitrogen and phosphorus were removed at a rate of 35.3 mg L\(^{-1}\) d\(^{-1}\) and 3.8 mg L\(^{-1}\) d\(^{-1}\), respectively.

3.1 Introduction

Anaerobic digestion (AD) is a technology to decompose organic matter under oxygen-free conditions and involves a variety of anaerobic microorganisms. The final products of AD are biogas (a mixture of methane (40–70% of total biogas volume) and CO\(_2\)) and liquid effluent (rich in nitrogen and phosphorus with total solids less than
10%). The technology is implemented in the treatment of agricultural wastes, food wastes, and wastewater sludge to reduce the pollution and produce bioenergy. Due to its nitrogen and phosphorus content, the effluent is typically used as a fertilizer or composted and used as a soil amendment. However, the use of the effluent as a fertilizer may be limited due to an insufficient amount of farmland within the economic transportation distance for the amount of effluent produced. Thus, the need for cost effective technologies for removing nitrogen and phosphorus from the effluent or for alternative uses remains a challenge for the AD industry.

Researchers have been working on nutrient removal and recovery from AD effluent by chemical and physical methods. Lei et al. (2007) successfully applied ammonium (NH$_4^+$) stripping on AD effluent and the removal rates for nitrogen, phosphorus, chemical oxygen demand (COD), and suspended solids were 78%, 99.9%, 82.1%, and 91%, respectively. This method requires a large amount of calcium hydroxide and it is suggested that the remained precipitates after NH$_4^+$ stripping be composted and can be used as a fertilizer. The method of struvite (MgNH$_4$PO$_4$) crystallization is effective for phosphorus removal from wastewater but not for other nutrients (Battistoni et al., 1997; Song et al., 2011). Maekawa et al. (1995) investigated nutrient removal and recovery from wastewater using an intermittent aeration batch reactor followed by an ammonium (NH$_4^+$) crystallization process and obtained a removal efficiency of 91% for total nitrogen (TN) and 99% for total ammonium nitrogen (TAN). Membrane filtration is one of the most important and commonly employed technologies for the purification of wastewater and effluent streams because of its high removal efficiency and water recovery capacity (Ahmad et al., 2005; Fan et al., 2008). Suspended solids,
microorganisms, and macromolecules in AD effluent can be removed by microfiltration or ultrafiltration, while nanofiltration and reverse osmosis can be applied for the removal of smaller organic molecules (Waeger et al., 2010).

The use of microalgae to remove nutrients from AD effluent has been recently studied and is argued to be effective (Wang et al., 2010a; Phang et al., 2000; Sooknah and Wilkie, 2004; Kebede-Westhead et al., 2003). The capability of microalgae to remove nitrogen and phosphorus from the AD effluent depends on a variety of factors, such as the species, feed concentration, temperature, and pH. Wang et al. (2010a) reported the effectiveness of using digested dairy manure as a nutrient supplement for the cultivation of microalgae *Chlorella* sp. The digested manure was diluted before being fed to the microalgae. The removal rates of TAN, TN, and total phosphorus (TP) varied with the dilution ratio. Biomass yield and total fatty acid content of the dry algal biomass also changed with the increasing dilution ratio. Park et al. (2010) used *Scenedesmus* sp. to remove NH$_4^+$ from AD effluent of livestock waste. Their study showed that *Scenedesmus* sp. can tolerate up to 100 mg L$^{-1}$ NH$_4^+$. Previous studies on *Nannochloropsis* mostly focused on the lipid content and composition related to its nutritional components such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and gamma-linolenic acid (GLA) (Emdadi and Berland, 1989; Zou et al., 2000; Zou and Richmond, 1999; Hu et al., 1997; Christian et al., 2009). For *Nannochloropsis salina*, studies showed that the lipid content could be as high as 50% of the dry weight during the stationary phase (Emdadi and Berland, 1989). The biomass productivity of *N. salina* reached up to 24.5 g m$^{-2}$ d$^{-1}$ in outdoor ponds (Boussiba et al., 1987) and its lipid content was enhanced up to 70% of its dry weight by nitrogen.
starvation (Shifrin and Chisholm, 1981). However, high lipid content is often offset by low growth rate, which leads to low overall lipid productivity (Huerlimann et al., 2010; Rodolfi et al., 2009). Therefore, for continuous microalgae cultivation, it is important to determine an optimal feeding frequency and harvesting ratio according to the growth rate and lipid content of the microalgae in order to achieve maximum overall lipid production.

The purpose of this study was to test the feasibility of growing *N. salina* in AD effluent. The AD effluent was collected from a commercial anaerobic digester after the removal of solids using a centrifuge. The performance of *N. salina* was evaluated by comparing its growth rate and nutrient removal efficiency at different effluent loadings. Batch experiments were conducted to find the optimal effluent loading. The optimal harvesting ratio and frequency were determined via semi-continuous laboratory experiments in order to provide basic reference data for future pilot scale production.

### 3.2 Material and Methods

#### 3.2.1 AD effluent and algae inoculum cultures

Four liter AD effluent was collected from a commercial-scale wet anaerobic digester (KB Compost Services, Akron, OH, USA) coupled with a D5LL solid bowl decanter centrifuge (ANDRITZ AG, Graz, Austria). The feedstock for this digester was the municipal wastewater from the city of Akron. The centrifuge ran continuously at 3200 rpm. The effluent was kept in 4°C before use.

The marine microalga *N. salina* (CCAP 849/6) was obtained from Culture Collection of Algae and Protozoa (CCAP, Oban, Scotland). Cultures were cultivated in f/2 medium (Guillard and Ryther, 1962) containing the following ingredients: 0.075 g L\(^{-1}\) NaNO\(_3\), 0.00565 g L\(^{-1}\) NaH\(_2\)PO\(_4\)
2H\(_2\)O, 1 ml L\(^{-1}\) trace elements stock solution, and 1 ml
L^{-1}; vitamin mix stock solution. The minor ingredients in the trace element stock solution included Na$_2$EDTA, FeCl$_3$·6H$_2$O, CuSO$_4$·5H$_2$O, ZnSO$_4$·7H$_2$O, CoCl$_2$·6H$_2$O, MnCl$_2$·4H$_2$O, Na$_2$MoO$_4$·2H$_2$O, and biotin, while the vitamin stock solution contained cyanocobalamin (vitamin B$_{12}$), and thiamine HCl (vitamin B$_1$). These solutions were used in the media following recipes provided in the CCAP website (CCAP). Seed cultures were cultured under continuous light (200 µmol m$^{-2}$ s$^{-1}$) at 25±1°C.

3.2.2 Batch and semi-continuous cultivation of N. salina in AD effluent

Each reactor consisted of a 2-L glass bottle capped with a rubber stopper and stainless steel tubing with a 4.76 mm (0.188 in) inner diameter inserted as an air inlet for the reactor. A polypropylene tee barb was connected at the bottom of the reactor to evenly disperse the incoming air in a horizontal direction and prevent the microalgae from adhering to the reactor wall. The walls of the chamber were coated white to thoroughly distribute the illumination within. The 32-W GE F32T8-SPX50 fluorescent lamps (GE Lighting, Ravenna, OH, USA) were used to provide a constant photosynthetic photon flux of approximately 200 µmol m$^{-2}$ s$^{-1}$, measured by a BQM quantum meter (Apogee Instruments, Logan, UT, USA). Ambient air was provided at a pressure of 27.6 kPa (4.0 psi) through a custom-made polyvinyl chloride (PVC) manifold, which provided a constant air flow of 175 ml min$^{-1}$ to each reactor through clear PVC tubing.

The AD effluent was diluted before being fed to the microalgae. As shown in Table 4, loading ratios ranging from 3% to 24% were tested to find the optimal loading of effluent for microalgal growth. Deionized (DI) water was added to adjust the final working volume of the reactor to 1 L. The salinity of each reactor was adjusted to 20% with Instant Ocean$^\text{®}$ sea salt (Spectrum Brands, Madison, WI, USA). Microalgae were
inoculated to each reactor to have an initial optical density of 0.1. DI water was added daily to make up the water lost through evaporation before taking samples for analysis. By the end of each batch experiment, the microalgal biomass was obtained using a Sorvall RC 6 Plus centrifuge (Thermo Scientific, Waltham, MA, USA). The supernatant remaining after centrifugation was analyzed for the nitrogen and phosphorus content. TAN, TN, and TP removal efficiencies during the 10-day cultivation were calculated based on the initial and final concentrations of TAN, TN, and TP in the supernatant.

<table>
<thead>
<tr>
<th>AD effluent loading ratio (%)</th>
<th>TAN (mg L⁻¹)</th>
<th>TN (mg L⁻¹)</th>
<th>TP (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>68</td>
<td>80</td>
<td>11.43</td>
</tr>
<tr>
<td>6</td>
<td>137</td>
<td>160</td>
<td>22.86</td>
</tr>
<tr>
<td>12</td>
<td>273</td>
<td>320</td>
<td>45.72</td>
</tr>
<tr>
<td>18</td>
<td>409</td>
<td>480</td>
<td>68.58</td>
</tr>
<tr>
<td>24</td>
<td>546</td>
<td>640</td>
<td>91.44</td>
</tr>
</tbody>
</table>

In the semi-continuous experiments, the effects of harvesting frequency (1-, 2- and 3-day intervals) and harvesting ratio (25% and 50% of total volume) on algal biomass yield and nutrient removal were studied at a 6% effluent loading ratio (TN loading level of 160 mg L⁻¹). Table 5 shows the experimental design of the semi-continuous experiment. The reactors were replenished with fresh effluent medium after harvesting. For each reactor, the optical density of the culture and the nitrogen and
phosphorus concentration in the medium was analyzed daily. All experiments were carried out in duplicate and average values were reported.

Table 5: Experimental design of semi-continuous experiment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Harvesting frequency (d)</th>
<th>Harvesting ratio (%)</th>
<th>Hydraulic retention time (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>25</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>50</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>25</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>50</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>50</td>
<td>6</td>
</tr>
</tbody>
</table>

3.2.3 Analytical methods

The optical density peak of 440 nm was used to establish a relationship with cell concentration. A Biomate 3 spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA) was used to measure the absorbance of cells at 440 nm. Microalgal biomass was determined gravimetrically and was reported as its ash-free dry weight (AFDW). A 50 g sample was placed in a clean centrifuge tube and centrifuged at 10,000 rpm for 15 min. The supernatant was used for determining nutrient removal efficiencies. The precipitate was washed with 25 ml of 0.5 M NH₄HCO₃ solution and then centrifuged at 10,000 rpm for 15 min. The precipitate was washed again with 10 ml of 0.5 M NH₄HCO₃ and transferred to a clean, ignited, and tared porcelain crucible and dried in a Thelco Model...
18 oven (Precision Scientific, Chennai, India) at 95°C for 12 hours. After the sample was
dried to constant weight, it was cooled in a desiccator and weighed. Ash weight was
obtained by igniting the sample in an Isotemp muffle furnace (Fisher Scientific,
Dubuque, IA, USA) at 500°C for 4 hours. The AFDW was calculated as the difference
between the dry weight and the weight of the residual ash.

The concentrations of 13 elements (Na, Mg, Al, P, K, Ca, Fe, Mn, Ni, Cd, Cu, Zn,
and Mo) in the culture medium were measured using an inductively coupled plasma-mass
spectrometry (ICP-MS) unit 7500cx (Agilent Technologies, Santa Clara, CA, USA)
following US EPA method 6062A (EPA, 2007). Prior to ICP-MS analysis, the sample
was digested in a microwave accelerated reaction system (CEM Corporation, Matthews,
NC, USA) at 190°C for 10 minutes. The digested sample was diluted 50-fold and
analyzed via ICP-MS. TAN, TN, total phosphorus (TP), and chemical oxygen demand
(COD) were determined using a 3900 spectrophotometer (Hach Company, Düsseldorf,
Germany) coupled with a DRB200 dual block reactor (Hach Company, Düsseldorf,
Germany).

Lipid content of the algal biomass was analyzed using a modified version of Bligh
and Dyer’s method (Bligh and Dyer, 1959). Before extraction, the cells were disrupted
with a UP400S ultrasonic processor (Hielscher Ultrasonics, Teltow, Germany) and an
H22 titanium sonotrode with a 22 mm tip. The frequency of the ultrasound was 24 kHz
and the output was set at 100 W. Lipids were extracted by mixing chloroform-methanol
(2:1 v/v) with the samples in a proportion of 1:1. The samples were placed in a stoppered
test tube to which solvents were added. The tube was closed and shaken manually for 10
min. Water and methanol were added to give a final solvent ratio of chloroform:
methanol: water of 1:1:0.9. The mixture was mixed for 5 min and then left to settle overnight. The top layer of the mixture (water and methanol) was removed with a pipette. The biomass layer and the chloroform layer were filtered using Whatman No. 1 filter paper (GE Healthcare, Maidstone, UK). The tube and filter paper were washed with 30 ml of chloroform. The chloroform was evaporated using a rotary evaporator. The weight of the crude lipid obtained from each sample was measured using an electronic scale.

3.2.4 Statistical analysis

Statistical significance was determined by analysis of variance (ANOVA) using SAS software (Version 8.1, SAS Institute Inc., Cary, NC, USA) with a threshold p-value of 0.05.

3.3 Results and Discussion

3.3.1 Characteristics of AD effluent

The chemical composition of the AD effluent is shown in Table 6. The AD effluent mainly contained carbon (2014 mg L⁻¹), nitrogen (2930 mg L⁻¹), and phosphorus (381 mg L⁻¹), which were higher concentrations than those in the original municipal wastewater. About 85% of the nitrogen was in the form of NH₄⁺, which was readily available for the microalgae to use. The nitrogen-to-phosphorus (N/P) ratio of the effluent was 7, which was lower than the atomic ratio of 16 for *N. salina*, indicating a nitrogen limitation for microalgal growth (Hecky and Kilham, 1988). The relatively low total solids (0.287%) of the effluent indicated a low turbidity (EPA, 2006), which was beneficial to the photosynthesis of *N. salina*. The effluent also contained other essential ions for microalgal growth, such as Na⁺, K⁺, Mg²⁺, Ca²⁺, Fe²⁺, and Al³⁺.
Table 6 Characteristics of the effluent

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Unit</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS</td>
<td>%</td>
<td>0.287±0.005</td>
</tr>
<tr>
<td>TVS</td>
<td>%</td>
<td>0.208±0.002</td>
</tr>
<tr>
<td>Total carbon</td>
<td>mg L(^{-1})</td>
<td>2014±65</td>
</tr>
<tr>
<td>TN</td>
<td>mg L(^{-1})</td>
<td>2667±30</td>
</tr>
<tr>
<td>NH(_4)-N</td>
<td>mg L(^{-1})</td>
<td>2276±45</td>
</tr>
<tr>
<td>TP</td>
<td>mg L(^{-1})</td>
<td>381±6</td>
</tr>
<tr>
<td>COD</td>
<td>mg L(^{-1})</td>
<td>2661±75</td>
</tr>
<tr>
<td>Na</td>
<td>mg L(^{-1})</td>
<td>89±4</td>
</tr>
<tr>
<td>K</td>
<td>mg L(^{-1})</td>
<td>121.7±3.5</td>
</tr>
<tr>
<td>Ca</td>
<td>mg L(^{-1})</td>
<td>32.95±1.95</td>
</tr>
<tr>
<td>Mg</td>
<td>μg L(^{-1})</td>
<td>680±26</td>
</tr>
<tr>
<td>Al</td>
<td>μg L(^{-1})</td>
<td>1166±43</td>
</tr>
<tr>
<td>Fe</td>
<td>μg L(^{-1})</td>
<td>4141±58</td>
</tr>
<tr>
<td>Mn</td>
<td>μg L(^{-1})</td>
<td>151.4±19.5</td>
</tr>
<tr>
<td>Ni</td>
<td>μg L(^{-1})</td>
<td>25.69±3.64</td>
</tr>
<tr>
<td>Co</td>
<td>μg L(^{-1})</td>
<td>&lt;0.000</td>
</tr>
<tr>
<td>Cu</td>
<td>μg L(^{-1})</td>
<td>26.75±2.39</td>
</tr>
<tr>
<td>Zn</td>
<td>μg L(^{-1})</td>
<td>105.7±12.3</td>
</tr>
<tr>
<td>Mo</td>
<td>μg L(^{-1})</td>
<td>20.93±0.56</td>
</tr>
</tbody>
</table>

3.3.2 Batch study

3.3.2.1 The effects of effluent loading on microalgae growth and biomass yield

Five effluent loadings (3%, 6%, 12%, 18% and 24%) were tested and the nitrogen levels at each loading are shown in Table 4. The microalgal growth curves in terms of optical density at different effluent loadings are shown in Figure 2. *N. salina* survived at all five effluent loadings. A 1-d lag phase was observed at effluent loadings from 3%-18%. It took 4 d for *N. salina* to adapt at the highest effluent loading of 24%. After the
sixth day, the microalgal growth curve at 3% loading started to level off due to exhaustion of nutrients.

Microalgal growth rate (d\(^{-1}\)) was calculated by plugging the OD values in the following equation:

\[
GR = \frac{(\ln OD_t - \ln OD_0)}{t} = \frac{\ln(OD_t/OD_0)}{t}
\]

where OD\(_0\) is the optical density at the beginning day of cultivation, and OD\(_t\) is the optical density measured on the fifth day of cultivation. The average specific growth rates at loadings of 3%, 6%, 12%, 18% and 24% were 0.624, 0.645, 0.643, 0.591 and 0.334 d\(^{-1}\), respectively (Table 7). These growth rates were higher than that of *N. oculata* (0.14 d\(^{-1}\)) and *Nannochloropsis* sp. (0.28 d\(^{-1}\)) grown in f/2 medium using NaNO\(_3\) as the sole source of nitrogen (Converti et al., 2009; Brown et al., 1998). However, the growth rates in this study are comparable to that of *N. oculata* (0.571 d\(^{-1}\)) with 2% CO\(_2\) aeration (Chiu et al., 2009) and that of *Nannochloropsis* sp. (0.54 d\(^{-1}\)) grown in 50% municipal wastewater (Jiang et al., 2011).
Figure 2 Algae growth curves at different effluent loadings

Table 7 Growth rate of *N. salina* at different effluent loadings

<table>
<thead>
<tr>
<th>Effluent loading (%)</th>
<th>Growth rate (d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.624±0.006</td>
</tr>
<tr>
<td>6</td>
<td>0.645±0.001</td>
</tr>
<tr>
<td>12</td>
<td>0.643±0.001</td>
</tr>
<tr>
<td>18</td>
<td>0.591±0.006</td>
</tr>
<tr>
<td>24</td>
<td>0.334±0.019</td>
</tr>
</tbody>
</table>
As shown in Figure 3, the final microalgal biomass concentrations at each effluent loading varied in the range of 0.68-0.92 g L\(^{-1}\), where the highest concentration of 0.92 g L\(^{-1}\) was obtained at an effluent loading of 6%. The biomass concentration dropped from 0.82 g L\(^{-1}\) to 0.68 g L\(^{-1}\) when the effluent loading increased from 18% to 24%, suggesting an inhibition of microalgal growth in high effluent concentrations. Other studies have found likewise results. The growth of *Nannochloropsis* sp. was inhibited when the loading of municipal wastewater increased from 50% to 80% (Jiang et al., 2011). The maximum biomass concentration of *Nannochlorophsis* sp. cultivated in municipal wastewater was 0.212 g L\(^{-1}\) (Jiang et al., 2011) which was lower than that obtained in this study. However, Gouveia and Oliveira (2009) reported an average biomass concentration of 1.6 g L\(^{-1}\) for *Nannochlorophosis* sp. grown in commercial medium. The biomass concentration of *N. oculata* was greatly increased from 0.268 g L\(^{-1}\) to 1.277 g L\(^{-1}\) when aerated with 2% CO\(_2\) instead of air (Chiu et al., 2009).
3.3.2.2 The effects of effluent loading on nutrient removal and lipid production

It has been shown that nitrate (NO$_3^-$), nitrite (NO$_2^-$), and NH$_4^+$ are the main forms of inorganic nitrogen that are directly available for microalgae (Barsanti and Gualtieri, 2006). Although NO$_3^-$ is the nitrogen source commonly used in culture media, NH$_4^+$ is actually the preferential form for many types of microalgae because it does not need to be reduced before amino acid synthesis. However, it was noted that NH$_4^+$ at concentrations greater than 450 mg L$^{-1}$ is often toxic to microalgae (Barsanti and Gualtieri, 2006). Most of the nitrogen in the effluent used in this study was in the form of NH$_4^+$ (Table 6). As shown in Figure 4, N. salina was able to remove the NH$_4^+$ completely at all effluent loadings studied. Therefore, the toxicity of NH$_4^+$ to microalgae (Abeliovich and Azov, 1976) was not observed for N. salina at NH$_4^+$ levels less than 546 mg L$^{-1}$. This result is also in agreement with a study by Hii et al. (2011) in which Nannochloropsis sp. was able
to grow in f/2 medium enriched with 400-900 µM NH$_4^+$ during 10-day cultivation. A complete TN removal was observed at 3% and 6% effluent loadings. TN removal efficiency decreased to 87% as the effluent loading increased from 6% to 24%, indicating that there was still some organic nitrogen not consumed by *N. salina*. The nitrogen removal efficiency of *N. salina* in this study was much higher than that from other studies. For example, Wang et al. (2010a) studied the nutrient removal efficiency of microalgae *Chlorella* sp. in anaerobically digested dairy manure. The highest TKN removal efficiency was 82.5% with an initial TKN of 250 mg L$^{-1}$ after 22 days of cultivation. Another study of *Chlorella* sp. grown in municipal wastewater for 15 days showed a TN removal rate of 89.1% with an initial TN of 116 mg L$^{-1}$ (Li et al., 2011). Microalga *Chlamydomonas reinhardtii* grown in municipal wastewater was only able to removal 55% TKN (initial concentration was 128.6 mg L$^{-1}$) after 10 d of culture (Kong et al., 2010).

As shown in Figure 4, more than 99% of the phosphorus in the medium was removed in the 10-d batch study. However, the N/P ratio of natural phytoplankton is about 15, and the initial N/P ratio of the effluent was 7. As the uptake of phosphorus was higher than expected, based on N/P ratios, *N. salina* might have the ability to uptake excess phosphorus. At N/P ratios of 2 and 4 in the growth medium, the nutrient uptake ratios (N: P) of *Scenedesmus* sp. LX1 were around 2:1 and 4:1, respectively, after 13 days of cultivation, indicating an over-uptake of phosphorus in microalgal cells (Xin et al., 2010b). Another explanation of the high phosphorus removal might be attributed to an increase in the pH of the culture medium from 7 to more than 10 during the cultivation.
period. High pH (>10) may have caused the precipitation of phosphorus with other elements such as calcium, thus facilitating the phosphorus removal (Wang et al., 2010b).

**Figure 4** TAN, TN, TP removal rates at different effluent loadings

Figure 5 shows the effects of effluent loading on lipid content of *N. salina*. The lipid content reached up to 35% of dry cell weight and then decreased as the AD effluent loading in the culture medium increased from 3% to 18%, which is consistent with other research showing that the lipid content of microalgae cells could be increased as the nutrient loading decreased (Emdadi and Berland, 1989; Converti et al., 2009; Suen et al., 1987). The lipid content of *N. oculata* and *Chlorella vulgaris* increased from 7.9% to 15.9% and from 5.9% to 16.4%, respectively, when the NaNO₃ concentration decreased from 300 mg L⁻¹ to 75 mg L⁻¹ (Converti et al., 2009). The lipid content of *Chlorella* sp. was also enhanced from 9% to 13.7% as the loading ratio of the anaerobic digested manure decreased from 10% to 4% (Wang et al., 2010a). The lipid content of
Nannochloropsis sp. is usually in the range of 21 – 36% of dry cell weight (Gong and Jiang, 2011), which is in agreement with this study.

![Figure 5 Algal lipid contents at different effluent loadings](image)

The lipid productivities at different effluent loadings are shown in Figure 6. As the effluent loading increased, the lipid productivity dropped from 29.2 mg L\(^{-1}\) d\(^{-1}\) to 14 mg L\(^{-1}\) d\(^{-1}\). However, the lipid productivities at 3% and 6% effluent loading were not significantly different (p>0.05) because the biomass yield at 6% loading was slightly higher than that at 3% loading. The lipid productivity obtained in this study was much higher than for *N. salina* fed with artificial sea water in outdoor ponds which produced lipids at a rate of 0.03 mg L\(^{-1}\) d\(^{-1}\) (Boussiba et al., 1987). It was reported by Hu and Gao (2006) that *Nannochloropsis* sp. aerated with 2,800 µl CO\(_2\) L\(^{-1}\) in f/2-enriched artificial seawater had a highest lipid production of 13.64 mg L\(^{-1}\) d\(^{-1}\) in 10-d cultivation. The lipid content and lipid productivity of microalgae were not only affected by nutrient loading,
but also by other factors such as temperature, irradiance, and salinity (Converti et al., 2009; Su et al., 2011). An increase of irradiance from 100 to 500 µmol photons m$^{-2}$ s$^{-1}$ led to an increase in the lipid content of *N. oculata* from 22.5% to 44.5%, resulting in an increase in the lipid productivity from 103 mg L$^{-1}$ d$^{-1}$ to 324 mg L$^{-1}$ d$^{-1}$ (Su et al., 2011). The lipid content of *C. vulgaris* decreased from 20% to 8.2% when the temperature increased from 25 to 35 °C, causing a decrease in lipid productivity as well (Converti et al., 2009).

![Figure 6 Algal lipid yields at different effluent loadings](image)

*Figure 6 Algal lipid yields at different effluent loadings*
3.3.3 Semi-continuous study

3.3.3.1 The effects of harvesting frequency and harvesting ratio on algae growth and biomass yield

The batch study showed that *N. salina* could adapt to effluent loadings between 3% and 18%. The effluent loading of 6% was chosen for the semi-continuous study because of the relatively high growth rate and biomass yield of *N. salina* under this condition. Different harvesting frequencies (1-, 2-, and 3-day intervals) and harvesting ratios (25% and 50%) were tested for an 18-d culture of *N. salina* (Table 5). As shown in Figure 7, at different harvesting frequencies, when half of the culture was harvested each time, the cultures were able to reach steady state earlier than those with 25% of the culture harvested. At a harvesting interval of 1 d, the reactors reached steady state on the second day when half of the culture was harvested; whereas, for those with 25% culture harvested, the reactors did not reach steady state until the sixth day. Both harvesting interval and ratio affected the accumulated biomass yield. According to Figure 8, at a 25% harvesting ratio, the biomass yield decreased as the harvesting interval increased from 1 d to 3 d, as the hydraulic retention time (HRT) increased from 4 d to 12 d. However, at a 50% harvesting ratio, the highest biomass yield was obtained with a 2-d harvesting frequency at a HRT of 4 days. Although lower harvesting ratios resulted in higher algal cell density, the highest biomass yield during the 18-d continuous cultivation was obtained at a harvesting ratio of 50% and a harvesting interval of 2 d. Similar results were also presented by Min et al. (2011) in a study of *Chlorella* sp. using municipals wastewater as a nutrient supplement. The biomass productivity of *Chlorella* sp. was increased by 22.8–66.7% when the harvesting ratio increased from 25% to 50%.
Figure 7 Algal growth curves at different harvesting frequencies and harvesting ratios: (a) 1-d harvesting interval with 25% or 50% culture removed; (b) 2-d harvesting interval with 25% or 50% culture removed; (c) 3-d harvesting interval with 25% or 50% culture removed
Figure 7 continued

(c)

\[ \text{OD440} \]

- △ 3d_25%
- ● 3d_50%

Cultivation time (day)
3.3.3.2 The effects of harvesting frequency and harvesting ratio on nutrient removal and lipid productivity

As shown in Table 8, at the same harvesting ratio, the TN removal rate decreased as the HRT increased. Higher harvesting ratios led to higher TN removal rates at each harvesting frequency. The TN removal efficiency was significantly increased as the culture was harvested less frequently (p < 0.05) because less frequency resulted in a longer HRT during which more algae biomass would be accumulated and more nutrients could be assimilated. The highest biomass productivity (155.3 mg L⁻¹ d⁻¹), lipid content (24.9% AFDW), and lipid productivity (38.7 mg L⁻¹ d⁻¹) were obtained at a harvesting ratio of 50% and harvest interval of 2 d (Table 8). The maximum lipid productivity of 38.7 mg L⁻¹ d⁻¹ obtained in the semi-continuous study was 1.33 times of that in the batch study, showing an advantage of semi-continuous cultivation for *N. salina*. The average
lipid productivity of *Nannochloropsis* sp. has been reported to be 37.6–90 mg L\(^{-1}\) d\(^{-1}\) (Gong and Jiang, 2011; Mata et al., 2010). Thus, this study demonstrated the technical feasibility of growing *N. salina* in AD effluent for both nutrient removal and lipid production.
Table 8 Nutrient removal rate and lipid productivity of *N. salina* at different harvesting frequencies and ratios

<table>
<thead>
<tr>
<th>Harvesting frequency (d)</th>
<th>Harvesting ratio (%)</th>
<th>TN removal rate (mg L$^{-1}$ d$^{-1}$)</th>
<th>TN removal efficiency (%)</th>
<th>TP removal rate (mg L$^{-1}$ d$^{-1}$)</th>
<th>TP removal efficiency (%)</th>
<th>Biomass productivity (mg L$^{-1}$ d$^{-1}$)</th>
<th>Lipid content (% AFDW)</th>
<th>Lipid productivity (mg L$^{-1}$ d$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>32.9±3.0</td>
<td>53.5±4.3</td>
<td>4.1±0.3</td>
<td>33.6±2.8</td>
<td>132.1±8.9</td>
<td>18.0±0.6</td>
<td>23.7±2.4</td>
</tr>
<tr>
<td>1</td>
<td>50</td>
<td>56.5±4.8</td>
<td>55.2±4.6</td>
<td>4.3±0.4</td>
<td>22.9±2.2</td>
<td>143.0±4.8</td>
<td>19.0±0.4</td>
<td>27.1±1.5</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>19.0±2.9</td>
<td>73.4±4.5</td>
<td>3.4±1.1</td>
<td>67.6±3.1</td>
<td>104.3±2.7</td>
<td>19.8±0.9</td>
<td>20.6±1.5</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>35.3±4.2</td>
<td>77.8±4.0</td>
<td>3.8±1.2</td>
<td>45.7±6.5</td>
<td>155.3±1.8</td>
<td>24.9±0.5</td>
<td>38.7±2.4</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>13.4±2.5</td>
<td>84.7±2.0</td>
<td>2.3±1.6</td>
<td>72.5±5.7</td>
<td>87.4±1.9</td>
<td>18.1±1.1</td>
<td>15.9±1.3</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>26.2±2.5</td>
<td>89.0±1.7</td>
<td>3.9±1.3</td>
<td>82.8±1.8</td>
<td>121.4±4.1</td>
<td>22.7±0.4</td>
<td>27.6±1.4</td>
</tr>
</tbody>
</table>

Data are the means ± SE
3.4 Conclusion

It is feasible to culture microalgae *N. salina* in AD effluent for nutrient removal and lipid production. However, proper dilution was necessary to mitigate the negative effects of toxic components on microalgal growth. *N. salina* was able to tolerate up to 480 mg L\(^{-1}\) TN (18% effluent loading). *N. salina* fed with 6% effluent had the highest biomass yield, along with 29% lipid content and 100% nitrogen and phosphorus removal efficiencies. The biomass and lipid productivity of *N. salina* was further improved in semi-continuous cultivation due to frequent replenishment of culture medium. The optimal operation parameters for semi-continuous cultivation are: 2-d harvesting interval and 50% harvesting ratio.
Chapter 4 Cultivation of *Synechocystis* sp. PCC6803 in AD Effluent for Nutrient Removal and Lipid Production

This study tested the growth of cyanobacteria *Synechocystis* sp. PCC6803 in AD effluent for nutrient removal, lipid and biomass production and compared its performance with that of *N. salina* presented in previous chapter. The results from batch study showed that *Synechocystis* sp. PCC6803 produced biomass at a higher rate (150 mg L\(^{-1}\) d\(^{-1}\)) than *N. salina* with 3% effluent loading. However, the lipid productivity of *Synechocystis* sp. PCC6803 was lower due to its low lipid content. It was noted that *Synechocystis* sp. PCC6803 was less efficient than *N. salina* in removing nitrogen and phosphorus. The growth rate of *Synechocystis* sp. PCC6803 was also inhibited by high effluent loading. The biomass productivity of *Synechocystis* sp. PCC6803 was further improved to 212.4 mg L\(^{-1}\) d\(^{-1}\) in semi-continuous study when 50% culture was harvested every day, resulting a lipid productivity of 29.1 mg L\(^{-1}\) d\(^{-1}\).

4.1 Introduction

In order to reduce dependence on fossil fuel and to mitigate global warming, researchers have been working to develop biofuels and biomaterials as renewable substitutes (Rittmann, 2008; Chisti, 2008; Farrell et al., 2006). Efficient photosynthetic organisms such as algae and cyanobacteria are among the most popular research subjects for production of biofuels and biomaterials. Cyanobacteria (blue green algae) are a group of the oldest forms of organisms on the earth and play important roles in biogeochemical
cycles of nitrogen, carbon, and oxygen (Rippka et al., 1979; Gademann and Portmann, 2008). Cyanobacteria contribute to 30% of the annual oxygen production on the earth (Sharma et al., 2011). Some cyanobacteria have unique metabolic pathways that can fix atmospheric nitrogen through photosynthesis (Karl et al., 2002). Most of the secondary metabolites of cyanobacteria are useful chemical compounds that have biological activities, such as antibacterial, antifungal, antitumoral, anti-HIV, antiviral, and anti-inflammatory; thus, they have been widely applied in clinical therapies (Sharma et al., 2011; Abed et al., 2009). Although a major focus on cyanobacteria in previous decades has been on the negative roles they have played in the eutrophication of lakes, oceans, rivers, and other water bodies, interest has shifted to biofuel production from cyanobacteria due to their fast growth rate, high biomass yield, and simple growth needs (Parmar et al., 2011; Heisler et al., 2008; Smith, 2003; Anderson et al., 2002).

*Synechocystis* sp. PCC6803 is a promising cyanobacteria strain which is attractive because of its high biomass yield and robustness. *Synechocystis* sp. PCC6803 is able to survive in a wide range of temperature, salinity, and pH conditions (Schubert et al., 1993; Antal and Lindblad, 2005; Anderson and McIntosh, 1991; Ohkawa et al., 2000; Ogawa and Kaplan, 2003; Kanesaki et al., 2002). Its genomic, biochemical, and physiological data have been extensively reported, offering valuable information for genetic modification of this strain to improve its potential for bioenergy production (Huang et al., 2002). The high biomass yield of *Synechocystis* sp. PCC6803 could provide a large volume of feedstock for production of bioenergy, such as bio-ethanol, methane, biodiesel, or hydrogen. Unlike most microalgae, the lipid content of cyanobacteria will not increase under nitrogen starvation. Instead, *Synechocystis* sp. PCC6803 has the highest lipid
content during the exponential phase when its growth rate is the highest (Kim et al., 2011; Hu et al., 2008). Similarly, the nutrient (nitrogen and phosphorus) uptake rates of *Synechocystis* sp. PCC6803 are also the highest during this optimal growth phase. Therefore, theoretically, optimal lipid production and nutrient removal rates could be achieved at the same time when cultivating *Synechocystis* sp. PCC6803.

Cyanobacteria are able to utilize both organic and inorganic carbon. *Synechocystis* sp. PCC6803 can fix CO₂ and perform photosynthesis in sunlight. It is also capable of heterotrophic growth with supply of organic carbon. Although nitrogen can be utilized in the forms of ammonium, nitrite, nitrate, or N₂, cyanobacteria prefer using ammonium nitrogen because it is in a less reduced form and takes less energy to consume (Converti et al., 2006). Phosphorus is another essential element for growth of cyanobacteria although, compared to nitrogen, a smaller amount is required. The uptake rate of phosphorus is affected by the pH of the medium and the existence of other elements such as calcium (Ritchie et al., 1997). Lack of ions such as Mg²⁺, K⁺, Na⁺ will also decrease the phosphorus uptake rate (Ritchie et al., 1997; Seale et al., 1987).

The effectiveness of *Synechocystis* sp. PCC6803 for optimal uptake of nutrients concurrent with optimal lipid production, suggests that it could be a cost effective treatment for wastewater, such as the effluent from an anaerobic digestion (AD). AD effluent has a sufficient amount of nutrients (nitrogen, phosphorus, organic carbon, and other trace elements) that could support the growth of *Synechocystis* sp. PCC6803 (Table 6) at a relatively low cost. According to analysis, more than 80% of the nitrogen in AD effluent is ammonium. The nitrogen/phosphorus ratio of 7 indicates excessive
phosphorus supply in the medium. Therefore, the growth of *Synechocystis* sp. PCC6803 will not be limited by phosphorus.

The objectives of this study were to: 1) evaluate the growth of *Synechocystis* sp. PCC6803 in AD effluent; 2) compare the biomass yield, nitrogen and phosphorus removal efficiencies, and lipid productivity of *Synechocystis* sp. PCC6803 with those of *N. salina* in batch and semi-continuous cultivation.

**4.2 Material and Methods**

**4.2.1 AD effluent and inoculum cultures**

The AD effluent used in this study was the same as the one used in the study discussed in Section 3.2.1 of Chapter 3. It was collected from a commercial-scale wet anaerobic digester (KB Compost Services, Akron, OH, USA) coupled with a D5LL solid bowl decanter centrifuge (ANDRITZ AG, Graz, Austria). The feedstock for this digester was the municipal wastewater from the city of Akron. The centrifuge ran continuously at 3200 rpm. The effluent was kept in 4°C before use.

The cyanobacteria strain used in this study was obtained from Touchstone Research Laboratories (Tridelphia, WV). DNA sequencing was used to identify the strain and it was identified as *Synechocystis* sp. PCC6803. The identification results are shown in Figure 9 (This part of work was done by Dr. Jiyoung Lee’s lab in OSU, Columbus, OH).
4.2.2 Batch and semi-continuous cultivation of *Synechocystis* sp. PCC6803 in AD effluent

The reactor configuration used to grow the *Synechocystis* sp. PCC6803 in the AD effluent was the same as the configuration described in Section 3.2.2 of Chapter 3.

In batch cultivation, different dilutions were applied to the AD effluent before being fed to *Synechocystis* sp. PCC6803 (Table 5). Deionized (DI) water was added to adjust the final working volume of the reactor to 1 L. The salinity of each reactor was adjusted to 20‰ with Instant Ocean® sea salt (Spectrum Brands, Madison, WI, USA). Seed cultures were inoculated to each reactor to have an initial optical density of 0.1. DI water was added daily to make up the water lost through evaporation before taking samples for analysis. By the end of each batch experiment, the biomass was obtained using a Sorvall RC 6 Plus centrifuge (Thermo Scientific, Waltham, MA, USA). The supernatant remaining after centrifugation was analyzed for the nitrogen and phosphorus content. TAN, TN, and TP removal efficiencies during the 10-day cultivation were
calculated based on the initial and final concentrations of TAN, TN, and TP in the supernatant.

According to the study of *N. salina* in semi-continuous cultivation in Chapter 3, the harvesting ratio of 50% led to higher biomass and lipid productivity than the ratio of 25%. Thus, only a 50% harvesting ratio was used for the semi-continuous cultivation of *Synechocystis* sp. PCC6803 and the harvesting interval varied between 1-3 days.

4.2.3 Analytical methods

The optical density peak of 440 nm was used to establish a relationship with cell concentration. Biomass was determined gravimetrically and is reported on an ash-free dry weight (AFDW) basis. The concentrations of 13 elements (Na, Mg, Al, P, K, Ca, Fe, Mn, Ni, Cd, Cu, Zn, and Mo) in the culture medium were measured using an ICP-MS unit 7500cx (Agilent Technologies, Santa Clara, CA) following US EPA method 6062A. TAN, TN, and TP and COD were determined using a HACH 3900 spectrophotometer (Düsseldorf, Germany) coupled with a HACH DRB200 dual block reactor (Düsseldorf, Germany) following the manufacturer’s instruction manual. Lipid content of the algal biomass was analyzed using a slightly modified version of Bligh and Dyer’s method (Bligh and Dyer, 1959). The procedures used for determination of the above parameters were the same as those described in Section 3.2.3 of Chapter 3.

4.2.4 Statistical analysis

Statistical significance was determined by analysis of variance (ANOVA) using SAS software (Version 8.1, SAS Institute Inc., Cary, NC, USA) with a threshold p-value of 0.05.

4.3 Results and Discussion
4.3.1 Characterization of AD Effluent

The chemical composition of the AD effluent is shown in Table 6. The AD effluent mainly contained nitrogen and phosphorous, which are both required for growth of cyanobacteria. There were also various kinds of metal ions in the AD effluent because the feedstock for the digester was municipal wastewater which contained the ions. The effects of N/P ratio on growth of cyanobacteria have been widely evaluated. It has been shown that an N/P ratio of less than 29 tends to trigger a large population of cyanobacteria (Smith, 1983). The N/P ratio of AD effluent used in this study was about 7, which was favored by Synechocystis sp. PCC6803 (Kim et al., 2011).

4.3.2 Growth curve and biomass yield of Synechocystis sp. PCC6803 in batch cultivation

Figure 10 illustrates the growth curves of Synechocystis sp. PCC6803 at five effluent loadings (3%, 6%, 12%, 18% and 24%). As the effluent loading increased, the inhibition on the growth of Synechocystis sp. PCC6803 gradually increased. No obvious lag phase was observed at effluent loadings of 3%-12%. A 4-day and 7-day lag phase was observed for effluent loadings of 18% and 24%, respectively. N. salina had a shorter lag phase (2 days) when grown with 18% effluent loading. Although the rate slowed down, Synechocystis sp. PCC6803 grown in 3% effluent continued to grow after the 6th day; whereas, N. salina began to go into the stationary phase on the 6th day (Chapter 3, Figure 2) due to nutrient depletion. The average specific growth rates of Synechocystis sp. PCC6803 at the five effluent loadings are shown in Table 9. The growth rate of Synechocystis sp. PCC6803 decreased significantly (p<0.05) as the effluent loading increased. However, N. salina was able to grow fast in effluent loading 3%-18% with the growth rate decreased only by 0.054 d⁻¹ (Table 7). The growth rate of Synechocystis sp.
PCC6803 was higher than that of *N. salina* at an effluent loading of 3%, but was lower at all other four effluent loadings. This result demonstrated that *Synechocystis* sp. PCC6803 is more sensitive than *N. salina* to the toxic components in the effluent. *Synechocystis* sp. PCC6803 tends to grow better in low nutrient loading conditions. The growth rates of *Synechocystis* sp. PCC6803 in effluent loadings of 3% - 12% were higher than those of *Synechocystis* sp. PCC6803 (0.41 d\(^{-1}\)) cultivated in BG-11 medium in photobioreactors Kim et al. (2010). It was reported that the maximum specific growth rates of PCC6803 could be 2.0-2.5 d\(^{-1}\), but the specific growth rate of *Synechocystis* sp. PCC 6803 was always lower than 1 d\(^{-1}\) in less favorable conditions (Ohkawa et al., 2000; Zhang et al., 1994). The specific growth rate of *Synechocystis* sp. PCC6803 depends on many factors such as temperature, pH, concentration of nitrogen and phosphorus, etc. In order to maintain high growth rate, the nutrient supply rate must match the rate of biomass synthesis (Zhang et al., 2008). Thus, semi-continuous cultivation could offer the benefit of continuous nutrient supply and help maintain the robust growth of *Synechocystis* sp. PCC6803.
Figure 10 The growth curve of *Synechocystis* sp. PCC6803 at different effluent loadings

Table 9 Growth rate of *Synechocystis* sp. PCC6803 at different effluent loadings

<table>
<thead>
<tr>
<th>Effluent loading (%)</th>
<th>Growth rate (d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.717±0.004</td>
</tr>
<tr>
<td>6</td>
<td>0.611±0.002</td>
</tr>
<tr>
<td>12</td>
<td>0.539±0.003</td>
</tr>
<tr>
<td>18</td>
<td>0.272±0.006</td>
</tr>
<tr>
<td>24</td>
<td>0.077±0.008</td>
</tr>
</tbody>
</table>
The final biomass concentrations by the end of the 10-day cultivation are presented in Figure 11. The biomass concentration decreased from 1.51 g L\(^{-1}\) to 0.41 g L\(^{-1}\) as the effluent loading increased from 3% to 24%, indicating an inhibition on growth at high effluent loading. The highest biomass yield 1.51 g L\(^{-1}\) was obtained at an effluent loading of 3% and it was 1.64 times the highest biomass yield of \textit{N. salina} which was achieved at 6% effluent loading in batch experiments. The biomass concentration of \textit{Synechocystis} sp. PCC6803 grown in BG-11 medium varied between 0.43-1.2 g L\(^{-1}\) as reported by Kim et al. (2010). Phang et al. (2000) cultivated another cyanobacteria, \textit{Spirulina platensis}, in digested sago starch factory wastewater and observed a biomass concentration of 0.528-0.610 g L\(^{-1}\), which is much lower than that obtained in this study. The growth of \textit{Synechocystis} sp. was also found to be closely related to light irradiance (Kim et al., 2011; Martinez et al., 2012). In a study of the effect of light on \textit{Synechocystis} sp., the highest biomass concentration of 4.53 g L\(^{-1}\) was achieved at an irradiance of 1600 \(\mu\text{mol photons m}^{-2}\text{s}^{-1}\) after 6 days of cultivation (Martinez et al., 2012).

![Figure 11 The biomass yield of Synechocystis sp. PCC6803 at different effluent loadings](image)

Figure 11 The biomass yield of Synechocystis sp. PCC6803 at different effluent loadings
4.3.3 Nutrient removal efficiency and lipid production of Synechocystis sp. PCC6803

The removal efficiencies of TAN, TN and TP during the 10-day cultivation were investigated and the results are shown in Table 10. Ammonium, which is the preferential nitrogen form, removal was 100% at effluent loadings of 3-18%, and was 83% at 24% a loading ratio. Both the TN and TP removal efficiency of Synechocystis sp. PCC6803 decreased as the effluent loading increased. Compared with Synechocystis sp. PCC6803, N. salina was more efficient in nutrient removal because the ammonium removal efficiency of N. salina was 100% at all loading ratios. N. salina also showed higher utilization of nitrogen and phosphorus. The uptake ratio (N/P) for Synechocystis sp. PCC6803 is about 6, close to the nitrogen to phosphorus ratio in biomass of Synechocystis sp. PCC6803 which is 8:1 (Kim et al., 2011). However, it has been noted that ammonium could be removed not only by active uptake during growth of organisms, but also by ammonia stripping into the atmosphere (Chaiklahan et al., 2010). Analysis of the cellular nitrogen content of Synechocystis sp. PCC6803 and N. salina is needed in order to determine the amount of nitrogen in the effluent that contributed to the their growth. Kim et al. (2011) studied the nitrogen uptake rate of Synechocystis sp. PCC6803 supplied with BG-11 medium and 2.5% CO2 in photo-bioreactors. The results showed that the nitrogen uptake rate was 0.46 g N g dry weight$^{-1}$ d$^{-1}$. It has also been suggested that stoichiometry be used to assess the necessary supply rate of nutrients for photosynthetic microorganisms to produce biomass. However, this method may not apply to using algae to remove nitrogen and phosphorus from wastewater because many factors can affect the nutrient removal such as ammonia stripping, phosphorus precipitation, etc. Therefore, the
nutrient loading should be determined based on the growth rate of microalgae, biomass yield, or apparent nutrient removal efficiency.

Table 10 Nutrient removal efficiency of algae culture system with *Synechocystis* sp. PCC6803 and *N. salina* in batch cultivation

<table>
<thead>
<tr>
<th>Effluent loading (%)</th>
<th><em>Synechocystis</em> sp. PCC6803</th>
<th>N. salina</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TAN removal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>100±0</td>
<td>100±0</td>
</tr>
<tr>
<td>6</td>
<td>100±0</td>
<td>100±0</td>
</tr>
<tr>
<td>12</td>
<td>100±0</td>
<td>100±0</td>
</tr>
<tr>
<td>18</td>
<td>100±0</td>
<td>100±0</td>
</tr>
<tr>
<td>24</td>
<td>82.54±2.51</td>
<td>100±0</td>
</tr>
<tr>
<td><strong>TN removal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>100±0</td>
<td>100±0</td>
</tr>
<tr>
<td>6</td>
<td>93.42±5.52</td>
<td>100±0</td>
</tr>
<tr>
<td>12</td>
<td>89.45±6.34</td>
<td>97.32±0.21</td>
</tr>
<tr>
<td>18</td>
<td>84.91±3.60</td>
<td>91.43±0.54</td>
</tr>
<tr>
<td>24</td>
<td>71.20±4.30</td>
<td>87.59±0.61</td>
</tr>
<tr>
<td><strong>TP removal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>100±0</td>
<td>100±0</td>
</tr>
<tr>
<td>6</td>
<td>100±0</td>
<td>100±0</td>
</tr>
<tr>
<td>12</td>
<td>97.47±0.51</td>
<td>99.8±0.13</td>
</tr>
<tr>
<td>18</td>
<td>94.32±1.75</td>
<td>99.4±0.14</td>
</tr>
<tr>
<td>24</td>
<td>83.57±5.00</td>
<td>99.3±0.23</td>
</tr>
</tbody>
</table>
As presented in Figure 12, the lipid content of Synechocystis sp. PCC6803 did not change significantly (p>0.05) as the effluent loading varied. In contrast, for N. salina, a decrease in lipid content was observed as effluent loading increased. This result is not surprising because many researchers have noticed that the reactions of cyanobacteria and microalgae to nutrient deficiency are quite different. Piorreck and Pohl (1984) studied and compared the effects of nitrogen starvation on the lipid metabolism of green algae (Chlorella vulgaris and Scenedesmus obliquus) and cyanobacteria (Anacystis nidulans, Microcystis aeruginosa, Oscillatoria rubescens, and Spirulina platensis). It was found that the lipid content and composition in the green algae changed during batch cultivation and was dependent on the nitrogen concentration in the media, but this effect was not observed in the cyanobacteria. Piorreck and Pohl (1984) used “endosymbiont theory” to explain these differences between blue green algae (cyanobacteria) and green algae. It is well-known that blue-green algae are prokaryotes and green algae are eukaryotes. According to the “endosymbiont theory”, the chloroplasts of eukaryotic organisms are considered to have originated from cyanobacteria through endosymbiosis. Blue-green algae and chloroplasts both contain the same kind of polar lipids and polyunsaturated fatty acids but little or no neutral lipids such as triacylglycerols, which are localized in the cytoplasm of eukaryotic algae. Nitrogen deficiency stimulates a decrease in the chlorophyll of green algae which results in a reduction in the chloroplast apparatus and in the polar lipids and polyunsaturated fatty acids in the chloroplast membranes, bringing an increase in the percentage of cytoplasmic neutral lipids and fatty acids in eukaryotic algae. However, the blue-green algae cell and its components are reduced evenly as the nitrogen supply decreases.
Although the lipid content of *Synechocystis* sp. PCC6803 was not altered significantly (p>0.05, Figure 12), its lipid yield decreased significantly (p<0.05) as the effluent loading increased because of the significant decrease in the biomass yield (p<0.05). The highest lipid yield of 20 mg L\(^{-1}\)d\(^{-1}\) is, however, lower than that of *N. salina* (29 mg L\(^{-1}\)d\(^{-1}\)) because the highest lipid content of *N. salina* was 35.38%, which was 2.68 times the lipid content of *Synechocystis* sp. PCC6803 at 3% effluent loading. Even though the biomass yield of *Synechocystis* sp. PCC6803 could be as high as 1.64 times that of *N. salina*, it is still not as competitive as *N. salina* for lipid production. Nonetheless, *Synechocystis* sp. PCC6803 is still a very good candidate for biomass production as a feedstock for biofuel because of its high biomass yield.

![Figure 12 The lipid content and lipid yields of *Synechocystis* sp. PCC6803 at different effluent loadings](image-url)
4.3.4 Performance of Synechocystis sp. PCC6803 in semi-continuous cultivation

It has been stated in previous sections that the growth rate of Synechocystis sp. PCC6803 was greatly affected by effluent loading, a result of the nutrient concentration. The highest growth rate, biomass yield, and lipid yield were all attained at the loading of 3%. Therefore, a semi-continuous experiment for Synechocystis sp. PCC6803 was conducted with 3% effluent loading with 1-, 2-, and 3-day harvesting intervals. As shown in Figure 13, the growth rate of Synechocystis sp. PCC6803 reached steady state within four days after inoculation, which was also observed in the study of N. salina. The optical density was maintained between 0.7-1.6, 1.2-3, and 2-4 for harvesting intervals of 1, 2, and 3 days, respectively. Longer harvesting intervals led to longer hydraulic retention times (HRT), allowing cells to grow more and reach higher cell densities. However, as the cell density increased, the self-shading effects became stronger and reduced the cell growth rate (Shigesada and Okubo, 1981). Therefore, the growth rate of Synechocystis sp. PCC6803 and N. salina decreased as the HRT increased (Table 11).
Figure 13 Growth curve of *Synechocystis* sp. PCC6803 at different harvesting intervals: (a) 1-day; (b) 2-day; (c) 3-day
Nitrogen and phosphorus removal rate (mg L\(^{-1}\) d\(^{-1}\)) decreased as the harvesting interval increased. The nutrient removal efficiency of *Synechocystis* sp. PCC6803 was more than 89%. Nitrogen and phosphorus were totally removed at 2 and 3 days harvesting interval, indicating that nutrient starvation might take place during cultivation. The effluent loading could be increased in the future in order to achieve higher nutrient removal rate. The nutrient removal rates of *Synechocystis* sp. PCC6803 obtained here was comparable with other cyanobacteria, too. Ratana et al. (2010) used digested pig wastewater to cultivate *Spirulina platensis* in semi-continuous process and the average removal rates for TN and TP were 34 mg L\(^{-1}\) d\(^{-1}\) and 4 mg L\(^{-1}\) d\(^{-1}\), respectively. Another group of researchers studied the nutrient removal capability of *Spirulina* sp. in outdoor
raceways treating digested pig wastewater and reported that the NH₄-N removal efficiency was 84-96% with a rate of 13.6 mg L⁻¹ d⁻¹ (Olguin et al., 2003).

The growth rate, biomass productivity, and lipid content and productivity of *Synechocystis* sp. PCC6803 and *N. salina* are summarized in Table 11. The biomass productivity of *Synechocystis* sp. PCC6803 decreased as the harvesting interval increased. The lipid content and lipid productivity did not change significantly (p>0.05). This result was different for *N. salina*, where the highest lipid productivity was obtained with a 2-day harvesting interval, probably due to the high biomass productivity and lipid content under these conditions. In spite of its higher biomass productivity, *Synechocystis* sp. PCC6803 had lower lipid productivity because its lipid content was much lower than that of *N. salina’s*. The biomass productivity of *Synechocystis* sp. PCC6803 obtained in semi-continuous cultivation was much higher than for other cyanobacteria strains reported in the literature. Laliberte et al. (1997) investigated the growth and nutrient removal efficiency of *Phormidium bohneri* in domestic wastewater and recorded biomass productivities of 23-57 mg L⁻¹ d⁻¹, along with ammonium and phosphorus removal rates of up to 20 mg L⁻¹ d⁻¹. Monoculture of *Planktothrix isothrix* in municipal wastewater produced biomass at rates of 27-50 mg L⁻¹ d⁻¹ in one laboratory study (Margarita Silva-Benavides and Torzillo, 2012). The potential of cultivating cyanobacteria *Synechocystis* sp. PCC6803 in AD effluent for nutrient removal and biomass production was demonstrated in this study.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific growth rate (d(^{-1}))</th>
<th>Biomass productivity (mg L(^{-1}) d(^{-1}))</th>
<th>Lipid content (%)</th>
<th>Lipid productivity (mg L(^{-1}) d(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Synechocystis sp. PCC6803</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d_50%</td>
<td>0.720±0.004</td>
<td>212.4±5.6</td>
<td>13.7±1.8</td>
<td>29.1±2.3</td>
</tr>
<tr>
<td>2d_50%</td>
<td>0.346±0.002</td>
<td>204.6±2.5</td>
<td>13.6±2.4</td>
<td>27.7±1.7</td>
</tr>
<tr>
<td>3d_50%</td>
<td>0.237±0.007</td>
<td>186.4±4.3</td>
<td>13.5±1.5</td>
<td>25.1±1.6</td>
</tr>
<tr>
<td><strong>N. salina</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d_50%</td>
<td>0.688±0.005</td>
<td>143.0±2.7</td>
<td>19.0±0.4</td>
<td>27.1±1.5</td>
</tr>
<tr>
<td>2d_50%</td>
<td>0.353±0.004</td>
<td>155.8±1.8</td>
<td>24.9±0.5</td>
<td>38.7±2.4</td>
</tr>
<tr>
<td>3d_50%</td>
<td>0.233±0.002</td>
<td>121.4±4.1</td>
<td>22.7±0.4</td>
<td>27.6±1.4</td>
</tr>
</tbody>
</table>
4.4 Conclusion

The results from this study demonstrated the potential of growing *Synechocystis* sp. PCC6803 in AD effluent for biomass and lipid production. The growth of *Synechocystis* sp. PCC6803 was affected by effluent loading, thus proper dilution was needed to maintain optimal growth rate. The highest biomass productivity of *Synechocystis* sp. PCC6803 in the batch (150 mg L$^{-1}$ d$^{-1}$) and semi-continuous (212.4 mg L$^{-1}$ d$^{-1}$) studies was 1.6 times and 1.4 times, respectively, higher than that of *N. salina* in similar batch and semi-continuous cultivation studies. However, the lipid productivity of *Synechocystis* sp. PCC6803 became lower as the harvesting interval increased because of its relatively low lipid content. The lipid productivity of *Synechocystis* sp. PCC6803 in batch cultivation was 20 mg L$^{-1}$ d$^{-1}$, and increased by 45.5% in semi-continuous cultivation. The nitrogen and phosphorus removal rates of 34.5 mg L$^{-1}$ d$^{-1}$ and 5.7 mg L$^{-1}$ d$^{-1}$ were obtained at a 1-day harvesting frequency and 50% harvesting ratio.
Chapter 5 Conclusions and Recommendations

Anaerobic digested municipal wastewater was shown to be a suitable culture medium for both *Nannochloropsis salina* and *Synechocystis* sp. PCC6803. Proper dilution is necessary to mitigate the negative effects of toxic components in effluent on algae growth. The growth rate and biomass productivity of *N. salina* and *Synechocystis* sp. PCC6803 were substantially affected by effluent loading rate. *N. salina* had higher tolerance to ammonium and grew well under effluent loading of 3-18%, while the growth rate of *Synechocystis* sp. PCC6803 gradually decreased as the effluent loading increased. *N. salina* and *Synechocystis* sp. PCC6803 removed nitrogen and phosphorus effectively (>90% removal efficiency) under effluent loading of 3% and 6%, respectively. The highest biomass yield of *Synechocystis* sp. PCC6803 in 10-day batch study (1.5 g L\(^{-1}\)) was 1.6 times of that of *N. salina*. But the lipid productivity of *N. salina* was higher than *Synechocystis* sp. PCC6803 due to higher lipid content of *N. salina*.

The biomass and lipid productivity of *N. salina* and *Synechocystis* sp. PCC6803 were both improved in semi-continuous cultivation at their optimal effluent loading, 6% and 3%, respectively. The nutrient removal rate was also increased due to frequent medium replenishment. The optimal conditions recommended for *N. salina* and *Synechocystis* sp. PCC6803 were 2-day harvesting interval with 50% harvesting ratio and: 1-day harvesting interval with 50% harvesting ratio, respectively, where the highest biomass and lipid productivity were obtained. Although the lipid productivity of
Synechocystis sp. PCC6803 was lower than *N. salina*, its higher biomass yield still makes it a good candidate for biomass production and wastewater bio-treatment.

This study demonstrated the feasibility of growing *N. salina* and *Synechocystis* sp. PCC6803 in AD effluent under high salinity (20‰) for bioenergy production. The optimal operation parameters for semi-continuous cultivation attained in this study need to be tested in a large pond which is subjected to changes of many factors such as temperature fluctuation, limited light, larger surface area, poor mixing. The downstream processes such as harvesting and lipid extraction are also important for the large scale algal biofuel production system. Further study and improvement are required to make algal biofuel economically viable.


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