# CULTIVATION OF ALGAE ON HIGHLY CONCENTRATED MUNICIPAL WASTEWATER AS AN ENERGY CROP FOR BIODIESEL PRODUCTION

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

Yecong Li

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Adviser: Dr. Roger Ruan

June 2012

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# Acknowledgements

"It is good to have an end to journey toward; but it is the journey that matters, in the end." *Ursula K. LeGuin*

As I come to the end of this journey, completing the dissertation provides me a perfect time to reminisce many individuals who have impacted me along the way and made this journey possible. At this particular point of time, I first would like to express my heartfelt appreciation to my advisor and Chair of the defense committee, Professor Roger Ruan, for his patience and unremitting assistance throughout this project. Professor Ruan has provided me with all the opportunities to coordinate every aspect of this project, which have given me tremendous knowledge and experience in carrying out scientific research that will benefit me for years along my professional career. Without his encouragement, kind guidance and tireless effort, I wound not have been able to complete this journey. His great passion and insights are always inspiring to me, and one simply could not wish for a better or friendlier supervisor like him.

I also greatly appreciate my co-advisor Professor Jun Zhu for admitting me into the Department of Biosystems and Agricultural Engineering at University of Minnesota – Twin Cities, for his guidance and friendship, also for many constructive suggestions and helpful advice he has given me over many years. With a deep gratitude I owe him for leading me to the world of scientific research and being a scientist.

A special thank you goes to the other two committee members, Professors Paige Novak and Ping Wang, for their efforts and time in assisting me revising my dossier and dissertation. I am also thankful to Professor Paul Chen, for proofreading my writings, always being there for daily communication, and lending me constant courage to complete my degree with energy and optimism.

I cannot say enough thank you to my friends, colleagues and fellow graduate students for their friendship, and for helping creating a joyful and stimulating living and working environment. In particularly, I would like to thank Drs. Wenguang Zhou, Min Min, Yiqin Wan and Blanca Martinez from whom I have learned plenty about experimental design, bioreactor construction, chemical analysis, and science in general; Bing Hu, and Zhenyi Du for their assistance in experiments, helpful suggestions and discussion at various stages of this project.

My gratitude also extends to the University of Minnesota Initiative for Renewable Energy and the Environment (IREE), Metropolitan Council Environmental Services (MCES), Xcel Energy, the Legislative-Citizen Commission on Minnesota Resources (LCCMR) for their financial support, Adam Sealock and Robert Polta from Saint Paul MCES Wastewater Treatment Plant for helping with the sample collection.

Last but not least, I would like to take this opportunity to express my deepest love and everlasting appreciation to my dearest parents, grandparents, brother and husband for their endless caring, understanding, support, sacrifice and completely confidence on me

even during the moment when I have doubts on myself, and for always letting me know that they are there for me, wherever I am in the world.

THANK YOU ALL!

## Dedication

This dissertation is dedicated to my beloved father Zhengjun Li and mother Suqin Liu. Their unconditional love is every reason for what I am and where I am.

## Abstract

There has been renewed interest in bio-fuel production over the past decade due to the sharp rise in fossil fuel prices and increasing concerns about the global climate change. This dissertation was inspired by the idea of coupling algae based biofuel generation and municipal wastewaster treatment. The objectives of this study are to employ potential algae strains for efficient simultaneous biodiesel production and wastewater nutrients removal, and to develop an operational strategy optimal for biomass and biodiesel production as well as nutrient removal from a highly concentrated municipal wastewater stream, centrate, which is generated during the sludge thickening process.

In the first stage, the feasibility of growing *Chlorella sp.* in the centrate for simultaneous wastewater treatment and energy production was tested. The characteristics of algal growth, biodiesel production, wastewater nutrient removal and the viability of scale-up and the stability of continuous operation were examined. Two culture media, namely autoclaved centrate (AC) and raw centrate (RC) were used for comparison. The results showed that by the end of a 14-day batch culture, algae could remove ammonia, total nitrogen, total phosphorus, and chemical oxygen demand (COD) by 93.9%, 89.1%, 80.9%, and 90.8%, respectively from raw centrate, and the fatty acid methyl ester (FAME) content was 11.04% of dry biomass providing a biodiesel yield of 0.12g-biodiesel/L-algae culture solution. The system could be successfully scaled up, and continuously operated at 50% daily harvesting rate, providing a net biomass productivity of 0.92 g-algae/(L·day).

The second stage was targeted at screening one or several algae species/strains that can survive and grow well in centrate with high biomass and lipid productivity as well as superior nutrient removal efficiency, and investigating the significance of environmental factors including ligh intensity, light/dark cyle, and exogenous $CO_2$ on biomass accumulation and biodiesel production using Plackett-Burman experiment design. In this stage the study was carried out in two sections, namely using aglae strains collected from local lake areas and those purchased from algae commercial bank, respectivity. In the first section, sixty algae-like microorganisms collected from different sampling sites in Minnesota were examined using multi-step screening and acclimation procedures to select high-lipid producing facultative heterotrophic microalgae strains capable of growing on centrate for simultaneous energy crop production and wastewater treatment. Twenty-seven facultative heterotrophic microalgae strains were found, among which seventeen strains were proved to be tolerant to centrate. These seventeen top-performing strains were identified through morphological observation and DNA sequencing as *Chlorella sp.*, *Heynigia sp.*, *Hindakia sp.*, *Micractinium sp.*, and *Scenedesmus sp.*. Five strains were chosen for other studies because of their ability to adapt to centrate, high growth rates (0.455 - 0.498 $d^{-1}$) and higher lipid productivities (74.5 – 77.8 mg $L^{-1}$ $d^{-1}$). These strains are considered highly promising compared with other strains reported in the literature. In the second section, 143 different algae strains from the family of *Chlamydomonas, Chlorella*, *Scenedesmus*, *Ankistrodesmus, Euglena*, *Chloroccum,* and *Botryococcus*, etc. purchased from a number of U.S. institutions were screened use the same multi-step screening and

acclimation procedures. The results showed that 14 algae strains from the genus of *Chlorella*, *Haematococcus, Scenedesmus*, *Chlamydomonas,* and *Chloroccum* were capable of growing on centrate. Since the highest net biomass accumulation (2.0143 g/L) was observed with *Chlorella kessleri* (UTEX398) followed by *Chlorella protothecoides* (UTEX25, 1.3089g/L), the two strains were used in further studies. It is found that both algae strains, UTEX398 and UTEX25 were capable of mixotrophic growth when cultivated on centrate. Environmental factors had significant effect on algal biomass accumulation, wastewater nutrients removal and biodiesel production for both strains. Higher light intensity and exogenous $CO_2$ concentration with longer lighting period promote biomass accumulation, fatty acid methyl ester (FAME) formation, removal of chemical oxygen demand (COD) and total nitrogen (TN), while, lower exogenous $CO_2$ concentration promotes phosphorus removal due to higher pH increase.

In the third stage, the single effect of light intensity on biomass accumulation, wastewater nutrient removal through algae cultivation, and biodiesel productivity was investigated with algae species *Chlorella kessleri* and *Chlorella protothecoide.* The light intensities studied were 0, 15, 30, 60, 120, 200 $\mu molm^{-2}s^{-1}$. The results showed that light intensity had profound impact on tested responses for both strains, and the dependence of these responses on light intensity varied with different algae strains. For *Chlorella kessleri*, the optimum light intensity was 120 $\mu mol \cdot m^{-2} \cdot S^{-1}$ for all responses except for COD removal. For *Chlorella protothecoide*, the optimum light intensity was 30 $\mu mol \cdot m^{-2} \cdot S^{-1}$. The major components of the biodiesel produced from algae biomass

were 16-C and 18-C FAME, and the highest biodiesel contents were 24.19% and 19.48% of dried biomass for *Chlorella kessleri* and *Chlorella protothecoide*, respectively. Both species were capable of wastewater nutrients removal under all lighting conditions with high removal efficiencies. Since *Chlorella kessleri* showed superior capability of biomass accumulation and biodiesel production, it was used in the following sections.

The fourth stage was aimed at investigating the single effect of light/dark cycle at low light frequency on algae based biodiesel production using centrate wastewater stream with mixotrophic strain *Chlorella kessleri.* The data suggested that the length of lighting period during a day greatly affects the algal biomass accumulation, biodiesel production and wastewater nutrients removal. The biomass concentration, biodiesel content and the removal of ammonia, total nitrogen and total phosphorus all increased with increasing lighting period. However, the removal of COD showed different trend, with higher removal rate observed under shorter lighting period. The results showed that for maximum biomass accumulation, biodiesel production and wastewater nutrients removal in batch culture system, the optimum lighting period is 16 hours and the cultivation time should be controlled at three days.

In the fifth stage, the effect of supplying different concentrations of exogenous $CO_2$ uder various light intensities at simulated light/dark cycle on biomass accumulation and wastewater nutrients removal was tested with aglae strain *Chlorella kessleri*. The results confirmed that there was an optimum $CO_2$ level for algae growth, which is 2.5% for this

investigation. When the light intensity reached a certain lower limit, the effect of $CO_2$ supplementation became minimum because the photoautotrophic efficiency was significantly reduced. It is also found that a future study to investigate the effects of environmental factors on biomass and biodiesel production under continuous or fed-batch cultivation mode, where wastewater is continuously replenished and algae was harvested, would be valuable due to the fact that the nutrients was depleted and the algae biomass concentration decreased on Day 8.

The objective of the last stage was to optimize the operational parameters, including light intensity, light-dark cycle, exogenous carbon dioxide concentration, and hydraulic retention time (HRT), for high algae biomass accumulation, biodiesel production and wastewater nutrients removal in designed fed-batch culture system. The results showed that response surface methodology with central composite design is effective in system optimization for algal-based biodiesel production using centrate wastewater in fed-batch culture. The regression analysis illustrates that, at $p\text{-value} < 0.05$, harvesting rate, light intensity and lighting period had significant effect on biomass concentration, biomass and biodiesel yield, as well as ammonia removal rate. Hydraulic retention time and exogenous $CO_2$ concentration played important role in FAME content, while the removal rates of toal nitrogen (TN), total phosphorous (TP) and chemical oxygen demand (COD) could not be explained by quadratic models. The optimum conditions for biodiesel production are hydraulic retention time of 2.86 days, which means harvesting 35% of the total volume daily, light intensity of $190 \mu mol m^{-2} s^{-1}$ and lighting period of 10h.

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# CHAPTER 1. INTRODUCTION

## 1.1 Background and significance of the research

Due to the depletion of fossil fuel supplies and contribution to global warming caused by carbon dioxide emission, continuous use of conventional petroleum sourced fuels has been widely recognized as untenable. Therefore, a concentrated effort is being made to advance renewable energy alternatives (Chisti, 2007). Biodiesel derived from plant oils or animal fats can address issues of sustainability and energy security, thus, is considered as a promising substitute to petroleum fuels. Currently, the major source for commercial biodiesel production is soybean, and other oil sources including canola, corn, jatropha, waste cooking oil and animal fats (Chisti, 2007) are also being tested. However, these processes have caused concerns of competing with food sources, having marginal effect on reduction of greenhouse gas emission (Fargione et al., 2008), and are not capable of satisfying the existing demand for petroleum fuels (Chisti, 2007). Prior research suggests that microalgae, which have an areal productivity 20 to 40 times that of oil crops (Sheehan et al., 1998) and oil content up to 80% by weight of dry biomass (Spolaore et al., 2006; Chisti, 2007) have the potential to replace the current source for renewable biodiesel production (Sheehan et al., 1998; Banerjee et al., 2002; Chisti 2007). However, for this transition to happen, algal biomass must be produced economically. Algae growth requires nutrients, water, and light, which are the main factors involved in the cost analysis of algal biomass production (Chisti 2007). To minimize the expenses associated with algal biomass production, nutrients, water and light must be supplied by readily available sources with low economic input.

One of the methods to reduce costs of nutrients and water is to integrate algae biomass production with wastewater treatment, which was first suggested in 1960s (Oswald and Golueke, 1960), since some wastewater streams contain nutrients and water sources readily available for algae cells. In previous studies, municipal wastewater was collected from various stages during the treatment process to test the ability of supporting algae growth. These wastewater streams included primary clarifier effluent (Tam and Wong, 1989, 1990; Woertz et al., 2009; Lau et al. 1995), activated sewage filtrates (Tam and Wong, 1989, 1990), and effluent from secondary treatment tank (Oswald et al., 1978; Shelef et al., 1978). The results and findings from these studies are summarized in section 2.4. The centrate, which is the liquid generated from activated sludge thickening process, is rich in nutrient including carbon, nitrogen and phosphorus. With a daily process rate of one million gallons in the wastewater treatment plant located in Saint Paul, Minnesota and higher nutrient levels than wastewater streams from other stages, centrate provides a potential source for algae cultivation. So far, only a few studies have been conducted to test the suitability of centrate for algae cultivation, thus our current research effort in this area is necessary and important.

Environmental factors, particularly light, temperature, nutrients status, and salinity not only affect photosynthesis and productivity of cell biomass, but also influence the pattern, pathway, and activity of cellular metabolism. Since the ultimate goal of this project is growing algae on centrate wastewater in greenhouse using semi-continuous cultivation system, thus this study will first focus on screening potential strains grown

on centrate for high biomass and biodiesel production and then study the effect of operational parameters including light intensity, light-dark cycle, hydraulic retention time, and carbon $CO_2$ (if needed) on algae growth, biodiesel production and wastewater nutrient removal in semi-continuous system. Operational parameters not only affect the metabolic pathway for lipid production but also the cost of energy consumption (Tedesco and Duerr, 2006, Lu et, al., 2001; Solovchenko et, al., 2008; Lee and Tan, 1988; Danesi et, al., 2004). To facilitate the production of biodiesel from algae biomass, it is crucial to understand the influence of operation parameters on the production and accumulation of biomass and lipids by photosynthetic microalgae because they show considerable metabolic flexibility in response to changes in cultivation conditions (Shifrin and Chisholm, 1980; Chelf, 1990). Therefore, optimization of operation strategy for maximum biomass and biodiesel production as well as wastewater nutrient removal is desirable for the cultivation system.

The significance of the proposed work can be summarized in the following aspects: 1) it will advance the knowledge and understanding of cultivating algae with municipal wastewater, specifically centrate, for simultaneous biomass and biodiesel production and nutrient removal; 2) it will enhance manufacturing practice in applying algae cultivation systems by providing key information concerning lighting strategy and hydraulic retention time for improvement and/or optimization of biomass and lipid accumulation; 3) it will provide another pathway to recycle flue gas if supplement of exogenous $CO_2$ was found beneficial for algal biomass and lipid accumulation; 4) it will provide knowledge to municipal wastewater treatment plants in selecting an alternative

process for waste reduction and nutrient removal; and 5) it will partially pave the way for biodiesel production from renewable sources.

## 1.2 Objectives

The goal of this study is to employ potential algae strains for simultaneous biodiesel production and wastewater nutrient removal at high efficiency, and to develop and verify the optimum operation strategy that can facilitate maximum biomass and biodiesel production as well as nutrient removal from municipal wastewater in semi-continuous system. The specific objectives of this research are to:

(1) Study the possibility of using algae strains cultivated in municipal wastewater specifically centrate for simultaneous biodiesel production and wastewater nutrients removal.

(2) Screen one or several algae species that can survive and grow well in centrate with high biomass and lipid productivity as well as superior nutrients removal efficiency.

(3) Determine if the algal growth in centrate is autotrophic or heterotrophic to further decide if supplying exogenous $CO_2$ is necessary for biomass accumulation.

(4) Investigate the single effect of operational parameters including light intensity, light-dark cycle, and exogenous carbon dioxide concentration on biomass and biodiesel production.

(5) Optimize the operational parameters mentioned above to maximize biomass and biodiesel production.

# CHPATER 2. LITERATURE REVIEW

## 2.1 Current status of biodiesel production

Because of diminishing petroleum reserves and the deleterious environmental consequences caused by burning of fossil fuels, renewable and biodegradable biodiesel has attracted attention during the past few decades as an environmentally friendly fuel. Biodiesel is produced from triglycerides in which three fatty acid molecules are esterified with a molecule of glycerol. In making biodiesel, triglycerides are reacted with an alcohol such as methanol in the presence of a catalyst, such as alkali or acid, through a reaction known as transesterification or alcoholysis. Transestrification produces esters of fatty acids, which are biodiesel, and glycerol (Hoydoncx et al., 2004). Currently, the majority of biodiesel is produced from plant oils and animal fats, mainly soybean (Chisti, 2007) by alkali-catalyzed transesterification (Vasudevan and Briggs, 2008). Considerable research has been done on biodiesel produced from vegetable oils (e.g., soybean oil, sunflower oil, rapeseed oil) using alkali catalysts. In this process, the vegetable oil and alcohol must be substantially anhydrous and have low free fatty acid content, because the presence of water or free fatty acid or both promotes soap formation, which lowers the yield of esters and renders the downstream separation of the products difficult (Freedman et. al, 1984)

## 2.2 Potential of biodiesel production from algal biomass

The crisis caused by reserve shortage of fossil fuels triggered the research on cultivation of microalgae for biofuels as early as 1970s. Within the context of feedstock development for biodiesel production, algae have certain well-known characteristics of great interest that are superior to conventional oil crops. First, algae grow extremely fast

and commonly double their biomass within 24 hours. During exponential phase, biomass doubling time is as short as few hours (Chisti, 2007). Second, algae have higher lipid content than conventional oil crop as shown in Table 2.1. Lipid content between 10% and 30% is common in many algae species under general cultivation conditions (Chisti, 2007). However, this value can double or triple under stressed environment, and reach up to 80% by weight of dry biomass (Spolaore et al., 2006; Chisti, 2007). Thus, algae can provide an areal productivity 20 to 40 times that of oil crops (Sheehan et al., 1998). Some algae species are found containing mainly 16 to 18 carbon length, which is ideal for biodiesel production (Xu et al., 2004; Chen et al., 2009).

**Table 2.1** Lipid content of some microalgae species (Deng et. al, 2009; Chisti, 2007; Li et al., 2008; Mata et al., 2009; Sialve et al., 2009; Um and Kim, 2009).)

| Microalgae species | Lipid content (% by weight of dry biomass) | Microalgae species | Lipid content (% by weight of dry biomass) |
|---|---|---|---|
| *Ankistrodesmus sp.* | 24–31 | *Monodus subterraneus* | 16 |
| *Botryococcus braunii* | 25–75 | *Monallanthus salina* | 20–22 |
| *Chaetoceros muelleri* | 33 | *Nannochloris sp.* | 20–56 |
| *Chlamydomonas reinhardtii* | 21 | *Nannochloropsis oculata.* | 22–29 |
| *Chlorella emersonii* | 25–63 | *Nannochloropsis sp.* | 12–53 |
| *Chlorella minutissima* | 57 | *Neochloris oleoabundans* | 29–65 |
| *Chlorella protothecoides* | 14–57 | *Pyrrosia laevis* | 69.1 |
| *Chlorella sorokiniana* | 19–22 | *Pavlova salina* | 30 |
| *Chlorella .sp* | 10–48 | *Prostanthera incisa* | 62 |
| *Chlorella vulgaris* | 5–58 | *Prymnesium parvum* | 22-39 |
| *Crypthecodinium cohnii* | 20–51 | *Pavlova lutheri* | 35 |
| *Dunaliella salina* | 6–25 | *Phaeodactylum tricornutum* | 18–57 |
| *Dunaliella primolecta* | 23 | *Scenedesmus obliquus* | 11–55 |
| *Dunaliella tertiolecta* | 16–71 | *Skeletonema costatum* | 13–51 |
| *Dunaliella sp.* | 17–67 | *Scenedesmus dimorphus* | 16-40 |
| *Euglena gracilis* | 14-20 | *Schizochytrium sp.* | 50-77 |
| *Ellipsoidion sp.* | 27 | *Thalassiosira pseudonana* | 20 |
| *Haematococcus pluvialis* | 25.0 | *Isochrysis galbana* | 7–40 |
| *Isochrysis sp.* | 7–33 | *Zitzschia sp.* | 45-47 |

To have a significant impact on diverting demand away from petroleum based fuels, the feedstock for biodiesel production has to be produced in vast amount with limited land area. Table 2.2 illustrates the cultivation areas for major oil crops for meeting the existing U.S transportation fuel needs by biodiesel. Clearly, oil crops cannot significantly contribute to replacing petroleum derived liquid fuels in the foreseeable future. This scenario changes dramatically, if microalgae are used to produce biodiesel. Between 2.2 and 15% of the total U.S. cropping area would be sufficient for producing algal biomass that satisfies 100% of the transport fuel needs (Table 2.2).

**Table 2.2** Biodiesel sources comparison (Chisti, 2007)

| Crop | Oil Yield (L/ha) | Land area needed (M ha)[a] | Percent of existing U.S cropping area[a] |
|---|---|---|---|
| Corn | 172 | 3081 | 1692 |
| Soybean | 446 | 1188 | 652 |
| Canola | 1190 | 445 | 244 |
| Jatropha | 1892 | 280 | 154 |
| Coconut | 2689 | 197 | 108 |
| Oil palm | 5950 | 89 | 48 |
| Microalgae [b] | 136900 | 3.9 | 2.2 |
| Microalgae [c] | 58700 | 9.0 | 5 |
| Microalgae[d] | 19567 | 27.0 | 15 |

[a] For meeting 100% of all transport fuel needs of the United States.
[b] 70% oil (by wt) in biomass.
[c] 30% oil (by wt) in biomass
[d] 10% oil (by wt) in biomass

## 2.3 Fundamentals of biodiesel production from algae biomass

### 2.3.1 General description of divisions and classes of algae

Algae are defined as any organisms with chlorophyll a and a thallus not differentiated into roots, stem and leaves (Lee, 1989). Macroalgae and microalgae are two main classes if the division is based on the morphology and size of the organism (Chen et al., 2009). In the family of macroalgae, the organisms are usually composed of multiple

cells arranged to structures that are similar to roots, stems and leaves of the higher plants. In contrast, microalgae mostly presented as unicellular photosynthetic organisms, which are mainly distributed in the waters with a few species found on the surface of all types of soils (Chen et al., 2009; Richmond, 2004). In this dissertation, the subject studied is the family of microalgae, thus, the term of "microalgae" and "algae" is both referred to microalgae throughout this dissertation starting from this section. According to their cellular composition, microalgae are further divided into prokaryotic cyanobacteria (also known as blue-green algae) and eukaryotic algae. In prokaryotic cyanobacteria, which lack the structure of nucleus, the DNA lies freely in the cytoplasm together with the photosynthetic membranes and is not organized in the chromosomes. Eukaryotic algae possess a true membrane-bounded nucleus that contains the major part of the genome distributed on a set of chromosomes and the nucleolus (Richmond, 2004). Eukaryotic algae can be further categorized into at least 12 major classes including diatoms (*Bacillariophyceae*), green algae (*Chlorophyceae*), red algae (*Rhodophyceae*), yellow-green algae (*Xanthophyceae*), golden algae (*Chrysophyceae*), brown algae (*Phaeophyceae*), and Euglenoids (Chen et al., 2009). Microalgae can thrive in diverse ecological habitats such as freshwater, seawater, and wastewater, and also adapt to various extreme temperatures and pH conditions. In addition, many microalgae show rapid growth under optimal conditions. For example the doubling time of some *Chlamydomanos* species is as short as six hours. Due to their strong ability in adaptation, microalgae therefore become dominating among the organisms on the earth, exemplified by their diverse unusual features such as being rich in starches, oils, and proteins, and able to accumulate important secondary metabolites (e.g., carotenoids). It

was estimated the upper limit of algal species in the nature is about 10 million (Norton et al., 1996], only small portion of which is identified in taxonomy, including several thousand algal species collected and stored in institutions for research and only about 15 species used for industrial production of foods, feeds, drugs, and fine chemicals. Therefore, there is a huge potential to identify new algal species with economic values from natural environments. The major push for the algal domestication to overcome food and energy shortage occurred in the 1960-70's. Some algal species such as *Spirulina* containing abundant proteins and of high biomass production are cultivated in Asian countries and other regions of the world. It was hypothesized that microalgae like diatoms deposited millions years ago might be a source of petroleum and natural gas (North, 1985).

### 2.3.2 Photosynthesis in microalgae

Photosynthesis is a unique process of sunlight energy conversion, in which atmospheric $CO_2$ and light energy are converted to chemical energy and stored in organic matter. For eukaryotic specie, which is the case in this study, this process takes place in chloroplasts that are rich in organic pigments for harvesting light energy (Richmond, 2004). Photosynthesis is traditionally divided into two stages, namely light reaction and dark reaction. In the light reaction, which occurs on the thylakoid membranes, the light energy in the form of photon is converted to chemical energy providing a biochemical reductant NADPH and a high energy compound ATP. In the dark reaction, which occurs in the stroma, NADPH and ATP are utilized in the reduction of $CO_2$ to carbonhydrates. In most algae species, carbon fixation was carried out in four distinct

phases forming the so-called Calvin-Benson cycle. The first step is called the carboxylation phase, in which the enzyme Rubisco (ribulose-bisphosphate carboxylase/oxygenase) catalyzes the reaction of adding $CO_2$ to the 5-carbon sugar, ribulose bisphosphate, to form two molecules of phosphoglycerate. The second step is called the reduction phase, in which phosphoglycerate was first phosphorylated by ATP to form ADP and diphosphoglycerate, which was then reduced to phosphoglyceraldehyde by NADPH. The third step is called the regeneration phase, in which ribulose phosphate is regenerated for further $CO_2$ fixation in a complex series of reactions combing 3-, 4-, 5-, 6- and 7-carbon sugar phosphate through the action of transketolase and aldolase enzymes. The fourth step is called the production phase, in which primary end-product of carbohydrate, as well as fatty acids, amino acids and organic acids are synthesized (Richmond, 2004).

Photosynthesis can be blocked by inhibitors which hinder one or several specific steps in the photosynthetic process (Francoeur et. al, 2007). One commonly used inhibitor is called DCMU (3-(3,4-dichlorophenyl)-1,1-dimethyl urea), which blocks the plastoquinone binding site of photosystem II, disallowing the electron flow from where it is generated to plastoquinone. This interrupts the photosynthetic electron transport chain in photosynthesis and thus blocks the ability of the organism to turn light energy into chemical energy (ATP and reductant potential). The effect of DCMU is very specific and sensitive. It only blocks electron flow from photosystem II, and have no effect on photosystem I or other reactions in photosynthesis, such as light absorption or

carbon fixation (DeLorenzo et. al, 2001; Francoeur et. al, 2007). In this project, DCMU was used to determine if the algal growth in centrate is autotrophic or heterotrophic.

### 2.3.2 Metabolic pathways and their association with biomass and lipid production

Many algae species have the capability of turning inorganic carbon into organic molecules through photosynthesis process for production of energy and substances essential to self survival and growth, which is called photoautotrophy. Besides the function of photoautotrophy, some algae species also possess the ability of heterotropy, which means that they can take up some organic substrates from the environment and convert them into building blocks and storage compounds in the dark and under light. The mechanism involved in uptake of the organic carbon substrate is complicated. It is reported that hexoses are sterically incapable of diffusing across plasma membrane passively (Fiechter et al., 1981). Some algae species such as *Chlorella sp.* possess an inducible active transport system of glucose, which could be monitored by the preferential synthesis of cytoplasmic membrane-bound protein and a significant increase in glucose transport protein is 15 minutes and the process is energy dependent (Tanner, 1969; Haass and Tanner, 1974, Fenzl et al., 1977). Thus the energy starved algae cell could not be induced to take up glucose in the dark despite the presence of the glucose in the environment. Sauer (1986) reported that the transport protein is in the range of 30-40 kDa range. Uptake of glucose is accompanied by a proton uptake through proton/hexose symport in a 1:1 stoichiometry, and deplolarisation of the membrane potential (Komor and Tanner, 1978). Amino acids are actively transported across cell membranes involving periplasmic binding proteins. The small molecular size

of organic acids (lactic acid and acetic acid) and alcohol (ethanol) can easily diffuse into the cells. The free acids are more lipid soluble and less negatively charged than the ionized form, hence reduction in pH will favor penetration of the acids into the cells (Richmond, 2004). The heterotrophic culture circumvents technical and physiological difficulties associated with the supply and distribution of light and carbon dioxide involved in the photosynthetic mode of cultivation. It offers the possibility of increasing the cell concentration and productivity. It is reported that under heterotrophic growth supplied with acetate, glucose, or other organic compounds as carbon source, algae species can provide a high biomass yield and high content of lipid in cells (Endo et al., 1977; Wu et al., 1994, Xu, et al., 2004). Additionally, heterotrophic algae can grow well at high cell density, leading to the fact that the biomass production from heterotrophic algae is usually much higher than that from autotrophic algae. Gladue and Maxey (1994) reported a cell concentration of above 45g/L when using heterotrophic culture. Not all species possess the ability of heterotrohoic growth, thus it is necessary to screen and isolate microalgae in the presence of organic carbon substrates and from sources which are rich in organic materials. *Chlorella vulgaris* is one of the exceptions, in that the photosynthetic and heterotrophic maximum specific growth rates are comparable (Richmod, 2004).

Some algae species are capable of mixotrophic growth in which $CO_2$ and organic carbon are simultaneously assimilated, both respiratory and photosynthetic metabolism operating concurrently. The specific growth rate of the mixotrophic culture is, therefore, approximately the sum of the specific growth rates of cells grown under

photoautotrophic condition and heterotrophic conditions (Table 2.3, Richmond, 2004). Matrínez et al. (1997) found that when *Chlorella pyrenoidosa* was cultivated under moderate light condition, an exponential mixotrophic phase was observed first and when the organic substrate was completely consumed, an autotrophic phase followed after an adaptation period. Lalucat et al. (1984) reported that *Chlorella* sp. strain VJ79 could grow autotrophically, heterotrophically, and mixotrophically on a variety of organic substrate. Furthermore, glucose and serine could promote a mixotrophic growth from which the yield is higher than the sum of autotrophic and heterotrophic yields. Examples of microalgal species reported to be able to growth mixotrophically along with the suitable organic substrate are summarized in Table 2.4 (Richmond, 2004)

Apparently, for higher biomass and oil production, heterotrophic growth and mixotrophic growth are preferred, but the fact that sugar or other organic substrate is of high cost lowers the economic feasibility of biodiesel production from algae biomass. Therefore, finding an organic source that is of low cost, easy accessibility, high nutrients level, free of toxic inhibitor is desirable.

**Table 2.3** Comparison of the maximum specific growth rate of some algae species cultured photosynthetically, heterotrophically and mixotrophically.

| | Maximum specific growth rate ($h^{-1}$) | | | |
|---|---|---|---|---|
| Species | Potosynthetic | Heterotrophic | Mixotrophic | References |
| *Chlorella pyrenoidosa* | 0.082 | 0.038 (glucose) | - | Droop (1994) |
| *C.vulgaris* | 0.110 | 0.098 (glucose) | 0.198 (glucose) | Ogawa and Aiba (1981) |
| | 0.081 | 0.050 (glucose) | 0.131 (glucose) | Martinez and Orus (1991) |
| *Haematococcus pluvialis* | - | 0.014 (acetate) | 0.037 (acetate) | Droop (1955) |
| | 0.013 | 0.009 (acetate) | 0.024 (acetate) | Kobayashi et al. (1992) |
| *Scenedesmus acutus* | 0.061 | 0.040 (glucose) | 0.048 (glucose) | Ogwa and Aiba (1981) |

**Table 2.4** Examples of microalgae species that can be cultivated mixotrophically

| Microalgae species | Organic carbon substrate | References |
|---|---|---|
| *Anabaena variabilis* | Fructose, glucose | Pearce and Carr (1969)<br>Valiente et al. (1992) |
| *Brachiomonas submarina* | Acetate | Tsavalos and Day (1994) |
| *Chlorella minutisima* | Methanol | Kotzbasis et al. (1999) |
| *C.regularis* | Acetate | Endo et al. (1977) |
| *C. Sorokiniana* | Glucose | Lee et al. (1996) |
| *C.vulgaris* | Glucose | Ogwa and Aiba (1981) |
| *Cyclotella cryptica* | Glycerol, glucose | Wood et al. (1999) |
| *Euglena gracilis* | Glucose, glycerol, galactose, ethanol | Tanim and Tsumura (1989) |
| *Haematococcus pluvialis* | Acetate | Droop (1955) |
| *Nannochloropsis CCAP879/5* | Glycerol, glucose | Wood et al. (1999) |
| *Nannochloropsis CCAP211/78* | Glycerol | Wood et al. (1999) |
| *Navicula saprophila* | Acetate | Kitano et al. (1997) |
| *Phaeodactylum tricornutum* | Glycerol | Garcia et al. (2000) |
| *Rhodomonas reticulata* | Glycerol, glucose | Wood et al. (1999) |
| *R.salina* | Acetate | Kitano et al. (1997) |
| *Scenedesmus acutus* | Glucose | Ogwa and Aiba (1981) |
| *S.obliquus* | Methanol | Kotzabasis et al. (1999) |

### 2.3.3 Lipid accumulation in eukaryotic microalgae cells

In terms of biodiesel production, lipids, especially triglycerides (TAGs), are the most desirable products of algae cultivation in this dossier. In most algae cells, lipids such as a range of phospholipids and glycolipids are mainly used as structural components of organelles. Algae also contain neutral lipids, mostly as TAGs, as storage products with a small amount of mono- and diacylglycerols (Hardwood and Jones, 1989). Several other researchers showed that in some algae cells lipids also play roles in signal transduction (Chen et al., 2009). The major components of lipids are fatty acids, which are produced in chloroplasts. A pathway called *de novo* fatty acid synthesis in the stroma of chloroplasts followed by the assimilation of the fatty acid into the glycerol backbone and acyl transfers from acyl CoA in the endoplasmic reticulum is employed in the process (Chen et al., 2009). The 16- or 18-carbon fatty acids (or both) produced

through this pathway are then used as precursors for the synthesis of cellular and organellar membranes as well as TAGs. The TAGs synthesis pathway has not been fully understood at molecular level, but it is believed that TAGs are synthesized via a direct glycerol pathway in which positions 1 and 2 of glycerol-3-phosphate react with fatty acids produced in chloroplasts and a third fatty acid is transferred to the position 3 of the diacylglycerol to form triacylglycerol (TAG) catalyzed by diacylglycerol acyltransferase (Chen et al., 2009). When cultivated under stressed conditions such as nitrogen starvation and phosphorus starvation, temperature shock, and extreme light algae will enhance their ability of lipid accumulation and some lipids will present in the form of free droplet as TAGs (Chisti, 2007). Unfortunately, high TAGs content has been found in the company of low biomass productivity, thus one needs to compromise between biomass growth and oil accumulation.

**2.3.4 Effect of lighting condition on algae growth and cell composition**

The variation in cell composition in a single algae species can vary in many folds according to the culture condition under which it is grown. Effect of lighting condition including light intensity and light-dark cycle on biochemical composition of photosynthetic algae is mainly controlled by the process called photoacclimation or photoadaptation. In this process, algal cells undergo dynamic changes in cell composition, in company with alteration in ultrastructural, biophysical and physiological properties to augment photosynthesis and algal growth (Dubinsky et al., 1995).

Light intensity plays an important role on organic C-substrate metabolism. In previous studies, it is found that growth is slow on glucose alone in heterotrophically grown *Scenedesmus acutus* and *Spirulina platensis,* while in the presence of light, the algae cells were able to utilize glucose more efficiently (Ogawa and Aiba, 1981; Marquez et al., 1995). They researchers concluded that perhaps light facilitating improved assimilation of the sugar. Martinez and Orus (1991) reported that the specific growth rate of mixotrophic *Chlorella* increased with increasing of light intensity, however, a similar effect was not observed in photoautotrophic cultures exposed to the same light intensities. This was explained by the stimulatory effect of light on metabolism of sugar by mixotrophic cells. As mentioned in section 2.3.2, Lalucat et al. (1984) found that the growth yield and productivity of mixotrophic *Chlorella* sp. grown in glucose was found to be greatest when compared to heterotrophic and photoautotrophic cells. In addition, they did an extensive bioenergetic analysis and found that there was a significant decrease in the fraction of light energy used for carbon dioxide fixation by mixotrophic cells. Most of the light energy was instead used as an energy source for carbon assimilation. They conclude that mixotrophy resulted in high energetic efficiency since the amount of energy dissipated was minimal.

In addition to C-substrate metabolism, light intensity also greatly affects the composition of the algae cells. A common trend of cellular response to decreasing light intensity is to increase chlorophyll a and other light-harvesting pigments including chlorophyll b, chlorophyll c, phycobiliproteins and primary carotenoids. On the contrary, under high light intensity conditions, chlorophyll a and other pigments directly

involved in photosynthesis decrease, while the secondary carotenoids such as zeaxanthin, astaxanthin and β-carotene, which serve as photoprotective agents, increase. These carotenoids play an important role in preventing excess light energy from reaching the photosynthetic machinery (Ben-Amotz et al., 1982; Vechtel et al., 1992). Besides the content of pigments, polysaccharide and lipid also alter their content in response to different light intensities. High light intensities tend to increase polysaccharide production in algal cells. According to Friedman et al. (1991), *Porphyridium sp.* and *Porphyridium aerugineum* increased their polysaccharide content by 0.6 and 3 folds, respectively, when the environment light intensity increased from 75 to 300μmol $m^{-2}$ $s^{-1}$. Previous research with various algae species suggests that lipid content in algae cells as well as total polyunsaturated fatty acids such as eicosapentaenoic acid (EPA), arachidonic acid (AA), and docosahexaenoic acid (DHA) are also related to the environmental light intensity (Cohen, 1987; 1999), however, diverse trends were observed when using different algae species. In the study conducted by Sukenik et al. (1989), high lipid content and high portions of EPA were detected in *Nannochloropsis* cells under light-limiting conditions, whereas 16:0 and 16:1 fatty acids dominated when light intensity increased to a saturated level. Similar results was observed by Tedesco and Duerr (1989), which showed that total lipid content decreased with the increase of light intensity from 170 to 870 μmol $m^{-2}$ $s^{-1}$ while the composition of fatty acids remained regardless of the light intensity. In another study conducted by Lee and Tan (1988), the contents of polyunsaturated fatty acids (18:2, 20:4, 20:5) increased with increasing incident light intensity at the surface of the cultures when working with *Porphyridium cruentum*. Molina Grima et al. (1999) also reported that

strong light intensity enhanced the polyunsaturated fatty acids levels in certain species, which may due to the increase in oxygen-mediated lipid desaturation under high light conditions.

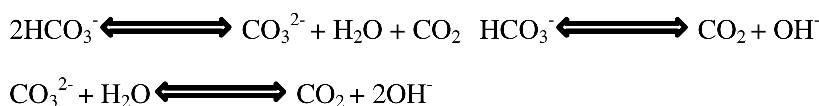
Light/dark cycle is another lighting regime that affects algae cell composition and biomass accumulation. However, the response of different algae species to light/dark cycle varies a great deal. Shifrin and Chisholm (1981) reported that the total lipid fraction in *O. polymorpha* was found to remain constant over the cell cycle in synchronized cultures regardless of the light regime, while, in the study carried out by Sicko-Goad (1988), it is reported that Chloroplast and lipid relative volume are greatest during the early part of the dark period. Total unsaturated fatty acids, including the C20:5 fatty acid, are lowest in the early part of the light period and highest in the dark. The sum of the C16 fatty acids remains constant at 70% of total fatty acids in the cells throughout the light-dark cycle, although percent composition of these two fatty acids shifts.

### 2.3.5 Effect of $CO_2$ concentration on algae growth and cell composition

For high rates of autotrophic production of algae biomass, supply of $CO_2$ and/or $HCO_3^-$ is the most important factor. Different from land plants, atmospheric $CO_2$ cannot satisfy the C-requirement of high yielding autotrophic algae production systems (Richmond, 2004). Previous research shows that higher biomass accumulation was observed when using $CO_2$-enriched air injection systems (Chiu et al., 2008; Yang and Gao, 2003; Kodama et al., 1993). Yang and Gao (2003) reported that increased $CO_2$ concentration enhanced significantly the growth rate of three species they tested including

*Chlamydomonas reinhardtii, Chlorella pyrenoidosa* and *Scenedesmus obliquus*, and the specific growth rates reached maximal values at 30, 100, and 60 μMCO2 in *C. reinhardtii, C. pyrenoidosa*, and *S. obliquus*, respectively. Chiu et al. (2008) conducted a set of experiments with *Chlorella sp.* and observed that during an 8-day interval cultures in the semi-continuous cultivation, at $CO_2$ concentrations of 2%, 5%, 10% and 15%, the rate of $CO_2$ reduction was 0.261, 0.316, 0.466 and 0.573 g $h^{-1}$, and efficiency of $CO_2$ removal was 58%, 27%, 20% and 16%, respectively. They also reported that in their semi-continuous culture, the optimum condition for biomass productivity was at 2% $CO_2$ aeration and lipid content was not affected even at high $CO_2$ aeration.

In addition to serve as carbon source for algae growth, the $CO_2$ - $H_2CO_3$ - $HCO_3^-$ -$CO_3^{2-}$ system also plays an important role in controlling and maintaining the pH levels of the culture solution. The bicarbonate-carbonate buffer systems can provide $CO_2$ for photosynthesis through the following reactions:



$$2HCO_3^- \Longleftrightarrow CO_3^{2-} + H_2O + CO_2 \quad HCO_3^- \Longleftrightarrow CO_2 + OH^-$$

$$CO_3^{2-} + H_2O \Longleftrightarrow CO_2 + 2OH^-$$

These reactions imply that during photosynthetic $CO_2$ fixation, $OH^-$ accumulates in the growth solution leading to a gradual rise in pH, thus, pH can reach as high as 11 in high algal density production systems where no additional $CO_2$ has been supplied (Richmond and Grobbelaar, 1986). Direct $CO_2$ sparging into the culture media is the best and most convenient method of pH control and at the same time supplying $CO_2$ for high yield in mass algal cultures (Richmond, 2004).

### 2.3.6 Effect of retention time on algal biomass yield and system stability

The retention times generally include hydraulic retention time (HRT) also known as hydraulic residence time and solid retention time (SRT). HRT is a measure of the average length of time that a soluble compound remains in a constructed bioreactor, while SRT measures the average length of time that the solids remain in a constructed bioreactor. For a single continuous stirred tank reactor, HRT is the same as SRT. In this study, the bioreactors we used were all single continuous stirred reactors, thus HRT, which is calculated according to Equation 2.1, will be used to represent the retention time.

$HRT=V_T/Q$ Equation 2.1

Where: HRT: hydraulic retention time

Q: daily wastewater flow rate, L/d

$V_T$: total reactor working volume, L

In the following chapters, another term, which is called harvesting rate and calculated by $Q/V_T$, will also be used to represent HRT. HRT is crucial for retaining the concentration of microorganism and available substrate, as well as for washing out of toxic compounds produced during the cultivation process. The shorter the HRT, the higher loading rate or a higher substrate concentration, and the higher rate of algae cells washing out. Generally, for algae cells with fast proliferation, short HRT is preferred in order to timely supply nutrients and decrease the cell concentration for better light penetration.

For commercial application, continuous or semi-continuous cultivation systems are more desirable than batch systems. Establish a stability boundary based on HRT is one of the most important tasks for the development of continuous or semi-continuous cultivation systems. Longer HRTs generally lead to higher biomass concentrations, which are attractive from a downstream processing point of view as they would minimize the energy required for harvesting leading to a higher net positive energy gain. However it may eventually fail the system if the nutrients or light are limited resulting in the death of the algae cells. Shorter HRTs are favorable for maintaining high concentrations of available nutrients and allowing better light penetration, however, for algae species with slow proliferation rate, too short a HRT will result in washing out of organism and fail the operation of the reactor eventually.

## 2.4 Integration of biomass and biodiesel production with municipal wastewater treatment through algae culture

Growing algae in wastewater has been under study for more than half a century (Oswald and Golueke, 1960). Previous studies demonstrated the success of using algae to clean wastewater rich in nitrogenous and phosphorus compounds (Oswald et al., 1978). Compared with the conventional wastewater treatment process, which applies activated sludge, a biological floc, to degrade organic carbonaceous matter to $CO_2$, algae can assimilate organic pollutants into cellular constituents such as lipid and carbohydrate, thus achieve pollutant reduction in a more environmental friendly way.

In previous studies, municipal wastewater was collected from various stages during the treatment process to test the ability of supporting algae growth as shown in Table 2.5. These wastewater streams included primary clarifier effluent (Tam and Wong, 1989, 1990; Woertz et al., 2009; Lau et al. 1995), activated sewage filtrates (Tam and Wong, 1989, 1990), effluent from secondary treatment tank (Oswald *et al.,* 1978; Shelef et al., 1978), and extract from activated and digested sludge (Cheung and Wong, 1981).

Tam and Wong (1989) conducted a study with algae species *Chlorella pyrenoidosa* and *Scenedesmus sp.* grown in settled and activated sewage filtrates, and found that *Chlorella* species were more feasible for wastewater treatment than *Scenedesmus*. They also reported that higher algal growth rate were recorded with higher inoculation size, and algae cells grew better in settled sewage than in activated sewage. In their study, more than 80% of total-N and inorganic N in settled sewage were removed by the end of experiment but such removal efficiency was lower in activated sewage. In their another study conducted in 1990, they grew *Chlorella pyrenoidosa* in settled and activated sewage under both batch and continuous systems, and reported that semi-continuous system with biomass recycling rate of 0.25 $day^{-1}$ support more biomass growth and appeared to be a more suitable and efficient way for wastewater treatment than the batch system. The nutrients removal efficiency for both settled and activated sewage were similar with about 62% reduction rate for phosphorus, around 80% reduction rate for $NH_4^+$-N, and 95% removal efficiency for $NO_3^-$-N. Woertz et al. (2009) investigated lipid productivity and nutrient removal by green algae grown during treatment of primary clarifier effluent supplemented with $CO_2$ in semi-continuous

indoor cultures with 2–4 days hydraulic residence times (HRT). They found that maximum lipid productivity in the municipal wastewater was 24 mg/day/L with 3-day HRT cultures, and over 99% removal of $NH_4^+$-N and phosphorus was achieved. Lau et al. (1995) examined the effect of initial sizes of algae on their activities and reduction of nutrient from the primary settled municipal sewage with green algae *Chlorella Vulgaris*. They reported that an initial algae density of $10^7$cells/mL was beneficial in this treatment, and the nutrient removal efficiency was more than 80% for total phosphorus, 90% for $NH_4^+$-N, 60% for total organic nitrogen (TON), and 50% for COD. They also concluded that COD and TON were mainly removed by indigenous bacteria instead of algae culture. In another paper published by same authors (Lau et al., 1998), they tested the operation mode of an immobilized algae cells batch system with same algae species and wastewater stream. They reported that immobilized algae cells was proved to be an efficient system in removing nutrients, which provided over 90% of $NH_4^+$-N removal and 99% of phosphorus removal. Cheung and Wong (1981) tested the growth of *Chlorella pyrenoidosa* on aqueous extracts of activated sludge and digested sludge. They suggested that digested sludge extract was better than activated sludge extract in supporting algal growth.

The wastewater streams tested for algal growth as well as the nutrients and final biomass concentrations are summarized in Table 2.5. From the literature cited above we can see that when combined with wastewater treatment, algae species from the family of *Chlorella* were most commonly used. In order to boost algae growth in wastewater streams, inoculation size needed to be controlled at relatively high level. The nutrients

removal efficiency for phosphorus, nitrogen and COD was satisfactory with reasonable hydraulic retention time, which provided encouraging information concerning the integration process of algae cultivation and wastewater treatment.

**Table 2.5** Summary of growth rate and final biomass concentration in previous studies
Note: P is short for total phosphorus, N is short for total nitrogen, COD is short for Chemical Oxygen Demand, and TOC is short for Total Organic Carbon.

| Waste used | Algae used | Nutrient concentration (mg/L) | | | Final biomass concentration (g/L) | Growth rate ($d^{-1}$) | Incubation time (day) | Reference |
|---|---|---|---|---|---|---|---|---|
| | | P | N | COD or TOC | | | | |
| Activated sludge extract | *Chlorella pyrenoidosa* | 28.33 | 0.588 | 20.99 (TOC) | 0.053 to 0.127 | | 11 | Cheung and Wong , 1981 |
| Digested sludge extract | *Chlorella pyrenoidosa* | 292.5 | 2.085 | 35.0 (TOC) | 0.116 to 0.57 | | 11 | Cheung and Wong , 1981 |
| Primary settled sewage | *Chlorella vulgaris* | 3.32-4.29 | 47.47-48.8 | 92-157.5 COD | | Highest at 0.2771 | 10 and 3 | Lau et al., 1995, 1998 |
| Settled sewage | *Chlorella pyrenoidosa* | 9.58-10.07 | 36.07-41.49 | 240 COD | 0.4-1.1 | 0.35 to 0.5 | 4 | Tam and Wong, 1989, 1990 |
| Activated sewage | *Chlorella pyrenoidosa* and *Scenedesmus sp.* | 10.04-12.18 | 24.17-39.2 | 112 COD | 0.35 to 1.2 | -- | 13 | Tam and Wong, 1989, 1990 |
| Primary clarifier effluent | A mixture of *Chlorella* and other algae species | 2.1 | 51 | -- | 0.317 to 0.843 | -- | 2-4 | Woertz et al., 2009 |
| Secondarily treated sewage | *Botryococcus braunii* | | | | 0.34-0.35 | -- | 10 | Sawayama et al., 1992 |

However, problems arise if we take a close examination of the data shown above. One common concern from Table 2.5 is that the algae biomass yield when cultivated in wastewater is very low and may not economically suitable for biodiesel production. Taken into consideration the fact that the nutrients concentrations in the wastewater streams listed in Table 2.5 were very low, one solution might be looking for alternative wastewater streams that contain high nutrients levels but not so high that the growth of algae will be inhibited. Another problem is that although algae stains from the family of *Chlorella* show excellent adaptation capability when cultivated in wastewater, they always contains low lipid content as shown in Table 2.1. Since this project is aimed at

cultivating algae cells for biodiesel production, species with high lipid content needs to be screened and tested on wastewater. The third problem is that the growth of the algae species was found so slow when using wastewater as substrate that longer hydraulic retention time needed to be employed, which increased the cost of operation. Clearly, fast proliferation is another desired property for the algae species used.

### 2.5 Potential of algae cultivation using centrate for simultaneous biomass and biodiesel production and wastewater treatment

In this project, the wastewater stream tested is called centrate, which is generated from thickening process of activated sludge and contains highest amount of ammonia nitrogen, total nitrogen, total organic carbon (TOC) and total phosphorus among wastewaters from several different stages in a municipal wastewater treatment plant (Figure 2.1). The centrate may be a viable alternative for the cultivation media for algae growth due to the following reasons. First, the concentrations of carbon, nitrogen and phosphorus are higher in the centrate than in any other wastewater streams obtained from a wastewater treatment plant, which can provide sufficient nutrients for algae growth. Second, centrate contains a variety of minerals such as K, Ca, Mg, Fe, Cu, and Mn, which are essential micronutrients for algae growth and metabolism. Third, the volume of the centrate produced daily is extremely large with no availability problem all year round, and the centrate need to be recycled to the activated sludge process for further treatment to avoid environmental contamination, which adds extra load for the treatment process, especially the high concentration of phosphorus. Thus the use of the centrate for algae cultivation could serve the dual role of waste reduction and biomass/bioenergy production.

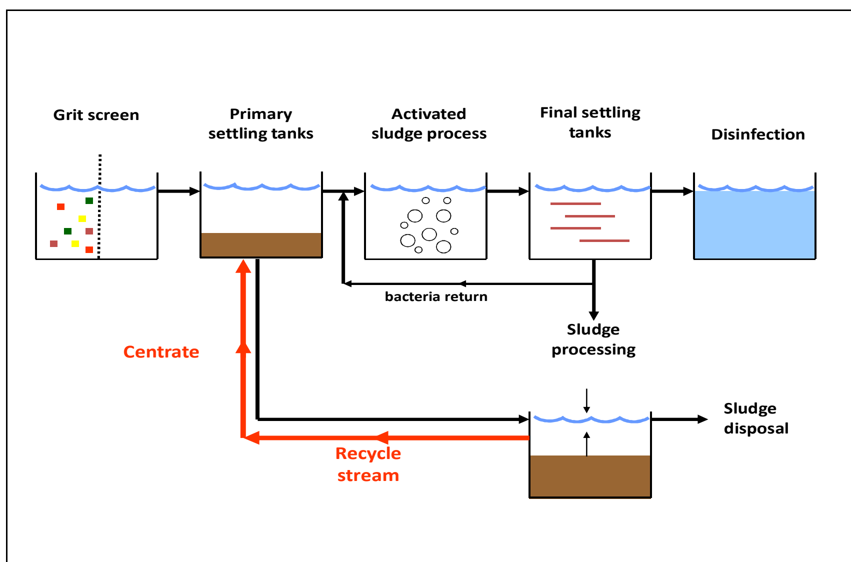



**Figure 2.1.** Typical wastewater treatment schematic in municipal wastewater treatment plant

With the above discussion comes the research hypothesis for this dissertation, which is that the centrate may be a candidate cultivation media that contains sufficient nutrients for algae cells to produce TAGs as feedstock for biodiesel production because the centrate contains carbon, nitrogen, phosphorus and minerals that can be utilized by algae cells to accumulate biomass and synthesize lipids. Algae species and strains diverge greatly in terms of biomass productivity, nutrient and light requirement, ability of lipids accumulation, etc. Since the aim of this project is to produce biodiesel from centrate, there are several criteria when choosing the right strains. The algae should be of high growth rate and lipid content, superior tolerance to environmental variables, capable of heterotrophic or mixotrophic growth and growing to high cell density.

# CHAPTER 3. FEASIBILITY ANALYSIS OF ALGAE CULTIVATION ON CENTRATE FOR SIMUTANEOUS BIODIESEL PRODUCTION AND WASTEWATER NUTRIENTS REMOVAL

## 3.1 Overview

The feasibility of growing *Chlorella sp.* in the centrate, a highly concentrated municipal wastewater stream generated from activated sludge thickening process, for simultaneous wastewater treatment and energy production was tested in this chapter. The characteristics of algal growth, biodiesel production, wastewater nutrient removal and the viability of scale-up and the stability of continuous operation were examined. Two culture media, namely autoclaved centrate (AC) and raw centrate (RC) were used for comparison. The results showed that by the end of a 14-day batch culture, algae could remove ammonia, total nitrogen, total phosphorus, and chemical oxygen demand (COD) by 93.9%, 89.1%, 80.9%, and 90.8%, respectively from raw centrate, and the fatty acid methyl ester (FAME) content was 11.04% of dry biomass providing a biodiesel yield of 0.12g-biodiesel/L-algae culture solution. The system could be successfully scaled up, and continuously operated at 50% daily harvesting rate, providing a net biomass productivity of 0.92 g-algae/(L·day).

## 3.2 Introduction

Petroleum based fuels are considered unsustainable because of the declining supply of fossil oils and their association with greenhouse gas emission. Therefore, discovering and constructing renewable, carbon neutral transportation fuel systems are possibly two

of the most vital issues for current society (Chisti, 2007). Biodiesel, a promising substitute for petroleum fuels, has the potential to address sustainability and energy security issues because it is derived from plant oils or animal fats, and has much lower greenhouse gas emission. Currently, soybean oil is the major feedstock for commercial biodiesel production; other oil feedstock including canola, corn, jatropha, waste cooking oil, and animal fats (Chisti, 2007) are also being tested. However, these processes have caused concerns of competing with food source, having little effect on green house gas emission (Fargione et al. 2008), and not capable of satisfying the existing demand for petroleum fuels (Chisti, 2007). Prior research suggested that microalgae, which has an areal productivity 20 to 40 times that of oil crops (Sheehan et al., 1998) and oil content up to 80% by weight of dry biomass (Spolaore et al., 2006, Chisti, 2007) has the potential to replace the current source for renewable biodiesel production (Sheehan et al., 1998; Banerjee et al., 2002; Chisti 2007). However for this transaction to happen, algae biomass must be produced practically and economically. One of the methods to reduce costs of algae mass cultivation is to integrate wastewater treatment with algae biomass production, which was first suggested in 1960s (Oswald and Golueke, 1960).

The research of growing algae in municipal wastewaters has been under investigation for more than half a century (Oswald *et al.,* 1978; Tam and Wong, 1989; Tam and Wong 1990; Lau et al. 1995; Woertz et al. 2009). Some researchers also investigated the feasibility of algal-bacterial symbiotic processes for the treatment of industrial and livestock wastewaters (Muñoz and Guieysse, 2006). Municipal wastewater collected from various stages during treatment process has been tested for their ability of

supporting algae growth in previous studies. These different types of wastewater include primary clarifier effluent (Tam and Wong, 1989, Tam and Wong 1990, Woertz et al. 2009, Lau et al. 1995), activated sewage filtrates (Tam and Wong, 1989, Tam and Wong 1990), and effluent from secondary treatment tank (Oswald *et al.,* 1978; Shelef et al. 1978). The centrate, which is the liquid from activated sludge thickening process, has the characteristics of rich nutrients including phosphorus, ammonium, and COD. With a daily process rate of one million gallons in the wastewater treatment plant located in Saint Paul, Minnesota and higher nutrients levels than liquid from other stages, centrate provided a potential source for algae cultivation. Up to now, rare studies have been conducted to test the suitability of growing algae in raw centrate, thus our current research effort in this area is necessary and important.

Lipid contents in algae cultivated in artificial media solution have been reported to exceed 80% with different lipid classes (Spolaore et al., 2006, Chisti 2007). However, few reports have explored the lipid contents of algae grown in municipal wastewaters (Woertz et al. 2009).

The objectives of this project are herein to (1) determine the growth rate, biomass yield, nutrients removal efficiency, and biodiesel productivity for algae grown in centrate, and (2) test the scalability and growth stability of algae cultivated in centrate.

## 3.3 Materials and Methods

### 3.3.1 Experiment layout

The experiments in this project were carried out in two consecutive stages. The first stage was aimed at providing baseline information on performance of microalgae alone in wastewater, and thus conducted using autoclaved centrate to eliminate bacterial contamination and excess heterotrophic growth. In this stage, TAP media was used as positive control to justify the performance of autoclaved centrate as media for algae cultivation. The second stage was targeted on testing the feasibility of growing algae as a practical wastewater nutrients removal and biomass accumulation process, and thus conducted in raw centrate after removing solid particles. The cultivation time for each condition was 14 days with three replications. The effect of both autoclaved and raw centrate was studied in terms of algal growth, wastewater nutrients removal, and biodiesel productivity. Scale-up experiment and the stability of continuous operation was tested with raw centrate. The scale-up experiments were first carried out in batch mode with 7-day cultivation time and then in continuous mode with 50% of algae culture solution harvested and same amount of raw centrate replenished daily for a period of 7 days.

### 3.3.2 Pretreatments and characteristics of wastewaters

The centrate was collected from the Metropolitan Wastewater Treatment Plant located in Saint Paul, Minnesota, and large solid particles were removed by sedimentation and filtration with filter cloth (Wypall X70, Kimberly-Clark Professional, Roswell, GA, United States). After filtration the centrate was divided into two equal portions. One

portion was autoclaved at 121℃, after which, the liquid was stored at 4℃ for 5 days to settle out any visible solid particles and the supernatant was used for algae cultivation. The other untreated portion was stored at 4℃ until use in experiments. Thus, there were two types of centrate used in this project, namely autoclaved centrate, and raw centrate. The characteristics of the two types of centrate are summarized in Table 3.1. It showed that similar nutrients characteristics were observed for both types of centrate.

**Table 3.1** Characteristics of centrate
Note: P is short for phosphorus, N is short for nitrogen, COD is short for Chemical Oxygen Demand, ND is short for not detected.

| Charateristics | Raw centrate | Autoclaved centrate |
|---|---|---|
| Ammonia (mg/L) | 82.5±2.2 | 85.9±1.1 |
| Total nitrogen (mg N/L ) | 116.1±3.8 | 132.3±5.1 |
| Total phosphorous (mg $PO_4^{3-}$-P /L) | 212.0±7.0 | 215.1±6.4 |
| COD (mg/L) | 2304.0±2.5 | 2389.5±57.3 |
| Total suspended solid (TSS) | 0.070±0.014 | 0.180±0.000 |
| Total volatile suspended solid (TVSS) | 0.050±0.014 | 0.120±0.000 |
| Al (mg/L) | 0.075 | 0.082 |
| B (mg/L) | 0.286 | 0.357 |
| Ca (mg/L) | 161.7 | 132.67 |
| Cu (mg/L) | 0.01 | 0.017 |
| Fe (mg/L) | 3.074 | 1.925 |
| K (mg/L) | 145.50 | 197.63 |
| Mg (mg/L) | 73.30 | 96.209 |
| Mn (mg/L) | 2.797 | 4.010 |
| Na (mg/L) | 160.70 | 199.34 |
| Ni (mg/L) | 0.0273 | 0.075 |
| Zn (mg/L) | 0.020 | 0.115 |

### 3.3.3 Algae strain and culture condition

A microalgal mixture, named 72205, was obtained from local Lake Minnetonka, Minnesota, U.S.A. Five unialgal strains, named 72205a to 72205e, were purified from the mixture on TAP medium solidified with 1.5% agar. One of the strains (72205e) grew well, initially on 50% TAP + 50% centrate, then on 100% (full) centrate. The strain was morphologically identified as a *Chlorella* species, renamed *Chlorella* sp. 10b,

and used in the entire investigations of this project. The algae strain was conserved in Tris-Acetate-Phosphorus (TAP) media (Harris, 1989) with the following solid ingredients: 400 mg/L $NH_4Cl$, 100 mg/L $MgSO_4 \cdot 7H_2O$, 50 mg/L $CaCl_2 \cdot 2H_2O$, 108 mg/L $K_2HPO_4$, 56 mg/L $KH_2PO_4$, and 2420 mg/L Tris (hydroxymethyl) aminomethane,. Liquid chemicals include: 1 mL/L glacial acetic acid, 1 mL/L trace elements solution consisted of 50 g/L $Na_2EDTA$, 22 g/L $ZnSO_4 \cdot 7H_2O$, 0.05 g/L $CaCl_2 \cdot 2H_2O$, 11.4 g/L $H_3BO_3$,; 5.06 g/L $MnCl_2 \cdot 4H_2O$, 4.99 g/L $FeSO_4 \cdot 7H_2O$, 1.61 g/L $CoCl_2 \cdot 6H_2O$, 1.57 g/L $CuSO_4 \cdot 5H_2O$, 1.10 g/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 16 g/L KOH,. The growth rate, nutrients consumption, lipid content and biodiesel productivity of the algae was studied with 250mL Erlenmeyer flasks containing 100mL TAP or two different centrate media. The 250ml Erlenmeyer flasks were kept on a shaker with 100 rpm rotation speed. The growth characteristics of the algae on raw centrate in a larger scale were further studied with a coil reactor, which was constructed with two pieces of 16 m long clear polyvinyl tubing (19-mm ID, 25-mm OD, and 9.0-L volume) coiled around a metal frame of 1.4 m in height and 0.4 m in diameter. The total volume of the algae culture solution was 25 L, 9 L of which was contained in the coil and the rest (16 L) was in a storage container. Light was provided by six 1.2-m-high Gro-Lux fluorescent tubes (40W/tube) residing on the inside of the coil. The tubes were placed 3 cm away from the reactor tubing. Dayton carbonator pump (4FG41, Dayton) was employed to circulate the solution of algae at 1.8-L $min^{-1}$. In all cases, *Chlorella sp.* were inoculated at 1:10 (v:v), and grown under 25±2°C with illumination at light intensity of 50 $\mu mol \cdot m^{-2}s^{-1}$.

### 3.3.4 Analytical procedures

#### 3.3.4.1 Sampling and nutrients analysis

A volume of 1.5mL algae suspension was collected daily from each flasks or coil reactor for nutrient consumption analysis starting from inoculation. The samples were first centrifuged at 5000 rpm for 15 min and then the supernatants were properly diluted and analyzed for chemical oxygen demand (COD), total phosphorus, ammonium ($NH_4$–N), and total nitrogen following the Hach DR 5000 Spectrophotometer Manual (Hach, 2008). The specific procedures used in this study are described below.

**Chemical Oxygen Demand (COD)**

(1) Preheat the COD digester to 150 °C. Dilute the sample for 10 times.

(2) Add 2 ml of the diluted sample to a COD reagent vial with barcode (HACH, TNT822) and invert gently several times to mix.

(3) Place the vial in the digester to heat for 2 hours. Turn off the digester.

(4) Wait the vial to cool to 120°C or below, and then invert the vial several times while still hot.

(5) Place the vial into a rack to cool to room temperature, and determined the COD result on a spectrophotometer (HACH DR 5000). Results are in mg/L COD.

The mg/L COD results are defined as the mg of $O_2$ consumed per liter of sample under conditions of this procedure. In this procedure, the sample is heated for two hours with a strong oxidizing agent, potassium dichromate. Oxidizable organic compounds react, reducing the dichromate ion ($Cr_2O_7^{2-}$) to green chromic ion ($Cr^{3+}$). When the 20–1500 mg/L colorimetric method is used, the amount of $Cr^{3+}$ produced is determined. The

COD reagent also contains silver and mercury ions. Silver is a catalyst and mercury is used to complex chloride interferences. Test results for the 20 to 1,500 mg/L COD range are measured at 620 nm, and then the OD value is converted to concentration through an internal standard curve.

**Total Phosphorus (TP)**

(1) Preheat the TP digester to 100 °C. Dilute the sample for 10 times.

(2) Add 0.4 ml of the diluted sample to TP reagent vial (HACH, TNT845), flip the cover and invert the vials several times to mix.

(3) Place the vial in the digester to heat for 1 hour. Turn off the digester.

(4) Wait the vial to cool to room temperature, add reagent B to the digestion vial, put cover C onto the vials, and invert the vial several times to mix.

(5) Place the vial into a rack, wait for 15 minutes.

(6) Determine the result on a spectrophotometer (HACH DR 5000). Results are in mg/L P.

Phosphate ions react with vanadate-molybdate reagent to form a yellow dye. Test results are measured at 435 nm.

**Ammonia ($NH_3$)**

(1) Add 0.2 ml of the diluted sample to ammonia nitrogen reagent vial (HACH, TNT832), flip the cover and invert the vials several times to mix.

(2) Place the vial into a rack, wait for 15 minutes to allow reaction to complete.

(3) Determine the result on a spectrophotometer (HACH DR 5000). Results are in mg/L ammonnia.

Ammonium ions react at pH 12.6 with hypochlorite ions and salicylate ions in the presence of sodium nitroprusside as a catalyst to form indophenol. The amount of color formed is directly proportional to the ammonia nitrogen present in the sample. Test results are measured at 690 nm.

**Total nitrogen (TN)**

(1) Preheat the TN digester to 100 °C. Dilute the sample for 10 times.

(2) Add 1.3 ml of the diluted sample, 1.3 ml of ragent A, 1 tablet of reagent B to a TN digestion vial.

(3) Place the vial in the digester to heat for 1 hour. Turn off the digester.

(4) Wait the vial to cool to room temperature, add reagent C to the digestion vial, and invert the vial several times to allow reagent C totally dissolve.

(5) Transfer 0.5 ml sample from digestion vial to TN reagent vial with barcode (HACH, TNT826), and then add 0.2 ml reagent D to the vial, invert several time to mix.

(6) Place the vial into a rack, wait for 15 minutes to allow reaction to complete.

(7) Determine the result on a spectrophotometer (HACH DR 5000). Results are in mg/L N.

Inorganically and organically bonded nitrogen is oxidized to nitrate by digestion with peroxodisulphate. The nitrate ions react with 2,6-dimethylphenol in a solution of sulphuric and phosphoric acid to form a nitrophenol. Test results are measured at 345 nm, then the OD value is converted to concentration through an internal standard curve.

#### 3.3.4.2 Algal growth determination

Algal growth was monitored daily by the total volatile suspended solids (TVSS), which represents biomass concentration and was determined according to the standard method (APHA,1995) using 5mL of algae suspension from the flasks or coil reactor. For algae grown in centrate, the algae growth was determined by the total biomass minus the biomass in control experiments, where only treated or untreated centrate was placed under light. Algae growth in batch culture is usually characterized by five reasonably defined phases: (1) lag, (2) exponential, (3) declined, (4) stationary, and (5) death. The growth rate ($k$) is usually determined from the exponential phase by following this equation:

$$\ln N = \ln N_0 + kt \qquad (3.1)$$

where $N_0$ and $N$ represent the biomass concentration at the beginning and any given time $t$, respectively. A plot of $\ln N$ against $t$ will yield a straight line which will allow us to determine $N_0$ and $k$ through linear regression. The specific procedure for TVSS measurement is listed below.

**Total Volatile Suspended Solids (TVSS)**

(1) Put a glass-fiber filter (Fisherbrand 09-804-42C) in a dry aluminum weighing boat and weigh for initial weight (a, g)

(2) Place the filter on a clean vacuum filtration setup and wet the filter with some distilled and deionized water (DDW), then filter 5 ml aqueous sample, and rinse down the funnel with a small amount of DDW.

(3) Dry to constant weight in an oven at 105°C.

(4) Cool in a desiccator and weigh to determine total weight of the boat and solids

(b, g).

(5) Run two blanks following procedures above using DDW instead of a sample, get results of a’, b’.

Calculation: TSS (g/L) = ((b-a-(b’-a’)*1000)/5

### 3.3.4.3 Fatty acid methyl ester (FAME) content analysis

The content of FAME was analyzed at the end of the batch experiments. Algae cells were harvested by centrifugation and then dried by a freeze dryer (Savant Instrument Inc., Holbrook, NY, United States) before analysis. Fatty acid content and composition analysis were performed by following two consecutive steps including preparation of FAME and GC-MS analysis. The FAME was prepared following a one step extraction-transesterification method by Indarti et al. (2005). The composition and content of FAME was analyzed with GC-MS (Agilent 7890-5975C, Agilent Technologies Inc., Santa Clara, CA, United States) equipped with a flame ionization detector and a DB-5-MS capillary column. The oven temperature was set at 80°C, held for 5 minutes, raised to 290°C at 4°C/minute, and held at 290°C for 5 minutes, and the temperature for injector and detector were set at 250°C and 230°C, respectively. The carrier gas (helium) was controlled at 1.2 ml/minute. Chromatographic data were recorded and integrated using the built-in Agilent data analysis software. The compounds were identified in the NIST Mass Spectral Database and quantified by comparing the peak area with that of the standard (C18:2) (Sigma-Aldrich, St. Louis, MO, United States).

All the experiments were carried out in triplicate and average values are reported. Results were analyzed using software SPSS 13.0. ANOVA analysis and Tukey's post-hoc analysis were used to determine the significance of difference wherever applicable.

## 3.4 Results and discussion

### 3.4.1 Algal growth

Figure 3.1a shows the growth curves for the three different culture media. These curves exhibit all characteristic growth phases except the lag phase of algae in batch culture. The exponential growth in all culture media lasted for 4 to 5 days, followed by a stationary phase until the end of cultivation. The lack of a visible lag phase may be because majority of the cells at the time when they were transferred to the culture media were already in exponential phase. The level of net biomass increase from raw and autoclaved centrate appears to be higher than that of TAP. However, the bacterial growth in the raw centrate complicates the interpretation of the results. We replotted the data from Figure 3.1a on a semi-log coordinates (Figure 3.1b), and fitted the first three growth data points (excluding day 0 data) into Eq. 3.1 through linear regression. The rate constant $k$ (growth rate) value obtained from Eq. 3.1 was 0.4614 for algae grown in TAP media, 0.4794 for algae grown in autoclaved centrate, and 0.1677 for algae grown in raw centrate. Algae grown on TAP and autoclaved centrate share similar growth rate which is much higher than that of algae grown on raw centrate. This suggests that autoclaved centrate is as good as the "ideal" TAP medium. However, autoclaving even a small portion of the wastewater in any size the municipal wastewater treatment plants is impractical. Therefore, attention should be paid to raw centrate. The facts that the

cells picked up their growth speed immediately after inoculation without any noticeable lag phase and that the raw centrate sustained algal growth at a reasonable rate and biomass accumulation level indicates that raw centrate is a promising culture medium. Nevertheless, centrate has a highly unbalanced N:P ratio (Table 3.1) compared with TAP media. The phosphorus concentration in centrate was ten times higher than that in TAP media while the COD, nitrogen and $NH_4$-N concentration was only about 60%, 34% and 38% of that in TAP media, respectively. Thus one probable explanation for the sooner onset of declining phase for centrate media is the depletion of certain nutrients, possibly nitrogen and carbon source in centrate (Table 3.1 and Figure 3.2), resulting in an unfavorable nutrients ratio and low carbon source due to the initial rapid growth and nitrogen uptake (Lau et al., 1995). The decreased algae biomass growth and net biomass increase in raw centrate might be due to the high levels of toxics and competitive bacteria presented in centrate. Another reason could be the low light availability arising from self-shading at high algal density and other particles present in the raw centrate (Fogg, 1975).

The growth rate and final biomass concentration obtained in previous studies using municipal wastewater as substrate are summarized in Table 3.2. The growth rates found in this study are comparable with others and the final biomass concentration obtained from treated and raw centrate in the present study ranked among the top ones of those from previous studies in terms of total suspended solid concentration. As described by Prescott (1968), green algae demand more nitrogen and phosphorous than do many other species, and they can take up generous nitrogen when the phosphorous content is

relatively high. Therefore, one possible explanation for the high biomass concentration in centrate could be that the nutrients level in centrate was higher than all the waste used in previous studies summarized in Table 3.2. It is worth noting that in raw centrate there were other bacteria growing simultaneously with algae, thus, the biomass concentration was the sum of algae and any survival bacterial that contributes to TVSS. However, in the control experiment where raw centrate was placed under light, the net growth of the bacteria accounted for only about 10% of the total biomass obtained in the algae containing cultivation broth, thus, the majority of the biomass was algae culture.

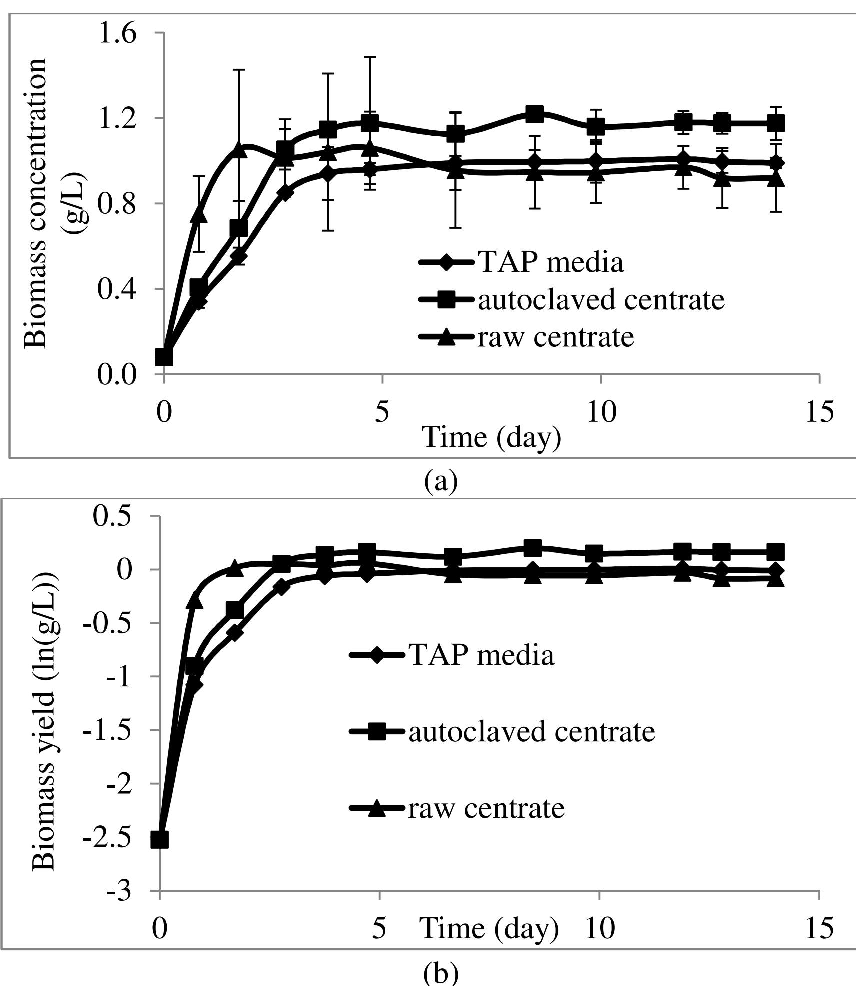



**Figure 3.1** (a) Growth curves for algae grown on the three different culture media. (b) Semi-log plot of growth data for algae grown on the three different culture media.

**Table 3.2.** Summary of growth rate and final biomass concentration in previous studies
Note: P is short for phosphorus, N is short for nitrogen, COD is short for Chemical Oxygen Demand, and TOC is short for Total Organic Carbon.

| Waste used | Algae used | Nutrient concentration (mg/L) | | | Final biomass concentration (g/L) | Growth rate ($d^{-1}$) | Incubation time (day) | Reference |
|---|---|---|---|---|---|---|---|---|
| | | P | N | COD or TOC | | | | |
| Activated sludge extract | *Chlorella pyrenoidosa* | 28.33 | 0.588 | 20.99 (TOC) | 0.053 to 0.127 | | 11 | Cheung and Wong , 1981 |
| Digested sludge extract | *Chlorella pyrenoidosa* | 292.5 | 2.085 | 35.0(TOC) | 0.116 to 0.57 | | 11 | Cheung and Wong , 1981 |
| Primary settled sewage | *Chlorella vulgaris* | 3.324.29 | 47.4748.8 | 92-157.5 COD | | Highest at 0.2771 | 10 and 3 | Lau et al., 1995, 1998 |
| Settled sewage | *Chlorella pyrenoidosa* | 9.58-10.07 | 36.07-41.49 | 240 COD | 0.4-1.1 | 0.35 to 0.5 | 4 | Tam and Wong, 1989, 1990 |
| Activated sewage | *Chlorella pyrenoidosa* and *Scenedesmus sp.* | 10.04-12.18 | 24.17-39.2 | 112 COD | 0.35 to 1.2 | -- | 13 | Tam and Wong, 1989, 1990 |
| Primary clarifier effluent | A mixture of *Chlorella* and other algae species | 2.1 | 51 | -- | 0.317 to 0.843 | -- | 2-4 | Woertz et al., 2009 |
| Secondarily treated sewage | *Botryococcus braunii* | | | | 0.34-0.35 | -- | 10 | Sawayama et al., 1992 |
| Autoclaved centrate | *Chlorella. Sp.* | 215 | 132 | 2389 | 1.175 | 0.4794 (exponential phase) | 14 days | Current study |
| Raw centrate | *Chlorella. Sp.* | 212 | 116 | 2304 | 1.06 at peak and 0.92 at the end | 0.677 (exponential phase) | 14 days | Current study |

### 3.4.2 Biodiesel productivity analysis

Fatty acid methyl esters derived from vegetable oil and animal fat through transesterification process is referred as biodiesel. Not all lipids can be converted to FAME, the chemical ingredient of biodiesel. Therefore, measurement of FAME in algal biomass is a direct indication of amount of lipids suitable for biodiesel production. Different from the longer chain hydrocarbons contained in other species like *Botryococcus braunii* (Banerjee et al.,2002) , the *Chlorella* species is found containing shorter chain fatty acids, mainly 16 to 18 carbon length, which is ideal for biodiesel

production (Miao and Wu, 2006; Xu et al., 2006). The FAMEs found in this study are in accordance with those reported in the paper written by Petkov and Garcia (2007). As shown in Table 3.3, the FAME yield for the algae cultivated in TAP media, is 0.11 g-biodiesel/g-dried algae, with octadecadienoic acid (C18:2) as the most abundant fatty acid derived from algae bodies, hexadecanoic acid (C16:0) as the second. For algae cultivated in autoclaved centrate, the FAME yield is around 0.1 g-biodiesel/g-dried biomass, which is similar to that in TAP media. The most abundant fatty acids obtained from algae were also octadecadienoic acid (C18:2) and hexadecanoic acid (C16:0). Different from algae cultivated in TAP media and autoclaved centrate, octadecatrienoic acid (C18:3) (18.79% of total FAME) and hexadecanoic acid (C16:0) (16.10% of total FAME) accounted for the majority of the fatty acids for algae cultivated in raw centrate, and the total FAME content was 11.04% of dried biomass. The biodiesel yield was 0.10g-biodiesel/L-culture solution in TAP media, 0.13 g-biodiesel/L-culture solution in autoclaved centrate, and 0.12 g-biodiesel/L-culture solution in raw centrate. The proportion of saturated fatty acids stayed at similar level for algae in all three media types, while the monoenoic fatty acids increased, and the polyenoic fatty acids decreased when algae were cultivated in treated and untreated centrate. It is reported that lipid content and fatty acid profile are affected by culturing situations, growth phase, and environmental conditions (Guschina and Harwood, 2006; Petkov and Garcia, 2007). Since the centrate has an unbalanced N:P ratio, higher levels of heavy metal and unknown bacteria, it is not surprising to see the different FAME profile. Statistic analysis shows that there was no significant difference in FAME content for algae cultivated in different media solution.

**Table 3.3** Summary of FAME profile for algae cultivated in different media

| FAME composition | | TAP media | Autoclaved centrate | Raw centrate |
|---|---|---|---|---|
| Saturated fatty acids (% of total FAME) | 12:0 | 2.93 | 2.48 | 3.78 |
| | 13:0 | 0.00 | 2.23 | 0.00 |
| | 14:0 | 2.52 | 0.00 | 0.00 |
| | 15:0 | 3.24 | 4.19 | 5.24 |
| | 16:0 | 19.06 | 15.15 | 16.10 |
| | 17:0 | 3.43 | 6.91 | 4.74 |
| | 18:0 | 3.61 | 3.34 | 4.35 |
| | 20:0 | 2.38 | 0.00 | 0.00 |
| | 22:0 | 0.00 | 2.61 | 3.53 |
| | Subtotal | 37.19 | 37.90 | 37.73 |
| Monoenoic fatty acids (% of total FAME) | 16:1 | 8.08 | 11.47 | 10.88 |
| | 18:1 | 6.80 | 13.89 | 8.45 |
| | Subtotal | 14.88 | 25.36 | 19.33 |
| Polyenoic fatty acids (% of total FAME) | 16:2 | 13.87 | 10.90 | 9.79 |
| | 18:2 | 22.43 | 16.15 | 14.36 |
| | 18:3 | 12.06 | 10.69 | 18.79 |
| | Subtotal | 48.35 | 36.73 | 42.94 |
| Total (% of TVSS) | | 10.56 | 9.98 | 11.04 |

The results in section 3.1 and 3.2 showed that the bacteria in raw centrate did not have negative effect on biomass yield and biodiesel productivity, thus raw centrate can be directly used for algae cultivation without any pretreatment except for removal of solid particles.

### 3.4.3 Wastewater nutrients removal by algae growth

The variation in total phosphorus, COD, $NH_4$-N and total nitrogen with time in different centrate for the 14-day batch culture is depicted in Figure 3.2 (a), (b), (c), and (d), respectively.

Total phosphorus was drastically reduced from 215 to 45.1 mg/L in autoclaved centrate, and from 212 mg/L to 40.4 mg/L in raw centrate during the first four days of cultivation, and stayed at the similar level until the end of experiment. During the 14-

day batch experiment, 79.0% total phosphorus in the autoclave centrate and 80.9% total phosphorus in the raw centrate were removed. The phosphorus removal was much greater than those reported in other studies using municipal wastewater (Lau et al., 1995; Tam and Wang, 1990; Tam and Wong, 1989; Tam and Wong, 2000; de-Bashan et al., 2008; Woertz et al., 2009), suggesting that the algae species used in this study is high phosphorus concentration tolerance. Previous researches (Hemens and Mason, 1968, Mcgriff and Mckinney, 1972, Song et al. 2002) reported that increase in pH values above 8 of the solution, algae system might cause coagulation and adsorption of inorganic phosphates. Since the pH in the centrate tested exceeded 9 at the end of experiment, it is reasonable to conclude that the removal of phosphorous is due to both algal metabolic uptake and phosphate precipitation. According to the reference, algae biomass typically contains 0.5% to 3.3% phosphorus (Richmond, 2004), thus it is reasonable to conclude that about 76% to 96% of phosphorus was removed by sedimentation and only about 3% to 23% of phosphorus was assimilated to algae biomass.

The concentration of COD decreased dramatically from 2390 mg/L to 230 mg/L in autoclaved centrate, and from 2304 mg/L to 210 mg/L in raw centrate within the first two days of cultivation. By the end of the experiment 90.3% and 90.8% of COD was removed in autoclaved centrate and raw centrate, respectively, suggesting that the algae species used in this study could rapidly utilize different organic compounds as carbon sources besides $CO_2$. Previous studies show that some *Chlorella* species are mixotrophic (Martínez et al., 1997; Lalucat et al., 1984), meaning that they can

simultaneously use light and organic source as energy sources and assimilate $CO_2$ and glucose or simple acids as carbon sources. Matrínez et al. (1997) found that when *Chlorella pyrenoidosa* was cultivated under moderate light condition, an exponential mixotrophic phase was observed first and when the organic substrate was completely consumed, an autotrophic phase followed after an adaptation period. Lalucat et al. (1984) reported that *Chlorella* sp. strain VJ79 could grow autotrophically, heterotrophically, and mixotrophically on a variety of organic substrate. Furthermore, glucose and serine could promote a mixotrophic growth from which the yield is higher than the sum of autotrophic and heterotrophic yields.

Over 93% of $NH_4$-N (93.0% in autoclaved centrate and 93.9 in raw centrate) and 89% of total nitrogen (89.9% in autoclaved centrate and 89.1% in raw centrate) were removed by the end of the experiments, with a majority of the removal occurring during the first four days of the cultivation. The concentration of $NH_4$-N dropped from 85.9 mg/L to 6.0 mg/L in autoclaved centrate, and from 82.5 mg/L to 5.0 mg/L in raw centrate. Although the removal efficiencies are lower than those found in other studies, the $NH_4$-N removal capacities observed in this study (above 75mg/L) are much higher than those in others using municipal wastewater (Lau et al., 1995; Tam and Wang, 1990; Tam and Wong, 1989; Tam and Wong, 2000; de-Bashan et al., 2008; Woertz et al., 2009), illustrating that the algae used in the present study can survive in municipal wastewater with high $NH_4$-N concentration and high pH value. The removal of $NH_4$-N from wastewater by algae culture was affected by two factors, namely direct utilization of $NH_4$-N and $NH_3$ stripping (Tam and Wong, 1898). It is reported that $NH_3$ stripping

occurred only under alkaline, elevated temperature (Reeves, 1972), and the existence of abundant urea in the wastewater (Matusiak et al., 1976). Since the temperature never exceed 27˚C and urea was not dominant in all types of centrate, the stripping process might not be significant in the present study, and the loss of $NH_4$-N could largely be attributed to the absorption by algae.

The total nitrogen dropped from 132.3mg/L to 13.3mg/L in autoclaved centrate, and from 116.1 mg/L to 12.7 mg/L in raw centrate after the cultivation of algae. The removal capacity of total nitrogen is larger than that of $NH_4$-N, suggesting that $NH_4$-N is not the only nitrogen form that can be assimilated by algae. Matusiak et al. (1976), Syrett, (1981), Barsanti and Gualtieri (2006) reported in their studies that, algae can assimilate $NH_4$-N, nitrate, and simple organic nitrogen such as urea and amino acids in the wastewater, but the complicated organic nitrogen
could not be directly used. At the end of the experiment, total nitrogen concentration is higher than $NH_4$-N concentration in all types of centrate, suggesting that some organic nitrogen was not converted to inorganic nitrogen and utilized by algae. It is worth mention that, while the removal of the nutrients was significant, the concentration remaining in the centrate was still high. In order to meet the wastewater discharge standards, further nutrients removal processes are required.

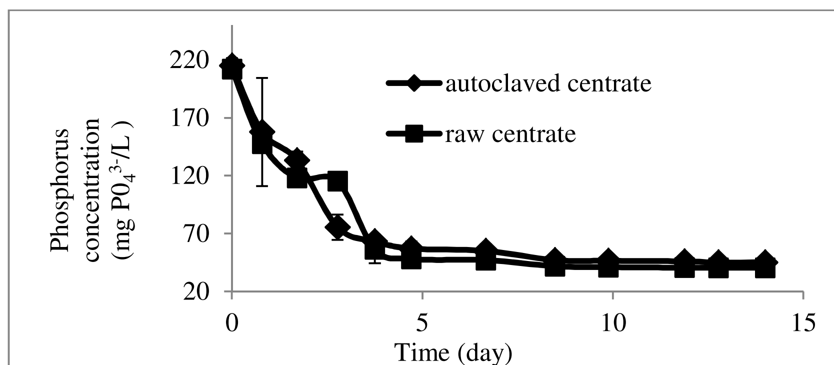



**Figure 3.2** (a) Total phosphorus removal profile

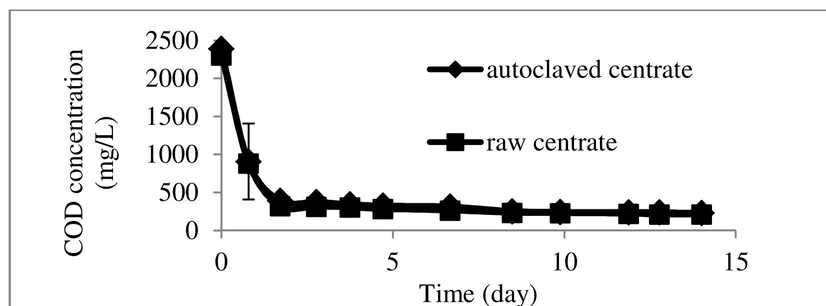



**Figure 3.2** (b) Total COD removal profile

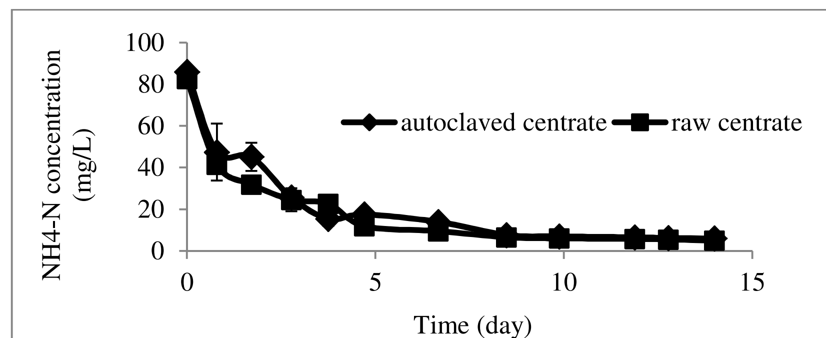



**Figure 3.2** (c) $NH_4$-N removal profile

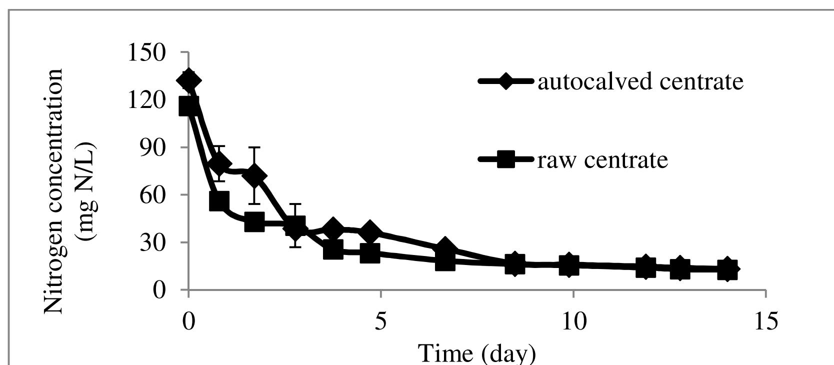



**Figure 3.2** (d) Total nitrogen removal profile

**3.4.4 Scale-up and continuous operation stability analysis**

Based on the fact that raw centrate can provide similar biomass yield and biodiesel productivity to TAP media, it is necessary to test the feasibility of scale-up and the stability of continuous operation since the daily process rate of centrate is as high as one million gallons in wastewater treatment plants. Figure 3.3 (a) shows the biomass accumulation profile in two different scales tested. For the first 3 days, algae culture in both scales showed exponential growth phase. From the 3$^{rd}$ day, the algal growth in 100mL flask started to level off while the growth in the larger scales continued growing. Algae growth in 25L coil peaked on day 5, after which the biomass yield dropped gradually. Despite the fact that the nutrients consumption for all scales was similar (data not shown), the main factor that caused the decrease in the growth might be light limitation. In the coil system, only 36% of the culture solution is illuminated at every given moment, and it took the system about 5 min to circulate the culture solution from the bottom of the light source to the top of the light source, and for the consecutive 8.9 min the solution sat in the dark in the storage tank, which means that the algae was exposed to light only 22% of the cultivation time. Another fact was that the light intensity was different for the inner part and the outer part of the coil reactor. In the inner section that was close to the light source, the light intensity was about 50 $\mu mol \cdot m^{-2}s^{-1}$, while in the outer section that was far away from the light source, the light intensity was only 3 $\mu mol \cdot m^{-2}s^{-1}$ , which was very low compared with the flask system. This was not a limiting factor when all the nutrients were in sufficient amount, but when the organic source was consumed to a certain level, phototrophic became dominant and light provided the only energy source, under which algae needed to adjust themselves to

autotrophic state from mixotrophic state (Matrínez et al. 1997). In this state, light was essential for algal growth. Due to self-shading in high algal density solution and the poor lighting condition in the coil reactor, algae in the coil system could not gain enough energy, and hence a decrease in biomass was observed. Biodiesel productivity was also determined at the end of the batch operation, and no significant difference was found between the small and large scales.

Figure 3.3 (b) shows the growth curve for continuous operation process. The reactor was maintained at batch mode from inoculation to day 5, starting from which half of the algae suspension was removed followed by the same amount of fresh centrate added. In this way, nutrients were supplemented every day, and self-shading effect was minimized. From the data we can see that during the 7-day continuous operation period, the algae could grow to a biomass concentration of about 0.9 to 1.0g/L in 1 day after the previous harvesting. The algae biomass concentration in the harvested solution during the continuous operation period was 0.92g/L on average, which provided a net productivity of the 0.92 g-dried algae/(L·day).

The results in this study imply that the centrate obtained from sludge thickening process presents promising opportunities for algae biomass production. A process of combing algae cultivation in wastewater further with algae biomass conversion would offer many environmental merits with production of valued added products including biodiesel, biooil, bio-ethanol, fertilizer etc. (Chen et. al, 2009).

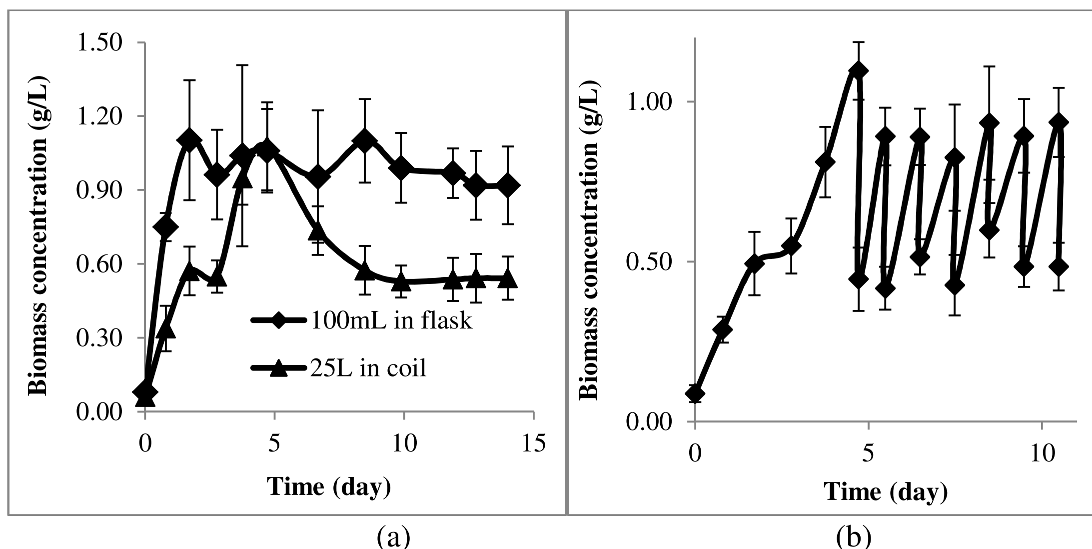



**Figure 3.3** (a) Comparison of growth curve of algae cultivated in centrate with different scales; (b) Growth curve during continuous operation process

### 3.5. Conclusions

Both autoclaved and raw centrate are proved to be able to support the growth of algal strain *Chlorella sp.*, and the growth rates and biomass yields are comparable with those reported in literature. Ammonia, total nitrogen, total phosphorus, and COD in raw centrate were removed by 93.9%, 89.1%, 80.9%, and 90.8%, respectively. The fatty acid methyl ester (FAME) content for algae cultivated in raw centrate was 11.04% of dry biomass, and the biodiesel productivity was 0.12g/L. The continuous operation of a coiled reactor with 50% daily harvesting and wastewater replenishing rate, providing a net biomass productivity of 0.92 g-algae/(L·day).

# CHAPTER 4 LOCAL BIOPROSPECTING FOR HIGH-LIPID PRODUCING MICROALGAL STRAINS TO BE GROWN ON CONCENTRATED MUNICIPAL WASTEWATER FOR BIOFUEL PRODUCTION

## 4.1 Overview

The results in last section showed that the process of cultivation algae in centrate for simultaneous biomass accumulation as feedstock for biodiesel production and wastewater treatment is achievable. However, the biomass concentration and biodiesl content were low. Since mass cultivation of microalgae for biofuel production depends heavily on the performance of the microalgae strains used, in this chapter, sixty algae-like microorganisms collected from different sampling sites in Minnesota were examined using multi-step screening and acclimation procedures to select high-lipid producing facultative heterotrophic microalgae strains capable of growing on concentrated municipal wastewater (centrate) for simultaneous energy crop production and wastewater treatment. Twenty-seven facultative heterotrophic microalgae strains were found, among which seventeen strains were proved to be tolerant to centrate. These seventeen top-performing strains were identified through morphological observation and DNA sequencing as *Chlorella sp.*, *Heynigia sp.*, *Hindakia sp.*, *Micractinium sp.*, and *Scenedesmus sp.*. Five strains were chosen for other studies because of their ability to adapt to centrate, high growth rates (0.455 - 0.498 $d^{-1}$) and higher lipid productivities (74.5 – 77.8 mg $L^{-1}$ $d^{-1}$). These strains are considered highly promising compared with other strains reported in the literature.

## 4.2 Introduction

There has been renewed interest in algal-based biofuels over the past few years due to sharp rise in fossil fuel prices and increasing concerns about the global climate change (Chisti, 2008). However, growing algae for energy use is still debatable in terms of sustainability and economic viability of such algae-derived biofuels (Van beilen, 2010). In fact, up to date, there remains a major price gap between microalgae-derived biofuels and fossil fuels despite the tremendous efforts to reduce the costs of algae production and processing. Coupling wastewater treatment with algae cultivation for biofuel production may offer an economically viable and environmentally friendly means for sustainable renewable algal based energy production since enormous amounts of water and nutrient (e.g. nitrogen and phosphorus) required for algae growth could be saved in such wastewater-based algal cultivation system (Sheehan et al., 1998; Clarens et al., 2010; Pittman et al., 2010).

Studies have been conducted to investigate algae growth and nutrient removal efficiency in industrial wastewater (Chinnasamy et al., 2010), municipal sewage (Tam and Wong, 1990; Wang et al., 2009), and animal manure wastewater (Wang et al., 2010). However, only a few strains within the *chlorella sp.* and *scenesmus sp.* have since been evaluated for their ability to grow in wastewater environments. In addition, research on strain differences and the methods to acclimate them to the wastewater environment are limited. In order to effectively couple the wastewater treatment with algae cultivation in the northern climate, selection and establishment of an adequate microalgae pool from local habitats becomes particularly important and necessary.

The idea of bioprospecting microalgae from local habitats for biofuel or high-value byproducts production is not new. National Renewable Energy Laboratory (NREL) launched the Aquatic Species Program (ASP) in 1978 and collected over 3,000 microalgae strains from the west, northwest, and southeastern regions of the continental U.S., as well as Hawaii to systematically screen for high oil content strains grown under normal or severe conditions (Sheehan et al., 1998). Yang et al. (2010) targeted local algal species from four types of marine habitats located in the west side of Taiwan with the aim to screen heterotrophic microalgal strains rich in docosahexaenoic acid (DHA, C22:6n-3). However, bioprospecting and establishment of an adequate microalgae pool specifically for wastewater treatment and algal biofuel production, to the best of our knowledge, has not yet been reported before.

"Centrate", a highly concentrated municipal wastewater (centrate) stream generated through dewatering of sludge from primary and secondary settling (Figure 2.1), is rich in nutrients including phosphorus, ammonia, and organic nitrogen. From our previous studies it is evident that such a nutrient-rich wastewater is the best municipal wastewater stream for cultivating algae for simultaneous high biomass yield for energy production and high efficiency in wastewater nutrient removal (Wang et al., 2009). In addition, we also observed that the high level of total organic carbon (TOC) in the centrate (Table 4.2) could be utilized by some algae species for fast growth under photoheterotrophic or mixotrophic mode in light or under chemoheterotrophic mode in

dark (Wang et al., 2009; Li et al., 2011 (a)). It was reported that algae grown in heterotrophic mode accumulate as high as 55% lipid (Xu et al., 2004).

The success of such an algae-based treatment system for organic-rich wastewater relies on the ability of the algal cells to effectively assimilate both organic carbons (heterotrophic growth) (Burrell et al., 1984) and the inorganic nutrients such as N, P, and inorganic carbons from wastewater for maximal algal biomass and lipid production and efficient nutrient removal (Lau et al., 1995).

The centrate had a high turbidity due to high concentration of total suspended solids. Thus it is probably not suitable for autotrophic algae growth to high cell density due to limited light penetration. Therefore, finding facultative heterotrophic strains for simultaneous treatment of high organic-rich centrate and sustainable biofuel production through screening is necessary.

Many researchers reported using BG-11 medium to enrich isolated microalgae species from various environments, particularly those derived from wastewater (Chinnasamy et al.,2010), suggesting that this medium could be used to enrich many kinds of strains derived from natural water bodies. In addition, BG-11 medium can also be used for culturing unicellular algae such as blue-green algae under photoheterotrophic and chemoheterotrophic modes by adding glucose, and was considered as a good candidate for screening algae with facultative heterotrophic algae from local isolated strains (Mckinley et al., 1979). Thus in this study, the candidate algal samples collected from

local waters were evaluated for facultative heterotrophic algae on BG-11 medium with added glucose during the screening process.

The present study was part of the ongoing major collaborative efforts on mass cultivation of algae on municipal wastewaters for fuels and products between the University of Minnesota and the Metropolitan Council Environmental Services of Minnesota. The specific objectives of this study were (1) to collect microalgae from freshwater and wastewater habitats in Minnesota; (2) to isolate and identify facultative heterotrophic microalgal strains grown robustly in the centrate streams; and (3) to characterize selected strains for their biomass and lipid productivities and fatty acid profiles when grown in centrate and determine the most promising strains for effective wastewater treatment as well as high oil productivity for biofuel production.

### 4.3. Material and methods

#### 4.3.1. Collection and isolation of algae samples from Minnesota

Microalgae used in this study were obtained from five different types of water bodies in Minnesota, including lakes, rivers, creeks, ponds and wastewaters. Samples from different locations were collected, labeled, and stored in sterile transparent plastic bottles and sent to the laboratory within one day for algal cell purification and amplification. In most cases, one water sample was taken from one water body, but several samples were collected from different locations in large lakes where multiple algal species might exist. The protocol for isolating the unialgal algae strains are as follows: 1) Suspensions containing algae from above collections were concentrated by centrifugation to increase the biomass concentration of candidate microalgae density; 2)

The concentrated biomass were then diluted in sterile water and passed through a 60 μm plankton net to remove zooplankton and collected again using a 0.45 μm glass filter; 3) The cells remained on the filter were rinsed several times with sterile salt water to remove as many bacteria as possible, then were inoculated onto sterile 12-well tissue culture plate containing BG-11 medium (Stanier, 1971, Rippka et al., 1979) with the following ingredients: glucose (1500mg/L), $Na_2CO_3$ (20 mg/L), $NaNO_3$ (1500 mg/L), $Na_2Mg$ EDTA (1 mg/L), Ferric ammonium citrate (6 mg/L), Citric acid · $1H_2O$ (6 mg/L), $CaCl_2 \cdot 2H_2O$ (36 mg/L), $MgSO_4 \cdot 7H_2O$ (75 mg/L), $K_2HPO_4$ (30.5 mg/L), $H_3BO_3$ (2.86 mg/L), $MnCl_2 \cdot 4H_2O$ (1.81 mg/L), $ZnSO_4 \cdot 7H_2O$ (0.222 mg/L), $CuSO_4 \cdot 5H_2O$ (0.079 mg/L), $COCl_2 \cdot 6H_2O$ (0.050 mg/L), $NaMoO_4 \cdot 2H_2O$ (0.391 mg/L) for enrichment; 4) After enrichment for 10 days, the candidate cells were transferred by loop, and streaked onto 1.5% agar BG-11 medium plates using standard aseptic procedure; 5) Unialgal strains were obtained by selecting individual green colonies for sequential subculturing; 6) Liquid cultures were grown by inoculating single colonies of purified strains from agar plates into to 12 well culture plate or conical flasks containing sterile BG-11 medium and placing on an orbital shaker at 150 rpm.

**4.3.2. Characterization of the centrate**

Same as 3.3.2

**4.3.3. Multi-step strategy for facultative strains screening**

In order to identify strains that showed superior growth on centrate, the following four-step process was conducted using solid media in petri dishes followed by liquid media in flasks on orbital shakers:

Step 1, BG-11 media (Rippka et al., 1979) was used to enrich the algae strains listed in Table 1. BG-11, when supplemented with glucose, is widely used for green algae cultivation, and has a nutrient profile similar to the centrate. Step 2, unialgal strains adapted well in BG-11 medium were placed in two types of media, namely BG-11 media for autotrophic growth (without glucose) and BG-11 media enriched with glucose without $NaHCO_3$ for heterotrophic growth. For autotrophic media, strains were cultivated under light at a light intensity of 50 μmol $m^{-2}s^{-1}$. For heterotrophic media, the strains were cultivated in light (50 μmol $m^{-2}$ $s^{-1}$) or completely dark. Step 3, strains that survived autotrophic media and heterotrophic media under both light and dark condition were screened in centrate under light intensity of 50 μmol $m^{-2}$ $s^{-1}$. Step 4, strains adapted well in centrate were further cultivated in centrate for at least 3 generations to determine stable growth characteristics. The same 4 steps were used first on solid media in petri dishes and then in liquid media on orbital shakers. The growth data for species survived in centrate was determined with 4-6 days batch cultures grown in liquid centrate under light intensity of 50 μmol $m^{-2}$ $s^{-1}$.

**4.3.4. Morphological analysis of microalgae**

Morphological characters, including cell dimensions, reproductive features, chloroplast shape and number, presence of pyrenoids/contractile vacuoles/starch/flagella, were evaluated in 3-week-old cultures using a microscope (Olympus IX70, USA). Features were compared with the published descriptions (Ettl and Ga¨rtner 1995) and incorporated with molecular analysis to aid the cell identification.

### 4.3.5 DNA extraction, polymerase chain reaction (PCR) amplification and sequencing

The genomic DNA of algal cells was extracted using Qiagen DNeasy® Plant Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instruction with minor modification. To disrupt the algal material a small portion of algae (ca. 0.25 g) and 200 µl of Buffer AP1 were placed in a 1.5 ml microcentrifuge tube and ground with a pestle (USA Scientific, Inc., Ocala FL). An additional 200 µl of Buffer AP1 plus 4 µl of RNase-A stock (100 mg/ml) were added. The mixture was then ground, mixed using a vortex, and incubated for 15 min at 65°C to lyse the cells (mixed 2-3 times during incubation). Next, 130 µl of Buffer AP2 was added to the lysate and mixed, incubated on ice for 5 min to precipitate proteins, polysaccharides, and detergents, and then centrifuged at >14,000 g for 5 min. The lysate was transferred to a QIA shredder spin column, and centrifuged at >14,000 g for 2 min. The supernatant was transferred to a fresh 1.5 ml microcentrifuge tube, to which 1.5 volumes of Buffer AP3/E was added and mixed. This mixture was applied to the DNeasy mini spin column, centrifuged at 6000 g for 1 min, and then placed in a fresh tube with 500 µl Buffer AW. The mixture was centrifuged at 6000 g for 1 min and the filtrate was discarded. Next, 500 µl of Buffer AW was added to the column and centrifuged for 2 min at >14,000 g for 2 min to dry the membrane. The column was placed in a fresh 1.5 ml microcentrifuge tube and 100 µl of Buffer AE at 65°C was added onto the membrane. The sample was next incubated for 5 min at room temperature, and then centrifuged at 6000 g for 1 min to elute the DNA. The elution step was repeated once to give a final volume of 200 µl. DNA was diluted 5, 10, 20, 50, or 100-fold for use in PCR amplification.

For PCR amplification, a diluted portion of extracted DNA was used to amplify the 18SrRNA gene with two primers, 16S1N (forward, 5′ TCCTGCCAGTAGTCATATGC-3′) and 16S2N (reverse, 5′ TGATCCTCT/CGCAGGTTCAC-3′) (Grzebyk et al. 1998). Genomic DNA template, 50-100 ng, was mixed with the polymerase chain reaction (PCR) mixture containing 50 μL 1× PCR buffer (TaKaRa, Otsu, Japan), 0.2 mM dNTP, 0.2 μM of 16S1N, and 16S2N primers respectively, and 2.5 U Taq polymerase (TaKaRa, Otsu, Japan), then denatured at 94°C for 5 min. The PCR program was run for 30 s at 94°C, 30 s at 50°C, 2 min at 72°C for 40 cycles, and a 7-min final extension at 72°C. The PCR products were purified using QiAquick gel extraction kit (Qiagen, Germantown, MD, USA), then sent to Biomedical Genomics Center at University of Minnesota (Saint Paul, Minnesota, USA) for DNA sequencing. The resulting 18S rRNA gene sequences were aligned and compared to the nucleotide sequences of some known microorganisms in GenBank database of the National Center for Biotechnology Information by using Basic Local Alignment Search Tool (BLAST).

**4.3.6. Growth and chemical analyses**

**4.3.6.1 Determination of algal growth**

Algal growth was monitored daily through testing the total volatile suspended solids (TVSS), which represents biomass concentration and was determined according to the standard method (APHA, 1995). Algae growth in batch culture usually include four phases as follows: lag phase, exponential phase, stationary phase, and death phase. During the exponential growth phase, the growth rate of the cells is proportional to the

biomass of cells. Since generally biomass can be measured more accurately than the number of cells, the basic microalgal growth equations are expressed in term of mass based on TVSS as follows:

$$R_{TVSS}= Ln(TVSS_t)-Ln(TVSS_0)/t \quad (4.1)$$

Where $R_{TVSS}$ is the accumulation rate based on TVSS. $TVSS_t$, and $TVSS_0$ are the TVSS at day t and day 0. t is the time interval (days) between $TVSS_t$ and $TVSS_0$.

**4.3.6.2 Nutrient analysis**

Same as 3.3.4.1

**4.3.6.3 Total lipid analysis**

Algae cells were harvested through centrifugation and then dried with a freeze dryer (Savant Instruments Inc., USA) for the analysis of lipid content. The lipids were extracted using a one-step extraction method adapted from Folch (Folch et al., 1956). About 0.1g dried algae powder were weighed into clean, 25 ml screw-top glass tubes, in which 10ml 2:1 chloroform-methanol (v/v) mixture was added. Extraction was carried out in 30 °C water bath (Cole-Parmer, USA) for 30 min with 100 rpm rotation speed. Upon completion of the reaction, the algal solid residues were removed by passing the suspension through a Whatman 934-AH glass fiber filter (Whatman Inc., USA). The filtrate was transferred to another clean, 25 ml screw-top glass tube containing 2 ml 0.9% NaCl solution to wash out water-soluble components. After centrifugation, a biphasic system without any interfacial fluff was obtained. The volume of the lower phase containing essentially all the algal lipids extracted was measured, and 3ml of the lower phase was transferred into a weighed, clean, 5ml glass tube, and then organic solvent was dried off under the N-EVAP Analytical Nitrogen Evaporator

(Organomation Associates, Inc., USA). Duplicate lipid determination was made for every sample. The lipid content of dry weight was calculated according to the following formula:

$LW\ (g/g) = (m_2 - m_0) \times V/(3 \times m_1)$

where LW stands by "lipid content based on dry weight", $m_1$ is the weight of the algae powder, $m_0$ is the weight of the empty 5ml glass tube, $m_2$ is the weight of the 5ml tube with the dried lipids, and V is the total volume of the lower phase after washing.

The total lipid concentration (TC) was calculated by mutiplying the lipid content of dry weight by TVSS on the 4th day. The equation is listed below:

$$TC\ (g/L) = LW \times TVSS$$

#### 4.3.6.4 Fatty acid profile analysis

Same as 3.3.4.3

## 4.4. Results and discussion

### 4.4.1. Collection and isolation of microalgal strains in Minnesota

Algae containing samples collected from diverse habitats in different lakes, rivers, creeks, ponds and wastewater plants in Minnesota, are shown in Table 1. Unialgal strains were obtained through a 6-step purification and isolation procedure. Sixty strains of algae-like microorganisms capable of growing in BG-11 agar plate were isolated from above mentioned water bodies. In nature, very diverse groups of freshwater algae grown in variety of habitats are the *Chlorophytes* or green algae (Duran et al. 1977). It was observed that the colony color of all 60 isolated strains grown on BG-11 agar-plate are green, suggesting all of strains collected from local area belong to unicellular chlorophytic microalgae. On the other hand, it was reported that unicellular

chlorophytic microalgae have been shown to be particularly tolerant to many wastewater conditions and very efficient in removing nutrients from wastewater (Aslan and Kapdan, 2006; Gonzalez et al., 1997; Ruiz-Marin et al., 2010). Therefore, we focused on screening above isolated candidate strains to obtain the most promising ones which could survive centrate and accumulate high biomass and lipid content.

**Table 4.1.** Collection dates and sites of microalgal strains established in this study

| Species | Collection date | Collection site |
|---|---|---|
| UM220 | Oct, 2006 | A Twin Cities lake |
| UM221 | Apr, 2006 | RoseLawn Pond |
| UM222 | Apr, 2006 | Como Park Golf Course Pond #1 |
| UM223 | Apr, 2006 | Como Park Golf Course Pond #2 |
| UM224 | Apr,2006 | Como Lake |
| UM225 | Apr, 2006 | McCarrons Lake |
| UM226 | Apr, 2006 | Rosville Park, Lexington south of County Rd C |
| UM227 | Apr, 2006 | Oxford and County Rd C |
| UM228 | Apr, 2006 | County Rd C and Victoria: north Pond |
| UM229 | Apr, 2006 | County Rd C and Victoria: north Pond |
| UM230 | Apr, 2006 | Falls to the lake on County Rd C and Victoria |
| UM231 | Apr, 2006 | Lake Johanna, west side |
| UM232 | May, 2006 | swamp in west side of Lake Johanna across the road |
| UM233 | May, 2006 | next swamp across Lake Johanna |
| UM234 | May, 2006 | drainage in Lake Johanna going north |
| UM235 | May, 2006 | Drainage 2 in Lake Johanna |
| UM236 | May, 2006 | Snelling Ave/skiles north of the road |
| UM237 | May, 2006 | under the bridge between Snelling and Hamline west side |
| UM238 | May, 2006 | Lake Josephine east side |
| UM239 | May, 2006 | Drainage to Lake Josephine #1 |
| UM240 | May, 2006 | Drainage to Lake Josephine #2 |
| UM241 | May, 2006 | Pond #1 across Rosville High School |
| UM243 | Jun, 2006 | Como Park lake |
| UM244 | Jun, 2006 | Channel on Ripley road, Litchfield (at the golf course) |
| UM245 | Jun, 2006 | Lake Ripley picnic area, Lithfield |
| UM246 | Jun, 2006 | Pond between County Rd 1 and County Rd 23, Litchfield |
| UM247 | Jun, 2006 | Lake Hope, Litchfield |
| UM248 | Jun, 2006 | Marlenes's drainage at the park |
| UM249 | Jun, 2006 | Long Lake, Blanca's place, Litchfield |
| UM250 | Jun, 2006 | Theodore Wirth Parkway, Pond #3 on the right coming from 394 |
| UM251 | Jun, 2006 | Theodore Wirth Parkway, left, right Pond #2b around the bridge |
| UM252 | Jun, 2006 | Theodore Wirth Lake 1, farther than the beach |
| UM253 | Jun, 2006 | Theodore Wirth Lake 2 on the beach |
| UM254 | Jun, 2006 | Lake Calhoun |
| UM255 | Jun, 2006 | Moore Lake |
| UM256 | Jun, 2006 | Malenes's creek |
| UM257 | Jun, 2006 | Rice Creek after the bridge on Mississippi River |
| UM258 | Jun, 2006 | Rice Creek after the bridge inside the park |
| UM259 | Jun, 2006 | MayFlower drainage pond |
| UM260 | Jun, 2006 | Drainage pond behind Bachmans |

| | | |
|---|---|---|
| UM261 | Jun, 2006 | Pond of Assisting Living banfill acrss apartments |
| UM263 | Jun, 2006 | Marlenes's drainage at the park |
| UM264 | Jun, 2006 | Metro Wastewater Treatment Plant dreft side |
| UM265 | Jun, 2006 | 3M Innovation Plant lake |
| UM266 | Jun, 2006 | Kaller Lake |
| UM267 | Jun, 2006 | Pond on Keller Lake |
| UM268 | Jun, 2006 | Maplewood: Lakewood and Maryland |
| UM269 | Jun, 2006 | Mcarron's lake |
| UM270 | Jun, 2006 | Margolis pond on Lapenteur |
| UM271 | Jul, 2006 | Loon Lake, Waseca |
| UM273 | Jul, 2006 | White Bear Lake |
| UM274 | Jul, 2006 | Bold Lake, east site |
| UM275 | Jul, 2006 | Amelia Lake |
| UM276 | Jul, 2006 | Coon Rapids Dam #1 |
| UM277 | Jul, 2007 | Pond at Marine City |
| UM279 | Jul, 2007 | Spring brook 1, Fridley |
| UM280 | Jan, 2010 | Municipal wastewater plant |
| UM281 | May, 2006 | Itasca main lake |
| UM282 | Jun, 2006 | Bryant lake, Dbc Pond |
| UM284 | Jan, 2010 | Municipal wastewater plant |

**Table 4.2.** Characteristic of the autoclaved centrate used in this study

| Parameter | Concentration (mg/L) | Parameter | Concentration (mg/L) |
|---|---|---|---|
| Soluble COD | 2324 ± 40.1 | $PO_4^{3-}$-P | 212± 7.2 |
| TOC | 960±30.50 | $NH_3$-N | 91±1.8 |
| pH | 6.31 ± 0.11 | TKN | 134± 6.8 |
| $NO_3$-N | 0.35 ± 0.36 | $NO_2$-N | <0.03 |
| Total suspended solid | 0.14±0.11 | | |

### 4.4.2. Screening facultative heterotrophic microalgae grown in centrate

BG-11 medium, which was commonly used for identifying autotrophy and heterotrophy of unicellular algae such as blue-green algae (Mckinley et al., 1979) was used to enrich the isolated microalgae species in this study and the results demonstrated that all sixty strains could grow well in such medium, which coincides with other reports (Ignacio et al., 2008; Chinnasamy et al., 2010). A multi-step screening strategy was adopted to identify candidate strains which showed facultative heterotrophic capability and were capable of surviving a centrate-based culture system. By this screening strategy, twenty seven strains which could grow photoautotrophically and heterotrophically under light

and in dark were obtained, among which only seventeen of them survived in centrate without further acclimation (Table 3). These candidate strains were further investigated for their stability in small flask with autoclaved centrate as media and cultivated in flasks on an orbital shaker for 6 days. The growth data in terms of total suspended volatile solid (TVSS) for the species that could survive centrate is summarized in Fig 3. The results further confirmed that the selected 17 candidate strains adapted well in the centrate.

It seems reasonable to hypothesize that species that naturally develop in wastewater should perform better than most others in commercial scale cultivation on wastewaters. That is the reason we also chose the metropolitan wastewater treatment plant as one sampling site in this study. Our results showed that two strains isolated from above wastewater environment, named UM280 and UM284, ranked among the top ones in terms of maximal grow rate and biomass productivity (Table 3), which coincided with other report that algae isolated from wastewater treatment plant sites or real water bodies can usually adapt to culture conditions similar to where they are found and grow better (Jiménez-Pérez et al, 2004).

**Table 4.3.** Strain classification, cell size, growth rate and biomass productivity in centrate

| Code | Species | Size (um) | Maximal Growth rate ($d^{-1}$) | Biomass productivity (mg $L^{-1}d^{-1}$) | Lipid productivity (mg $L^{-1}d^{-1}$) |
|---|---|---|---|---|---|
| UM 221 | *Heynigia. sp* | 6-9 | 0.431 | 210.4 | 50.8 |
| UM 224 | *Chlorella. sp* | 6-10 | 0.455 | 231.4 | 77.5 |
| UM 280 | Auxenochlorella protothecoides | 6-9 | 0.492 | 268.8 | 77.7 |
| UM 231 | *Chlorella. sp* | 7-9 | 0.391 | 179.2 | 41.7 |

| | | | | | |
|---|---|---|---|---|---|
| UM 235 | *Chlorella. vulgaris* | 2-4 | 0.293 | 120.8 | 21.0 |
| UM 281 | *Micractinium. sp* | 5-7 | 0.455 | 231.4 | 42.6 |
| UM 258 | *Scenedesmus. sp* | 13-15 | 0.411 | 193.8 | 49.8 |
| UM 259 | *Chlorella. vulgaris* | 3-5 | 0.367 | 162.5 | 0.0369 |
| UM 265 | *Hindakia sp* | 6-9 | 0.498 | 275.0 | 77.8 |
| UM 268 | *Chlorella. sp* | 5-7 | 0.325 | 137.5 | 36.9 |
| UM 269 | *Chlorella. sp* | 5-7 | 0.317 | 137.5 | 65.4 |
| UM 270 | *Chlorella. sorokiniana* | 6-9 | 0.402 | 187.5 | 49.4 |
| UM 253 | *Chlorella. sp* | 6-8 | 0.466 | 241.7 | 74.7 |
| UM 271 | *Chlorella. sp* | 5-7 | 0.434 | 212.5 | 58.5 |
| UM 273 | *Chlorella. sp* | 7-9 | 0.416 | 197.9 | 41.3 |
| UM 277 | *Chlorella. sorokiniana* | 5-7 | 0.397 | 183.3 | 94.8 |
| UM 284 | *Scenedesmus. sp* | 13-15 | 0.472 | 247.5 | 74.5 |

### 4.4.3. Classification of top performing strains

A freshwater strain chlorella sp. (obtained from the University of Texas; UTEX 2714) was used as a positive control (Figure 4.2:  Lanes 1) and $dH_2O$ was used as a negative control (Figure 4.2:  Lane 6) for the PCR reaction.

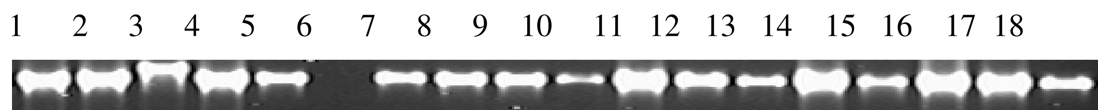



**Figure 4.2.** PCR amplification of the 18S RNA gene in selected seventeen freshwater strains.  Positive control (UTEX 2714, Lane 1) and negative control (Lane 6), other Lane from 2 to18 as UM221, UM 224, UM 231, UM 235, UM 253, UM 258, UM 259, UM 265, UM 268, UM 269, UM 270, UM 271, UM 273, UM 277, UM 280, UM 281, UM 284, respectively.

The 18SrDNA genes of candidate strains were successfully amplified from the whole genomic DNA using the following primer pairs: 16S1N and 16S2N (Figure 4.2: Lane 2-18). According to the BLAST result from algae gene library combination with

morphological observation, the seventeen strains were identified as Chlorella sp., Heynigia sp., Hindakia sp., Micractinium sp., and Scenedesmus sp., respectively (Table 4.3).

It is not surprising that among these selected strains, more than 60% were identified as *chlorella sp.* Previous researches (Tam and Wong, 1990; Chinnasamy et al., 2010; Wang et al., 2010) also reported that *chlorella sp*. can be used for treatment of different wastewater streams such as primary settled wastewater, settled and activated sewage, animal manure and industrial wastewater etc, suggesting such species could tolerate and survive different types of wastewater streams. For *Scenedesmus* species, numerous studies reported their ability to grow in above wastewaters (Tam and Wong, 1989; Gonzalez et al., 1997; Woertz et al., 2009). In this study, it was also observed that two strains, namely UM258 and UM284, which belong to *Scenedesmus* species, adapted well in centrate (Table 5), showed their great potential for wastewater treatment.

It is worth noting that there were a few studies reported using algal species identified in this study, *Heynigia sp., Hindakia sp.,* and *Micractinium sp.* for wastewater treatment. Shelef (1982) reported high rate algal ponds for wastewater treatment using *Micractinium sp*. implying such algal species also could be applied to wastewater treatment for nutrient recovery and environment protection. Bock et al. (2010) demonstrated the *Heynigia sp.*and *Hindakia sp.*, frequent morphospecies of the genus in inland waters, have the polyphyletic origin of morphotype within the Chlorellaceae,

suggesting these species normally exist in freshwater bodies and have potential for wastewater treatment as alternative species to *chlorella* and *scenedesmus*.

### 4.4.4. Comparison of biomass concentration, total lipid and fatty acid profile of candidate microalgae

For the algae grown on wastewater, selection of fast-growing and high-lipid content microalgal strains is essential to the success of commercial wastewater-algae-based biofuel application. Thus the biomass, total lipid, and profiles of fatty acid of selected strains were systematically examined in this study.

#### 4.4.4.1. Comparison of growth rate and biomass productivity of candidate microalgae

As shown in Table 4.2, the centrate can supply enough nutrients such as nitrogen and phosphorous for autotrophic growth, and TOC for photo-heterotrophic or chemo-heterotrophic growth under light/dark condition. Therefore, it was hypothesized that it could be used as substrate to replace artificial media and to support chemo-heterotrophic and photo-heterotrophic growth for candidate strains under light/dark condition. Therefore all candidate strains were grown in such an organic-rich centrate for 6 days to evaluate their growth rate and biomass concentration. It was observed that all strains reached steady-state biomass concentration during the first 4 days without obvious lag phases and the highest net biomass concentration achieved ranged from 0.48 g/L to 1.08 g/L (TVSS) at day 4, except for strain UM 269, which reached the stationary phase after 6 days of operation. The reason could be the lag phase occurred at initial day for this strain (Fig.4.3). It was also found that the strain UM224, UM253, UM265, UM280, and UM284 showed faster growth rates than other candidate strains

with the average specific growth rates of 0.455, 0.466, 0.498, 0.492 and 0.472 $d^{-1}$, respectively, during the first 4 days (Table 4.3). These growth rates were comparable with those grown in municipal primary settled sewage (0.277 $d^{-1}$) and in municipal settled sewage (0.35-0.5 $d^{-1}$) wastewaters (Tam and Wong. 1990; Lau, et al. 1995).

The biomass productivity obtained in previous studies using various wastewater resources as substrate was summarized in Table 4.5. It is clear that the biomass productivity found in this study ranked among the top ones in terms of total suspended solid concentration. There are reported evidences indicating that some heterotrophic algae utilize organic carbons more efficiently under light condition than in dark (McKinley and Wetzel, 1979; Boichenko et al., 1992). Furthermore, it was suggested that the growth rate for algae grown in mixotrophic way was almost the sum of autotrophic and heterotrophic growth (Lee, 2007). Thus the high growth rate and high biomass productivity of candidate strains in present study probably were mainly due to the photohetrotrophy or mixotrophy. The TOC results shown in Figure 4.4 also confirmed that TOC was almost completely consumed by the candidate strains in the first 3 days, suggesting these strains could assimilate almost all organic carbon source for photoheterotrophic growth.

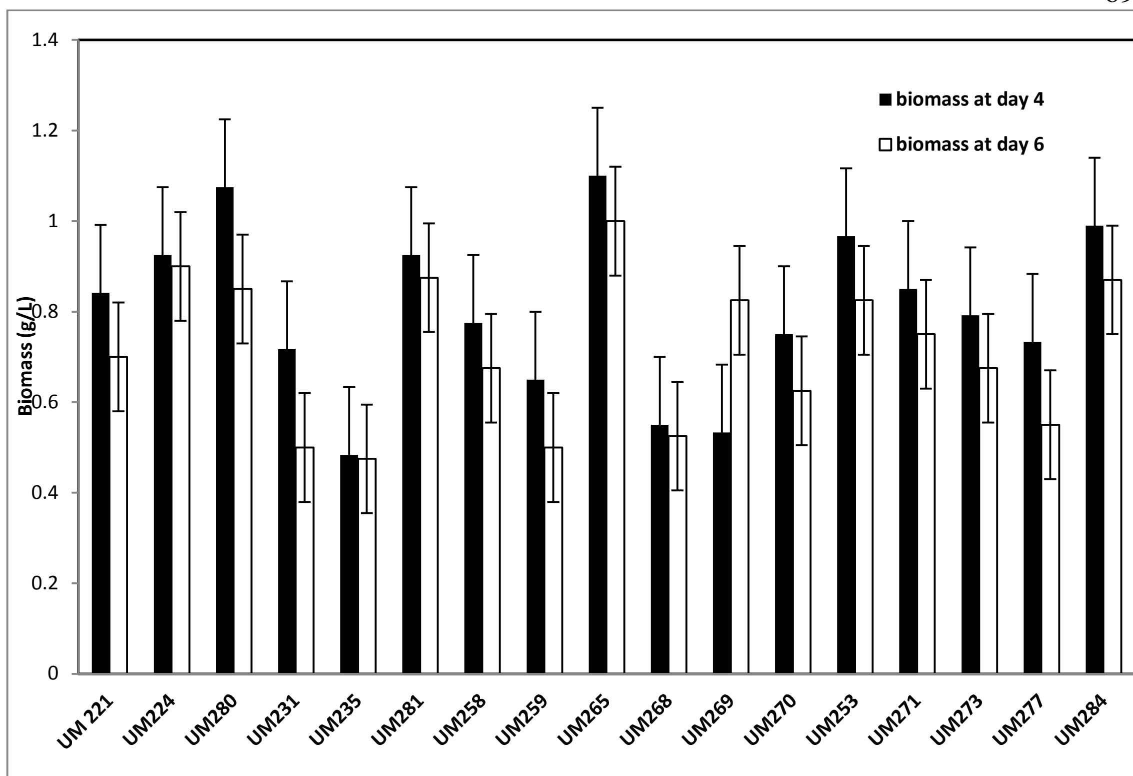



**Figure 4.3.** Biomass concentration (g/L) at day 4 and day 6 for batch culture grown in centrate

After 4 days cultivation, almost all candidate algae grew slower and the concentration of the algal cell started to decline (Figure 3). The factors affecting algae growth are numerous including nutrients depletion, pH variation, self shading, etc. However, in this case, nutrients such as nitrogen and phosphorus were not limiting factors because the concentration of nitrogen and phosphorus still remained very high after 3 days operation (Figure 4.4 (c) and Figure 4.4 (d)), which coincided with other report (Wang et al., 2009; Li et al., 2011 (a)). Our previous study also proved these candidate strains can tolerate different pH value ranged from 6 to 9 (data not shown). Thus, the only probable reason for growth decline in this case may be the shortage of carbon source (including organic and inorganic carbon). It was observed that TOC was almost used up in the first three days (Figure 4.4 (a)), indicating that the algae could not maintain heterotrophic

growth without continuous supply of organic carbon, or grow autotrophically without the addition of inorganic carbon or $CO_2$. In addition, COD, which is commonly used to indirectly measure the amount of organic compounds in wastewater, was tested in this study. As shown in Figure 4.4 (b), the consumpation of COD was occurred during the first 3 days, which further confirmed that the usable organic carbon was assimilated by algae due to the heterotrophic growth.

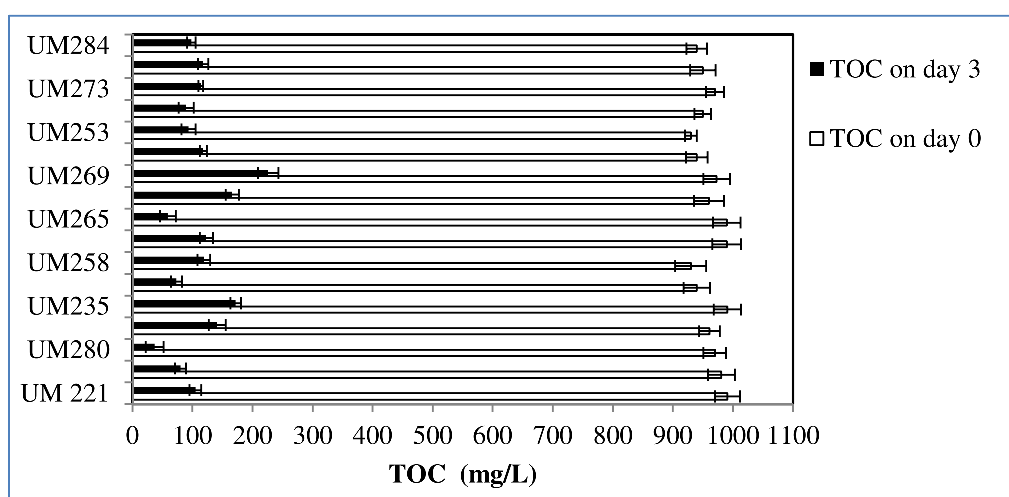



**Figure 4.4 (a).** TOC concentration (mg/L) at initial day and at day 3 for batch culture grown in centrate

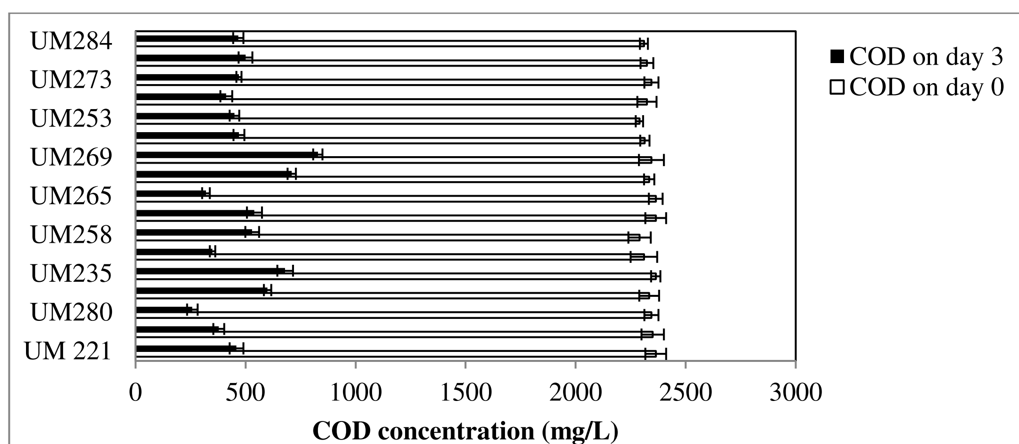



**Figure 4.4 (b).** COD concentration (mg/L) at initial day and at day 3 for batch culture grown in centrate

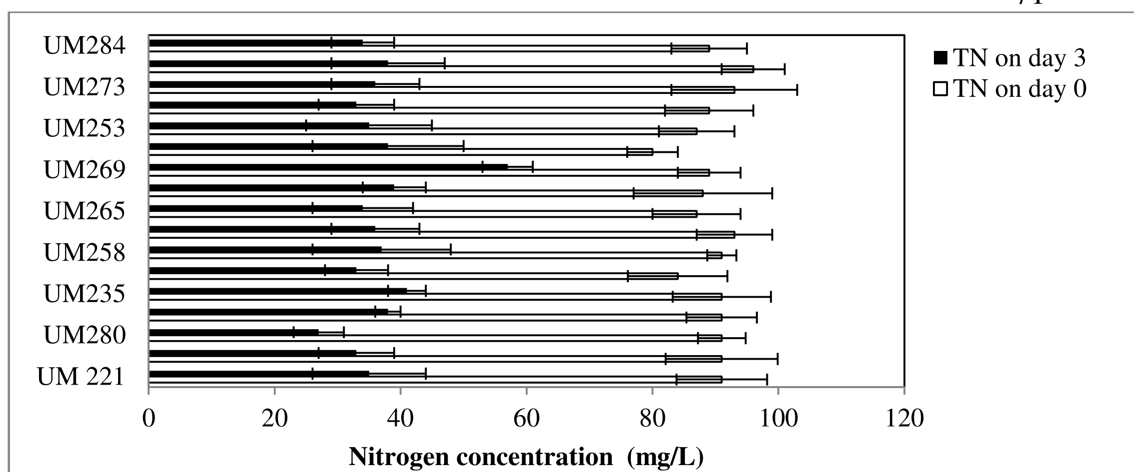



**Figure 4.4 (c).** Nitrogen concentration (mg/L) at initial day and at day 3 for batch culture grown in centrate

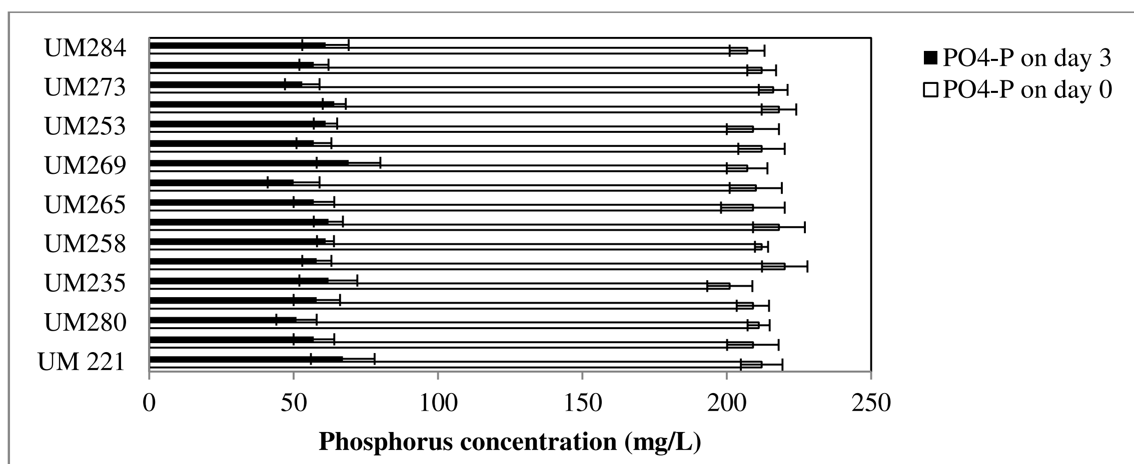



**Figure 4.4 (d)** Phosphorus concentration (mg/L) at initial day and at day 3 for batch culture grown in centrate

#### 4.4.4.2. Comparison of lipid content and fatty acid profile of candidate microalgae

The total lipid contents of the candidate algae grown in centrate ranged from 17.41% to 33.53% of TVSS based weight (Table 4.3). Among them, the strains UM224, UM280, UM265, UM269, UM253, UM271 and UM284 showed higher lipid content of 33.53%, 28.90%, 28.30%, 31.73%, 30.91%, 27.51% and 30.09%, respectively, than the rest, suggesting that these candidates could accumulate higher lipid content than other strains through photoheterotrophic pathway. It was also found that TOC was reduced

significantly for all strains after cultivated for 3 days with removal efficiency between 82.27% and 96.18%, suggesting that all selected algae strains can utilize the organic carbon in concentrated wastewater for photo-heterotrophic growth. These results also coincided with previous reports that photoheterotrophy could produce higher biomass and oil yields than photoautotrophy or chemoheterotrophy alone (Lee 2007; Xu et al. 2004).

The profile of fatty acids components are shown in Table 4.4. GC-MS analysis showed that the main fatty acid components of the candidate microalgae were composed of C16-C18 fatty acids, accounting for 80.23 -94.45% of total fatty acids (Table 4.4). C16-C18 fatty acids are suitable for biodiesel production (Xu et al. 2004; Miao et al., 2006). It is interesting to note that the strains UM231 and UM258 could also produce a very unique fatty acid-eicosapentaenoic acid (EPA, C20:5), which account for 5.05% and 7.58% of total fatty acid, respectively (Table 4.4). EPA is an important ω-3 polyunsaturated fatty acid which plays an important role in the prevention of various human diseases (Nettleton et al; 1995). Thus, it may be feasible to extract this high-value product to improve the overall economic viability of algae to fuels technologies. Based on their ability to grow in centrate and high biomass and lipid producctivity, UM224, UM253, UM265, UM280, and UM284 were considerably highly promising and chosen for further research to improve the technologies for sustainable algae-biofuel production and effective wastewater treatment.

**Table 4.4** Main fatty acid content and the total lipid content in screened microalgae strains

| Fatty acid | Fatty acid content (%) in screened microalgae strain | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | UM 221 | UM 224 | UM 280 | UM 231 | UM 235 | UM 281 | UM 258 | UM 259 | UM 265 |
| C12:0 | 0.23 | | 0.21 | - | - | - | - | - | 0.59 |
| C14:0 | - | 1.87 | - | 0.89 | 2.93 | 1.41 | 4.12 | 1.24 | 1.12 |
| C16:0 | 26.29 | 24.28 | 27.85 | 22.15 | 30.68 | 25.18 | 26.92 | 26.45 | 27.37 |
| C16:1 | 0.95 | - | 0.18 | 0.2 | 6.36 | 0.66 | 5.77 | | 4.62 |
| C16:2 | 8.66 | 6.25 | 0.59 | 2.86 | 4.61 | 5.48 | 2.75 | 6.82 | 10.58 |
| C16:3 | - | - | 0.59 | - | - | - | - | - | - |
| C18:0 | 0.86 | 1.95 | 4.62 | 3.74 | 2.81 | 3.27 | 0.87 | 1.70 | 1.42 |
| C18:1 | 11.88 | 4.74 | - | 10.40 | 0.23 | 9.14 | 31.73 | 37.42 | 5.86 |
| C18:2 | 31.08 | 30.28 | 28.75 | 22.81 | 19.38 | 34.98 | 15.86 | 23.88 | 32.15 |
| C18:3 | 18.90 | 29.31 | 36.89 | 30.16 | 28.92 | 17.78 | 0.56 | 0.11 | 14.51 |
| C20:0 | - | - | - | - | - | - | 0.14 | - | 0.43 |
| C20:1 | - | 0.2 | - | 0.23 | - | 0.16 | 0.13 | - | - |
| C20:5 | - | - | - | 5.05 | - | - | 7.58 | - | - |
| C22:0 | - | - | - | - | 0.17 | 0.11 | - | - | 0.38 |
| C24:0 | 0.19 | 0.28 | | | 0.12 | 0.28 | - | 0.19 | 0.47 |
| satu[a] | 27.91 | 28.78 | 32.96 | 13.00 | 37.88 | 31.38 | 35.62 | 30.00 | 32.01 |
| mono[b] | 13.45 | 5.38 | 0.18 | 10.66 | 9.20 | 10.31 | 37.62 | 39.19 | 10.70 |
| poly[c] | 58.64 | 65.84 | 66.86 | 76.34 | 52.92 | 58.31 | 26.76 | 30.81 | 57.28 |
| C16-C18 | 91.61 | 94.45 | 94.177 | 85.35 | 81.94 | 93.46 | 80.23 | 90.54 | 94.9 |
| Total lipid | 24.16 | 33.53 | 28.90 | 23.28 | 17.41 | 18.41 | 25.70 | 22.68 | 28.30 |

| Fatty acid | Fatty acid content (%) in screened microalgae strain | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | UM268 | UM269 | UM270 | UM253 | UM271 | UM273 | UM277 | UM284 |
| C12:0 | 0.31 | - | - | 0.19 | - | 0.1 | - | - |
| C14:0 | 5.13 | - | 0.77 | | 1.03 | 1.48 | 4.09 | 0.44 |
| C16:0 | 35.66 | 22.97 | 27.57 | 28.59 | 27.31 | 30.59 | 34.64 | 31.59 |
| C16:1 | 0.12 | 17.72 | 0.27 | | 6.89 | 6.43 | 3.12 | 0.72 |
| C16:2 | 3.78 | 5.33 | 8.70 | 8.79 | 9.61 | 6.23 | 5.25 | 3.19 |
| C16:3 | - | - | 8.67 | 8.57 | - | - | - | 6.44 |
| C18:0 | 2.24 | 0.51 | 1.28 | 1.44 | 2.80 | 3.79 | 1.81 | 2.29 |
| C18:1 | - | - | 4.54 | 4.24 | 5.24 | 8.18 | 8.46 | 1.22 |
| C18:2 | 23.91 | 18.37 | 28.67 | 29.28 | 31.57 | 19.09 | 25.98 | 10.12 |
| C18:3 | 26.30 | 31.85 | 18.18 | 17.26 | 13.03 | 22.06 | 15.65 | 38.19 |
| C20:0 | - | 0.15 | 0.34 | 0.40 | 0.44 | 0.14 | 0.24 | 0.15 |
| C20:1 | - | - | - | - | - | - | - | 0.45 |
| C20:5 | - | - | - | - | - | - | - | 0.13 |
| C22:0 | 0.33 | 0.11 | 0.14 | 0.18 | 0.39 | 0.19 | - | - |
| C24:0 | 0.51 | | 0.20 | 0.38 | 0.54 | 0.36 | 0.34 | |
| satu[a] | 44.56 | 24.78 | 30.81 | 31.46 | 33.07 | 37.72 | 41.47 | 40.47 |
| mono[b] | 1.35 | 19.67 | 4.91 | 4.57 | 12.61 | 14.89 | 11.58 | 2.39 |

| poly[c] | 54.09 | 55.55 | 64.28 | 63.97 | 54.31 | 47.39 | 46.94 | 57.14 |
|---|---|---|---|---|---|---|---|---|
| C16-C18 | 81.76 | 92.15 | 94.71 | 94.14 | 93.64 | 90.01 | 86.85 | 93.76 |
| Total lipid | 26.85 | 31.73 | 26.34 | 30.91 | 27.51 | 20.89 | 26.99 | 30.09 |

a: saturated fatty acids; b: monoenoic fatty acids; c: polyenoic fatty acid

**Table 4.5** Comparison of biomass and lipid productivities in microalgae grown in different wastewater resources

| Wastewater type | Microalgae species | Biomass productivity (mg $L^{-1}d^{-1}$) | Lipid content (%DW) | Lipid productivity (mg$L^{-1}d^{-1}$) | reference |
|---|---|---|---|---|---|
| Carpet mill | *Chlorella asccharophila* | 23 | 18.10 | 4.2 | Chinnasamy et al.(2010) |
| Carpet mill | *Scenedesmus sp* | 126.54 | 12.80 | 16.2 | Chinnasamy et al.(2010) |
| Dairy wastewater ,25X | *Polyculture of chlorella sp., Micractinium sp., Actinastrum sp* | NA | 29.00 | 17 | Woertz et al.(2009) |
| Primary clarifier effuent | *Polyculture of chlorella sp., Micractinium sp., Actinastrum sp* | NA | 9.00 | 24.4 | Woertz et al.(2009) |
| Activated sludge extract | *Chlorella pyrenoidosa* | 11.55 | NA | NA | Cheung and Wong.(1981) |
| Digested sludge extract | *Chlorella pyrenoidosa* | 51.82 | NA | NA | Cheung and Wong.(1981) |
| Settled sewage | *Chlorella pyrenoidosa* | 275 | NA | NA | Tam and Wong.( 1989,1990) |
| Activated sewage | *Chlorella pyrenoidosa and scenedesmus sp* | 92.31 | NA | NA | Tam and Wong.( 1989,1990) |
| Secondarily treated sewage | *Botryococcus braunii* | 35.00 | NA | NA | Sawayama et al.,1992 |
| Artificial wastewater | *Scenedesmus sp* | 126.54 | 12.80 | 16.2 | Voltolina et al.(1999) |
| centrate | UM 224 | 231.4 | 33.53 | 77.5 | This study |
| centrate | UM 280 | 268.8 | 28.90 | 77.7 | This study |
| centrate | UM 265 | 275.0 | 28.30 | 77.8 | This study |
| centrate | UM 253 | 241.7 | 30.91 | 74.7 | This study |
| centrate | UM 284 | 247.5 | 30.09 | 74.5 | This study |

## 4.5. Conclusions

Our bioprospecting procedures involving collecting algae samples from local water habitats and screening and acclimation using different culture media and lighting conditions resulted in a pool of algal strains having high biomass and lipid productivities and high wastewater nutrient removal efficiency. Many selected microalgal strains could grow well in wastewater with mixotrophic mode under light/dark conditions to achieve high biomass concentration and high lipid content simultaneously. The candidate strains could potentially be used in cultivation on other wastewater sources such as industrial and agricultural wastewaters. The bioprospecting procedures may be extended to selection of algae strains for different cultivation environments.

# CHAPTER 5. IDENTIFICATION OF TOP-PERFORMING STRAIN FOR ALGAE BASED BIODIESL PRODUCTION AND SIGNIFICANCE EVALUATION OF GROWTH CONDITIONS

## 5.1 Overview

In last chapter the growth of the algae strains collected from local lake areas were test. In this chapter algae strains purchased from commercial algae banks were evaluated.

The objectives of this chapter were to find the robust strains for the centrate cultivation system and to evaluate the effects of environmental factors including light intensity, light/dark cycle, and exogenous $CO_2$ concentration on biomass accumulation, wastewater nutrient removal and biodiesel production. The results showed that 14 among 143 algae strains, which were purchased from commercial algae banks, from the genus of *Chlorella, Haematococcus, Scenedesmus, Chlamydomonas,* and *Chloroccum* were able to grow on centrate. The highest net biomass accumulation (2.01 g/L) was observed with *Chlorella kessleri* followed by *Chlorella protothecoides* (1.31 g/L), and both of them were proved to be capable of mixotrophic growth when cultivated on centrate. Environmental factors had significant effect on algal biomass accumulation, wastewater nutrients removal and biodiesel production. Higher light intensity and exogenous $CO_2$ concentration with longer lighting period promote biomass accumulation, biodiesel production, as well as the removal of chemical oxygen demand and nitrogen, while, lower exogenous $CO_2$ concentration promotes phosphorus removal.

**5.2 Introduction**

With the increasing amount of waste originating from human activities comes the negative impact on the environment and in particular the water quality. Waste streams, which are rich in carbon, nitrogen and other minerals, have potential for use as a substrate for microalgae cultivation (Hammouda et al., 1995; Hoffmann, 1998), which will greatly reduce the cost involved in nutrients and water supply. The centrate, which is generated from centrifuging of activated sludge (Zhou et al., 2011), may be a viable alternative as the cultivation media for algae. First, the concentrations of carbon, nitrogen and phosphorus are higher in the centrate than in any other wastewater streams obtained from a wastewater treatment plant, which can provide sufficient nutrients for algae growth. Second, centrate contains a variety of minerals such as K, Ca, Mg, Fe, Cu, and Mn (Li et al., 2011 (a)), which are essential micronutrients for algae growth and metabolism. Third, the volume of the centrate produced daily is extremely large with no availability problem all year round, and the centrate need to be recycled to the activated sludge process for further treatment to avoid environmental contamination, which adds extra load for the treatment process, especially the high concentration of phosphorus. Thus the use of the centrate for algae cultivation could serve the dual role of waste reduction and biomass/bioenergy production.

The concept of algae cultivation as engineered systems in wastewater treatment and recycling has extended the microalgae based biofuel production to its maximum potential. Previous studies demonstrated the success of using algae to clean municipal wastewater rich in nitrogenous and phosphorus compounds collected from different

stages during the treatment process (Li et al., 2011 (a); Zhou et al., 2011). When combined with wastewater treatment, only a few microalgae species from the family of *Chlorella* were most commonly used such as *Chlorella pyrenoidosa* (Tam and Wong, 1989, 1990; Cheung and Wong, 1981), and *Chlorella Vulgaris* (Lau et al., 1995, 1998), while strains from other genus were not well studied. Although these tested strains show excellent adaptation in wastewater, the concentration of the algae cells and the lipid content are not satisfactory for biomass and biodiesel production (Deng et al., 2009; Mata et al., 2009; Sialve et al., 2009; Um and Kim, 2009; Li et al., 2008; Chisti, 2007). For the objective of algae cultivation on centrate as feedstock for biodiesel production, algae strains to be used need to meet several criteria including ability of survival in wastewater, capabilities of growing to high cell density robustly, high content of lipids, preferably triacylglycerol (TAG), and capability of heterotrophic or mixotrophic growth since centrate contains both organic carbon and inorganic carbon. Up to now rare research into the screening and acclimation of microalgae to adapt to the centrate environments is available (Zhou et al., 2011). Therefore, the first task of this study was to find the robust strains for the specific cultivation systems.

The variation in cell composition in a single algae species can vary in many folds according to the culture condition under which it is grown. Environmental factors, particularly light input such as light intensity and light–dark cycle, temperature, nutrients status such as the concentration of carbon and nitrogen, and salinity not only affect photosynthesis and productivity of cell biomass, but also influence the pattern, pathway, and activity of cellular metabolism (Richmond, 2004; Yang and Gao, 2003;

Lee and Lee, 2001; Martínez et al., 1997). However, different species and strains show various responses when the cultivation condition changes. The second objective of this study was to evaluate the significance of environmental factors including light intensity, light–dark cycle, and exogenous $CO_2$ concentration on biomass accumulation and biodiesel production.

## 5.3. Materials and Methods

### 5.3.1 Experimental design and data analysis

The experiments in this study were carried out in three consecutive stages. The first stage was aimed at testing the survival capability of all selected algae strains and determining the best candidates to grow in centrate cultivation system. The strategy used in this stage is described in section 4.3.3. The second stage was targeted at determination of the growth type (autotrophic growth, heterotrophic growth or mixotrophic growth) of the selected top-performing candidate algae strains when cultivated on centrate wastewater, in order to further investigate if supplement of exogenous $CO_2$ is necessary for improvement of algae growth. The third stage was designed to test which environmental factors would have significant effect on algae growth, wastewater nutrients removal and biodiesel production. To that end, the Plackett–Burman statistical experimental design, which is a very efficient screening design when only main effects are of interest (Plackett and Burman, 1946), was applied. This design allows the investigation of N-1 factors, with 2 levels (+ and -) for each factor, using N experiments, where N is a multiple of four. Moreover, the design is orthogonal in nature and thus gives pure effect of each variable not confounded with interactions among variables (Chauhan et al., 2007). The parameters tested in this

study included light intensity, light–dark cycle and exogenous $CO_2$ concentration. The coded levels and real values for each parameter are shown in Table 5.1a, and the design matrix is shown in Table 5.1b. In Table 5.1b, each row represents a trial run and each column represents an independent variable.

All the experiments were carried out in triplicate and average values are reported. Results were analyzed using Excel and software SPSS.

**Table 5.1 (a)** Coded levels and real values of the independent variables

| Variables | Environmental Factors | + Values | - Values |
|---|---|---|---|
| $X_1$ | $CO_2$ concentration (%) | 0.039 (air) | 5 |
| $X_2$ | Light intensity ($\mu molm^{-2}s^{-1}$) | 30 | 200 |
| $X_3$ | Lighting period (hours) | 4 | 24 |

**Table 5.1 (b)** Matrix for Plankett-Burman experimental design method

| Experiment runs | $X_1$ | $X_2$ | $X_3$ | $CO_2$ conc. (%) | Light intensity ($\mu molm^{-2}s^{-1}$) | Lighting period (hours) |
|---|---|---|---|---|---|---|
| 1 | - | - | + | 0.039 (air) | 30 | 24 |
| 2 | + | - | - | 5 | 30 | 4 |
| 3 | - | + | - | 0.039 (air) | 200 | 4 |
| 4 | + | + | + | 5 | 200 | 24 |

### 5.3.2 Source and pretreatment of wastewater

Same as 3.3.2

### 5.3.3 Algae strain collection and culture condition

In this chapter, 143 different algae strains from the family of *Chlamydomonas, Chlorella*, *Scenedesmus*, *Ankistrodesmus, Euglena*, *Chloroccum,* and *Botryococcus*, etc. were purchased from a number of U.S. institutions including the Culture Collection of Algae at the University of Texas, the Algae Culture Collection at the University of Hawaii and the Chlamydomonas Center at the University of Minnesota as shown in Table 5.2. They were selected because they are reported to be able to grow in media containing high concentration of phosphorus, nitrogen and organic carbon, have high

content of lipid, proliferate fast, or grow well in wastewater streams. Prior to be transferred on centrate wastewater, they were conserved in BG-11 medium as described in section 4.3.1. The experiments for the first stage were carried out in both solid media (on petri dishes) and liquid media (250mL Erlenmeyer flasks containing 100mL sterilized centrate). The experiments in the second stage were carried out with 250mL Erlenmeyer flasks containing 100mL sterilized centrate media in 5-day batch culture. The 250ml Erlenmeyer flasks were kept on a shaker at 100 rpm rotation speed under light intensity of 100µmolm$^{-2}$s$^{-1}$. The experiments planned according to the Plackett-Burman experimental design method were performed in 1L Roux culture bottles (Corning Inc., USA) containing 550mL sterilized centrate media with a magnetic stir bar in the bottom to mix the solution at 100 rpm in 11-day batch culture under designed conditions. In all cases, algae strains were grown at 25±2°C. In the first stage, the biomass concentration was controlled at around 0.4 g/L after inoculation to ensure that there was enough seed culture in the system. In the second and third stage, the biomass concentration was controlled at around 0.1 g/L after inoculation.

**Table 5.2.** Algae strains purchased from the commercial algae banks

| Genus | Species | Strain ID |
|---|---|---|
| *Chlamydomonas* | *Chlamydomonas reinhardtii* | CC-124 |
| | *Chlamydomonas reinhardtii* | CC-125 |
| | *Chlamydomonas reinhardtii* | CC-407 |
| | *Chlamydomonas reinhardtii* | CC-408 |
| | *Chlamydomonas reinhardtii* | CC-1009 |
| | *Chlamydomonas reinhardtii* | CC-1010 |
| | *Chlamydomonas reinhardtii* | CC-1373 |
| | *Chlamydomonas reinhardtii* | CC-1690 |
| | *Chlamydomonas reinhardtii* | CC-2290 |
| | *Chlamydomonas reinhardtii* | CC-2342 |
| | *Chlamydomonas reinhardtii* | CC-2343 |
| | *Chlamydomonas reinhardtii* | CC-2344 |

| | | |
|---|---|---|
| | *Chlamydomonas reinhardtii* | CC-2931 |
| | *Chlamydomonas reinhardtii* | CC-2932 |
| | *Chlamydomonas reinhardtii* | CC-2935 |
| | *Chlamydomonas reinhardtii* | CC-2936 |
| | *Chlamydomonas reinhardtii* | CC-2937 |
| | *Chlamydomonas reinhardtii* | CC-2938 |
| | *Chlamydomonas reinhardtii* | CC-3268 |
| | *Chlamydomonas reinhardtii* | UTEX 2337 |
| | *Chlamydomonas incerta* | CC-3871 |
| | *Chlamydomonas applanata* | UTEX 230 |
| | *Chlamydomonas debaryana var. cristata* | UTEX 1344 |
| | *Chlamydomonas hydra* | UTEX 4 |
| | *Chlamydomonas dorsoventralis* | UTEX 228 |
| | *Chlamydomonas moewusii* | UTEX 97 |
| | *Chlamydomonas moewusii var. microstigmata* | UTEX 1054 |
| | *Chlamydomonas noctigama* | UTEX 1338 |
| | *Chlamydomonas zebra* | UTEX 1904 |
| | *Chlamydomonas oblonga* | UTEX 839 |
| | *Chlamydomonas sphaeroides* | UTEX 208 |
| | *Chlamydomonas peterfii* | UTEX 2400 |
| *Chlorella* | *Chlorella sorokiniana* | UTEX 246 |
| | *Chlorella sorokiniana* | UTEX 260 |
| | *Chlorella sorokiniana* | UTEX 261 |
| | *Chlorella sorokiniana* | UTEX 1230 |
| | *Chlorella sorokiniana* | UTEX 2805 |
| | *Chlorella fusca var. fusca* | UTEX 343 |
| | *Chlorella fusca var. vacuolata* | UTEX 251 |
| | *Chlorella fusca var. vacuolata* | UTEX 252 |
| | *Chlorella vulgaris* | UTEX 2714 |
| | *Chlorella vulgaris* | UTEX 26 |
| | *Chlorella protothecoides* | UTEX 25 |
| | *Chlorella protothecoides* | UTEX 256 |
| | *Chlorella ellipsoidea* | UTEX 20 |
| | *Chlorella kessleri* | UTEX 398 |
| | *Chlorella minutissima* | UTEX 2341 |
| | *Chlorella saccharophila* | UTEX 2911 |
| | *Chlorella zofingiensis* | UTEX 32 |
| | *Chlorella sp.* | UTEX 580 |
| | *Chlorella sp.* | UTEX 2168 |
| | *Chlorella sp.* | UTEX 2248 |
| *Ankistrodesmus* | *Ankistrodesmus braunii* | UTEX 244 |
| | *Ankistrodesmus braunii* | UTEX 245 |

| | | |
|---|---|---|
| | *Ankistrodesmus braunii* | UTEX 750 |
| | *Ankistrodesmus braunii* | UTEX 187 |
| | *Ankistrodesmus angustus* | UTEX 188 |
| | *Ankistrodesmus angustus* | UTEX 189 |
| | *Ankistrodesmus angustus* | UTEX 241 |
| | *Ankistrodesmus densus* | UTEX 190 |
| | *Ankistrodesmus falcatus var. acicularis* | UTEX B748 |
| | *Ankistrodesmus nannoselene* | UTEX 243 |
| | *Ankistrodesmus pseudobraunii* | UTEX LB1380 |
| *Scenedesmus* | *Scenedesmus dimorphus* | UTEX 417 |
| | *Scenedesmus dimorphus* | UTEX 746 |
| | *Scenedesmus dimorphus* | UTEX 1237 |
| | *Scenedesmus obliquus* | UTEX 78 |
| | *Scenedesmus obliquus* | UTEX 1450 |
| | *Scenedesmus obliquus* | UTEX B2630 |
| | *Scenedesmus armatus* | UTEX 2551 |
| | *Scenedesmus acuminatus* | UTEX 415 |
| | *Scenedesmus acutiformis* | UTEX 416 |
| | *Scenedesmus basiliensis* | UTEX 79 |
| | *Scenedesmus dispar* | UTEX 414 |
| | *Scenedesmus longus* | UTEX 1236 |
| | *Scenedesmus parisiensis* | UTEX 1584 |
| | *Scenedesmus subspicatus* | UTEX 2532 |
| | *Scenedesmus sp.* | UTEX 1591 |
| *Chloroccum* | *Chloroccum arenosum* | UTEX 1767 |
| | *Chloroccum aureum* | UTEX 1768 |
| | *Chloroccum aquaticum* | UTEX 2222 |
| | *Chloroccum citriforme* | UTEX 1769 |
| | *Chloroccum ellipsoidum* | UTEX 972 |
| | *Chloroccum isabeliense* | UTEX 1774 |
| | *Chloroccum loculatum* | UTEX 1776 |
| | *Chloroccum microstigmatum* | UTEX 1777 |
| | *Chloroccum minutum* | UTEX 117 |
| | *Chloroccum macrostigatum* | UTEX 109 |
| | *Chloroccum oviforme* | UTEX 1782 |
| | *Chloroccum paludosum* | UTEX 1779 |
| | *Chloroccum pinguiddeum* | UTEX 774 |
| | *Chloroccum pamirum* | UTEX 2498 |
| | *Chloroccum polymorphum* | UTEX 1242 |
| | *Chloroccum rugosum* | UTEX 1785 |
| | *Chloroccum scabellum* | UTEX 1233 |
| | *Chloroccum salsugineum* | UTEX 1786 |

| | | |
|---|---|---|
| | *Chloroccum sphacosum* | UTEX 1787 |
| | *Chloroccum texanum* | UTEX 1788 |
| | *Chloroccum typicum* | UTEX 1789 |
| | *Chloroccum vacuolatum* | UTEX 110 |
| | *Chloroccum sp.* | UTEX 2438 |
| *Euglena* | *Euglena gracilis* | UTEX LB367 |
| | *Euglena gracilis* | UTEX LB368 |
| | *Euglena gracilis* | UTEX 1717 |
| | *Euglena gracilis* | UTEX 1718 |
| | *Euglena gracilis* | UTEX 1719 |
| | *Euglena gracilis var. saccharophia* | UTEX 160 |
| | *Euglena anabaena* | UTEX 373 |
| | *Euglena stellata* | UTEX 372 |
| | Non-dominant species | |
| *Botryococcus* | *Botryococcus braunii* | UTEX 572 |
| | *Botryococcus braunii* | UTEX 2441 |
| | *Botryococcus sudeticus* | UTEX 2629 |
| *Coelastrum* | *Coelastrum astroideum var. rugosum* | UTEX 2442 |
| | *Coelastrum microporum* | UTEX 280 |
| *Characium* | *Characium californicum* | UTEX 2097 |
| | *Characium typicum* | UTEX 2108 |
| *Dictyochloris* | *Dictyochloris fragrans* | UTEX 127 |
| | *Dictyochloris pulchra* | UTEX 2527 |
| *Dictyococcus* | *Dictyococcus schumacherensis* | UTEX 2252 |
| *Dimorphococcus* | *Dimorphococcus lunatus* | UTEX 69 |
| *Dunaliella* | *Dunaliella salina* | UTEX LB1644 |
| *Galdieria* | *Galdieria sulphuraria* | UTEX 2919 |
| *Haematoccus* | *Haematoccus droebakensis* | UTEX 55 |
| | *Haematoccus lacustris* | UTEX 16 |
| | *Haematoccus pluvialis* | UTEX 2505 |
| *Monodus* | *Monodus subterraneus* | UTEX 151 |
| *Nannochloris* | *Nannochloris eucaryotum* | UTEX 2502 |
| | *Nannochloris sp.* | UTEX B2378 |
| *Neochloris* | *Neochloris wimmeri* | UTEX 113 |
| | *Neochloris oleoabundans* | UTEX 1185 |
| *Oocystis* | *Oocystis marssonii* | UTEX 287 |
| *Pandorina* | *Pandorina morum* | UTEX 1727 |
| *Phaeodactylum* | *Phaeodactylum tricornutum* | UTEX 640 |
| *Selenastrum* | *Selenastrum minutum* | UTEX 326 |
| | *Selenastrum minutum* | UTEX 2459 |
| | *Selenastrum bibraianum* | UTEX 324 |

| | | |
|---|---|---|
| | *Selenastrum capricornutum* | UTEX 1648 |
| | *Selenastrum gracile* | UTEX 325 |
| | *Selenastrum sp.* | UTEX 235 |
| *Tetracystis* | *Tetracystis aplanosporum* | UTEX 773 |
| *Tetraedron* | *Tetraedron bitridens* | UTEX 120 |
| *Tetrallantos* | *Tetrallantos lagerheimii* | UTEX 1582 |
| *Tetrastrum* | *Tetrastrum heteracantum* | UTEX 2445 |

### 5.3.4 Determination of growth type (autotrophic, heterotrophic or mixotrophic growth) of algae cultivated in centrate

DCMU (3-(3,4-dichlorophenyl)-1,1-dimethyl urea) is commonly used as photosynthesis inhibitor, which blocks the plastoquinone binding site of photosystem II, disallowing the electron flow from where it is generated to plastoquinone. This interrupts the photosynthetic electron transport chain in photosynthesis and thus blocks the ability of the organism to turn light energy into chemical energy (ATP and reductant potential). The effect of DCMU is very specific and sensitive. It only blocks electron flow from photosystem II, and have no effect on photosystem I or other reactions in photosynthesis, such as light absorption or carbon fixation (DeLorenzo et. al, 2001; Francoeur et. al, 2007). In this section, DCMU was used to determine if the algal growth in centrate was autotrophic or heterotrophic.

Four sets of experiments for each algae strain were carried out in this stage. Experiment 1 and 2 were used as control and conducted in BG-11 media. Experiment 3 and 4 were carried out in centrate. In experiment 2 and 4, DCMU was used to block the photoautotrophic pathway. The biomass growth was analyzed for all 4 experiments. The

ratio of biomass growth by autotrophic pathway and heterotrophic pathway was calculated.

### 5.3.5 Control of light intensity and light-dark cycle

The light intensities used in this study were 30, 100 and 200 $\mu molm^{-2}s^{-1}$, and controlled by varying the number of fluorescent lamps as well as the distance between the lamps and the algae culture. The light-dark cycle investigated were 4h lighting period and 24h lighting period, and the dark condition was achieved by wrapping the cultivation system with aluminum foil.

### 5.3.6 Supply of exogenous $CO_2$ and adjustment of $CO_2$ concentration

Two concentrations of $CO_2$-enriched air aeration, namely 0% and 5% $CO_2$ at 0.5 vvm were applied in this study. The aeration system composed of a $CO_2$ tank, an air compressor, and two flow meters was constructed to supply ambient air or $CO_2$-enriched air to the cultivation system. The concentration of the exogenous $CO_2$ was adjusted by the flow rates of ambient air and $CO_2$ gas.

### 5.3.7 Analytical methods

#### 5.3.7.1 Determination of nutrients concentration and removal rates

Same as section 3.3.4.1

#### 5.3.7.2 Analysis of algal biomass concentration

Same as section 3.3.4.2

#### 5.3.7.3 Examination of fatty acid methyl ester (FAME) composition

Same as section 3.3.4.3

## 5.4 Results and Discussion

### 5.4.1 14 algae strains were able to grow on centrate wastewater stream

Previous researchers reported that BG-11 medium was commonly used for identifying autotrophy and heterotrophy of unicellular blue–green algae (Zhou et al., 2011; McKinley and Wetzel, 1979). For the algae species purchased from algae collection centers, 123 out of 143 adapted well to BG-11 media in the first step, while only 25 of them can grow photoautotrophically, heterotrophically under light and in dark in step 2, illustrating that the 25 strains were capable of using both inorganic and organic carbon source and assimilating them into biomass, which is ideal for growth in centrate wastewater stream since it contains both inorganic and organic carbon form. Among the 25 strains, 14 of them survived in centrate, and showed biomass accumulation during the 5-day batch culture. The biomass concentrations in terms of TVSS are summarized in Table 5.3.

**Table 5.3** Accumulation of biomass of 14 algae strains when cultivated on centrate wastewater stream

| Strain ID | Algae species | Net biomass growth in centrate (g TVSS/L) |
|---|---|---|
| UTEX 25 | *Chlorella protothecoides* | 1.3089 |
| UTEX 252 | *Chlorella fusca var. vacuolata* | 0.3810 |
| UTEX 256 | *Chlorella protothecoides* | 1.2000 |
| UTEX 398 | *Chlorella kessleri* | 2.0143 |
| UTEX 580 | *Chlorella sp.* | 1.0586 |
| UTEX 1230 | *Chlorella sorokiniana* | 0.7000 |
| UTEX 2240 | *Chlorella minutissima* | 0.7600 |
| UTEX 2805 | *Chlorella sorokiniana* | 0.7580 |
| UTEX 294 | *Haematococcus lacustris* | 0.3813 |
| UTEX 1236 | *Scenedesmus longus* | 0.4143 |
| UTEX 1338 | *Chlamydomonas noctigama* | 0.2286 |
| UTEX 1788 | *Chlorococcum texanum* | 0.4885 |
| UTEX 2222 | *Chlorococcum aquaticum* | 0.6370 |
| UTEX 2498 | *Chlorococcum pamirum* | 0.7570 |

The results show that the lowest net biomass accumulation (0.23 g/L) was observed with algae strain *Chlamydomonas noctigama* (UTEX 1338), and the highest net

biomass accumulation (2.0143 g/L)) was observed with algae strain *Chlorella kessleri* (UTEX 398). Among the 14 tested algae strains, 4 of them showed net biomass accumulation larger than 1.0 g/L including UTEX 580, UTEX 256, UTEX 25 and UTEX 398; 5 of them showed net biomass accumulation between 0.5 to 1.0 g/L including UTEX 2222, UTEX 1230, UTEX 2805, UTEX 2240 and UTEX 2498; and 5 of them showed net biomass accumulation lower than 0.5g/L including UTEX1338, UTEX 294, UTEX 252, UTEX 1236, UTEX 1788. Due to their better growth in centrate wastewater, UTEX 398 and UTEX 25 were chosen for further study and used in the second and third stages.

### 5.4.2 The top-perfoming candidates were capable of mixotrophic growth when cultivated on centrate wastewater stream

In experiments with DCMU added to the cultivation system, photosynthesis was completely blocked leaving only the pathway for heterotrophic growth. While, in experiments without DCMU, algae cells could perform both heterotrophic and autotrophic growth, namely mxiotrophic growth, in the cultivation system. The growth curves for both strains in all conditions are shown in Figure 5.1 (a). From the figure we can see that both strains were able to perform of mixotrophic growth, in which $CO_2$ and organic carbon are simultaneously assimilated. According to the statistical analysis, the biomass accumulated through mixotrophic growth is higher than that accumulated through heterotrophic growth alone, which is in accordance with previous studies (Richmond, 2004; Matrínez et al., 1997; Lalucat et al., 1984). The percentage of heterotrophic growth for both strains during the 5-day cultivation is shown in Figure 5.1 (b). Comparing the data from the first and last day of cultivation, we can see that with

the decrease of organic carbon concentration in the wastewater, the portion of heterotrophic growth decreased and that of the autotrophic growth increased gradually, illustrating that heterotrophic growth is preferred by both algae strains when organic carbon source was in sufficient amount, and autotrophic growth picked up the portion in total growth when organic carbon was consumed to low levels.

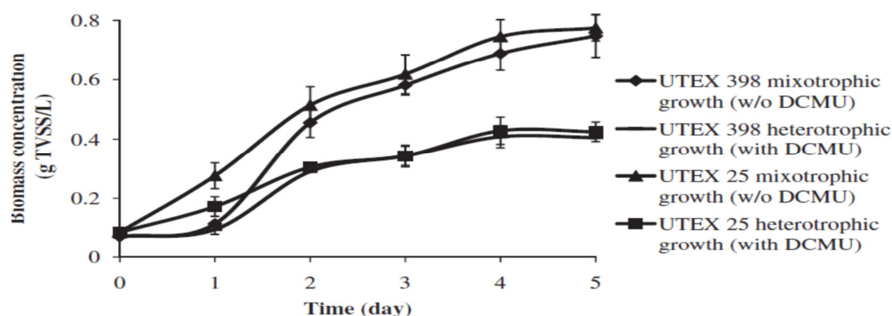



**Figure 5.1** (a) Growth curve for strains UTEX 398 and UTEX 25 under mixotrophic and heterotrophic growth conditions when cultivated on centrate wastewater stream

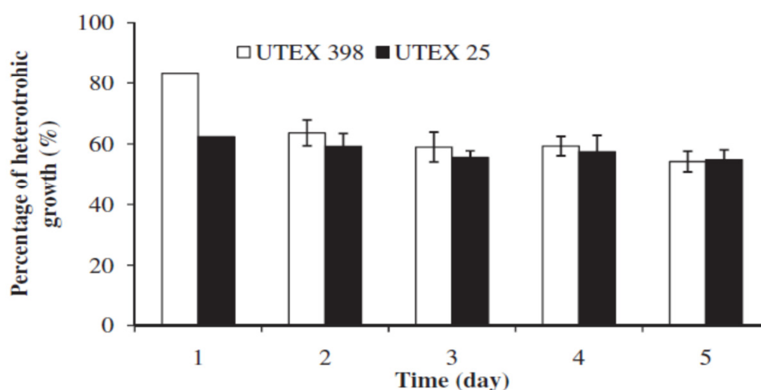



**Figure 5.1** (b) The portion of heterotrophic growth for strains UTEX 398 and UTEX 25 when cultivated on centrate wastewater stream

Matrínez et al. (1997) found that when *Chlorella pyrenoidosa* was cultivated under moderate light condition, an exponential mixotrophic phase was observed first and when the organic substrate was completely consumed, an autotrophic phase followed

after an adaptation period. Lalucat et al. (1984) reported that *Chlorella* sp. strain VJ79 could grow autotrophically, heterotrophically, and mixotrophically on a variety of organic substrate. Furthermore, glucose and serine could promote a mixotrophic growth from which the yield is higher than the sum of autotrophic and heterotrophic yields. Examples of algae strains from the family of *Chlorella* that are capable of mixotrophic growth are summarized in Table 5.4 along with the organic carbon sources tested. Since autotrophic growth presented in the cultivation system for both algae strains, supplying exogenous $CO_2$ might be helpful for biomass accumulation because the inorganic carbon source in the centrate is in very low concentration.

**Table 5.4** Examples of microalgae species that can be cultivated mixotrophically

| Microalgae species | Organic carbon substrate | References |
| --- | --- | --- |
| *Chlorella minutisima* | Methanol | Kotzbasis et al. (1999) |
| *Chlorellar egularis* | Acetate | Endo et al. (1977) |
| *Chlorella Sorokiniana* | Glucose | Lee et al. (1996) |
| *Chlorella vulgaris* | Glucose | Ogwa and Aiba (1981) |
| *Chlorella sp.* | Glucose, Serine | Lalucat et al. (1984) |
| *Chlorella pyrenoidosa* | Glucose | Matrínez et al., 1997 |
| | Centrate | Current study |
| *Chlorella kessleri* | Centrate | Current study |

**5.4.3 Environmental factors had significant effect on algal biomass accumulation, wastewater nutrients removal and biodiesel production**

The growth curves for both strains are shown in Figure 5.2 (a) and Figure 5.2 (b). From the figure we can see that both strains showed biomass accumulation under all conditions tested. For strain UTEX 398, the net biomass accumulation was 1.13 g/L in Run 1, 0.47 g/L in Run 2, 0.64 g/L in Run 3, and 2.44 g/L in Run 4. For strain UTEX 25, the net biomass accumulation was 0.87 g/L in Run 1, 0.39 g/L in Run 2, 0.53 g/L in Run 3 and 1.79 g/L in Run 4. In general, strain UTEX 398 showed better growth than

UTEX 25 under the same cultivation condition. Both strains showed the highest biomass accumulation in Run 4, which has light intensity of 200 $\mu molm^{-2}s^{-1}$, lighting period of 24 hours and exogenous $CO_2$ concentration of 5.0%, followed by that in Run 1, which has a light intensity of 30 $\mu molm^{-2}s^{-1}$, lighting period of 24 hour and exogenous $CO_2$ concentration of 0.039% (ambient air). The biomass accumulation was found similar in Run 2 and Run 3 for both algae strains, which are lower than that in Run 4 and Run 1. The FAME content and wastewater nutrient removal efficiency data is summarized in Table 5.5 (a) and Table 5.5 (b). The FAME content, around 25% of TVSS, was found highest in Run 4 for both strains. In Run 1, UTEX 25 provided higher FAME content (14.24%) than UTEX 398 (10.87%). The FAME content was found higher in Run 2 than that in Run 3 for both strains. Strain UTEX 398 was found to have higher FAME content than UTEX 25 in these two runs. For ammonium removal, both strains showed 100% removal efficiency under all conditions by the end of the batch experiments, illustrating that the environmental factors tested have no effect on ammonium nitrogen removal rate by the end of the cultivation. For strain UTEX 398, best COD, TP, TN removal was found in Run 2, Run 1, Run 4 respectively. For strain UTEX 25, COD and TN removal efficiency was similar for all for runs, while best TP removal was found in Run 1. According to our previous research (Li et al., 2011 (a); Zhou et al., 2011), phosphorus was removed mainly by precipitation at high pH levels, thus high removal rate of total phosphorus was found in the experiments with large increase of pH in the cultivation system.

The p-values showing the effect of all three environmental factors on biomass accumulation, FAME content and nutrient removal efficiency are summarized in Table 5.5 (c). Previous researchers used different critical values for the p-value to justify the significance of the tested independent variables (Chauhan et al., 2007; Djekrif-Dakhmouche et al., 2006). In this study, p-value of 0.30 was used as critical point as suggested by Djekrif-Dakhmouche et al. (2006), meaning that any parameter with p-value less than 0.30 was considered having significant influence on response. The data in Table 5.5 (c) shows that light intensity, lighting period and exogenous $CO_2$ concentration all have significant effect on biomass accumulation and FAME content. For COD removal, lighting period showed more significant effect than light intensity and exogenous $CO_2$ concentration, with longer lighting period improving COD removal. Only exogenous $CO_2$ concentration showed significant effect on TP removal, due to the fact that addition of $CO_2$ resulted in lowering the system pH, which lessened the precipitation of phosphorus. For strain UTEX 398, the removal of TN depends on both light intensity and exogenous $CO_2$ concentration, while for strain UTEX 25, the removal of TN was more dependent on lighting period than the other two factors. Ammonium was removed completely under all conditions by the end of the batch culture, and the removal rate was found independent of the three tested environmental factors. In general, higher light intensity, longer lighting period and higher exogenous $CO_2$ concentration promote biomass accumulation, FAME formation, removal of COD and TN, while, lower exogenous $CO_2$ concentration promotes phosphorus removal.

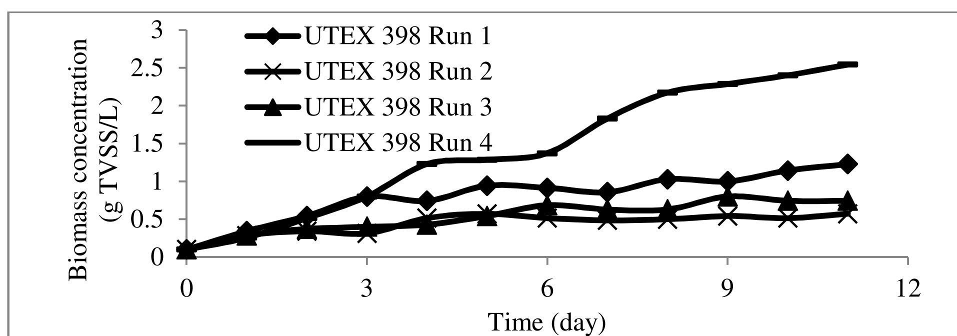



**Figure 5.2** (a) Growth curve for strain UTEX 398 in Plackett-Burman experiment design

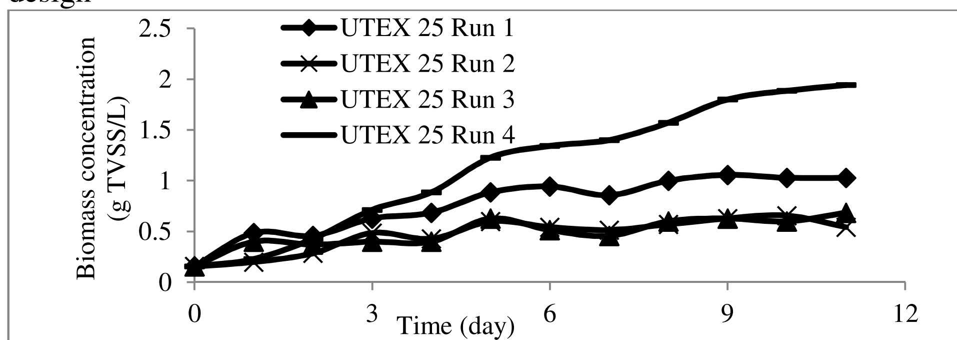



**Figure 5.2** (b) Growth curve for strain UTEX 25 in Plackett-Burman experiment design

**Table 5.5 (a)** Plackett-Burman design matrix along with the observed responses for UTEX 398

| Exp. Runs | Environmental factors and levles | | | Responses | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | FAME | Final | Nutrients removal rate (%) | | | |
| | $CO_2$ conc. (%) | Light intensity (µmolm$^{-2}$s$^{-1}$) | Lighting period (hours) | content (% of TVSS) | biomass conc. (TVSS) (g/L) | COD | TP | TN | $NH_4^+$ |
| 1 | 0.039 (air) | 30 | 24 | 10.87 | 1.2286 | 74.89 | 92.39 | 61.12 | 100 |
| 2 | 5.0 | 30 | 4 | 10.63 | 0.5714 | 89.96 | 12.44 | 71.73 | 100 |
| 3 | 0.039 (air) | 200 | 4 | 8.04 | 0.7429 | 87.21 | 77.46 | 69.95 | 100 |
| 4 | 5.0 | 200 | 24 | 25.86 | 2.5429 | 74.93 | 51.20 | 83.94 | 100 |

**Table 5.5 (b)** Plackett-Burman design matrix along with the observed responses for UTEX 25

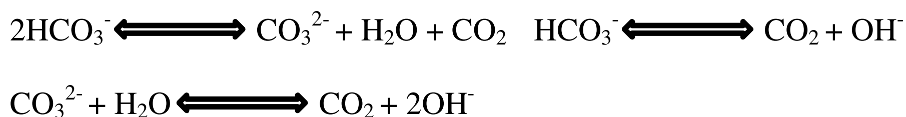
| Exp. Runs | Environmental factors and levels | | | Responses | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | FAME | Final | Nutrients removal rate (%) | | | |
| | $CO_2$ con. (%) | Light intensity (µmolm$^{-2}$s$^{-1}$) | Lighting period (hours) | content (% of TVSS) | biomass conc. (TVSS) (g/L) | COD | TP | TN | Ammonia |
| 1 | 0 | 30 | 24 | 14.24 | 1.0286 | 82.30 | 82.54 | 86.74 | 100 |
| 2 | 5 | 30 | 4 | 7.36 | 0.5429 | 87.77 | 13.17 | 84.43 | 100 |
| 3 | 0 | 200 | 24 | 5.64 | 0.6857 | 83.87 | 72.68 | 86.17 | 100 |
| 4 | 5 | 200 | 4 | 24.06 | 1.9429 | 80.78 | 55.12 | 86.65 | 100 |

**Table 5.5 (c)** Summary of p-values in ANOVA analysis

| | | | $CO_2$ conc. (%) | Light intensity ($\mu molm^{-2}s^{-1}$) | Lighting period (hours) |
|---|---|---|---|---|---|
| FAME content (% of TVSS) | | UTEX 398 | 0.104 | 0.160 | 0.097 |
| | | UTEX 25 | 0.219 | 0.298 | 0.016 |
| Final biomass conc. (g TVSS/L) | | UTEX 398 | 0.284 | 0.176 | 0.025 |
| | | UTEX 25 | 0.242 | 0.164 | 0.034 |
| Nutrients removal rate (%) | COD | UTEX 398 | 0.636 | 0.433 | 0.001 |
| | | UTEX 25 | 0.872 | 0.373 | 0.109 |
| | TP | UTEX 398 | 0.047 | 0.849 | 0.547 |
| | | UTEX 25 | 0.109 | 0.628 | 0.414 |
| | TN | UTEX 398 | 0.046 | 0.087 | 0.438 |
| | | UTEX 25 | 0.749 | 0.377 | 0.166 |
| | Ammonia | UTEX 398 | >>0.05 | >>0.05 | >>0.05 |
| | | UTEX 25 | >>0.05 | >>0.05 | >>0.05 |

In mixotrophic cultures, increase of the light intensity within certain range promotes the growth of algae cells, since light has stimulatory effect of on metabolism of organic carbons by mixotrophic cells (Martinez and Orus, 1991, Lalucat et al., 1984). Previous research shows that higher biomass accumulation was observed when using $CO_2$-enriched air injection systems (Chiu et al., 2008; Yang and Gao, 2003). Yang and Gao (2003) reported that increased $CO_2$ concentration enhanced significantly the growth rate of three species they tested including *Chlamydomonas reinhardtii, Chlorella pyrenoidosa* and *Scenedesmus oblique.* Chiu et al. (2008) conducted a set of experiments with *Chlorella sp.* and reported that in their semi-continuous culture, the optimum condition for biomass productivity was at 2% $CO_2$ aeration. In addition to serve as carbon source for algae growth, the $CO_2$ - $H_2CO_3$ - $HCO_3^-$ -$CO_3^{2-}$ system also

plays an important role in controlling and maintaining the pH levels of the culture solution. The bicarbonate-carbonate buffer systems can provide $CO_2$ for photosynthesis through the following reactions:



$$2HCO_3^- \Longleftrightarrow CO_3^{2-} + H_2O + CO_2 \quad HCO_3^- \Longleftrightarrow CO_2 + OH^-$$

$$CO_3^{2-} + H_2O \Longleftrightarrow CO_2 + 2OH^-$$

These reactions imply that during photosynthetic $CO_2$ fixation, $OH^-$ accumulates in the growth solution leading to a gradual rise in pH, thus, pH can reach as high as 11 in high algal density production systems where no additional $CO_2$ has been supplied (Richmond and Grobbelaar, 1986). Direct $CO_2$ sparging into the culture media is the best and most convenient method of pH control and at the same time supplying $CO_2$ for high yield in mass algal cultures (Richmond, 2004).

**5.5 Conclusions**

In this chapter, 14 algae strains from the family of *Chlorella*, *Haematococcus, Scenedesmus*, *Chlamydomonas,* and *Chloroccum* were capable of growing on centrate. The highest net biomass accumulation (2.01 g/L) was observed with strain *Chlorella kessleri* (UTEX 398) followed by *Chlorella protothecoides* (UTEX 25, 1.31g/L). Strains UTEX 398 and UTEX 25 were found to be capable of mixotrophic growth when cultivated on centrate. Environmental factors had significant effect on algal biomass accumulation, wastewater nutrients removal and biodiesel production. Higher light intensity and exogenous $CO_2$ concentration with longer lighting period promote biomass accumulation, FAME formation, removal of COD and TN, while, lower exogenous $CO_2$ concentration promotes phosphorus removal.

# CHAPTER 6. EFFECT OF LIGHT INTENSITY ON ALGAL BIOMASS ACCUMULATION AND BIODIESEL PRODUCTION FOR MIXOTROPHIC STRAINS *Chlorella kessleri* AND *Chlorella protothecoide* CULTIVATED ON HIGHLY CONCENTRATED MUNICIPAL WASTEWATER

## 6.1 Overview

The results in Chapter 5 show that environmental factors including light intensity, light/dark cycle, and exogenous $CO_2$ concentration had significant effect on algal based biodiesel production process using highly concentrated municipal wastewater as cultivation media. In the following three chapters, the single effect of these factors on biomass accumulation, wastewater nutrient removal through algae cultivation, and biodiesel productivity was investigated with the top-performing strains identified in Chapter 5. In this chapter, the single effect of light intensity was investigated using strains *Chlorella kessleri* and *Chlorella protothecoide.* The light intensities studied were 0, 15, 30, 60, 120, 200 $\mu molm^{-2}s^{-1}$. The results showed that light intensity has profound impact on tested responses for both strains, and the dependence of these responses on light intensity varied with different algae strains. For *Chlorella kessleri*, the best light intensity is 120 $\mu mol \cdot m^{-2} \cdot S^{-1}$ for all responses except for COD removal. For *Chlorella protothecoide*, the best light intensity is 30 $\mu mol \cdot m^{-2} \cdot S^{-1}$. The major components of the biodiesel produced from algae biomass are 16-C and 18-C FAME, and the highest biodiesel contents are 28.19% and 19.48% of TVSS for *Chlorella kessleri* and *Chlorella protothecoide*, respectively. Both species were capable of wastewater nutrients removal under all lighting conditions with high removal efficiencies.

### 6.2 Introduction

The use of algae as biofuel feedstock has been attracting more and more interest, effort and investment recently due to the concerns of limited reserve of fossil fuel, the problems of global warming owing to increased use of fossil fuels, as well as the higher areal oil yield from algal biomass than conventional oil crops (Chisti, 2007). However, commercial applications of microalgae have still been limited by the low yield and high cost involved in microalgae production process (Yeh et al, 2010). Therefore, an efficient and economical algae production strategy is of urgent need.

One way to reduce the cultivation cost is growing algae in wastewater with minimum energy input since wastewater contains readily available water source and major nutrients required by algae proliferation (Sheehan et al, 1998). In previous studies, we compared the effect of different wastewater streams collected from a wastewater treatment plant on the growth of algae biomass and found that the centrate, which is generated from the thickening process of activated sludge and contains higher content of carbon, nitrogen and phosphorus than other wastewater streams, has the best ability of supporting algae biomass accumulation (Wang et al., 2010, Li et al., 2011(a)). Thus, further investigations concerning cultivation parameters such as light input, temperature control and reactor design are necessary when using such type of wastewater for algal biomass production.

Among all the environmental factors, light supply is one of the most important environmental factors that not only greatly affect algae photosynthesis and productivity as well as cell composition and metabolic pathway, but also the economic efficiency of the algae cultivation process (Cuaresma et al., 2009), and consequently, the supply and efficient utilization of light energy have been the greatest scientific and technological challenge in research and development on cultivation of photosynthetic microorganism. Many strains of photosynthetic algae are capable of heterotrophic growth, using organic carbon, under dark conditions and their heterotrophic culture can be used for efficient production of biomass and some metabolites. However, light is absolutely required for efficient production of biomass and some metabolites. In such cases, the requirements for light supply is lowered compared with completely photoautotrophic growth. Thus, in order to produce high biomass concentration with high cellular concentration of desirable metabolic products, one way of overcoming the problem of light supply to photobioreactors is to use cultures capable of both heterotrophic growth and photoautotrophic growth, namely mixotrophic culture, where both organic carbon sources and light energy are simultaneously supplied to the system. In this project, two algae strains approved to be capable of mixotrophic growth (Li et. al., 2011 (b)), namely *Chlorella kessleri* and *Chlorella protothecoide* were used for simultaneous wastewater treatment and biodiesel production.

The variation in cell composition in a single algae species can vary in many folds according to the culture condition under which it is grown (Richmond, 2004). In terms of biodiesel production, lipids, especially triglycerides (TAGs), are the most desirable

products of algae cultivation process in this article. Previous research with various algae species suggests that lipid content in algae cells as well as different fatty acid fractions are related to the environmental light intensity (Cohen, 1987; 1999), however, diverse trends with the change of light intensity were observed when using different algae strains from the family of *Chlorella* when cultivated in artificial media (Cao et al., 2010; Klyachko-Gurvich et al., 1999, Matrinez et al., 1997). The optimum light intensity for algae biomass accumulation and desired products production is not only strain-dependent, but also relies on the suitability of other factors of the environment such as temperature and nutrient supply (Sorokin and Krauss, 1958). To build an effective centrate-based wastewater algae production system, determination of the light intensity optimum for biomass accumulation and lipid production preferably TAGs is one of the crucial tasks. So far, little information is reported concerning the influence of light intensity on algae growth and lipid accumulation when cultivated in wastewater streams, specifically centrate used in this study. Thus, the objective of the current research is to investigate the effect of light intensity on algal biomass accumulation and biodiesel production, and determine the optimum light supply for two different algae strains when using centrate as cultivation media.

## 6.3 Materials and Methods

### 6.3.1 Algae strain and culture condition

Two algae strains used in this study, namely *Chlorella kessleri* and *Chlorella protothecoid,* were purchased from the Culture Collection of Algae at the University of Texas, USA, and was conserved in BG-11 medium as described in section 4.3.1. Prior to experiment, both algae strains were cultivated in centrate for three generations to

obtain stable characteristics. The seed culture was cultivated in the centrate for 3-4 days and reached to a biomass concentration of 1.0g dried biomass per liter. In all cases, algae strains were inoculated at 1:10 (volume of seed culture: volume of centrate) and grown under 25±2°C with designed light intensity. The experiments were carried out with 250mL Erlenmeyer flasks containing 100mL sterilized centrate media. The 250ml Erlenmeyer flasks were kept on a shaker with 100 rpm rotation speed.

### 6.3.2 Control of light intensity

The effect of light intensity on biomass growth, wastewater nutrient removal and biodiesel production was studied with two algae strains stated above. According to preliminary data (not shown), the light intensities studied were 0, 15, 30, 60, 120, 200 $\mu molm^{-2}s^{-1}$, and controlled by varying the number of fluorescent lamps as well as the distance between the lamps and the algae culture.

### 6.3.3 Pretreatment of wastewater

Same as section 3.3.2

### 6.3.4 Analytical methods

#### 6.3.4.1 Determination of nutrients concentration and removal rates

Same as 3.3.4.1

#### 6.3.4.2 Analysis of algal biomass concentration and electricity converstion efficiency

The methods for biomass concentration and growth rate analysis are same as 3.3.4.2

The electricity conversion efficiency was calculated based on biomass concentration by following equation ( Yeh, et al., 2010):

$$\text{Electricity conversion efficiency} = \frac{\text{Biomass concentration}}{\text{light intensity}}$$

Where biomass concentration (g/L) represents the highest accumulative biomass concentration obtained during the cultivation process, and the light intensity ($\mu molm^{-2}s^{-1}$) represents the light intensity applied under the specific condition. For dark condition, light intensity of 1 $\mu molm^{-2}s^{-1}$ was used to calculate the conversion efficiency.

**6.3.4.3 Examination of fatty acid methyl ester (FAME) composition**

Same as 3.3.4.2

All the experiments were carried out in triplicate and average values are reported.

Results were analyzed using software JMP 8.0. ANOVA analysis and Tukey's post-hoc analysis were used to determine the significance of difference wherever applicable.

## 6.4 Results and Discussion

When combined with wastewater treatment, microalgae species from the family of *Chlorella* were most commonly used (Tam and Wong, 1990; Li et al., 2011 (a)). In our previous research (Li et al., 2011 (a)), we also found that algae strain *Chlorella sp.* cultivated in centrate wastewater stream, provided comparable biomass accumulation and biodiesel productivity with those grown in standard cultivation media for blue-green algae. These strains showed excellent adaptation in wastewater (Li, et al., 2011 (a), (b)) and great potential to simultaneously treat wastewater and accumulate valuable compounds.

### 6.4.1 Effect of light intensity on algal biomass accumulation

Algal growth as indicated by daily TVSS concentration under light intensities of 0, 15, 30, 60, 120, 200µmolm$^{-2}$s$^{-1}$ with two algae strains were shown in Figure 6.1 (a) and (b). Both algae strains could survive in centrate under all light conditions without obvious lag phases observed. The growth under all light intensity, except under dark condition, for both algae strains lasted approximately 3 days, after which the biomass concentration either remained or dropped. Statistical analysis showed that light intensity had crucial effect on biomass accumulation for both strains (p-value $< 0.05$). The comparison of growth characteristics among all light intensities suggested that different algae strains showed different growth variation with the increase of light intensity from 0 to 200µmolm$^{-2}$s$^{-1}$. For algae strain *Chlorella kessleri,* it is noticed that the growth of biomass increased with the increase of light intensity from 0 to 120 µmolm$^{-2}$s$^{-1}$, while further increase of light intensity to 200 µmolm$^{-2}$s$^{-1}$ repressed the accumulation of biomass due to light inhibition (Ogbonna and Tanaka, 2000). While for algae strain *Chlorella protothecoide,* based on the accumulative biomass by day 2.75, the growth of algae increased with the ascending light intensity from 0 to 30µmolm$^{-2}$s$^{-1}$, and further increase of light intensity from 30 to 200µmolm$^{-2}$s$^{-1}$ did not obviously promote biomass accumulation, while the biomass accumulation improved greatly when the light intensity increased from 120 to 200µmolm$^{-2}$s$^{-1}$.

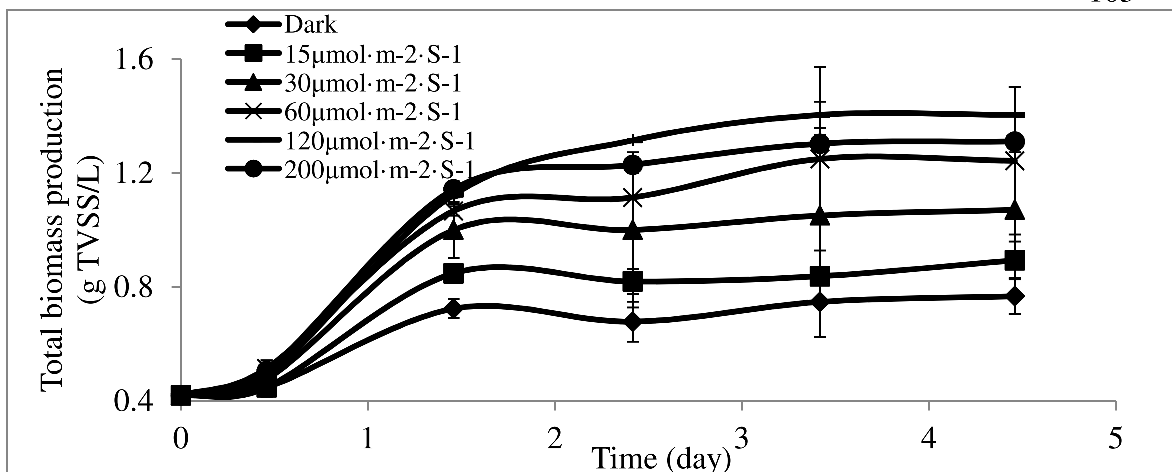



**Figure 6.1** (a), Effect of light intensity on algal biomass accumulation with strain *Chlorella kessleri*

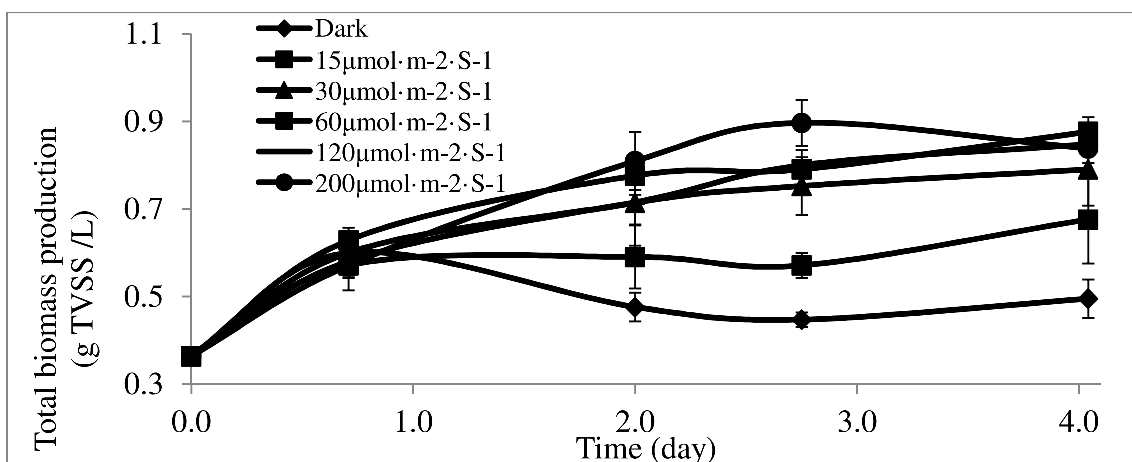



**Figure 6.1 (b)**, Effect of light intensity on algal biomass accumulation with strain *Chlorella protothecoide*

The accumulation of algae biomass as well as the composition of algal cells are known to be greatly dependent on the light supply, and in general, when the light intensity increases from zero to higher values, the effect of light intensity on microalgal growth could be classified as four phases, including lag phase in which growth rate stays similar as the increase of light intensity, light limitation in which growth rate rises with the increase of light intensity, light saturation in which growth rate is independent with

light intensity, and light inhibition in which the growth rate declines with the increase in light intensity (Ogbonna and Tanaka, 2000). In this study, both algae strains were grown under fluorescence light at different light intensities. The dependence of specific cell growth rate on the light intensity is shown in Figure 6.2 (a) and (b). Figure 6.2 (a) shows that for strain *Chlorella kessleri* three phases with the lag phase absent were observed when light intensity increased from 0 to 200 $\mu molm^{-2}s^{-1}$. When light intensity exceeded 120 $\mu molm^{-2}s^{-1}$, the inhibition on cell growth was clearly noticed. This photo-inhibition phenomenon was also observed in other studies (Ogbonna and Tanaka, 2000; Yeh et al., 2010). Total biomass production and growth rate reached maximal when light intensity was controlled around 120$\mu molm^{-2}s^{-1}$. Similar trend was also observed in previous studies. Yeh et al. (2010), reported that when working with *Chlorella vulgaris* ESP-31, light limitation phase occurred when the light intensity increased from 0 to 9 $W/m^2$, light saturation occurred when the light intensity increased from 9 to 18 $W/m^2$, and when light intensity exceeded 18 $W/m^2$, light inhibition was clearly observed.

For algae strain *Chlorella protothecoide,* different trend was observed as shown in Figure 6.2 (b). The specific growth rate increased when the light intensity increased from 0 to 30$\mu molm^{-2}s^{-1}$ without any lag phase noticed, and it reached a platform when the light intensity increased from 30 to 120$\mu molm^{-2}s^{-1}$. However, when the light intensity further increased to 200$\mu molm^{-2}s^{-1}$, the specific growth rate was considerably enhanced. The dependence of algae growth and cell composition on light intensity depends heavily on the genetic make up of the organisms, and on external conditions other than the change in light intensity (Sorokin and Krauss, 1958). In the study

performed by Sorokin and Krauss (1958), they also found that the dependence of algae growth on light intensity and the utilization efficiency of incident light energy differed widely with various strains when working with algae from the family of *Chlorella*, *Chlamydomonas* and *Scenedesmus*. Martinez and Orus (1991) reported that the specific growth rate of mixotrophic *Chlorella* increased with increasing of light intensity, however, a similar effect was not observed in photoautotrophic cultures exposed to the same light intensities. This was explained by the stimulatory effect of light on metabolism of sugar by mixotrophic cells. Lalucat et al. (1984) found that the growth yield and productivity of mixotrophic *Chlorella* sp. grown in glucose was found to be greatest when compared to heterotrophic and photoautotrophic cells. In addition, they did an extensive bioenergetic analysis and found that there was a significant decrease in the fraction of light energy used for carbon dioxide fixation by mixotrophic cells. Most of the light energy was instead used as an energy source for carbon assimilation. They conclude that mixotrophy resulted in high energetic efficiency since the amount of energy dissipated was minimal. The growth rates for both algae strains in this study were higher than those grown in municipal primary settled sewage (0.277 $d^{-1}$) and in municipal settled sewage (0.35-0.5 $d^{-1}$) wastewaters in previous research (Tam and Wong, 1990; Lau et al., 1995).

The electricity conversion efficiency for both strains at different light levels was listed in Table 6.1. The data shows that electricity conversion efficiency decreased as the light intensity increased for both algae strains. From the viewpoint of electricity saving, low light intensities should be applied. However, despite attaining the higher electricity

conversion efficiency, using low light intensities resulted in the lower specific growth rate (Figure 6.2), and having a lower biomass productivity than those under high light intensities, thus, the cost involved in harvesting and conversion process might not be economical. Therefore, one needs to compromise between high biomass production and low energy input when working with actual algae cultivation systems. Similar phenomena was also observed in previous research done by Yeh et al. (2010), they also reported decreased electricity conversion efficiency with increase light intensity when cultivating *Chlorella vulgaris* ESP-31.

**Table 6.1** Electricity conversion efficiency ((g/L)/(μmol·$m^{-2}$·$S^{-1}$)) for both strains under different light levels

| Algae strain | Light intensity (μmol·$m^{-2}$·$S^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | dark | 15 | 30 | 60 | 120 | 200 |
| *Chlorella kessleri* | 0.7677 | 0.0596 | 0.0357 | 0.0208 | 0.0117 | 0.0066 |
| *Chlorella protothecoide* | 0.6000 | 0.0451 | 0.0263 | 0.0146 | 0.0071 | 0.0053 |

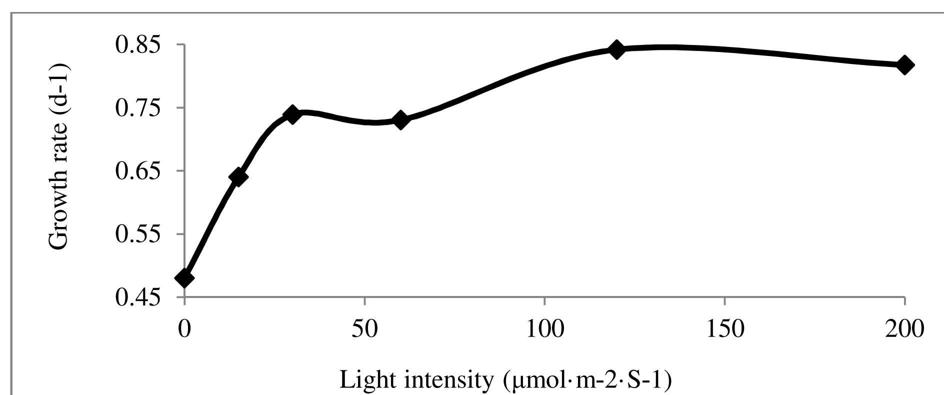



**Figure 6.2** (a), Effect of light intensity on algal growth rate with strain *Chlorella kessleri*

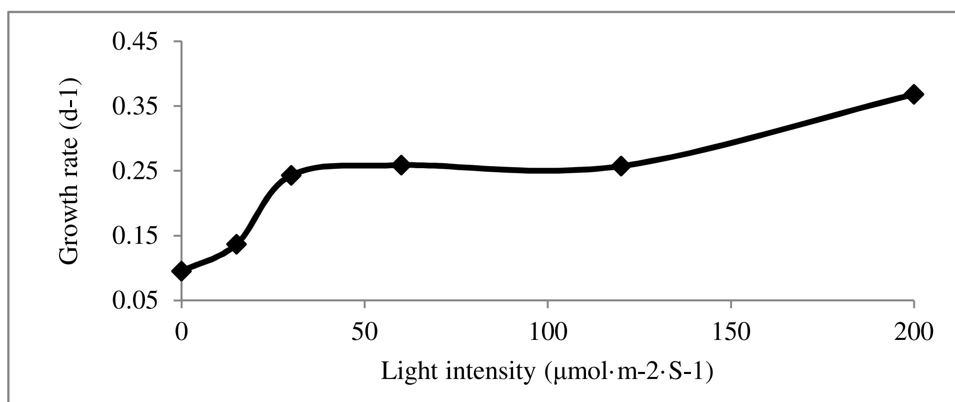



**Figure 6.2** (b), Effect of light intensity on algal growth rate with strain *Chlorella protothecoide*

### 6.4.2 Effect of light intensity on wastewater nutrient removal by algae cultivation process

The nutrient removal profile by algae cultivation process was shown in Figure 6.3 (a) to (h), and the removal rates for each nutrient form by the end of the batch culture under different light intensities are listed in Table 6.2. For all nutrient forms, the concentration dropped to the lowest point after 2-3 days of algae growth, while further cultivation of algae culture resulted in slight concentration increase due to nutrient release as the aging of the algae culture (Wang et al., 2010).

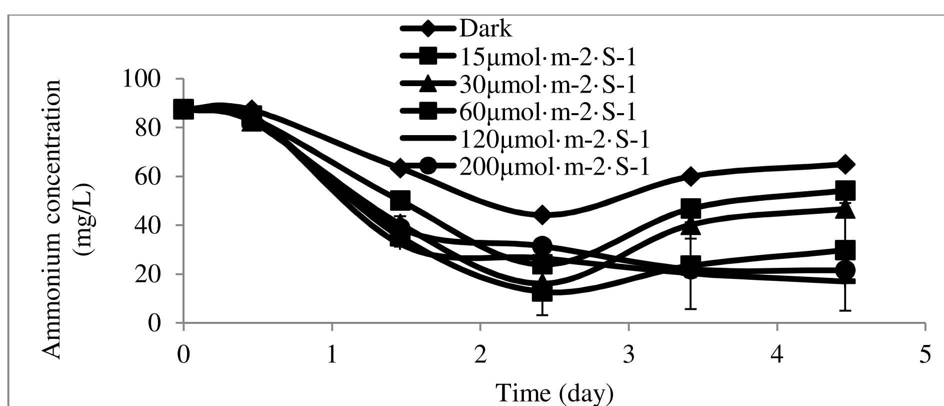



**Figure 6.3** (a) Effect of light intensity on ammonium consumption for species *Chlorella kessleri*

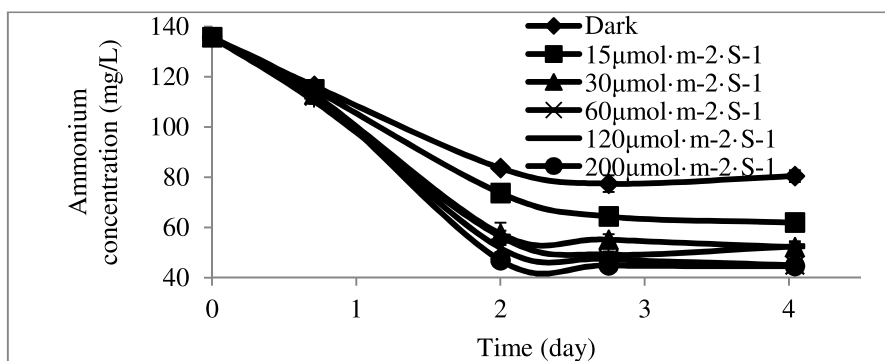



**Figure 6.3** (b) Effect of light intensity on ammonium consumption for species *Chlorella protothecoide*

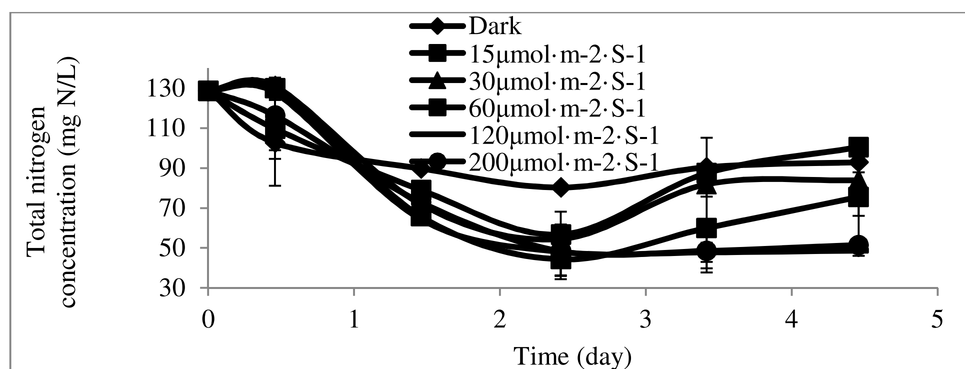



**Figure 6.3** (c) Effect of light intensity on total nitrogen consumption for species *Chlorella kessleri*

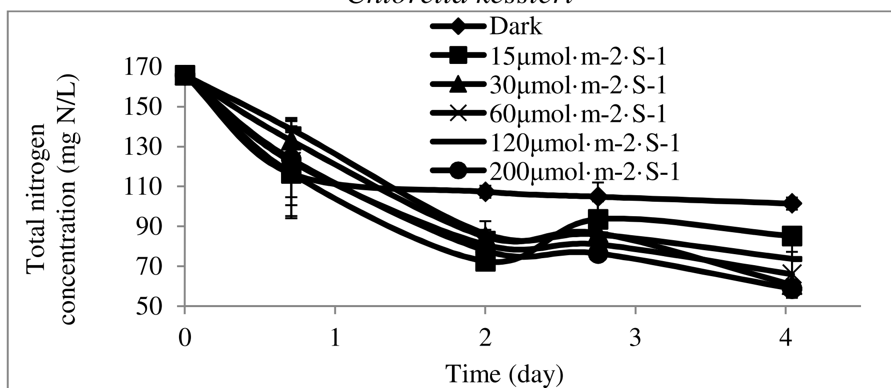



**Figure 6.3** (d) Effect of light intensity on total nitrogen consumption for species *Chlorella protothecoide*

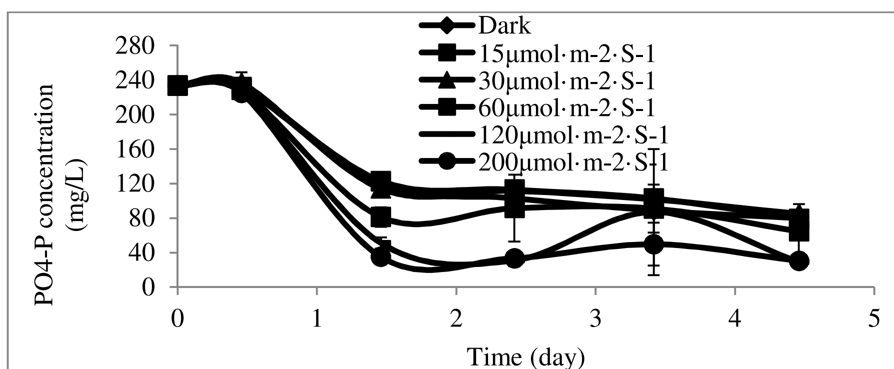



**Figure 6.3** (e) Effect of light intensity on total phosphorus consumption for species *Chlorella kessleri*

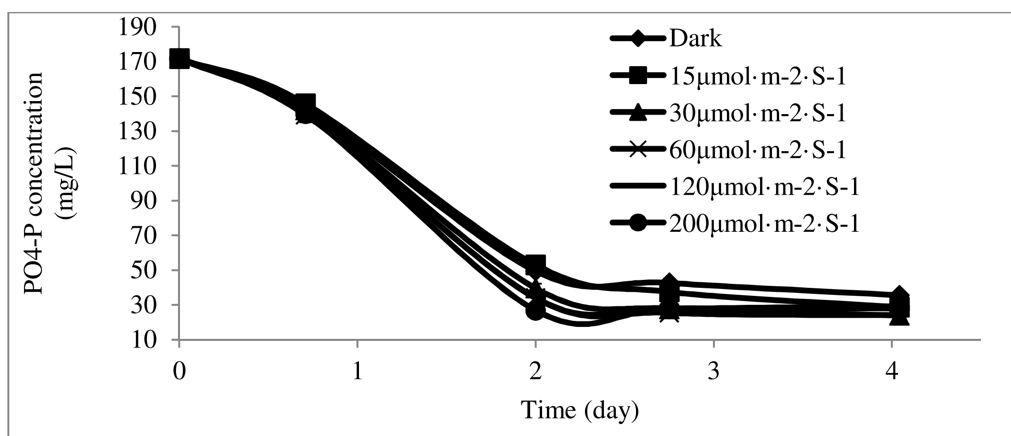



**Figure 6.3** (f) Effect of light intensity on total phosphorus consumption for species *Chlorella protothecoide*

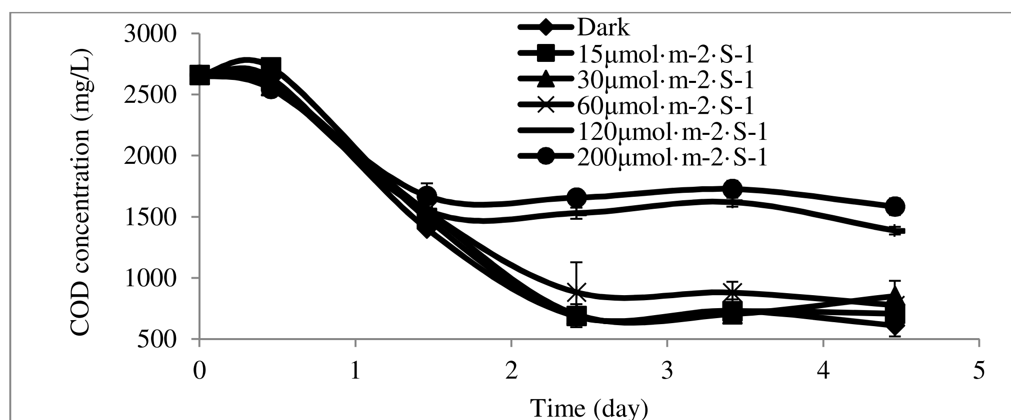



**Figure 6.3** (g) Effect of light intensity on COD consumption for species *Chlorella kessleri*

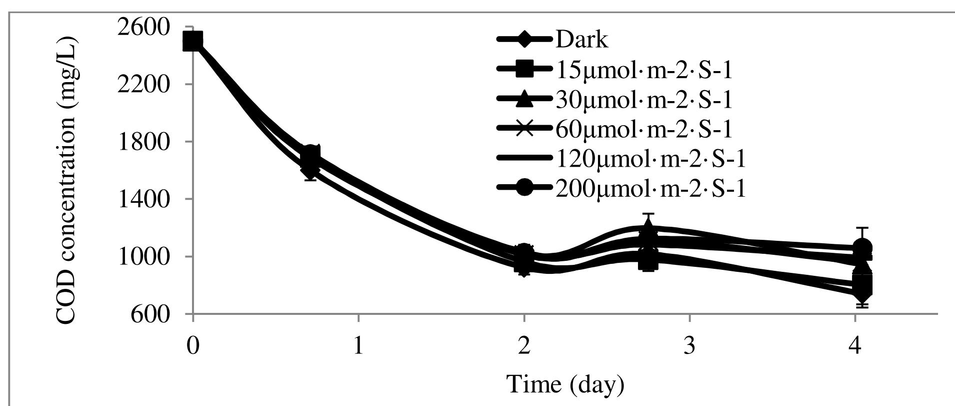



**Figure 6.3** (h) Effect of light intensity on COD consumption for species *Chlorella protothecoide*

**Table 6.2** Removal rates for each nutrient form under different light intensities for both algae strains.

| Nutrient | | Removal rate (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | Light intensity (μmol·m$^{-2}$·S$^{-1}$) | 0 | 15 | 30 | 60 | 120 | 200 |
| Ammonia | *Chlorella kessleri* | 25.8 | 38.0 | 46.5 | 66.0 | 80.5 | 75.3 |
| | *Chlorella protothecoide* | 40.7 | 54.4 | 61.6 | 66.8 | 61.3 | 67.1 |
| TN | *Chlorella kessleri* | 27.8 | 21.9 | 34.8 | 41.3 | 62.2 | 60.0 |
| | *Chlorella protothecoide* | 38.8 | 48.6 | 63.4 | 60.1 | 55.5 | 64.5 |
| TP | *Chlorella kessleri* | 65.8 | 66.2 | 63.6 | 72.3 | 87.4 | 87.0 |
| | *Chlorella protothecoide* | 79.3 | 83.3 | 86.1 | 86.0 | 83.7 | 83.0 |
| COD | *Chlorella kessleri* | 77.0 | 73.4 | 68.0 | 70.8 | 47.9 | 40.5 |
| | *Chlorella protothecoide* | 70.3 | 67.6 | 62.2 | 61.6 | 60.4 | 57.7 |

Ammonium, the only inorganic nitrogen form detected in centrate, was significantly removed by both species. As high as 80.5% of ammonium was removed by strain *Chlorella kessleri,* and 67.1% was removed by strain *Chlorella protothecoide*. Under all conditions, *Chlorella protothecoide* showed better ammonium assimilation into biomass than *Chlorella protothecoide* by the end of the batch culture. In general, with the increase of light intensity, the variation of ammonium removal is in accordance with the variation of biomass accumulation, meaning that most of the ammonium consumed by algae culture was converted to biomass. Statistic analysis shows that for algae strain *Chlorella kessleri,* the best ammonium removal occurred under light intensities of 60,

120 and 200 $\mu mol \cdot m^{-2} \cdot S^{-1}$, while, for algae strain *Chlorella protothecoide,* the best ammonium removal occurred under light intensities of 60 and 200 $\mu mol \cdot m^{-2} \cdot S^{-1}$. Thus, in terms of ammonia removal, light intensity of 60 $\mu mol \cdot m^{-2} \cdot S^{-1}$ is enough for both algae strains.

Total nitrogen, 60-80% of which is ammonium, was also greatly consumed by algae cultivation process. Through the algae cultivation process, approximately, 62.2% of nitrogen was removed by *Chlorella kessleri,* and 64.5% of nitrogen was removed by *Chlorella protothecoide*. With the variation of the light intensity, the nitrogen removal profile was also in accordance with the biomass accumulation profile, suggesting that most of the nitrogen was assimilated to biomass. The best nitrogen consumption by algae culture was found under light intensities of 120 and 200 $\mu mol \cdot m^{-2} \cdot S^{-1}$ for strain *Chlorella kessleri,* and 30, 60, 200 $\mu mol \cdot m^{-2} \cdot S^{-1}$ for strain *Chlorella protothecoide*.

The consumption of total phosphorus is independent on light intensity for algae strain *Chlorella protothecoide.* For algae strain *Chlorella kessleri*, the general trend is higher light intensity led to higher phosphorus removal rate. It is approved that major part of phosphorus is removed by precipitation under high pH (Li, et al., 2011 (a)). In this study it is also observed that higher growth rate resulted in larger pH increase, which led to higher phosphorus removal rate.

The COD removal profile is of particular interest in this study. It showed that the consumption of COD is independent on light intensity for algae strain *Chlorella*

*protothecoide.* For algae strain *Chlorella kessleri*, the removal profile was similar for algae cells cultivated under light intensity of 120 and 200 $\mu mol \cdot m^{-2} \cdot S^{-1}$, which were lower than those cultivated under 0, 15, 30 and 60 $\mu mol \cdot m^{-2} \cdot S^{-1}$, indicating that under light intensity of 120 and 200 $\mu mol \cdot m^{-2} \cdot S^{-1}$, the consumption of COD was inhibited, which is in accordance with the light inhibition process observed in Figure 2 (b). Since COD is an indirect indicator of carbon content in the wastewater, it is suspected that light intensity plays an important role in carbon consumption for algae strain *Chlorella kessleri*. In previous studies, it is found that in the presence of light, the algae cells were able to utilize organic carbon more efficiently (Ogawa and Aiba, 1981; Marquez et al., 1993). The researchers concluded that perhaps light facilitating improved assimilation of the sugar. However, organic carbon uptake in mixotrophic cultures of some strains of *Chlorella* was inhibited by high light intensity (Lalucat et al., 1984; Kamiya and Kowallik, 1987), and it is reported that this is due to the inhibition of synthesis of organic carbon transporter under high light intensity. Photoinhibition of the organic carbon uptake is observed strain-dependent in this study. Thus, if outdoor cultivation is the ultimate goal, only those algae strains which are not sensitive to photoinhibition of organic-C-uptake are suitable for mixotrophic cultivation in centrate.

### 6.4.3 Effect of light intensity on biodiesel composition and productivity

Light is the inducer and regulator for the production of some algal products such as pigments and fatty acids (Cohen, 1999; Sukenik et al., 1989). Light intensity greatly affects the composition and content of biodiesel derived from algae cells cultivated under different lighting conditions as shown in Table 6.3.

**Table 6.3** Biodiesel composition and productivity under different light intensities

| | | Light intensity ($\mu mol \cdot m^{-2} \cdot S^{-1}$) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 15 | 30 | 60 | 120 | 200 |
| Content of 16-C FAME | *Chlorella kessleri* | 22.93 | 34.77 | 53.98 | 38.65 | 25.08 | 26.38 |
| (% of total FAME) | *Chlorella protothecoide* | 14.40 | 11.29 | 11.90 | 13.02 | 14.95 | 15.69 |
| Content of 18-C FAME | *Chlorella kessleri* | 77.06 | 58.37 | 24.40 | 58.45 | 74.55 | 73.26 |
| (% of total FAME) | *Chlorella protothecoide* | 85.60 | 88.71 | 88.09 | 86.98 | 85.05 | 84.31 |
| FAME content | *Chlorella kessleri* | 14.42 | 15.73 | 14.39 | 17.88 | 26.76 | 28.19 |
| (% of TVSS) | *Chlorella protothecoide* | 12.94 | 19.58 | 17.27 | 17.82 | 18.36 | 19.48 |

The total FAME content based on TVSS increased with the increase of light intensity for both species in general, and the major component of FAME derived from algae cells for both species were 16-C and 18-C FAME. For species *Chlorella kessleri,* similar FAME content was detected for algae cells cultivated under light intensity of 0, 15, and 30 $\mu mol \cdot m^{-2} \cdot S^{-1}$, then it increased gradually with the ascending light intensity from 30 to 200 $\mu mol \cdot m^{-2} \cdot S^{-1}$, and the maximum FAME content was found in algae cells under light intensity of 120 $\mu mol \cdot m^{-2} \cdot S^{-1}$ (28.19 % of TVSS). The percentage of 16-C FAME increase from 22.93% under dark to 53.98 % under 30 $\mu mol \cdot m^{-2} \cdot S^{-1}$, and then decreased to 26.38 under 200 $\mu mol \cdot m^{-2} \cdot S^{-1}$, while, the percentage of 18-C FAME decreased from 77.06% under dark to 24.40% under 30 $\mu mol \cdot m^{-2} \cdot S^{-1}$, and increased to 73.26 under 200 $\mu mol \cdot m^{-2} \cdot S^{-1}$. For algae strain *Chlorella protothecoide,* the FAME content increased from 12.94% (of TVSS) under dark to 19.58 % (of TVSS) under 30 $\mu mol \cdot m^{-2} \cdot S^{-1}$, and stayed at similar level afterwards, while the percentage of 16-C and 18-C FAME remained regardless of the light intensity. The dependence of lipid composition on light intensity is highly strain-dependent. In the study conducted by Sukenik et al. (1989), high lipid content and high portions of EPA were detected in *Nannochloropsis* cells under light-limiting conditions, whereas 16:0 and 16:1 fatty acids dominated when light intensity increased to a saturated level. Similar results was observed by Tedesco and

Duerr (1989), which showed that total lipid content decreased with the increase of light intensity from 170 to 870 μmol $m^{-2}$ $s^{-1}$ while the composition of fatty acids remained regardless of the light intensity.

**6.5 Conclusions**

Light intensity has profound impact on algae biomass accumulation, wastewater nutrient removal through algae cultivation, and biodiesel productivity for both *Chlorella kessleri* and *Chlorella protothecoide*, and the dependence of these responses on light intensity varied with different algae strains. For *Chlorella kessleri*, the best light intensity is 120 $\mu mol \cdot m^{-2} \cdot S^{-1}$ for all responses except for COD removal. For *Chlorella protothecoide*, the best light intensity is 30 $\mu mol \cdot m^{-2} \cdot S^{-1}$. The major component of the biodiesel produced from both algae strains are 16-C and 18-C FAME, and the highest biodiesel contents are 28.19% and 19.48% of TVSS for *Chlorella kessleri* and *Chlorella protothecoide*, respectively.

# CHAPTER 7. EFFECT OF LIGHT/DARK CYCLE AT LOW LIGHT FREQUENCY ON ALGAL BIOMASS ACCUMULATION AND BIODIESEL PRODUCTION FOR MIXOTROPHIC STRAIN *Chlorella kessleri* CULTIVATED ON HIGHLY CONCENTRATED MUNICIPAL WASTEWATER

## 7.1 Overview

Due to its high biomass accumulation, biodiesel productivity and growth stabiligy in centrate cultivation system, algae strain *Chlorella kessleri* was used in the following chapters. In this chaper, the single effect of light/dark cycle at low light frequency on algal biomass accumulation, wastewater nutrients removal through algae cultivation and biodiesel production for mixotrophic strain *Chlorella kessleri* using centrate wastewater stream as cultivation media was investigated. The data suggested that the length of lighting period during a day greatly affects the algal biomass accumulation, biodiesel production and wastewater nutrients removal. The biomass concentration, biodiesel productivity and the removal of ammonium, total nitrogen and total phosphorus all increased with increasing lighting period. However, the removal of COD showed different trend, with higher removal rate observed under shorter lighting period. It is found that for maximum biomass accumulation, biodiesel production and wastewater nutrients removal in batch culture system, the optimum lighting period is 16 hours and the cultivation time should be controlled at three days.

**7.2 Introduction**

The interest in algal oil as a promising supplemental oil source for biodiesel production has surged during the recent decade (Li et al., 2008). Researchers found that many specific algae species are capable of using inorganic or organic carbons for oil accumulation under certain cultivation conditions (Li et al., 2008; Meng et al., 2009). Furthermore, they found that triglyceride (TAG) and fatty acids are the dominating components of algal oil, from which biodiesel can be prepared by transesterification, yielding monoalkyl esters of fatty acids and alcohols (Miao and Wu, 2007; Xu et al., 2006; Cheng et al., 2009).

Despite the fact that algae cells are good sources of many biofuel products, their commercial application has been limited by high production cost (Richmond, 2004; Ogbonna and Tanaka, 1996). Although many efforts are being made to reduce the production costs by developing efficient photobioreactors (Ogbonna et al., 1996), installing and operating artificial light sources remains expensive. In this regard, the utilization of solar energy is thought to be the only way of achieving commercial production of most cheap algal products (Ogbonna and Tanaka, 1996). However, depending on the location and season, the length of the day ***(i.e.,*** the number of hours when the intensity of solar radiation is sufficient to support cell growth) can be very different. Thus it is crucial to investigate the effect of light/dark on algae biomass accumulation and lipid content. Light/dark cycle is one of the important environmental factors that not only affect the pattern, pathway, and activity of cellular metabolism, but also affect the economical feasibility of the algae based biodiesel production process

(Richmond,2004). As reported by Grobbelaar (1991), the fluctuating light-/dark cycles could be classified into three categories (Ogbonna and Tanaka, 1996); (i) high-frequency fluctuations of 100 ms (10 Hz) and less, (ii) medium-frequency of seconds to minutes, and (iii) low-frequency of hours to days and years. Several studies have been conducted using high and medium frequencies (Grobbelaar, 1991; Kok, 1953; Friedrickson and Tsuchiya; 1970; Terry, 1986), but studies using low frequency are quite limited (Ogbonna and Tanaka, 1996).

When growing under autotrophic conditions using $CO_2$ as carbon source and sunlight as energy supply, both biomass productivity and oil content of the cells are extremely low (Richmond, 2004; Cheng et al., 2009). On the contrary, heterotrophic growth, under which organic carbon is used as carbon source, and mixotrophic growth, under which both inorganic and organic carbon are simultaneously assimilated into biomass, allow algae to accumulate a much higher proportion of oil within less time and scale-up is much easier (Cheng et al., 2009; Eriksen,2008). However, the fact that sugar or other organic substrate is of high cost lowers the economic feasibility of biodiesel production from algae biomass. Therefore, finding an organic source that is of low cost, easy accessibility, high nutrients level, free of toxic inhibitor is desirable. Centrate, which is generated from the sludge thickening process in a typical municipal wastewater treatment plant, has the characteristics of high nutrients level including inorganic and organic carbon, nitrogen phosphorus as well as minerals, large quantity and yearly availability has the potential for algae based biodiesel production as evidenced by

previous studies both in lab and pilot scales (Li et al, 2011(a), (b), Zhou et al, 2011, Min et al., 2011).

The major objective of the present study was to evaluate the effect of light/dark cycle at low frequency on algae biomass accumulation, biodiesel productivity and wastewater nutrients removal with centrate wastewater stream as feedstock, and find the optimum light/dark cycle that should be applied.

## 7.3 Material and Methods

### 7.3.1 Algae strain and culture condition

The algae strain used in this study, namely *Chlorella kessleri,* was purchased from the Culture Collection of Algae at the University of Texas, USA, and was conserved in BG-11 medium as described in section 4.3.1. Prior to experiment, the algae strain was cultivated on centrate for three generations to obtain stable characteristics. The experiments were carried out with 250mL Erlenmeyer flasks containing 100mL sterilized centrate media. The 250ml Erlenmeyer flasks were kept on a shaker with 100 rpm rotation speed. In all cases, algae strains were inoculated at 1:10 (v:v) and grown under 25±2°C with light intensity of 120 μmolm$^{-2}$s$^{-1}$ at designed light/dark.

### 7.3.2 Control of light/dark cycle

The effect of light/dark cycle on biomass growth, wastewater nutrient removal and biodiesel production was studied with two algae strains stated above. The lighting period studied were 0, 4, 8, 16 and 24 hours, and the dark condition was achieved by wrapping the flasks with aluminum foil.

### 7.3.3 Pretreatment of wastewater

The procedure used is same as section 3.3.2. The sterilized centrate contains 48 mg/L ammonia, 71 mg/L total nitrogen (TN), 66 mg/L total phosphorus (TP) and 2000 mg/L chemical oxygen demand (COD).

### 7.3.4 Analytical methods

#### 7.3.4.1 Determination of nutrients concentration and removal rates

Same as 3.3.4.1

#### 7.3.4.2 Analysis of algal biomass concentration and growth rate

Same as 4.3.6.1

#### 7.3.4.3 Examination of fatty acid methyl ester (FAME) composition

Same as 3.3.4.3

All the experiments were carried out in triplicate and average values are reported.

Results were analyzed using Excel and software JMP 8.0.

## 7.4 Results and Discussion

### 7.4.1 Effect of light/dark cycle on biomass accumulation

Algal growth as indicated by daily TVSS concentration under lighting periods of 0, 4, 8, 16 and 24 hours was shown in Figure 7.1. The algae strain used in this study showed excellent adaptation in the centrate cultivation system. It could survive on centrate under all lighting periods with no obvious lag phases observed. The growth under all conditions lasted approximately 3 days, after which the biomass concentration either remained or dropped. The results of statistical analysis showed that lighting period had crucial effect on biomass accumulation ($p<0.05$). In general, long lighting period promotes algae growth. Since the growth under all conditions lasted three days, the

discussion will be based on the biomass accumulation during the first three days in the rest of this section. It is found that during the first three days, the biomass accumulation under 24 hours lighting period was similar with that under 16 hours lighting period and no statistically significant difference was observed, illustrating that during the exponential phase 16 hours lighting is enough for algae growth. When the lighting period decreased from 16 to 0 hours, the biomass accumulation declined gradually. The growth rates were 0.18, 0.22, 0.26, 0.34, and 0.35 $d^{-1}$ for lighting period of 0, 4, 8, 16 and 24 hours, respectively, which also increased with the increasing lighting period from 0 to 16 hours and stayed at stable level when lighting period increased from 16 to 24 hours.

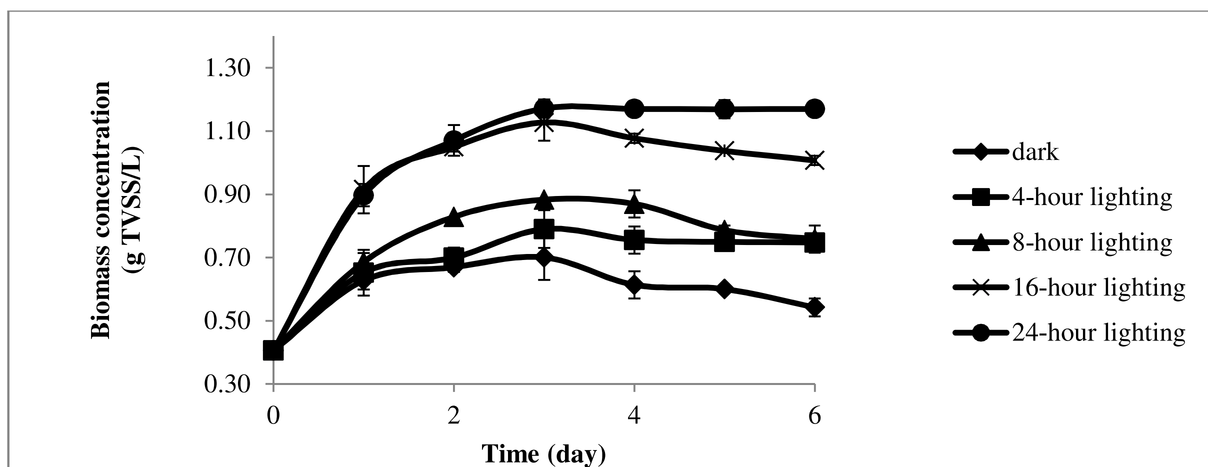



**Figure 7.1.** Biomass accumulation profile at different light/dark cycles

The algae strain used in this study was found capable of mixotrophic growth, which represents the use of light as energy coupled with a simultaneous assimilation of $CO_2$ and organic carbon as carbon sources (Richmond, 2004). Light is essential for autotrophic growth and it can also improve the use of organic carbon (Richmond, 2004), thus, under dark condition only reduced growth from organic carbon resumed.

Furthermore, when organic carbon was completely consumed, only inorganic carbon could be used as carbon source. Under this condition, algae growth could only perform when light energy is present (Richmond, 2004). In this study, organic carbon was consumed completely during the first three days, thus, from day 3, the biomass accumulation is mainly from autotrophic growth. It is reported that exogenous organic carbon enhanced respiration both in light and dark (Martinez and Orus, 1991; Valiente, 1992). Ogbanna and Tanaka (1996) reported that when light is absent biomass concentration was detected to be decreasing. Similar results were also observed under high and medium frequencies of light fluctuations. Richmond and Vonshak (1980) reported that the growth rate of *Spirulina* increased significantly in mass algal cultures when the exposure to the light source increased.

**7.4.2 Effect of light/dark cycle on biodiesel composition and productivity**

As reported by Cohen et al. (1999) and Sukenik et al. (1989), the formation of some algal products such as pigments and fatty acids needs light as inducer and regulator. As shown in Table 7.1, the composition and content of biodiesel were greatly affected by the lighting period. The total FAME content based on TVSS increased with the increase of lighting period in general. For species *Chlorella kessleri,* the FAME content increased from around 10% to 14% when the lighting period increased from 0 to 4 hours and stayed at similar level when the lighting period increased from 4 to 8 hours. When the lighting period increased from 8 hours to 16 hours the FAME content increased from 14 to 16% and stayed at around 16% afterwards. Previous researchers reported that during night the carbohydrate contents of the cells decreased while their protein content increased, implying that in the absence of light energy, intracellularly

stored compounds are metabolized as an energy source (Ogbonna and Tanaka, 1996). As shown in Table 7.1, the major components of biodiesel derived from algae cells were 16-C and 18-C FAME. The content of 16-C and 18-C FAME in terms of total FAME increased when the lighting period increased from 0 to 4 hours and stayed at stable level when the lighting period increased from 4 to 24 hours. Previous studies show that the response of different algae species to light/dark cycle varies a great deal. Shifrin and Chisholm (1980) reported that the total lipid fraction in *O. polymorpha* was found to remain constant over the cell cycle in synchronized cultures regardless of the light regime, while, in the study carried out by Sicko-Goad et al. (1988), it is reported that the sum of the C16 fatty acids remains constant at 70% of total fatty acids in the cells throughout the light-dark cycle.

Table 7.1 Biodiesel composition and productivity under different lighting period

| | Lighting period (hours) | | | | |
|---|---|---|---|---|---|
| | 0 | 4 | 8 | 16 | 24 |
| Content of 16-C FAME (% of total FAME) | 26.46 | 33.94 | 34.25 | 35.54 | 32.51 |
| Content of 18-C FAME (% of total FAME) | 54.41 | 60.42 | 57.96 | 60.73 | 58.39 |
| Total FAME content (% of TVSS) | 9.81 | 13.90 | 13.80 | 15.85 | 16.86 |

### 7.4.3 Effect of light/dark cycle on wastewater nutrients removal through algae cultivation

Figure 7.2 (a)-(d) show the nutrient removal profiles by algae cultivation process at different light/dark cycle settlings. The removal rates for each nutrient form by the end of the batch culture under different lighting period are listed in Table 7.2. For all nutrient forms, the concentration dropped to the lowest point after 2-3 days of algae growth, while further cultivation of algae culture resulted in slight concentration

increase due to nutrient release as the aging of the algae culture, which was in accordance with our previous results (Li et al., 2011 (a), Wang et al., 2010).

The profile of the ammonia and total nitrogen consumption corresponded well with the growth curves shown in Figure 7.1, meaning that higher growth rate and biomass concentration correlated with higher ammonia and total nitrogen removal rate and removal efficiency, which suggests that most of the ammonia and total nitrogen consumed by algae culture was converted to biomass. The majority of the removal was observed during the first day of algae cultivation. Statistic analysis shows that for algae strain *Chlorella kessleri,* the best ammonia and total nitroten removal occurred under lighting period of 16 and 24 hours, which were around 95% and 60%, respectively. Thus in terms of ammonia and total nitrogen removal, 16 hours lighting period is enough to reach the maximum ammonia removal rate.

The consumption of total phosphorus in aqueous solution is also dependent on the lighting period in this study. Most of the phosphorus was removed during the first two days of algae cultivation with higher removal rate found at longer lighting period. By the end of the cultivation, the lowest removal rate was found at dark condition, the highest removal rate was found at 24 hours lighting period, and the removal rate was similar at 4, 8 and 16 hours lighting.

The COD removal profile is different from other nutrients stated above. It showed that most of the COD is consumed during the first day of cultivation, and the removal

profile is similar under all lighting conditions. But by the end of the cultivation, the lowest removal rate was found at 24 hour of lighting, while the removal rate was similar under other conditions, indicating that under light intensity of 120μmol·$m^{-2}$·$S^{-1}$and lighting period of 24 hours, the consumption of COD was inhibited, which suggests a light inhibition process observed by other researchers (Lalucat et al., 1984) and in our previous studies (Li et al. 2012). In previous studies (Li et al. 2012), it is found that organic carbon uptake in mixotrophic cultures of some strains of *Chlorella* was inhibited by light at certain level, and it is reported that this is due to the inhibition of synthesis of organic carbon transporter under high light intensity (Lalucat et al., 1984; Kamiya and Kowallik, 1987).

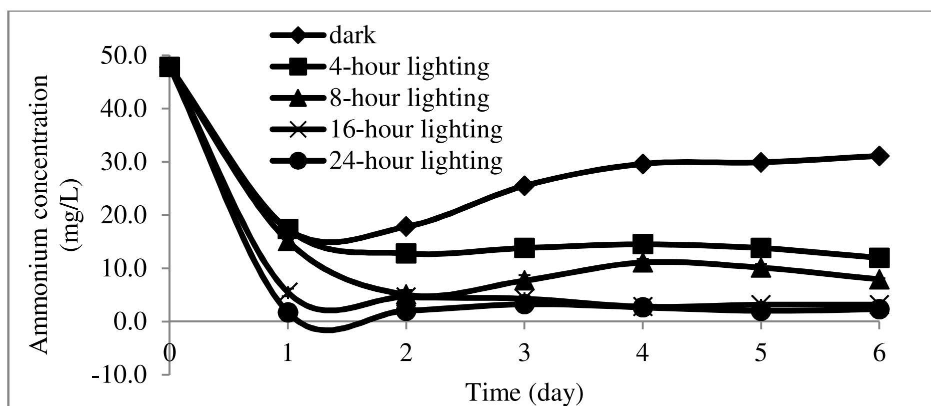



**Figure 7.2 (a).** Ammonium removal profile at different light/dark cycles

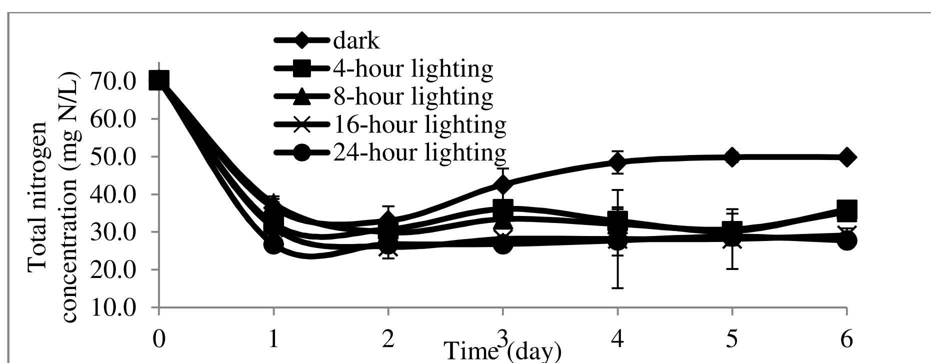



**Figure 7.2 (b).** Total nitrogen removal profile at different light/dark cycles

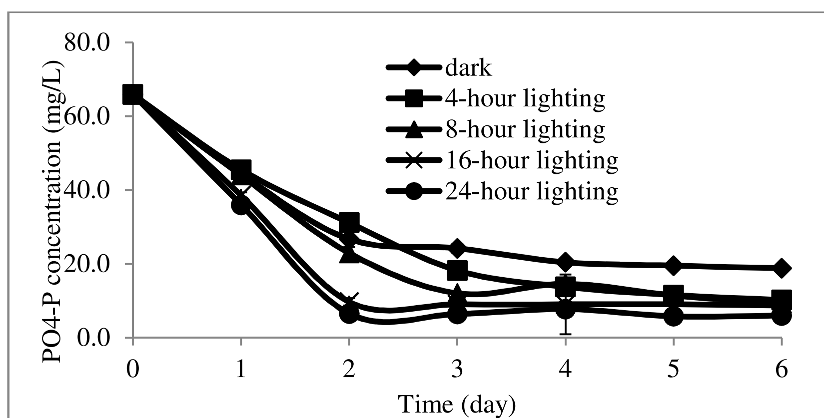



**Figure 7.2 (c).** Total phosphorus removal profile at different light/dark cycles

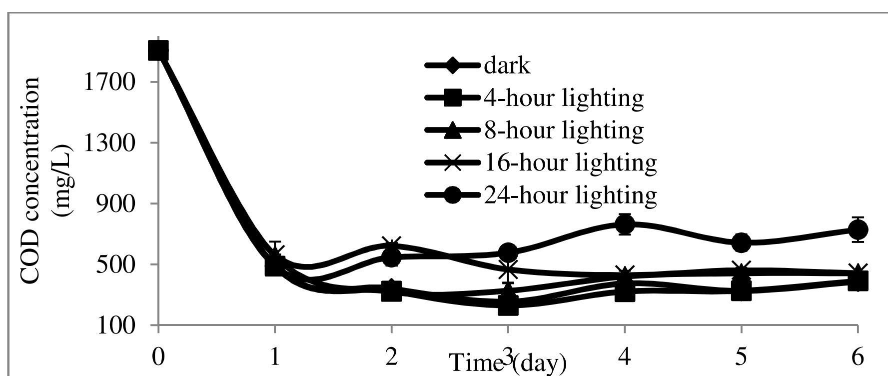



**Figure 7.2 (d).** COD removal profile at different light/dark cycles

Table 7.2 Removal rates for each nutrient form under different light intensities for both algae

| Nutrients | dark | 4h lighting | 8h lighting | 16h lighting | 24h lighting |
|---|---|---|---|---|---|
| Ammonia | 34.98 | 75.05 | 83.47 | 93.44 | 95.18 |
| TN | 29.11 | 48.91 | 49.73 | 58.57 | 60.49 |
| TP | 71.45 | 84.53 | 85.99 | 86.75 | 90.92 |
| COD | 79.70 | 79.48 | 76.84 | 76.79 | 61.80 |

### 7.5 Conclusions

This study is the first to report the effect of light/dark cycle at low light frequency on algae based biodiesel production using centrate wastewater stream with mixotrophic strain *Chlorella kessleri.* The results showed that the length of lighting period during a day greatly affects the algal biomass accumulation, biodiesel production and wastewater

nutrients removal. The biomass concentration, FAME content and the removal of ammonia, total nitrogen and total phosphorus all increased with increasing lighting period. However, the removal of COD showed reverse trend, with higher removal rate observed under shorter lighting period. Taken all responses into consideration, the optimum lighting period is 16 hours and the cultivation time should be controlled at three days for maximum biomass and biodiesel production as well as wastewater nutrients removal.

# CHPATER 8 THE EFFECT OF ECOGENOUS $CO_2$ AT SIMULATED NATURAL LIGHT/DARK CYCLE ON ALGAE BIOMASS ACCUMULAITON AND WASTEWATER NUTEIENTS REMOVAL WITH ALGAE STRAIN *Chlorella kessleri*

## 8.1 Overview

In the last two chapters, the single effects of light intensity and light/dark cycle on algal biomass accumulation and biodiesel production were studied. In this chapter, the effect of supplying different concentrations of exogenous $CO_2$ uder various light intensities at simulated light/dark cycle on biomass accumulation and wastewater nutrients removal was tested with aglae strain *Chlorella kessleri*. The results confirmed that there was an optimum $CO_2$ level for algae growth, which is 2.5% for this investigation. When the light intensity reached a certain lower limit, the effect of $CO_2$ supplementation became minimum because the photoautotrophic efficiency was significantly reduced. It is also found that a future study to investigate the effects of environmental factors on biomass and biodiesel production under continuous or fed-batch cultivation mode, where wastewater is continuously replenished and algae was harvested, would be valuable due to the fact that the nutrients was depleted and the algae biomass concentration decreased on Day 8.

## 8.2 Introduction

Some algae species such as *Chlorella* have been successfully used in wastewater treatment and have proven to be able to utilize and remove nitrogen, phosphorus, and

chemical oxygen demand (COD) under different conditions (Lau et al., 1998; Hernandez et al., 2006). The idea of combining wastewater treatment with mass algae cultivation, which could potentially greatly reduce the cost of growing microalgae feedstock, has been investigated by several research groups (Yun et al., 1997; Sawayama et al., 1995; Wang 2010). Their results have also demonstrated that certain algae species have higher specific growth rate under mixotrophical conditions than either under photoautotrophical or heterotrophical condition alone in wastewater. Sawayama et al., (1995) showed that the continuous cultivation of *Botryococcus braunii* Berkeley strain in secondary treated sewage (STS) with aeration of 1% $CO_2$ reduced inorganic nutrients, including Cr, As, and Cd, as well as producing algae for algal oil production. Therefore, introducing exogenous $CO_2$ in organic carbon-rich wastewater may increase carbon assimilation and algal biomass production by providing additional inorganic carbon source to the culture system, especially during the period when organic carbon is completely consumed. Our previous data also showed inceasing algal biomass concentration under exogenous $CO_2$ bubbling condtions. Thus, the objective of this study is to investigate the growth characteristics of algae strain *Chlorella kessleri* when cultivated in centrate wastewater stream under simulated natural sunlight cycle with two levels of light intensity and three concentrations of exogenous $CO_2$. The assumption was that higher light intensity up to certain limit would promote the utilization of inorganic $CO_2$, hence, enhance the growth rate of algae biomass. Also, there is an optimum level of $CO_2$ at which the algae presents optimum growth.

## 8.3 Material and Methods

### 8.3.1 Experimental design and data analysis

A full factorial design (3x2) with three replications was used in this chapter. Three levels of exogenous $CO_2$ tested were 0%, 2.5%, and 5% (v/v) at 0.5 vvm, and 2 levels of light intensities used were 30 and 190µmolm$^{-2}$s$^{-1}$. The light/dark cycle was controlled at 16hours of lighting with 8 hours of dark. Eighteen 1L Roux culture bottles (Fisher Scientific, USA) were used as the bioreactors, each containing 560ml culture solution. The initial algae cell concentration was approximate $2.1\times10^{6}$ cells/ml. An aeration system composed of a 1ml pipette connected to ambient air or $CO_2$-enriched air supplies was inserted from the top of the bioreactor to the bottom. All the cultures were cultivated at ambient temperature (25±2ºC). A magnetic stir bar was placed at the bottom of the bioreactor operating at 100 rpm. All experiments were carried out in 8-day batch culture.

Data analysis was done by Excel and software JMP 8.0. Treatments with p-value less than 0.05 were considered as statistically significant.

### 8.3.2 Wastewater Preparation

The procedure is same as 3.3.2 and the characteristic of the centrate is shown is Table 8.1.

**Table 8.1** Charateristics of autoclaved centrate

| Parameters | pH | TVSS (g/L) | Ammonia (mg/L) | TN (mg-N/L) | TP (mg-$PO_4^{3-}$-P/L) | COD (mg/L) |
|---|---|---|---|---|---|---|
| Values | 5.6 | 0.1095 | 72.73 | 119 | 70.73 | 2027.5 |

#### 8.3.3 Algae strain and culture condition

Same as 7.3.1

#### 8.3.4 Control of light intensity, light/dark cycle and exogenous $CO_2$ concentration

Same as 5.3.5 and 5.3.6

#### 8.3.5 Analytical methods

##### 8.3.5.1 Determination of nutrients concentration and removal rates

Same as 3.3.4.1

##### 8.3.5.2 Analysis of algal biomass concentration and growth rate

Same as 4.3.6.1

### 8.4 Results and discussion

#### 8.4.1 Effect of exogenous $CO_2$ on algae growth

Figure 8.1 shows the algae biomass growth curves as indicated by TVSS during the 8-day batch culture. The data shown for the first 3 days suggests that there is no obvious difference observed among the growth curves under all 6 conditions. It is reasonable to state that the strain used adapted well to the centrate wastewater culture system. For most conditions, the biomass reached their peaks on day 7, and the highest biomass concentration was observed under light intensity of 190μmol$m^{-2}s^{-1}$and exogenous $CO_2$ concentration at 2.5%.

Statistic analysis shows that under light intensity of 190μmol$m^{-2}s^{-1}$$CO_2$ had significant effect on algae growth, while under light intensity of 30μmol$m^{-2}s^{-1}$, $CO_2$ did not show any positive effect on algae biomass accumulation. Thus, the interaction between the light intensity and exogenous concentration also needs to be considered. The two way

ANOVA results showed that both $CO_2$ and light intensity are statistically significant (p-value<0.05) affecting the biomass growth, so is the interaction between exogenous $CO_2$ and light intensity. In autotrophic culture, light is necessary for inorganic assimilation as stated in previous studies (Richmond, 2004), and when light is supplied higher biomass accumulation was observed when using $CO_2$-enriched air injection comparing with no $CO_2$ injection (Chiu et al., 2008; Yang and Gao, 2003).

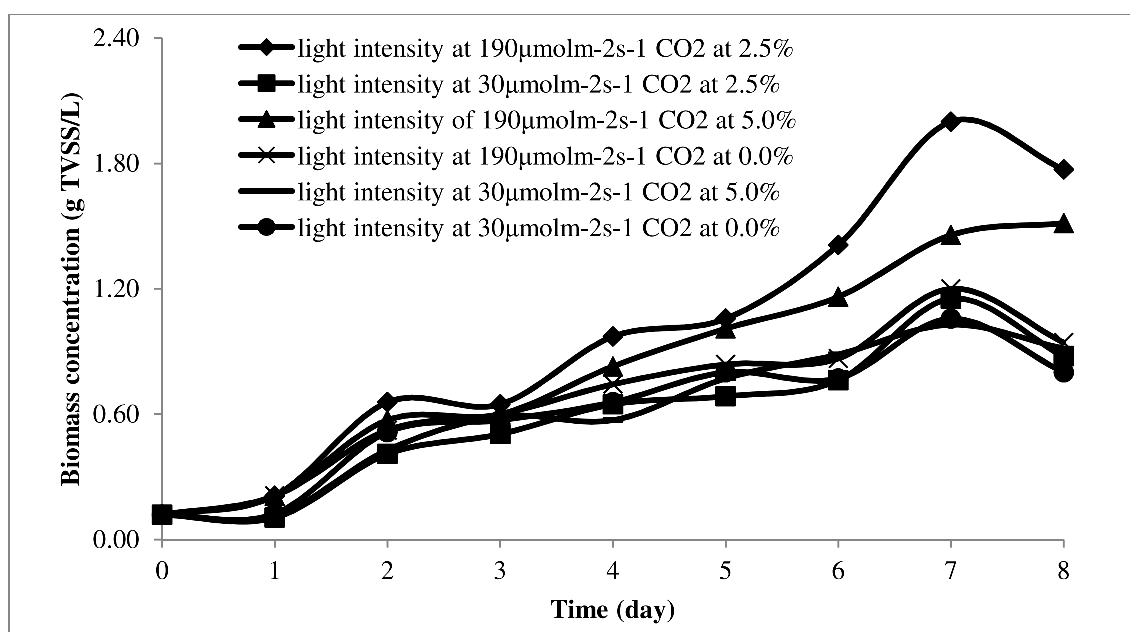



**Figure 8.1** Growth curves for 8-day batch cultures under all designed conditions

### 8.4.2 Effect of exogenous $CO_2$ on pH change during algae cultivation process

The variation of pH during the 8-day cultivation was also monitored. In experiments where no exogenous $CO_2$ added to the system the pH incrasesed up to 9.0 by the end of the cultivation, while for those with $CO_2$ supplement, the pH was controlled well below 7.5 during the cultivation process. Thus addition of exogenous $CO_2$ not only provides a carbon source but also offers a buffer effect and stabilizes the pH of the wastewater

(Richmond, 2004). These experiments also prove that *Chlorella kessleri* has an ability to tolerate high pH value. The pH of the cultivation solution was found crutial for phosphorus removal as stated in the earlier chapters, and higher pH improved phosphorus removal.

### 8.4.3 Effect of exogenous $CO_2$ on COD removal

COD is an indirect indicator of the amount of organic compounds in water. Table 8.2 lists the average COD removal rates by the end of the 8-day culture. The average COD removal achieved 71.4% to 78.2%. Statistical analysis shows that the effect of exogenous $CO_2$ on COD removal is marginally significant under both light intensities (p-value close to 0.05), with higher COD removal efficiencies corresponses with high exogenous $CO_2$ concentration. The mechanism how $CO_2$ affected COD removal was not clear. One possible reason is that $CO_2$ addition moderated the pH value of the wastewater. Proper pH values enhanced the heterotrophic algae growth and COD removal. As shown in Figure 8.2, the major removal of COD was observed during the first 6 days of cultivation, after which, the removal rate decreased. The organic carbon was mainly used by algae strain during the first 6 days, after which, autotrophic process picked up its portion and at the end of the experiment, autotrophic growth using exogenous $CO_2$ was the dominant mode.

**Table 8.2** COD Removal Rate (%) under all designed conditions

| | Exogenous $CO_2$ Level (%) | | |
|---|---|---|---|
| | 0.0% | 2.5% | 5.0% |
| 190μmol$m^{-2}s^{-1}$ | 71.4 | 74.2 | 78.2 |
| 30 μmol$m^{-2}s^{-1}$ | 73.9 | 76.4 | 77.9 |

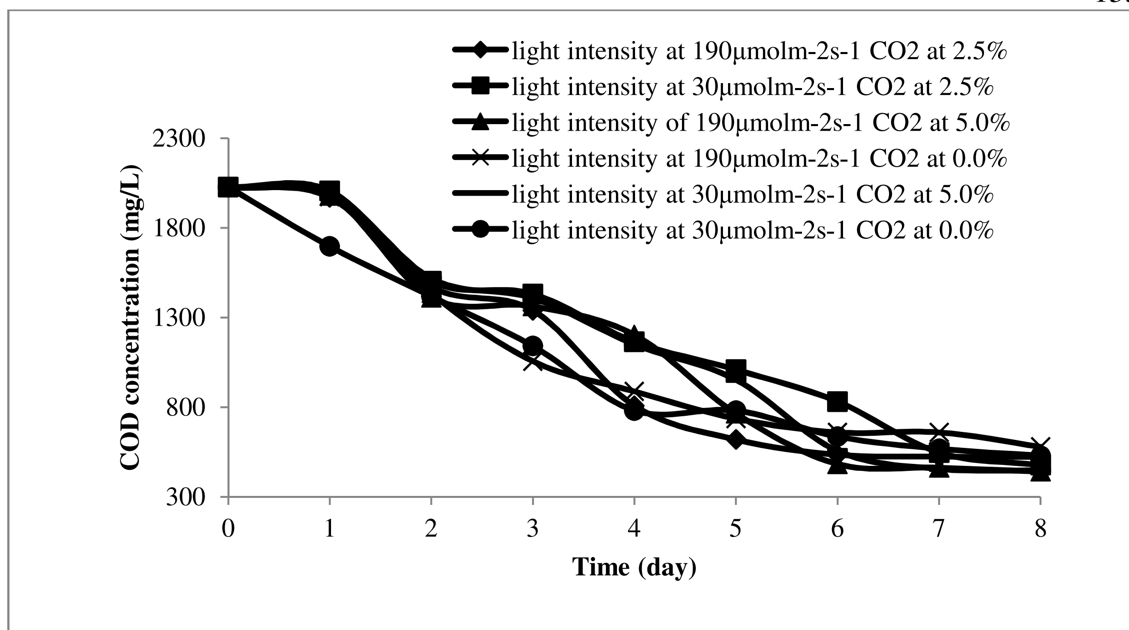



**Figure 8.2** COD removal profile under all designed conditions

### 8.4.4 Effect of exogenous $CO_2$ on ammonium and total nitrogen removal

The removal profile of ammonium is shown in Figure 8.3, and the ammonium removal rate by the end of the cultivation is shown in Table 8.3. Statistical analysis shows that at the same light intensity level, $CO_2$ did not show significant effect on ammonia removal rate by the end of the cultivation. The majority of the ammonia was removed during the first five days. It is found that high ammonia removal rate corresponeded with high bomass accumulation, which is in accordance with previous results.

**Table 8.3** Ammonia removal rate (%) under designed conditions

| | Exogenous $CO_2$ Level | | |
|---|---|---|---|
| | 0.0% | 2.5% | 5.0% |
| 190 $\mu molm^{-2}s^{-1}$ | 99.7 | 99.9 | 99.9 |
| 30 $\mu molm^{-2}s^{-1}$ | 88.4 | 87.6 | 89.2 |

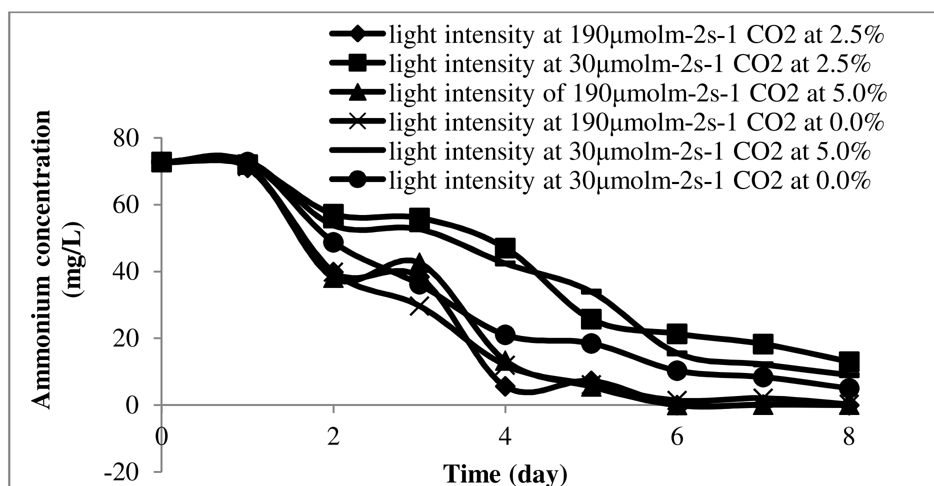



**Figure 8.3** Ammonium removal profile under all designed conditions

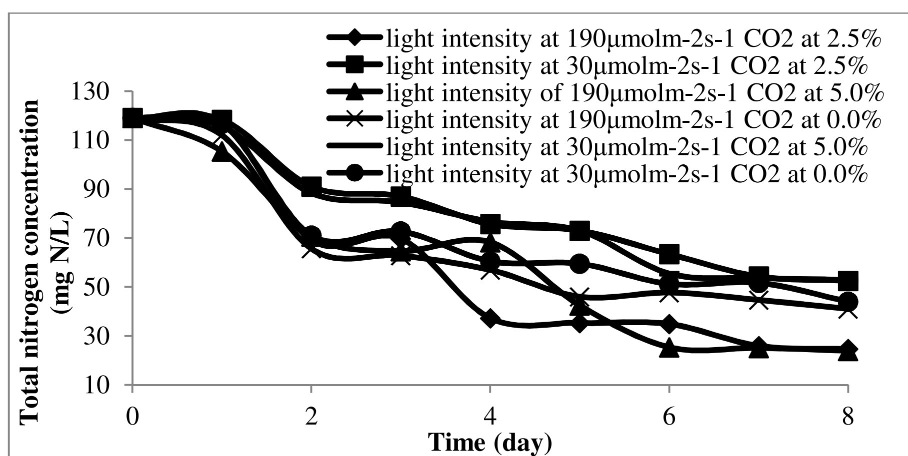



Figure 8.4 Total nitrogen removal profile under designed conditions

Table 8.4 Total nitrogen removal rate under designed conditions

| | Exogenous $CO_2$ Level | | |
|---|---|---|---|
| | 0.0% | 2.5% | 5.0% |
| 190 $\mu molm^{-2}s^{-1}$ | 65.5 | 78.2 | 78.9 |
| 30 $\mu molm^{-2}s^{-1}$ | 55.5 | 54.4 | 54.9 |

Figure 8.4 shows the variation of total nitrogen concentration over 8-day cultivation period. Table 8.4 lists the average percentage of total nitrogen removal rate. Since the majority of the nitrogen is ammonium, it is not surprising to see similar trends of total

nitrogen removal with ammonium removal profile. Highe total nitrogen removal also corresponse with high biomass accumulation. The removal of ammonium and total nitrogen is more dependent on light intensity than exogenous $CO_2$. The metabolisms of nitrogen and carbon in plants such as algae are linked because they share energy and carbon sources (Reizer et al. 1992), thus, withholding either $CO_2$ or light would inhibit nitrogen assimilation.

### 8.4.5 Effect of exogenous $CO_2$ on total phosphorus removal

As stated in previous studies, the aglae strain used in this study is capable of removing phosphorus from wastewater both by metabolic uptake and by chemical coagulation and adsorption (Li et al., 2011 (a); Wang et al., 2010; Bogan, 1961). Under high pH levels, phosphate becomes unavailable for algae growth due to forming of sediment in forms of insoluble inorganic complex salts (Wang et al., 2010).

Figure 8.5 shows the removal profile of taotal phosphorus during the 8-day culture. From the figure we can see that for the first two days, the curve is similar in all conditions. It was believed the driving force in the first two days were mainly biological adsorption and consumption since the pH during the first two days stayed below the critical value for phosphosrus sedimentation. For experiment conducted with 2.5% and 5.0% exogenous $CO_2$, it is also believed due to the metabolic uptake by algae cells due to the low pH (<7.5). While in experiment with 0.0% exogenous $CO_2$, it is the combination of metabolic pathway and phosphates settlements as complex salts under high pH conditions that caused the removal of phosphorus.

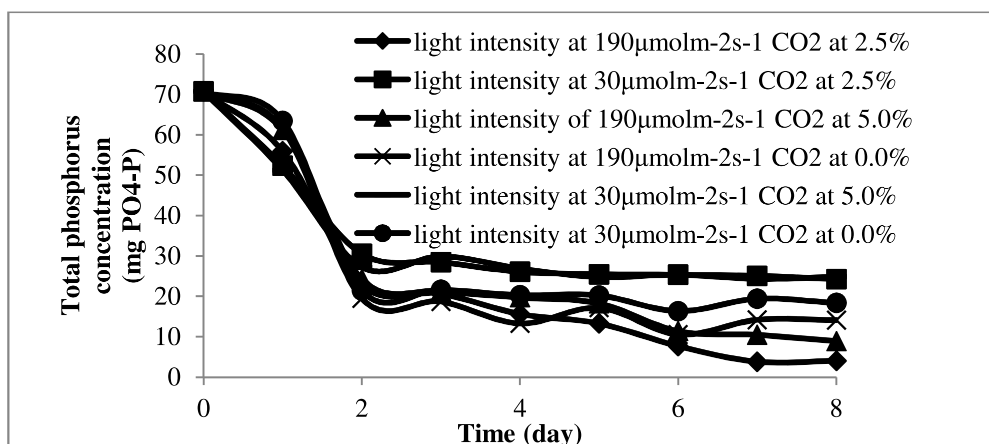



**Figure 8.5** Removal profile of total phosphorus under designed condition

**Table 8.5** Removl rate of total phosphorus (%) under designed conditions

| | Exogenous $CO_2$ Level | | |
|---|---|---|---|
| | 0.0% | 2.5% | 5.0% |
| 190 $\mu molm^{-2}s^{-1}$ | 80.0 | 94.2 | 87.4 |
| 30 $\mu molm^{-2}s^{-1}$ | 74.0 | 65.6 | 64.7 |

### 8.5 Conclusions

In this section, algae strain *Chlorella kessleri* was approve to be capable of quickly adapting to centrate wastewater supplemented with exogenous $CO_2$ in batch reactors in a simulated natural light cycle. The exogenous $CO_2$ concentration had significant effect on biomass accumulation and wastewater nutrients removal under high light intensitie (190$\mu molm^{-2}s^{-1}$ in this study). A 2.5% $CO_2$ level was found better than 0.0 and 5.0% $CO_2$ level for algal biomass accumulation and wastewater nutrients removal in this study. When the light intensity reaches certain lower limit, the effect of $CO_2$ bubbling became minimum because the autotrophic process efficiency was reduced. Thus in later studies, the interaction between light intensity and exogenous $CO_2$ should also be considered.

# CHAPTER 9 OPTIMIZAION OF LIGHT INTENSITY, LIGHT/DARK CYCLE, EOXGENOUS $CO_2$ AND HYDRAULIC RETENTION TIME FOR HIGH BIOMASS AND BIODIESEL PRODUCTION USING MICROALGAE STRAIN *Chlorella kessleri* CULTIVATED ON HIGHLY CONCENTRATED MUNICIPAL WASTEWATER

## 9.1 Overview

It is found that the interactions among the factors tested above also affect the algal biomass accumulation, biodiesl production and wastewater nutrients removal. Furthermore, continous cultivation system is also suggested by previous data. Thus, the objective of this chapter was to optimize the operational parameters, including light intensity, light/dark cycle, exogenous $CO_2$ concentration, and hydraulic retention time (HRT), for high algae biomass accumulation, biodiesel production and wastewater nutrients removal. The results showed that response surface methodology with central composite design is effective in system optimization for algal-based biodiesel production using centrate wastewater in fed-batch culture. The regression analysis illustrates that, at $p$-value$< 0.05$, HRT, light intensity and lighting period had significant effect on biomass concentration, biomass and biodiesel yield, and ammonium removal rate. HRT and exogenous $CO_2$ concentration played important role in FAME content, while the removal rates of TN, TP and COD could not be explained by quadratic models. The optimum conditions for biodiesel production are HRT of 2.86 days, which means harvesting 35% of the total volume daily, light intensity of 190µmolm$^{-2}$s$^{-1}$ and lighting period of 10h.

## 9.2 Introduction

In order to build a sustainable cultivation system to produce liquid fuel from algae biomass using wastewater streams, it is essential to optimize the environmental factors on the production of biomass and lipids by microalgae cells since it is well known that microalgae show substantial metabolism flexibility in response to changes in cultivation conditions (Shifrin and Chisholm, 1981; Cohen et al., 1987, Chelf, 1990; Harrison et al., 1990; Richmond, 2004). Environmental factors, such as light input, temperature, and nutrients status not only affect photosynthesis and productivity of cell biomass, but also influence the pattern of the pathway, the activity of cellular metabolism, and the cellular content of lipids and total amount of triglycerides (TAGs) (Richmond, 2004). For biodiesel production, the content of TAGs is of particular interest. According to previous data, light intensity, light-dark cycle, and exogenous carbon dioxide concentration are all potentially important variables in algae growth and TAGs production process. However, it is lacking in literature so far about the work systematically examining these variables, and most of previous work examined the effects of single variables or pairs of variables. Moreover, when working with different algae strains, researchers found varying results (Richmond, 2004). Thus, optimization of operational factors in the specific cultivation system in this study is crucial.

Batch culture is the most common method for cultivation of microalgae cells (Richmond, 2004), however, in this mode, the algae cells are grown in a condition where ambient factor, such as nutrient concentration or cell density, or a physiological factor may change simultaneously, which may strongly affect the development of the

culture. On the other hand, in continuous cultures it is possible to maintain environmental parameters at constant values and make an independent assessment of the effect of each parameter on algae growth and TAGs production (Saoudi-Helis et al., 1994). The fed-batch culture is the most widely used industrial continuous flow culture process (Lee, 1997). In this cultivation mode, a quasi steady state is reached when the biomass concentration and other culture parameters vary in a repeating pattern within a fed-batch cycle (Richmond, 2004). In the fed-batch culture, hydraulic retention time (HRT) is crucial for retaining the concentration of microorganism and available substrate, as well as for washing out of toxic compounds. Establishing a stability boundary based on HRT is one of the most important tasks for the development of semi-continuous cultivation systems. Longer HRTs generally lead to higher biomass concentrations, which are attractive from a downstream processing point of view as they would minimize the energy required for harvesting. However it may eventually fail the system if the nutrients or light are limited resulting in the death of the algae cells. Shorter HRTs are favorable for maintaining high concentrations of available nutrients and allowing better light penetration, however, for algae species with slow proliferation rate, too short a HRT will result in washing out of organism and fail the operation of the reactor (Zhu et al., 2009).

Response surface methodology (RSM), which was introduced by Box and Wilson in 1951, is an effective statistical technique for the investigation of complex process parameters and it has been successfully adapted to many scientific studies (Hill and Hunter, 1966; Shieh et al., 1995; Basri et al., 2007; Cheng et al., 2009). The graphical

representations of these equations, namely response surface, can be used to describe the individual and cumulative effect of the test variables on the response and to determine the mutual interactions between the test variables and their subsequent effect on the response. Previous researchers reviewed its applications in processing, experimental design and data analysis, and they reported that the main advantage of RSM is the reduction of experiment runs needed to provide sufficient information for statistically accepted results (Hill and Hunter, 1966; Myers, 1999). It is a faster and less-expensive method for collecting results than classic full factorial experimentation (Shieh et al., 1995). In this study RSM and the predictive polynomial quadratic equation were used to evaluate the effect of several variables on algae biomass accumulation and biodiesel production.

The objectives of this study are to better understand the relationship among the operational parameters (light intensity, light-dark cycle, exogenous carbon dioxide concentration, and HRT) affecting algae biomass accumulation, biodiesel production and wastewater nutrients removal, and to determine the optimum conditions for the above mentioned responses.

## 9.3 Materials and Methods

### 9.3.1 Experimental design and data analysis

The experiments were designed according to RSM with four factors and three-levels as described by Shieh et al. (1995). In this project, only significant independent variables are involved, therefore, a central composite design was directly employed to estimate a second-degree polynomial model, which can be used to optimize a response.

Central composite design is a design method, useful in response surface methodology, for building a second order model for the response variables without employing a complete full factorial experiment design. In this project a face-centered central composite design was employed to design the experiments for the following four independent variables (1) harvesting rate, which is 1/HRT, $X_1$; (2) light intensity ($\mu molm^{-2}s^{-1}$), $X_2$; (3) lighting period (hours), $X_3$; and (4) exogenous $CO_2$ concentration (%),$X_4$. For the ease of recording, harvesting rate, which is calculated by volume of harvested algae broth divided by total cultivation volume times 100, is used in the place of HRT. The responses in this project were biomass content, biomass yield, biodiesel content, biodiesel yield and the removal rates of $NH_3$, total nitrogen (TN), total phosphorus (TP) and chemical oxygen demand (COD). The coded levels and actual values of the variables are listed in Table 9.1 (a). A total of 27 experiments were performed for the four independent variables according to the design matrix shown in Table 9.1 (b). To avoid bias, 27 runs were performed in a totally random order.

Data was collected after the cultivation system reached quasi steady state and average values are reported. Upon the completion of the experiments, the results were analyzed by Excel and Design Expert 8.0 (Static Made Easy, Minneapolis, MN) (Cheng et al., 2009). Regression analysis was first done with all linear and quadratic terms, insignificant terms were then eliminated with backward elimination, and finally a second order quadratic model was obtained to evaluate the effects of four variables, $X_1$, $X_2$, $X_3$, and $X_4$ on the responses. The coefficient and the fitted regression model were

checked with the statistical F test. If the calculated p value was less than 0.05, then the coefficient was significant and a statistically significant regression model was obtained. Significant items in the model were then used in optimization study.

**Table 9.1 (a)** Coded levels and real values of the independent variables

| Variables | | Coded and real values | | |
|---|---|---|---|---|
| | | -1 | 0 | 1 |
| $X_1$ | Harvesting rate | 1/8 | 3/8 | 5/8 |
| $X_2$ | Light intensity ($\mu molm^{-2}s^{-1}$) | 30 | 110 | 190 |
| $X_3$ | Lighting period (hours) | 4 | 14 | 24 |
| $X_4$ | Exogenous $CO_2$ concentration (%) | 0 | 2.5 | 5 |

**Table 9.1 (b)** Full factorial central composite design matrix

| Experimental runs | $X_1$ | $X_2$ | $X_3$ | $X_4$ | Harvesting rate | Light intensity ($\mu molm^{-2}s^{-1}$) | Lighting period (hours) | Exogenous $CO_2$ concentration (%) |
|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 1 | 0 | 0 | 5/8 | 190 | 14 | 2.5 |
| 2 | 1 | -1 | 0 | 0 | 5/8 | 30 | 14 | 2.5 |
| 3 | -1 | 1 | 0 | 0 | 1/8 | 190 | 14 | 2.5 |
| 4 | -1 | -1 | 0 | 0 | 1/8 | 30 | 14 | 2.5 |
| 5 | 0 | 0 | 1 | 1 | 3/8 | 110 | 24 | 0 |
| 6 | 0 | 0 | 1 | -1 | 3/8 | 110 | 24 | 5.0 |
| 7 | 0 | 0 | -1 | 1 | 3/8 | 110 | 4 | 0 |
| 8 | 0 | 0 | -1 | -1 | 3/8 | 110 | 4 | 5.0 |
| 9 | 0 | 0 | 0 | 0 | 3/8 | 110 | 14 | 2.5 |
| 10 | 1 | 0 | 0 | 1 | 5/8 | 110 | 14 | 5 |
| 11 | 1 | 0 | 0 | -1 | 5/8 | 110 | 14 | 0 |
| 12 | -1 | 0 | 0 | 1 | 1/8 | 110 | 14 | 5 |
| 13 | -1 | 0 | 0 | -1 | 1/8 | 110 | 14 | 0 |
| 14 | 0 | 1 | 1 | 0 | 3/8 | 190 | 24 | 2.5 |
| 15 | 0 | 1 | -1 | 0 | 3/8 | 190 | 4 | 2.5 |
| 16 | 0 | -1 | 1 | 0 | 3/8 | 30 | 24 | 2.5 |
| 17 | 0 | -1 | -1 | 0 | 3/8 | 30 | 4 | 2.5 |
| 18 | 0 | 0 | 0 | 0 | 3/8 | 110 | 14 | 2.5 |
| 19 | 1 | 0 | 1 | 0 | 5/8 | 110 | 24 | 2.5 |
| 20 | 1 | 0 | -1 | 0 | 5/8 | 110 | 4 | 2.5 |
| 21 | -1 | 0 | 1 | 0 | 1/8 | 110 | 24 | 2.5 |
| 22 | -1 | 0 | -1 | 0 | 1/8 | 110 | 4 | 2.5 |
| 23 | 0 | 1 | 0 | 1 | 3/8 | 190 | 14 | 5.0 |
| 24 | 0 | 1 | 0 | -1 | 3/8 | 190 | 14 | 0 |
| 25 | 0 | -1 | 0 | 1 | 3/8 | 30 | 14 | 5.0 |
| 26 | 0 | -1 | 0 | -1 | 3/8 | 30 | 14 | 0 |
| 27 | 0 | 0 | 0 | 0 | 3/8 | 110 | 14 | 2.5 |

### 9.3.2 Algae strain and culture condition

In this study, *Chlorella kessleri* was used due to its high biomass concentration and biodiesel productivity in previous studies. Prior to be transferred on centrate wastewater, algae cells were conserved in BG-11 medium as described in section 4.3.1. Before the start of the experiments, all algae strains were cultivated in centrate for three generations to obtain stable characteristics. The experiments were performed in 1L Roux culture bottles (Corning Inc., USA) containing 560mL cultivation solution with a magnetic stir bar on the bottom mixing the solution at 100 rpm in semi-continuous culture under designed conditions. In all experiments, algae cells were grown under 25±2°C.

### 9.3.3 Source and pretreatment of wastewater

The procedure is same as 3.3.2. The sterilized centrate contains 75.8 mg/L ammonia, 11.4 mg/L total nitrogen (TN), 123.4 mg/L total phosphorus (TP) and 2170 mg/L chemical oxygen demand (COD).

### 9.3.4 Control of light intensity, light-dark cycle and HRT

The light intensities studied were 30, 110 and 190 $\mu molm^{-2}s^{-1}$, and controlled by varying the number of fluorescent lamps as well as the distance between the lamps and the algae culture. The light-dark cycle investigated were 4h, 14h and 24h lighting period, and the dark condition was achieved by wrapping the cultivation system with aluminum foil.

In this study, fed-batch culture was used in which the algae cells were harvested followed by same amount of media added to the system. The HRT is controlled by daily harvesting rate. For example, if the HRT is 8 days, then the daily harvesting rate is 1/8 of the total cultivation solution.

**9.3.5 Supply of exogenous CO2 and adjustment of CO2 concentration**

Three concentrations of $CO_2$-enriched air aeration, namely 0.0% (ambient air), 2.5% $CO_2$ and 5.0% at 0.5 vvm were applied in this study. The aeration system composed of a $CO_2$ tank, an air compressor, and two flow meters was constructed to supply ambient air or $CO_2$-enriched air to the cultivation system. The concentration of the exogenous $CO_2$ was adjusted by the flow rates of ambient air and $CO_2$ gas.

**9.3.6 Analytical methods**

**9.3.6.1 Determination of nutrients concentration and removal rates**

Same as 3.3.4.1.

**9.3.6.2 Analysis of algal biomass concentration and biomass yield**

Algal growth was measured daily by the total volatile suspended solids (TVSS), which represents biomass concentration based on dry weight and was determined according to the standard method (APHA,1995) using 5mL of algae suspension from the flasks. The biomass yield was calculated by biomass concentration times the daily harvested volume.

**9.3.6.3 Determination of fatty acid methyl ester (FAME) composition and yield**

The content of FAME was analyzed at the end of the batch experiments. Algae cells were harvested by centrifugation and then dried by a freeze dryer (Savant Instruments Inc., USA) before analysis. Fatty acid content and composition analysis were performed

by following two consecutive steps including preparation of FAME and GC–MS analysis. The FAME was prepared following a one step extraction-transesterification method by Indarti et al. (2005). The composition and content of FAME was analyzed with GC-MS (Agilent 7890-5975C, USA) equipped with a flame ionization detector and a DB-5-MS capillary column. The oven temperature was set at 80°C, held for 5 minutes, raised to 290°C at 4°C/minute, and held at 290°C for 5 minutes, and the temperature for injector and detector were set at 250°C and 230°C, respectively. The carrier gas (helium) was controlled at 1.2 ml/minute. Chromatographic data were recorded and integrated using the built-in Agilent data analysis software. The compounds were identified in the NIST Mass Spectral Database and quantified by comparing the peak area with that of the standard (C18:2) (Sigma-Aldrich, MO). The biodiesel (FAME) yield was expressed by the content of FAME times the biomass yield.

## 9.4. Results and Discussion

To evaluate the influence of the four variables, harvesting rate, $X_1$; light intensity, $X_2$; lighting period, $X_3$; and exogenous $CO_2$ concentration, $X_4$ on the algae based biodiesel production process towards system optimization, a rapid and reliable approach is to compare the biomass concentration, FAME content, biomass and biodiesel yield and wastewater nutrients removal rate, which are most concerned indicators over those statistically designed conditions. The results of the 27 experiment runs are summarized in Table 9.2. When doing the regression analysis, the data in Run 24 was identified as outliers, thus deleted from the list for further investigation.

**Table 9.2** Responses in 27 experiment runs

| Exp. runs | Biomass concentration (g TVSS/L) | FAME content (% of TVSS) | Biomass yield (g/day) | Biodiesel yield (g/L) | Wastewater nutrient removal rate (%) | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | $NH_4^+$ | TN | TP | COD |
| 1 | 0.1102 | 4.08 | 0.0386 | 0.0016 | 38.97 | 17.84 | 19.43 | 52.56 |
| 2 | 0.1071 | 3.77 | 0.0375 | 0.0014 | 31.87 | 15.78 | 21.52 | 40.57 |
| 3 | 1.4634 | 12.66 | 0.1024 | 0.0130 | 99.86 | 79.73 | 77.26 | 77.82 |
| 4 | 1.0189 | 13.58 | 0.0713 | 0.0097 | 81.29 | 61.83 | 40.43 | 84.61 |
| 5 | 0.6571 | 14.05 | 0.1380 | 0.0194 | 57.66 | 64.90 | 34.67 | 90.71 |
| 6 | 0.3143 | 12.83 | 0.0660 | 0.0085 | 56.58 | 63.77 | 77.14 | 90.61 |
| 7 | 0.3771 | 11.74 | 0.0792 | 0.0093 | 50.30 | 60.55 | 31.89 | 90.72 |
| 8 | 0.2971 | 7.17 | 0.0624 | 0.0045 | 52.35 | 59.64 | 73.89 | 91.35 |
| 9 | 0.4514 | 13.80 | 0.0948 | 0.0131 | 56.00 | 61.60 | 30.55 | 90.72 |
| 10 | 0.3714 | 11.69 | 0.1300 | 0.0152 | 58.78 | 59.63 | 34.12 | 89.85 |
| 11 | 0.1600 | 0.07 | 0.0560 | 0.0000 | 59.37 | 60.06 | 60.70 | 87.90 |
| 12 | 1.3200 | 11.52 | 0.0924 | 0.0106 | 100.00 | 88.26 | 38.75 | 87.42 |
| 13 | 0.8914 | 9.09 | 0.0624 | 0.0057 | 80.26 | 71.18 | 91.74 | 86.20 |
| 14 | 1.0114 | 24.18 | 0.2124 | 0.0514 | 65.33 | 49.02 | 56.51 | 77.77 |
| 15 | 0.4686 | 12.43 | 0.0984 | 0.0122 | 53.23 | 41.58 | 32.86 | 86.08 |
| 16 | 0.6514 | 13.90 | 0.1368 | 0.0190 | 58.80 | 46.53 | 53.36 | 75.97 |
| 17 | 0.3714 | 10.96 | 0.0780 | 0.0086 | 44.37 | 35.73 | 27.31 | 83.42 |
| 18 | 0.5829 | 15.64 | 0.1224 | 0.0191 | 61.39 | 64.74 | 33.08 | 90.55 |
| 19 | 0.2629 | 7.84 | 0.0920 | 0.0072 | 56.89 | 61.08 | 34.77 | 91.49 |
| 20 | 0.2114 | 7.13 | 0.0740 | 0.0053 | 54.84 | 60.47 | 32.72 | 91.26 |
| 21 | 1.6000 | 7.43 | 0.1120 | 0.0083 | 100.00 | 88.63 | 52.03 | 86.97 |
| 22 | 0.7486 | 9.40 | 0.0524 | 0.0049 | 71.95 | 68.79 | 25.95 | 89.64 |
| 23 | 0.8857 | 6.33 | 0.1860 | 0.0118 | 66.90 | 54.18 | 52.68 | 76.85 |
| 24 | 0.5314 | 5.41 | 0.1116 | 0.0060 | 64.10 | 49.51 | 63.21 | 84.57 |
| 25 | 0.5486 | 12.48 | 0.1152 | 0.0144 | 57.99 | 45.22 | 34.40 | 84.04 |
| 26 | 0.4514 | 10.87 | 0.0948 | 0.0103 | 59.37 | 47.60 | 42.22 | 84.26 |
| 27 | 0.5829 | 13.26 | 0.1224 | 0.0162 | 57.88 | 63.41 | 33.46 | 90.73 |

### 9.4.1 Biomass concentration and yield

The design matrix with the corresponding results for biomass concentration and biomass yield in Table 9.2 were subjected to regression analysis. The generated ANOVA tables are shown in Table 9.3 (a) and Table 9.3 (b) and the quadratic model equations are shown in equation 9.1 and 9.2, where $R_1$ and $R_2$ stand for biomass concentration and biomass yield, respectively. The variables, $X_1$, $X_2$, $X_3$, and $X_4$, are in the form of actual values throughout the results and discussion section.

$$R_1=0.5602-1.9321X_1+0.0034X_2+0.0469X_3-0.0055X_1X_2-0.0800X_1X_3+2.2922X_1^2 \quad 9.1$$

$$R_2=0.0609+0.5316X_1+3.654\times10^{-4}X_2+0.0042X_3-3.7500\times10^{-4}X_1X_2-0.0042X_1X_3-0.6050X_1^2 \quad 9.2$$

**Table 9.3** (a). ANOVA of the response surface quadratic model for biomass concentration

| Source | Sum of Squares | df | Mean square | F Value | p-value (Prob > F) |
|---|---|---|---|---|---|
| Model | 3.6541 | 6 | 0.6090 | 25.1287 | < 0.0001 (significant) |
| $X_1$-harvesting rate | 0.1337 | 1 | 0.1337 | 5.5175 | 0.0292 |
| $X_2$-light intensity | 0.0481 | 1 | 0.0481 | 1.9828 | 0.1745 |
| $X_3$-lighting period | 0.1581 | 1 | 0.1581 | 6.5252 | 0.0189 |
| $X_1X_2$ | 0.0487 | 1 | 0.0487 | 2.0097 | 0.1717 |
| $X_1X_3$ | 0.1600 | 1 | 0.1600 | 6.6000 | 0.0183 |
| $X_1$^2 | 0.1368 | 1 | 0.1368 | 5.6455 | 0.0276 |
| Residual | 0.4847 | 20 | 0.0242 | | |
| Lack of Fit | 0.4732 | 18 | 0.0263 | 4.5608 | 0.1948 (not significant) |
| Pure Error | 0.0115 | 2 | 0.0058 | | |
| Correlation Total | 4.1389 | 26 | | | |

**Table 9.3** (b). ANOVA of the response surface quadratic model for biomass productivity

| Source | Sum of squares | df | Mean square | Value | p-value (Prob > F) |
|---|---|---|---|---|---|
| Model | 0.0226 | 6 | 0.0038 | 3.5839 | 0.0141(significant) |
| $X_1$-harvesting rate | 0.0095 | 1 | 0.0095 | 9.0879 | 0.0068 |
| $X_2$-light intensity | 0.0002 | 1 | 0.0002 | 0.2075 | 0.6537 |
| $X_3$-lighting period | 0.0004 | 1 | 0.0004 | 0.3983 | 0.5351 |
| $X_1X_2$ | 0.0002 | 1 | 0.0002 | 0.2143 | 0.6484 |
| $X_1X_3$ | 0.0004 | 1 | 0.0004 | 0.4121 | 0.5282 |
| $X_1$^2 | 0.0095 | 1 | 0.0095 | 9.0792 | 0.0069 |
| Residual | 0.0210 | 20 | 0.0010 | | |
| Lack of Fit | 0.0205 | 18 | 0.0011 | 4.4823 | 0.1978 (not significant) |
| Pure Error | 0.0005 | 2 | 0.0003 | | |
| Correlation Total | 0.0436 | 26 | | | |

For the response of biomass concentration, since the p-value for the model was less than 0.05 and the value of $R^2$ (0.8829) was close to 1, the regression model was considered to be a good representation of the experimental data. The p-values for coefficients of the terms $X_1$, $X_3$, $X_1X_3$ and $X_1^2$ were less than 0.05 while those for others were larger, meaning that harvesting rate and lighting period had more significant effect on the biomass concentration. The effect of light intensity only showed in linear term with

positive coefficient meaning that higher light intensity promoteS higher biomass accumulation. The effect of harvesting rate and lighting period on biomass concentration was plotted at three tested light intensity levels with constant exogenous carbon dioxide concentration at 2.5% as shown in Figure 9.1. Such application allows us to compare all of the factors simultaneously. From the figures we can see that the effects of $X_1$ and $X_3$ on biomass concentration at different light intensity levels were similar with high biomass concentration at long lighting period and low harvesting rate, which is in agreement with our previous data in batch culture. Within the tested ranges, the highest biomass concentration was found around 1.4g/L at harvesting rate of 1/8, lighting period of 24 hours, and light intensity of 190μmolm$^{-2}$s$^{-1}$.

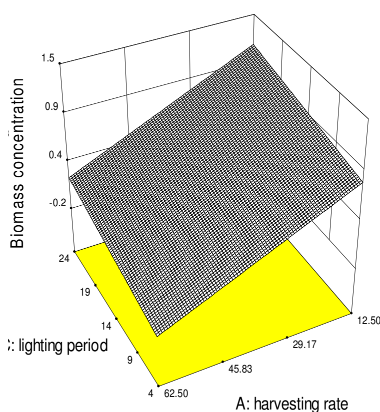



Figure 9.1 (a)

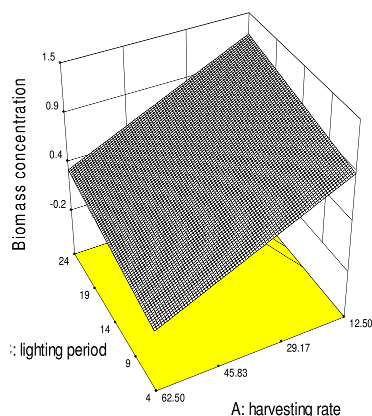



Figure 9.1 (b)

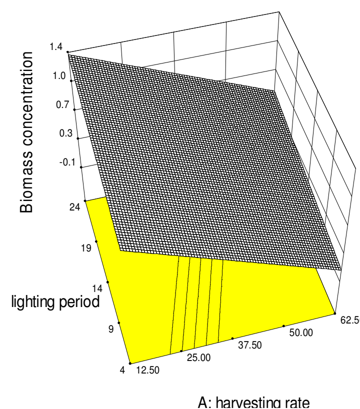



Figure 9.1 (c)

**Figure 9.1** Response surface plot representing the effects of harvesting rate and lighting period on biomass concentration. (a) light intensity at 30 μmolm$^{-2}$s$^{-1}$, 2.5 % $CO_2$; (b) light intensity at 110 μmolm$^{-2}$s$^{-1}$, $CO_2$ 2.5%; (c) light intensity at 190 μmolm$^{-2}$s$^{-1}$, $CO_2$ 2.5%

For biomass yield, the p-value for the regression model was less than 0.05 suggesting that the fitted model is significant in illustrating the data. The p-values for the

coefficients reveal that the quadratic form of harvesting rate had the most significant effect on biomass yield. The coefficients of the regression equation are used as a basis for judging statistical significance and illustrating the relative effects of linear, quadratic and interaction between the variables. A negative value of coefficient for $X_1^2$ suggests the plot of biomass yield on harvesting rate would be a parabola facing down as shown in Figure 9.2. Since the calculated optimum harvesting rate is much larger than the upper limit tested, the optimization analysis was done according to the plots shown in Figure 2. According to the analysis, the optimum harvesting rate for biomass yield within the tested range is 1/3, and when the harvesting rate was controlled between 12.5% and 50%, 85% of maximum biomass yield would be obtained. The effects of light intensity and lighting period on biomass yield were found similar to that on biomass concentration with high biomass yield occurred under long lighting period and high light intensity.

In the regression analysis shown in equation 9.1 and 9.2, exogenous carbon dioxide concentration was eliminated from the model, suggesting that in the fed-batch culture system exogenous carbon dioxide concentration had insignificant effect on biomass concentration at significance level of 0.05. Previous research shows that higher biomass accumulation was observed when using $CO_2$-enriched air injection systems with autotrophic cultures (Chiu et al., 2008; Yang and Gao, 2003). However, for the cultivation system in this study, our pervious data shows the algae cells perform a mixotrophic growth, in which organic and inorganic carbon source are both used for algae growth (Richmond, 2004). Matrínez et al. (1997) found that when *Chlorella*

*pyrenoidosa* was cultivated under moderate light condition, an exponential mixotrophic phase was observed first and when the organic substrate was completely consumed, an autotrophic phase followed after an adaptation period. It is suspected that the algae strain used in this study had similar growth mode with the above stated algae species. Since the nutrient in the cultivation system was added daily, it is reasonable to assume that organic carbon and the inorganic carbon in the centrate is sufficient to support algae growth, and there is no need to add exogenous carbon dioxide for the purpose of improving biomass concentration and yield.

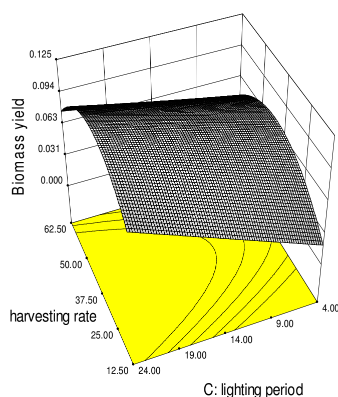



Figure 9.2 (a)

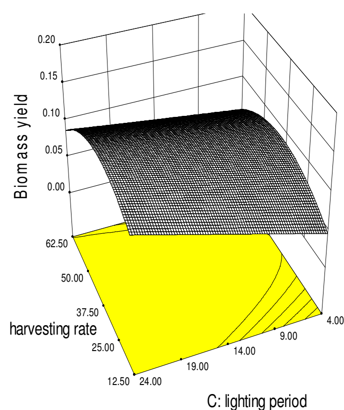



Figure 9.2 (b)

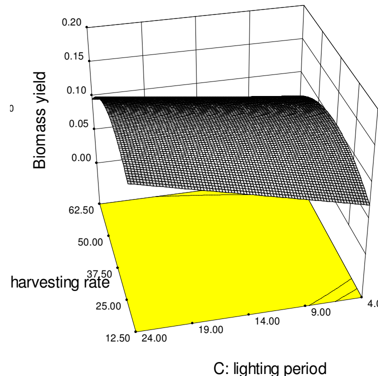



Figure 9.2 (c)

**Figure 9.2** Response surface plot representing the effects of harvesting rate and lighting period on biomass yield. (a) light intensity at 30 µmolm$^{-2}$s$^{-1}$, 2.5 % $CO_2$; (b) light intensity at 110 µmolm$^{-2}$s$^{-1}$, $CO_2$ 2.5%; (c) light intensity at 190 µmolm$^{-2}$s$^{-1}$, $CO_2$ 2.5%

### 9.4.2 Biodiesel (FAME) content and yield

Modeling of the biodiesel content and yield with the four variables was also done by a response surface analysis. Table 9.4 (a) and (b) summarize the ANOVA results for biodiesel content and yield, respectively. The quadratic model equations are shown in

equation 9.3 and 9.4, where $R_3$ and $R_4$ stand for biodiesel content and biodiesel yield, respectively.

$$R_3=3.1349-46.9038X_1+2.6343X_4-75.4717X_1^2-0.4549X_4^2 \quad 9.3$$

$$R_4=8.6833\times10^{-4}+0.0911X_1-9.2042\times10^{-5}X_2-3.5875\times10^{-4}X_3-1.5000\times10^{-4}X_1X_3+9.0000\times10^{-6}X_2X_3-0.1282X_1^2 \quad 9.4$$

**Table 9.4** (a) ANOVA of the response surface quadratic model for biodiesel content

| Source | Sum of squares | df | Mean square | F value | p-value (Prob > F) |
|---|---|---|---|---|---|
| Model | 246.7313 | 4 | 61.6828 | 4.2169 | 0.0110 (significant) |
| $X_1$-harvesting rate | 142.8919 | 1 | 142.8919 | 9.7687 | 0.0049 |
| $X_4$-carbon dioxide conc. | 9.7020 | 1 | 9.7020 | 0.6633 | 0.4241 |
| $X_1$^2 | 142.3993 | 1 | 142.3993 | 9.7351 | 0.0050 |
| $X_4$^2 | 51.7373 | 1 | 51.7373 | 3.5370 | 0.0733 |
| Residual | 321.8039 | 22 | 14.6274 | | |
| Lack of Fit | 318.6900 | 20 | 15.9345 | 10.2345 | 0.0927(not significant) |
| Pure Error | 3.1139 | 2 | 1.5569 | | |
| Correlation Total | 568.5352 | 26 | | | |

**Table 9.4** (b) ANOVA of the response surface quadratic model for biodiesel yield

| Source | Sum of squares | df | Mean Square | Value | p-value (Prob > F) |
|---|---|---|---|---|---|
| Model | 0.0012 | 6 | 0.0002 | 3.1138 | 0.0255 (significant) |
| $X_1$-harvesting rate | 0.0004 | 1 | 0.0004 | 6.9042 | 0.0161 |
| $X_2$-light intensity | 0.0001 | 1 | 0.0001 | 1.4268 | 0.2463 |
| $X_3$-lighting period | 0.0000 | 1 | 0.0000 | 0.0073 | 0.9328 |
| $X_1$ $X_3$ | 0.0000 | 1 | 0.0000 | 0.0091 | 0.9251 |
| $X_2$ $X_3$ | 0.0002 | 1 | 0.0002 | 3.3406 | 0.0825 |
| $X_1$^2 | 0.0004 | 1 | 0.0004 | 6.8938 | 0.0162 |
| Residual | 0.0012 | 20 | 0.0001 | | |
| Lack of Fit | 0.0012 | 18 | 0.0001 | 7.5492 | 0.1232 (not significant) |
| Pure Error | 0.0000 | 2 | 0.0000 | | |
| Correlation Total | 0.0024 | 26 | | | |

The regression analysis for the biodiesel content shows that harvesting rate and exogenous carbon dioxide concentration had significant effect on the response at a significant level of 0.05. The calculated optimum harvesting rate is 31.07% and optimum exogenous carbon dioxide concentration is 2.90% according to equation 9.3.

The accumulation of TAGs is closely related to the nutrients status, especially nitrogen and C/N ratio (Richmond, 2004). When growing under nitrogen-limited conditions, the most striking effect is the active and specific degradation of phycobilisomes, and the flow of the carbon fixed in photosynthesis under these circumstances is diverted from protein synthesis to that leading to lipid or carbohydrate synthesis (Richmond, 2004; Collier and Grossman, 1992). Richmond (1986) reported that some *Chlorella* strains showed significant neutral lipid accumulation under nitrogen starvation conditions. Harvesting rate directly affected the available nutrients in the cultivation system and exogenous carbon dioxide concentration has influence on C/N ratio, thus the two factors had significant effects on FAME content.

The effect of lighting period and harvesting rate on biodiesel yield at different light intensity levels at constant exogenous carbon dioxide concentration (2.50%) is shown in Figure 3. The regression analysis shows that harvesting rate, light intensity and lighting period should all be included in the mode and the optimum value is 34.93% for harvesting rate, 10.22 hours for lighting period. The optimum light intensity was obtained from the plots, which is 190μmol$m^{-2}s^{-1}$, since the calculated optimum light intensity is well above 190μmol$m^{-2}s^{-1}$. Under all light intensity levels, the response surface were dome shape, but at different light intensity levels tested, the effect of harvesting rate and lighting period on the response varied. Generally, the higher the light intensity, the higher the biodiesel yield.

One concern about the regression model for biomass yield, FAME content and biodiesel yield is that the values of $R^2$ (0.5181 for biomass yield, 0.5902 for FAME content and 0.4829 for biodiesel yild) were well below 1, meaning that the experimental and predicted values are not in very good agreement and the model is not adequate enough to predict the data. Thus, one needs to be cautious in using these models.

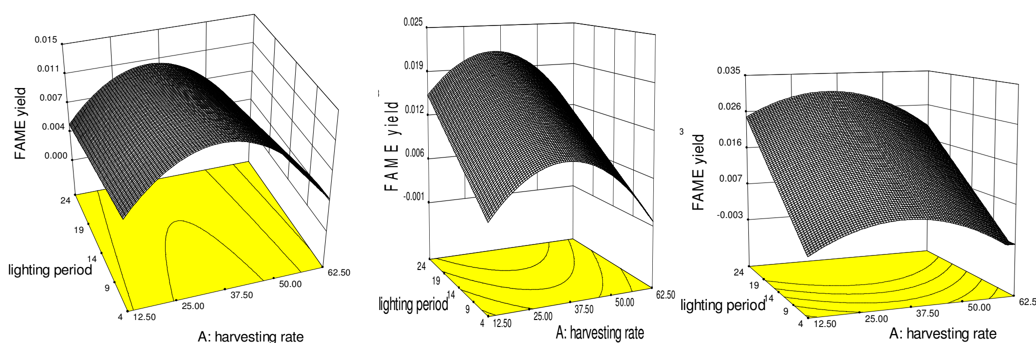



Figure 9.3 (a) Figure 9.3 (b) Figure 9.3 (c)

**Figure 9.3** Response surface plot representing the effects of harvesting rate and lighting period on biodiesel yield. (a) light intensity at 30 µmolm$^{-2}$s$^{-1}$, 2.5 % $CO_2$; (b) light intensity at 110 µmolm$^{-2}$s$^{-1}$, $CO_2$ 2.5%; (c) light intensity at 190 µmolm$^{-2}$s$^{-1}$, $CO_2$ 2.5%

### 9.4.3 Wastewater nutrients removal rate

Similarly, by applying regression analysis on the experimental data, the effects of the four variables on nutrients removal rates were evaluated. The ANOVA tables are listed in Table 9.5 (a) to (d).

**Table 9.5** (a) ANOVA of the response surface quadratic model for $NH_4^+$ removal rate

| Source | Sum of squares | df | Mean Square | Value | p-value (Prob > F) |
|---|---|---|---|---|---|
| Model | 6312.9129 | 6 | 1052.1521 | 18.7978 | < 0.0001 |
| $X_1$-harvesting rate | 953.2951 | 1 | 953.2951 | 17.0316 | 0.0005 |
| $X_2$-light intensity | 32.1893 | 1 | 32.1893 | 0.5751 | 0.4571 |
| $X_3$-lighting period | 167.0126 | 1 | 167.0126 | 2.9839 | 0.0995 |
| $X_1 X_2$ | 32.8902 | 1 | 32.8902 | 0.5876 | 0.4523 |
| $X_1 X_3$ | 169.0000 | 1 | 169.0000 | 3.0194 | 0.0977 |
| $X_1$^2 | 963.7370 | 1 | 963.7370 | 17.2182 | 0.0005 |
| Residual | 1119.4407 | 20 | 55.9720 | | |
| Lack of Fit | 1104.4718 | 18 | 61.3595 | 8.1983 | 0.1141 |

| | | | | | |
|---|---|---|---|---|---|
| Pure Error | 14.9689 | 2 | 7.4844 | | |
| Correlation Total | 7432.3536 | 26 | | | |

**Table 9.5** (b). ANOVA of the response surface quadratic model for TN removal rate

| | Sum of | | Mean | F | p-value |
|---|---|---|---|---|---|
| Source | Squares | df | Square | Value | Prob > F |
| Model | 6460.5583 | 14 | 461.4684 | 5.1314 | 0.0036 (significant) |
| $X_1$-harvesting rate | 71.8565 | 1 | 71.8565 | 0.7990 | 0.3890 |
| $X_2$-light intensity | 62.0310 | 1 | 62.0310 | 0.6898 | 0.4225 |
| $X_3$-lighting period | 91.4316 | 1 | 91.4316 | 1.0167 | 0.3332 |
| $X_4$-carbon dioxide concentration | 76.3171 | 1 | 76.3171 | 0.8486 | 0.3751 |
| $X_1 X_2$ | 62.7264 | 1 | 62.7264 | 0.6975 | 0.4199 |
| $X_1 X_3$ | 92.4482 | 1 | 92.4482 | 1.0280 | 0.3306 |
| $X_1 X_4$ | 76.6500 | 1 | 76.6500 | 0.8523 | 0.3741 |
| $X_2 X_3$ | 2.8224 | 1 | 2.8224 | 0.0314 | 0.8623 |
| $X_1 X_4$ | 12.4256 | 1 | 12.4256 | 0.1382 | 0.7166 |
| $X_3 X_4$ | 0.0121 | 1 | 0.0121 | 0.0001 | 0.9909 |
| $X_1$^2 | 73.8875 | 1 | 73.8875 | 0.8216 | 0.3825 |
| $X_2$^2 | 2105.5135 | 1 | 2105.5135 | 23.4128 | 0.0004 |
| $X_3$^2 | 0.6596 | 1 | 0.6596 | 0.0073 | 0.9332 |
| $X_4$^2 | 36.7383 | 1 | 36.7383 | 0.4085 | 0.5347 |
| Residual | 1079.1594 | 12 | 89.9299 | | |
| Lack of Fit | 1074.1912 | 10 | 107.4191 | 43.2427 | 0.0228 (significant) |
| Pure Error | 4.9682 | 2 | 2.4841 | | |
| Correlation Total | 7539.7176 | 26 | | | |

**Table 9.5** (c). ANOVA of the response surface quadratic model for TP removal rate

| Source | Sum of squares | df | Mean Square | Value | p-value (Prob > F) |
|---|---|---|---|---|---|
| Model | 2773.7503 | 4 | 693.4376 | 2.3290 | 0.0879 (not significant) |
| $X_1$-harvesting rate | 25.3391 | 1 | 25.3391 | 0.0851 | 0.7732 |
| $X_4$-carbon dioxide conc. | 15.0752 | 1 | 15.0752 | 0.0506 | 0.8240 |
| $X_1$^2 | 26.2251 | 1 | 26.2251 | 0.0881 | 0.7694 |
| $X_4$^2 | 1492.8971 | 1 | 1492.8971 | 5.0141 | 0.0356 |
| Residual | 6550.3378 | 22 | 297.7426 | | |
| Lack of Fit | 6545.3334 | 20 | 327.2667 | 130.7898 | 0.0076 (significant) |
| Pure Error | 5.0045 | 2 | 2.5022 | | |
| Correlation Total | 9324.0882 | 26 | | | |

**Table 9.5** (d). ANOVA of the response surface quadratic model for COD removal rate

| | Sum of | | Mean | F | p-value |
|---|---|---|---|---|---|
| Source | Squares | df | Square | Value | Prob > F |
| Model | 2262.4692 | 14 | 161.60494 | 1.4469338 | 0.2636(not significant) |
| $X_1$-harvesting rate | 274.4583 | 1 | 274.4583 | 2.4574 | 0.1430 |
| $X_2$-light intensity | 88.2294 | 1 | 88.2294 | 0.7900 | 0.3916 |
| $X_3$-lighting period | 2.0412 | 1 | 2.0412 | 0.0183 | 0.8947 |
| $X_4$-carbon dioxide concentration | 0.1298 | 1 | 0.1298 | 0.0012 | 0.9734 |
| $X_1 X_2$ | 88.1721 | 1 | 88.1721 | 0.7895 | 0.3917 |
| $X_1 X_3$ | 2.1025 | 1 | 2.1025 | 0.0188 | 0.8931 |
| $X_1 X_4$ | 0.1332 | 1 | 0.1332 | 0.0012 | 0.9730 |
| $X_2 X_3$ | 0.1849 | 1 | 0.1849 | 0.0017 | 0.9682 |
| $X_1 X_4$ | 14.0625 | 1 | 14.0625 | 0.1259 | 0.7289 |
| $X_3 X_4$ | 0.1332 | 1 | 0.1332 | 0.0012 | 0.9730 |

| | | | | | |
|---|---|---|---|---|---|
| $X_1$^2 | 273.1938 | 1 | 273.1938 | 2.4460 | 0.1438 |
| $X_2$^2 | 1102.5945 | 1 | 1102.5945 | 9.8721 | 0.0085 |
| $X_3$^2 | 41.9753 | 1 | 41.9753 | 0.3758 | 0.5513 |
| $X_4$^2 | 36.5170 | 1 | 36.5170 | 0.3270 | 0.5780 |
| Residual | 1340.2543 | 12 | 111.6879 | | |
| Lack of Fit | 1340.2338 | 10 | 134.0234 | 13096.7476 | < 0.0001 (significant) |
| Pure Error | 0.0205 | 2 | 0.0102 | | |
| Correlation Total | 3602.7235 | 26 | | | |

For ammonium ($NH_4^+$) removal rate, statistical test results showed that the p-value of the F-test was well below 0.05 and the $R^2$ of this equation was 0.7601 (close to 1), reflecting the high significance of the model, and meaning that the regression model was suitable for accurately explaining the experimental data. The fitted quadratic model was shown in equation 9.5. A close examination at equation 9.5 reveals that it is in the similar mode with equation 9.1, which in turn proves that ammonia is mainly assumed by algae growth to synthesize biomass (Li, et al., 2011)

$$R_5=79.8249-169.6554X_1+0.1107X_2+1.5435X_3-0.1434X_1X_2-2.6000X_1X_3+192.3733 \quad 9.5$$

The contour plots with light intensity and lighting period as variables at different harvesting rate are shown in Figure 9.4. The analysis performed with contour plots shows that the maximum ammonia removal rate of 100% could be achieved by control the harvesting rate below 16% (Figure 9.4 (a)) and more than 85% of ammonia removal rate was achieved if the harvesting rate could be controlled below 25%. When the harvesting rate increased to 35%, the removal rate of ammonia would be decreased to 70%.

For total nitrogen removal rate, it shows that although the fitted model is significant, it is not accurate enough to explain the data obtained since p-value the lack-of-fit is well below 0.05, suggesting that a higher order model or a transform of response is required.

The p-values of the fitted model for total phosphorus and COD removal rate are larger than 0.05, illustrating that the regression models are not significant. As mentioned in previous study, total phosphorus is mainly removed by sedimentation caused by high pH (Li, et al., 2011), thus, the pH in the culture solution plays an important role than other parameters.

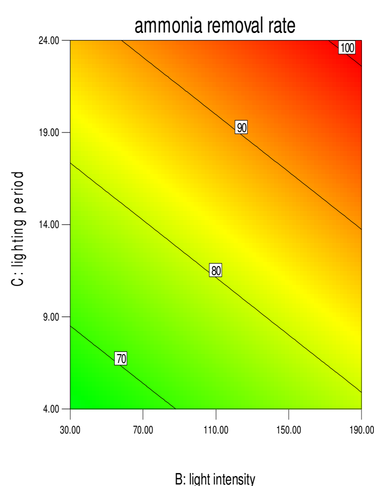



Figure 9.4 (a)

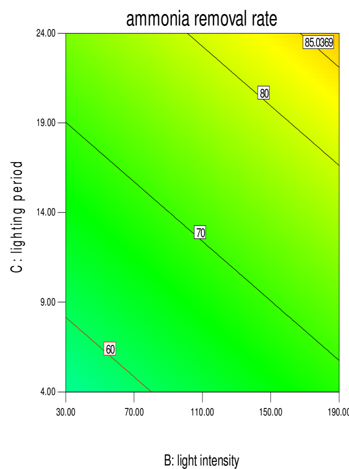



Figure 9.4 (b)

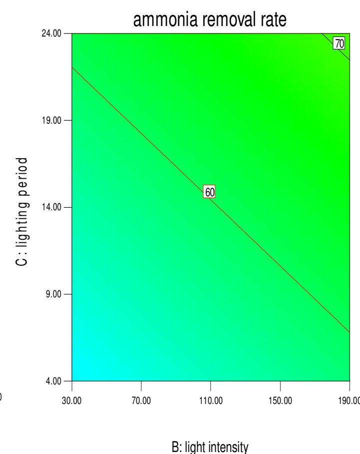



Figure 9.4 (c)

**Figure 9.4** Contour plots representing the effects of light intensity and lighting period on ammonia removal rate(a) harvesting rate at 16%, 2.5 % $CO_2$; (b) harvesting rate at 25%, $CO_2$ 2.5%; (c) harvesting rate at 35%, $CO_2$ 2.5%;

The optimum conditions for each response mentioned above are summarized in Table 9.6. From the table we can see that, the optimum condition varies with different objectives, and one needs to pick their own conditions according to his specific target. For the purpose of biodiesel production from algal biomass cultivated in highly

concentrated municipal wastewater, the optimum condition is obtained at harvesting rate of 35%, light intensity at 190μmolm$^{-2}$s$^{-1}$ and lighting period of 10 hours. Under this condition over 70% of ammonia could be removed.

**Table 9.6** Optimal conditions calculated from regression functions
Note: if optimum value is larger than the tested levels, the upper limit was determined by the contour or response surface plots.

| | Biomass concentration (g TVSS/L) | Biomass yield (g/day) | FAME content (% of TVSS) | FAME yield (g/day) | Wastewater nutrient removal rate (%) | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | $NH_4^+$ | TN | TP | COD |
| Opt. Harvesting rate | 12.50% | 33.00% | 31.07% | 34.93% | 16.00% | N/A | N/A | N/A |
| Opt. light intensity (μmolm$^{-2}$s$^{-1}$) | 190 | 190 | 2.90 | 190 | 190 | N/A | N/A | N/A |
| Opt. lighting period (hours) | 24 | 24 | | 10.22 | 24 | N/A | N/A | N/A |
| Opt. exogenous $CO_2$ conc. (%) | 0.0-5.0% | 0.0-5.0% | 2.9% | 0.0-5.0% | 0.0-5.0% | N/A | N/A | N/A |

### 9.5 Conclusions

RSM with central composite design is effective in system optimization for algal-based biodiesel production using centrate in fed-batch culture. The regression analysis shows that, at p-value< 0.05, harvesting rate, light intensity and lighting period were significant in the models for biomass concentration, biomass and biodiesel yield, and ammonia removal rate. Harvesting rate and exogenous carbon dioxide concentration were significant in the model for FAME content. The removal rates of TN, TP and COD could not be explained by quadratic models. The optimum conditions for biodiesel production are HRT of 2.86 days, which means harvesting 35% of the total volume daily, light intensity of 190μmolm$^{-2}$s$^{-1}$ and lighting period of 10h.

# CHPATER 10 SUMMARY

## 10.1 Introduction

Despite the potential of algae as feedstock for biofuel production, the high cost of algae production remains a hurdle. An algal strain with high lipid content and high growth rate, a cost-effective cultivation system, and low cost harvesting and lipid extraction systems are critical to the future of commercial scale algae to fuel production. In this dissertation, we focused on reduing the cost involved in cultivation process.

Taking into account the fact that the centrate wastewater stream contains various nutrients for micoorganism growth and is available year round, we hypothesized that the centrate is a candidate media suitable for algae cultivation and TAGs accumulation as feedstock for biodiesel production, and we verified this hypotheis mainly by algae strain screening and operational parameter optimization in batch and fed-batch culture system, respectively.

By way of completing the research in this dissertation, several significant accomplishments were achieved: 1) it advanced the knowledge and understanding of cultivating algae with municipal wastewater, specifically centrate, for simultaneous biomass and biodiesel production and nutrient removal; 2) it enhanced manufacturing practice in applying algae cultivation systems by providing key information concerning lighting strategy and hydraulic retention time for improvement and/or optimization of biomass and lipid accumulation; 3) it provided another pathway to recycle flue gas

since supplement of exogenous $CO_2$ was found beneficial for algal biomass and lipid accumulation; 4) it provided knowledge to municipal wastewater treatment plants in selecting an alternative process for waste reduction and nutrient removal; and 5) it delivered valuable information for biodiesel production from renewable sources.

**10.2 Summary of this dissertation**

The objectives of this study are to employ potential algae strains for efficient simultaneous biodiesel production and wastewater nutrients removal, and to develop an operational strategy optimal for biomass and biodiesel production as well as nutrient removal from a highly concentrated municipal wastewater stream, centrate, which is generated during the sludge thickening process.

Chapter 1 of this dissertation describes the background and significance of the study. In Chapter 2, literature is reviewed for the current needs of algae based biodiesel production, the related fundamentals and principals, the factors significantly impacting the algae cultivation process including light intensity, light/dark cycle, exogenous carbon dioxide and hydraulic retention time (HRT), as well as the potential of centrate as cultivation media. Information on the detailed operational parameters is defined for the algae based biodiesel production.

In Chapter 3, the feasibility of growing *Chlorella sp.* on the centrate wastewater stream for simultaneous wastewater treatment and energy production was tested. The characteristics of algal growth, biodiesel production, wastewater nutrient removal and the viability of scale-up and the stability of continuous operation were examined. Two

culture media, namely autoclaved centrate (AC) and raw centrate (RC) were used for comparison. The results showed that by the end of a 14-day batch culture, algae could remove ammonia, total nitrogen, total phosphorus, and chemical oxygen demand (COD) by 93.9%, 89.1%, 80.9%, and 90.8%, respectively from raw centrate, and the fatty acid methyl ester (FAME) content was 11.04% of dry biomass providing a biodiesel yield of 0.12g-biodiesel/L-algae culture solution. The system could be successfully scaled up, and continuously operated at 50% daily harvesting rate, providing a net biomass productivity of 0.92 g-algae/(L·day).

Chapter 4 and Chapter 5 were targeted at screening one or several algae species/strains that can survive and grow well on centrate with high biomass and lipid productivity as well as superior nutrient removal efficiency, determing the growth type (autotrophic growth, heterotrophic growth and mixotropic growth) of the algae cells cultivated on centrate, and investigating significance of environmental factors including ligh intensity, light/dark cyle, and exogenous $CO_2$ on biomass accumulation and biodiesel production using Plackett-Burman experiment design. In this stage the study was carried out in two sections, using aglae strains collected from local lake areas and those purchased from algae commercial bank, respectivity. In Chapter 4, sixty algae-like microorganisms collected from different sampling sites in Minnesota were examined using multi-step screening and acclimation procedures to select high-lipid producing facultative heterotrophic microalgae strains capable of growing on centrate for simultaneous energy crop production and wastewater treatment. Twenty-seven facultative heterotrophic microalgae strains were found, among which seventeen strains

were proved to be tolerant to centrate. These seventeen top-performing strains were identified through morphological observation and DNA sequencing as *Chlorella sp*., *Heynigia sp.*, *Hindakia sp.*, *Micractinium sp.*, and *Scenedesmus sp*.. Five strains were chosen for other studies because of their ability to adapt to centrate, high growth rates (0.455 - 0.498 $d^{-1}$) and higher lipid productivities (74.5 – 77.8 mg $L^{-1}$ $d^{-1}$). These strains are considered highly promising compared with other strains reported in the literature. In Chapter 5, 143 different algae strains from the family of *Chlamydomonas, Chlorella*, *Scenedesmus*, *Ankistrodesmus, Euglena*, *Chloroccum,* and *Botryococcus*, etc. purchased from a number of U.S. institutions were screened use the same multi-step screening and acclimation procedures. The results showed that 14 algae strains from the genus of *Chlorella*, *Haematococcus, Scenedesmus*, *Chlamydomonas,* and *Chloroccum* were capable of growing on centrate. Since the highest net biomass accumulation (2.0143 g/L) was observed with *Chlorella kessleri* (UTEX398) followed by *Chlorella protothecoides* (UTEX25, 1.3089g/L), the two strains were used in further studies. It is found that both algae strains, UTEX398 and UTEX25 were capable of mixotrophic growth when cultivated on centrate. Environmental factors had significant effect on algal biomass accumulation, wastewater nutrients removal and biodiesel production for both strains. Higher light intensity and exogenous $CO_2$ concentration with longer lighting period promote biomass accumulation, FAME formation, removal of COD and TN, while, lower exogenous $CO_2$ concentration promotes phosphorus removal due to higher pH increase.

In Chapter 6, the single effect of light intensity on biomass accumulation, wastewater nutrient removal through algae cultivation, and biodiesel productivity was investigated with algae species *Chlorella kessleri* and *Chlorella protothecoide.* The light intensities studied were 0, 15, 30, 60, 120, 200 μmolm$^{-2}$s$^{-1}$. The results showed that light intensity had profound impact on tested responses for both strains, and the dependence of these responses on light intensity varied with different algae strains. For *Chlorella kessleri*, the optimum light intensity was 120 μmol·m$^{-2}$·S$^{-1}$ for all responses except for COD removal. For *Chlorella protothecoide*, the optimum light intensity was 30 μmol·m$^{-2}$·S$^{-1}$. The major components of the biodiesel produced from algae biomass were 16-C and 18-C FAME, and the highest biodiesel contents were 24.19% and 19.48% of TVSS for *Chlorella kessleri* and *Chlorella protothecoide*, respectively. Both species were capable of wastewater nutrients removal under all lighting conditions with high removal efficiencies. Since *Chlorella kessleri* showed superior capability of biomass accumulation and biodiesel production, it was used in the following sections.

Chapter 7 was aimed at investigating the single effect of light/dark cycle at low light frequency on algae based biodiesel production using centrate wastewater stream with mixotrophic strain *Chlorella kessleri.* The data suggested that the length of lighting period during a day greatly affects the algal biomass accumulation, biodiesel production and wastewater nutrients removal. The biomass concentration, biodiesel content and the removal of ammonia, total nitrogen and total phosphorus all increased with increasing lighting period. However, the removal of COD showed different trend, with higher removal rate observed under shorter lighting period. The results showed that for

maximum biomass accumulation, biodiesel production and wastewater nutrients removal in batch culture system, the optimum lighting period is 16 hours and the cultivation time should be controlled at three days.

In Chapter 8, the effect of supplying different concentrations of exogenous $CO_2$ uder various light intensities at simulated light/dark cycle on biomass accumulation and wastewater nutrients removal was tested with aglae strain *Chlorella kessleri*. The results showed that the optimum $CO_2$ level for algae growth and nutrients removal is 2.5% under light intensity of 190 $\mu mol \cdot m^{-2} \cdot S^{-1}$. When the light intensity reaches certain lower limit, the effect of exogenous $CO_2$ became minimum because the autotrophic process efficiency was reduced, thus the interaction beween the light intensity and exogenous $CO_2$ also need to be considered.

The objective of Chapter 9 was to optimize the operational parameters, including light intensity, light-dark cycle, exogenous carbon dioxide concentration, and hydraulic retention time, for high algae biomass accumulation, biodiesel production and wastewater nutrients removal in designed fed-batch culture system. The results showed that response surface methodology with central composite design is effective in system optimization for algal-based biodiesel production using centrate wastewater in fed-batch culture. The regression analysis illustrates that, at $p\text{-value} < 0.05$, harvesting rate, light intensity and lighting period had significant effect on biomass concentration, biomass and biodiesel yield, and ammonia removal rate. Harvesting rate and exogenous carbon dioxide concentration played important role in FAME content, while the removal rates

of TN, TP and COD could not be explained by quadratic models. The optimum conditions for biodiesel production are harvesting rate of 35%, light intensity of 190µmolm$^{-2}$s$^{-1}$ and lighting period of 10h.

**10.3 Directions for future research**

In view of growth conditions optimization, the process of algae cultivation for simultaneous biodiesel production and wastewater treatment appeared to have great potential to be developed as a practical algae and biodiesel procution system. Substantial economic improvements, however, can be made through algae strain screening and genetic modification, derect or *in situ* transesterification for biodiesel production, and development of biorefinary based production system.

**10.3.1 Algae strain screening and genetic modification**

Algae species and strains vary greatly in terms of growth rate and productivity, nutrient and light requirement, ability to accumulate lipids or other desirable compounds, ability to adapt to adverse conditions, etc (Chen et al., 2009). For the specific cultivation system mentionted in this study, the criteria for the selected algae strains can be summarized as ability of mixotrophic growth on centrate, high growth rate, high cell concentration, high lipid content especially TAGs, and superior capability of nutrients removal from wastewater. According to previous studies, three approaches could be proposed. One is to select strains from environments where the strains grow under similar conditions, i.e., wastewater treatment plants. In this research, we also collected several strains from the metro plant, which showed some desired charactreristics. However, low lipid content and biomass accumulation were found among those strains. Another strategy is to acclimate the natural strains to adapt to the centrate environment

in which these strains do not normally grow well. For example, in this study, to adapt an algal strain to concentrated wastewater environment, one may gradually increase the amount of wastewater added to an artificial medium and ultimately grow the strain in 100% wastewater. A third strategy is to use genetic approach to probe, understand, and modify regulation of key metabolisms pathways important to all performance parameters in the above mentioned screening criteria. Research in this area is still at its very early stage and slow in progress. In some early studies, researchers were able to isolate Acetyl CoA Carboxylase (ACCase) from a diatom, an enzyme found to be responsible for catalyzing a key metabolic step in lipid synthesis in algae (Roessler and Ohlrogge, 1993; Dunahay et al., 1996) .However, over-expression of the ACCase gene did not demonstrate increased oil production in the cells (Sheehan et al., 1998). Some basic researches such as genome sequence (Merchant et al., 2007), insertional mutagenesis (Tam and Lefebvre, 1993), RNA interference (RNAi) methods (Fuhrmann et al., 2001; Sineshchekov et al., 2002), molecular map (Kathir et al., 2003), annotations of lipid genes (Riekhof, 2005; 2008) are expected to speed up the genetic modification of algae.

### 10.3.2 Direct or In situ transesterification

Conventional transesterification process requires costly oil extraction and separation. If fatty acid containing lipids are simultaneously extracted and transesterified, it would eliminate the need to extract and separate the lipids and fatty acids contained in the algae (Chen et al., 2009). Direct or *in situ* transesterification has been proven in a number of feedstocks including marine tissues (Meier et al., 2006), yeast and fungi (Liu and Zhao, 2007), bacteria (Dionisi, 1999), microheterotrophs (Lewis et al., 2000), algae

fatty acid (Pyle et al., 2008), and municipal primary and secondary sludge (Mondala et al., 2009). Johnson and Wen (2009) compared *in situ* transesterification of dry and wet algae with conventional transesterification of extracted oil. *In situ* transesterification of dry algae with extraction solvent (chloroform, hexane, or petroleum ether) added resulted in higher crude biodiesel yield than the conventional transesterification process. *In situ* transesterification of wet algae compared unfavorably with conventional process. A very recent study by Ehimen et al. (2010) examined the key variables such as alcohol volume, moisture content, temperature, reaction time, and mixing on an acid-catalyzed *in situ* transesterification process for production of biodiesel from microalgae lipids. They found that the *in situ* transesterification was inhibited when the biomass water content was greater than 115% w/w (based on oil weight). More efforts to evaluate and improve this method are worthwhile.

### 10.3.3 Biorefinery based production system

In addition to lipids (30%-50%), algal biomass also contains 20%-40% protein, 20% carbohydrate, and 10% other compounds (Chen et al., 2009). Depending on the conversion processes, a range of products can be obtained from algal biomass. Biorefining is a system approach, in which every component of the biomass raw material was used to produce useable products (Chisti, 2007). It is capable of producing multiple product streams and thus multiple income streams from a single biomass feedstock and, therefore, more economically viable than single product-based production schemes. A microalgal biorefinery can simultaneously produce biodiesel, animal feed, biogas and electrical power as shown in Figure 10.1. It is also feasible to extract other high-value products such as DHA or EPA, depending on the specific

microalgae used. Some of the residual biomass after biodiesel produciton may be used to produce methane by anaerobic digestion, for generating the electrical power necessary for running the microalgal biomass production facility (Chen et al., 2009; Chisti, 2007). Development of new processes, design of the system, and life cycle analysis are necessary for the development and implementation of algae based biorefineries.

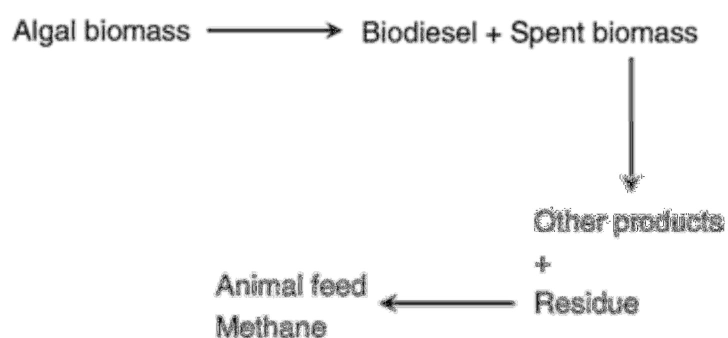



**Figure 10.1** Algae biorefinary process (Chisti, 2007)

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