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Microalgal Lipids Production and N/P Removal Using the Green Alga *Neochloris Oleoabundans*

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Department of Chemical and Biological Engineering

Microalgal Lipids Production and N/P Removal Using the Green Alga *Neochloris Oleoabundans*

Thèse de MASc

Département de génie chimique et biologique

Production de lipides et enlèvement de l'azote et du phosphore à l'aide de l'algue verte *Neochloris Oleoabundans*

by/par

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ABSTRACT

A *Neochloris oleoabundans* strain screened in our lab was demonstrated to have the ability to accumulate large quantities of triglycerides, the feedstock of biodiesel production, and to remove nitrogen and phosphorus from wastewater efficiently. Box-Behnken experimental design (BBD) and response surface method (RSM) were used to study the effects of a large number of nutrients on cell growth and lipid accumulation. The optimized medium supported a lipid concentration of 641.4 mg/l and a cell density of 2.54 g/l, which were 4.5-fold and 2.2-fold of that obtained with the basic medium, modified Bristol medium (MBM), respectively. The capacity of the strain for phosphorus removal and nitrogen removal was investigated using artificial wastewater and municipal wastewater effluents. It was demonstrated that the strain could achieve near-zero residual phosphate and nitrogen at the end of cultivation when artificial wastewater contained up to 140 mg $N\text{-}NO_3^-$/l and 47 mg $P\text{-}PO_4^{3-}$/l. The rates of the removal of $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$, are 27.5 mg/l·d, and 9.4 mg/l·d, respectively under optimal conditions. It was observed that the nitrogen content of the wastewater effluent was not sufficient and additional nitrogen source (e.g., $NaNO_3$) of 70 mg/l was required for optimal cell growth, CO_2 fixation, and phosphate removal.

RÉSUMÉ

L'algue verte *Neochloris oleoabundans* cultivée à partir de souche sélectionnée dans le laboratoire est capable d'accumuler de grandes quantités de triglycerides, matières premières pour la production du biodiésel, en plus de métaboliser l'azote et le phosphore permettant ainsi son utilisation à des fins de Polissage d'effluents d'eau usée. L'approche expérimentale Box-Behnken (BBD) ainsi que la méthode des surfaces de réponses (RSM) furent utilisées dans le choix des conditions expérimentales et de l'analyse des résultats expérimentaux. L'utilisation du milieu de culture optimisé a permis d'obtenir une densité cellulaire de 2.54 g/l et un rendement de 641.4 mg lipide/l soit 2.2 et 4.5 fois les rendements obtenus en utilisant le milieu de culture traditionnel soit le milieu de Bristol modifié. La capacité de *N. oleoabundans* à métaboliser l'azote et le phosphore des eaux usées fut aussi investiguée et les résultats démontrent que *N. oleoabundans* peut retirer la quasi-totalité de l'azote et du phosphore d'une eau usée synthétique contenant 140 mg/l $N\text{-}NO_3^-$ et 47 mg $P\text{-}PO_4^{3-}$ /l à des taux de 27.5 mg/l·d, and 9.4 mg/l·d respectivement. Lorsqu'un effluent d'eau usée municipale fut utilisé pour la culture de l'algue, aucun effet délétère ne fut observé sur la croissance de l'algue. Cependant, afin de permettre une croissance optimale, la fixation du CO_2 ou l'assimilation du phosphore, 70 mg/l d'azote sous forme de $NaNO_3$ a dû être ajouté à l'effluent municipal.

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ABBREVIATIONS

TAG	triacylglycerol
DCW	dry cell weight
IPCC	Intergovernmental Panel on Climate Change
FAME	fatty acid methyl esters
BBD	Box-Behnken experimental design
RSM	response surface method
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
ATP	adenosine triphosphate
ADP	adenosine diphosphate
PS I	photosystem I
PS II	photosystem II
NADPH	nicotinamide adenine dinucleotide phosphate
MBM	modified Bristol medium
PUFAs	polyunsaturated fatty acids

NOTATIONS

μ_{max}	maximum specific growth rates
P_i	phosphate
Y	predicted response
x_i, x_j	independent variables
R^2	coefficient of determination
$\beta_0, \beta_i, \beta_{ii}, \beta_{ij}$	constant coefficients of Equation (4,5)
R_i	rate of the removal of a substrate of interest
S_i	the corresponding substrate concentration

Chapter 1: Introduction

Most the energy carriers on earth originate ultimately from the sun. Examples of different forms of sun-origin energy include fossil fuels, solar energy, biofuels, wind power, and hydropower. Hypothetically, fossil fuels such as coal, oil and natural gas were converted from biomass under high pressure high temperature condition at the absence of oxygen when large quantities of biomass were buried beneath the earth surface due to catastrophic changes of the earth shell and were accumulated over billions of years in the past of earth history and are therefore practically unrenewable. The other sun-origin energies are renewable and shall contribute to the solution of the sustainable energy cycle in the future. Wind energy and hydropower are available at limited scales and solar energy, when converted to electricity directly using solar panels, is ultimately limited by the availability of silicon, a rare element on earth. As a result, biofuels derived from different biomasses have been regarded as the most promising renewable energy sources.

A large diversity of different biofuels has been developed in the past few decades and some of them have achieved great commercial success. Most important examples of these biofuels include bio-ethanol/butanol, biodiesel, biohydrogen, bio-gas, bio-oil, and bio-syngas. Biomasses from different sources, including forestry, agricultural, and aquatic sources have been investigated as the feedstock for the production of different biofuels. However, burning fuels derived from existing biomass has an environmental impact similar to the combustion of fossil fuels in terms of the carbon cycle, i.e.,

conversion of fixed carbon into CO_2. In addition, depletion of certain existing biomasses (e.g., wood) without appropriate compensation (e.g., replanting) may result in massive biomass deficit, creating serious environmental problems (e.g., deforestation).

Conventional terrestrial plants are not very efficient in capturing solar energy. It was estimated that switch grass, the fastest-growing terrestrial crops, can convert solar energy to biomass-energy at a yearly rate of no more than 1 W/m^2, less than 0.5% of the solar energy received at a typical mid-latitude region (200–300 W/m^2) (Lewis and Nocera, 2006, UN, 2003). On the other hand, studies have shown the photosynthetic efficiency of microalge could well be in the range of 10 - 20% or higher (Richmond, 2000)(Huntley and Redalje, 2007). Furthermore, recent studies showed that the extra N_2O entering the atmosphere as a result of using nitrogen fertilizers to produce crops for biofuels, when calculated in CO_2-equivalent global warming terms, and compared with the quasi-cooling effect of saving emissions of fossil fuel derived CO_2, could contribute as much or more to global warming by N_2O emissions than cooling by fossil fuel savings (Crutzen et al., 2007). These concerns may be addressed by using fast-growing microalgal species for biofuel production.

Microalgae have high growth rates and photosynthetic efficiencies due to their simple structures. It is estimated that the biomass productivity of microalgae can be 50 times more than that of switch grass (Nakamura, 2006, Demirbas, 2006). Biofuels production using microalgal farming offers the following advantages: 1) high growth rate of microalgae makes it possible to satisfy the massive demand on biofuels using

limited land resources without causing potential biomass deficit; 2) microalgal cultivation consumes less water than land crops; 3) tolerance of microalgae to high-CO_2 content in gas streams allows high-efficiency CO_2 mitigation; 4) nitrous oxide release could be minimized when microalgae are used for biofuel production; 5) microalgal farming could be potentially more cost effective than conventional farming. 6) nitrogen and phosphorus in wastewater could be removed by microalgae. Microalgae was initially examined as a potential replacement fuel source for fossil fuels in the 1970's amidst the gas scare (W. Barkley 1987), but prohibitive production costs and limitations discouraged the commercial development of algae-based fuel production. Subsequent studies, continued through the 1980's and heightened in the last 15 years, illustrate that research developments are enabling the commercial potential of microalgae to shift from aquaculture, fine chemicals, and health food (De la Noue 1988) to fuel production.

Neochloris oleoabundans belongs to the oil-rich group of microalgae due to its characteristics of producing more than 30% total lipids, which are potential feedstock for biodiesel production. Besides, this strain has high nitrogen and phosphorus removal capacity, which enables cost-effective nitrogen and phosphorus removal from wastewater effluents.

The objectives of this thesis are twofold: 1) maximize lipid production by optimizing medium components, and 2) investigate the capacity of N/P removal of this strain under different conditions. This thesis is composed of five chapters: chapter 1 gave brief introduction of biofuels from microalgae; chapter 2 summarized

a comprehensive literature review. Chapter 3, which is entitled "Optimizing the lipid production of the green alga *Neochloris oleoabundans* using Box-Behnken experimental design in combination with factor grouping, reported the results regarding medium optimization aiming to improve lipid production of *N. oleoabundans*. In Chapter 4, nitrogen and phosphorus removal capacity of this strain was investigated in order to explore the potential of applying the combined biofuel production, CO_2 bio-sequestration, and wastewater treatment strategy. Finally, conclusions of this study were summarized in Chapter 5.

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Chapter 2: Literature review

2.1 Microalgae

2.1.1 Microalgal classification and common microalgal divisions

Microalgae, defined conventionally for the purpose of this book as all unicellular and simple multi-cellular photosynthetic microorganisms including both prokaryotic microalgae (cyanobacteria) and eukaryotic microalgae, are the most important primary producer of the oceans. They are also widely found in other habitats such as lakes, rivers, ponds, wet lands, deserts and even the north and south poles. It was estimated that there are one to ten million microalgal species on the earth (Bold HC 1985) and more than 40,000 species have been identified to date. The vast diversity of microalgal species and their capability of high-efficiency photosynthesis for fast growing, solar energy capturing and CO_2 fixation make them the most promising bio-species for CO_2 bio-sequestration and biofuels production.

The microalgal species that have been identified to date can been classified into 11 divisions (10 eukaryotic microalgae plus the prokaryotic cyanobacteria) according to their photosynthetic pigment composition, biochemical constituents, ultrastructure, and life cycle as listed in Table 2.1. Among these microalgae, six classes of them are of primary importance to biofuel production: diatoms (*alias Bacillariophyceae*, belonging to the division Chrysophyta), green algae (Class Chlorophyceae), golden-brown algae (Class Chrysophyceae), prymnesiophytes (Class Prymnesiophyceae), eustigmatophytes (Class Eustigmatophyceae), and blue-green algae or cyanobacteria (Class Cyanophyceae)(Sheehan et al., 1998).

Table 2.1 Main pigments, storage products, and cell coverings of different divisions of microalgae (Laura Barsanti, 2006)

Division	Pigments					
		Chloro -phylls	Phycobilins	Carotenoids	Xanthophylls	Storage Products
Cyanophyta	blue-green algae	A	c-Phycoerythrin c-Phycocyanin Allophycocyanin Phycoerythrocyanin	β-Carotene	Myxoxanthin Zeaxanthin	Cyanophycin (argine & asparagine polymer) Cyanophycean starch (α-1,4-glucan)
Prochlorophyta	blue-green algae	a,b	Absent	β-Carotene	Zeaxanthin	Cyanophycean starch (α-1,4-glucan)
Glaucophyta	green algae	A	c-Phycocyanin Allophycocyanin	β-Carotene	Zeaxanthin	starch (α-1,4-glucan)
Rhodophyta	Red Algae	A	r,b-Phycoerythrin r-Phycocyanin Allophycocyanin	α- and β-Carotene	Lutein	Floridean starch (α-1,4-glucan)
Cryptophyta	blue-green algae	a,c	Phycoerythrin -545 r-Phycocyanin	α-,β-, and ε-Carotene	Alloxanthin	starch (α-1,4-glucan)
Heterokontophyta	Golden or Brown algae	a,c	Absent	α-,β-, and ε-Carotene	Fucoxanthin Violaxanthin	Chrysolaminaran (β-1,3-glucan)
Haptophyta	green algae	a,c	Absent	α- and β-Carotene	Fucoxanthin	Chrysolaminaran (β-1,3-glucan)
Dinophyta	dinoflagellates	a,b,c	Absent	β-Carotene	Peridinin, Fucoxanthin, Diadinoxanthin Dinoxanthin Gyroxanthin	starch (α-1,4-glucan)
Euglenophyta	flagellate	a,b	Absent	β- and γ-Carotene	Diadinoxanthin	Paramylon (β-1,3-glucan)
Chlorarachniophyta	mainly green algae	a,b	Absent	Absent	Lutein Neoxanthin Violaxanthin	Paramylon (β-1,3-glucan)
Chlorophyta	green algae	a,b	Absent	α-,β-, and γ-Carotene	Lutein Prasinoxanthin	starch (α-1,4-glucan)

Diatoms

Diatoms, also called Bacillariophyceae, is a class belonging to division Chrysophyta. The cells of diatoms are golden-brown because of the presence of high level of fucoxanthin, a photosynthetic accessory pigment. Several other xanthophylls are present at lower levels, as well as β-carotene, chlorophyll α and chlorophyll c. The main storage compounds of diatoms are triglycerides (TAGs) and chrysolaminarin, a β-1, 3-linked carbohydrate. Diatom cell wall contains substantial quantities of polymerized Si. This unique feature has important implications for media preparation and costs in a commercial production facility, because silicate is a relatively expensive chemical. On the other hand, deficiency of silicate can promote lipid (TAG) accumulation in diatoms. It can be employed to provide a controllable means to induce lipid synthesis in a two-stage production process. Diatoms are the most common and widely distributed groups of microalgae on earth. They dominate the phytoplankton of the oceans and are also commonly found in fresh- and brackish waters.

Green Algae

Green algae, including divisions Prochlorophyta and Chlorophyta, have chlorophyll a and chlorophyll b as photosynthetic pigments. Green algae are believed to be the evolutionary progenitors of higher plants and have received more attention than other groups of algae. *Chlamydomonas reinhardtii* (and closely related species), a member of this group, has been studied extensively. It was the first alga to be genetically transformed. Another genus of green algae that has been studied

extensively is Chlorella. Several green algae, for instance, *Neochloris oleoabundans*, are known to be able to accumulate large quantities of lipids and efficient in CO_2 fixation (Li, Horsman et al. 2008; Li, Horsman et al. 2008), making them attractive candidates for combined CO_2 fixation and biofuel production.

Golden-Brown algae

Golden-Brown algae include the chrysophytes and the synurophytes. They are similar to diatoms with respect to pigment composition. Some chrysophytes have lightly silicified cell walls. They are found primarily in freshwater habitats. Lipids and chrysolaminarin are the most common carbon storage materials of this group.

Prymnesiophytes

Prymnesiophytes (haptophytes) are primarily marine organisms and account for a substantial proportion of the primary productivity of tropical oceans. Some prymnesiophytes produce algal blooms, which may cause serious problems. Prymnesiophytes are often of a golden-brown color because of the presence of the yellow-brown accessory pigments, diadinoxanthin and fucoxanthin. Lipids and chrysolaminarin are the major storage form of this group of algae.

Eustigmatophytes

This group represents an important component of the "picoplankton", which is comprised of a group of small microalgae with cell size in the range of 2-4μm in diameter. The genus Nannochloropsis is one of the few marine species in this class, and is common in the world's oceans. Chlorophyll a is the only chlorophyll present in Eustigmatophyte cells. They contain however several xanthophylls that serve as

accessory photosynthetic pigments.

Blue Green Algae (Cyanobacteria)

As mentioned previously, Cyanobacteria are not microalgae but a group of photosynthetic bacteria. They are treated here as microalgae in a broader sense for convenience. Cyanobacteria are prokaryotes that contain no nucleus, no chloroplasts, and have a different gene structure than all other microalgae. There are approximately 2,000 species of cyanobacteria, which have been found in a diversity of different habitats. Some members of this group can assimilate atmospheric N and therefore eliminate the need to provide fixed N for cell growth. A few commercial facilities have been built for the cultivation of cyanobacteria, (e.g., *Spirulina platensis*) for production of health foods and other novel products. No member of this class produces significant quantities of storage lipids.

2.1.2 Important species for biofuel production: *Neochloris oleoabundans*

Neochloris oleoabundans is a microalga belonging in the class Chlorophyceae. (Table 2.1) The genus Neochloris belongs to the oily group of microalgae due to its characteristic of producing more than 30% total lipids Containing polyunsaturated fatty acids C18:1(oleic acid) and C18:2 (linoleic acid). In conditions of osmotic shock, the cells excrete high quantities of polysaccharides into the medium and show an increase in the neutral lipids. It has been cultivated in mineral medium deficient in nitrogen and the yield of lipids was 35-54% of cell dry weight. Triglycerides comprised 80% of the total lipids. Aliphatic hydrocarbons, sterols, pigments, glycolipids and phospholipids comprised the remaining lipid fraction. Saturated,

monounsaturated and diunsaturated octodecanoic acid represented approximately one-half of the total fatty acids (2-3 weeks, nitrogen starvation).

2.2 Microalgal nutrition and cultivation

2.2.1 Nutrition

Most algal groups are photoautotrophs, i.e., depending entirely upon photosynthesis for cell growth and cell maintenance using sunlight as the source of energy and CO_2 the source of carbon. On the other hand, there are some colorless heterotrophic species that depends on organic carbon sources from the external environment either by taking up dissolved substances (osmotrophy) or by engulfing bacteria and other microorganisms (phagotrophy). A large group of algae can utilize both inorganic and organic carbon sources and is referred to as mixotrophs. Some mixotrophs are primarily photosynthetic and only use organic energy sources occasionally. Others meet most of their nutritional demand by phagotrophy but may use some of the products of photosynthesis from sequestered prey chloroplasts. Of particular importance to CO_2 mitigation and biofuels production are phototrophs and mixtrophs that are primarily photosynthetic.

Hydrogen (H), oxygen (O), carbon (C), nitrogen (N), phosphorus (P), and sulfur (S) are the most important elements constituting algal cells. Growth medium must provide sufficient nutrients for microalgal growth. While hydrogen and oxygen are usually provided in the form of water (H_2O) and molecular oxygen (O_2), other elements, including C, N, P, S, metal ions such as iron, magnesium, and trace elements, and in some cases, silicon (Rebolloso-Fuentes, Navarro-Perez et al. 2001)

must be supplied. It is important to develop balanced media for optimal microalgal cultivation and CO_2-fixation (Mandalam and Palsson 1998).

Carbon Sources

According to the mode of cell growth (heterotrophic, autotrophic, or mixotrophic), microalgae can utilize organic and/or inorganic carbon sources for cell growth. From the perspective of microalga cultivation, the most common organic carbon sources for heterotrophic and mixotrophic cultivation of microalgae are glucose, sucrose, and other sugars derived from starch, sugar cane, lignocellulosic biomass, and other sugar sources. There are three different sources of inorganic carbons sources: 1) CO_2 from the atmosphere; 2) CO_2 from industrial exhaust gases (e.g., flue gas and flaring gas); and 3) fixed CO_2 in the form of soluble carbonates (e.g., $NaHCO_3$ and Na_2CO_3).

Traditionally, microalgae are cultivated in closed systems or open ponds, which are aerated or exposed to air to allow microalgae to capture carbon dioxide from the atmosphere for cell growth. Since the atmosphere contains only 0.03 - 0.06% CO_2, it is expected that mass transfer limitation could slow down the cell growth of microalgae (Chelf, Brown et al. 1993). On the other hand, industrial exhaust gases such as flue gas contains up to 15% CO_2, providing a CO_2-rich source for microalgal cultivation and a potentially more efficient route for CO_2 bio-fixation. The third route is to fix CO_2 by chemical reaction to produce carbonates (e.g., Na_2CO_3) and use the latter as the carbon source for microalgal cultivation.

A number of microalgal species have been shown to be able to utilize carbonates

such as Na_2CO_3 and $NaHCO_3$ for cell growth (Ginzburg 1993; Merrett, Nimer et al. 1996; Emma Huertas, Colman et al. 2000). Some of these species typically have high extracellular carboanhydrase activities (Emma Huertas, Colman et al. 2000), which is responsible for the conversion of carbonate to free CO_2 to facilitate CO_2 assimilation. In addition, the direct uptake of bicarbonate by an active transport system has been found in several species (Colman and Rotatore 1995; Merrett, Nimer et al. 1996). Adoption of Carbonate-Utilizing strains for CO_2 fixation could be advantageous in many aspects: 1) CO_2 released in nighttime from industrial facilities could be converted to carbonate salts and stored for conversion in daytime; 2) since only a limited number of microalgal species thrive in media containing high concentration of carbonate salts, species control (i.e., preventing wild type microalgal species from contaminating the cultivation system) is relatively simple; 3) most of these species have high pH optima (in the range of 9.0 to 11.0), further simplifies species control (Ginzburg 1993).

Flue gases from power plant are responsible for more than 7% of the total world CO_2 emissions (Sakai, Sakamoto et al. 1995). Carbon dioxide in flue gas is available at little or no cost. As estimated by the IPCC criteria, the CO_2 concentration of flue gas is up to 15% (Maeda, Owada et al. 1995). Therefore, it would be beneficial if microalgae are tolerant to elevated CO_2 level should they be used for CO_2 fixation from flue gases (Maeda, Owada et al. 1995).

An early review on flue gas tolerance by microalgae indicated that high levels of CO_2 were tolerated by many microalgal species and that moderate levels of SO_x and

NO_x (up to 150ppm) were also well-tolerated (Matsumoto et al., 1997).

Chlorococcum littorale, a marine alga, showed exceptional tolerance to high CO_2 concentration of up to 40% (Iwasaki et al., 1998, Murakami and Ikenouchi, 1997). Microalgae *Scenedesmus obliquus* and *Chlorella kessleri*, separated from the waste treatment ponds of the Presidente Médici coal fired thermoelectric power plant, also exhibited good tolerance to high CO_2 contents (de Morais and Costa, 2007b). *Chlorella kessleri* showed maximum specific growth rate (μ_{max}) of $0.267d^{-1}$ and biomass productivity of approximately 0.087 g/l·d when cultivated with 6% (V/V) and 12% (V/V) CO_2 and with a maximum biomass productivity of 0.085 g/l·d was achieved at 6% CO_2. These two microalgae also grew well when the culture was supplemented with enriched air stream contained up to 18% CO_2, indicating their great potentials for CO_2 fixation from CO_2-rich streams. It was also reported (de Morais and Costa, 2007a) that *Scenedesmus obliquus* and *Spirulina sp.* showed good capacities to fix carbon dioxide when they were cultivated at 30°C in a temperature-controlled three-stage serial tubular photo-bioreactor. For *Spirulina sp.*, the maximum specific growth rate and maximum productivity were 0.44/d and 0.22 g/l·d, with both 6% (v/v) carbon dioxide and 12% (v/v) carbon dioxide, respectively, while the maximum cell concentration was 3.50 g DCW/l with both CO_2 concentrations. For *S. obliquus*, the corresponding maximum growth rate and maximum productivity were 0.22/d and 0.14 g/l·d, respectively. Murakami and Ikenouchi (1997) selected more than 10 strains of microalgae with high capability of fixing CO_2 by extensive screening. Two green algal strains, *Chlorella* sp. UK001 and

Chlorococcum littorale, showed high CO_2 fixation rates exceeding 1 g CO_2 g/l·d. *Botryococcus braunii* SI-30, which showed the ability of producing high content of hydrocarbons, was recommended as a promising candidate for combined CO_2 mitigation and biofuel production.

The tolerance of microalgae to relatively high temperature is very important in reducing cooling costs of the feeding flue gases released from industrial facilities at high temperature. These thermotolerant strains may also simplify species-control because the temperature optima of most microalgal species locate in the range of 20-30°C. A few thermotolerant strains have been selected. For instance, several unicellular green algal strains, identified as species of *Chlorella*, were isolated from hot springs in Japan (Sakai et al., 1995). These strains grew at temperatures up to 42 °C and in air containing more than 40% CO_2. Their tolerance to both high temperature and high CO_2 content makes them potentially the appropriate microbial cellar reactors for bio-CO_2 mitigation from flue gas.

Table 2.2 summarizes a few microalgal strains that have been studied for CO_2 bio-mitigation. Some of these strains can tolerate high temperature and high CO_2 in the gas stream. CO_2 fixed through photosynthesis are converted to different organic cell components including carbohydrates, lipids, proteins, and nucleic acids (Spolaore et al., 2006).

Table 2.2 Some microalgal strains studied for CO_2 bio-mitigation (Wang, et al., 2008)

Microalga	CO_2 %	T °C	P g/l·d	P_{CO2} g/l·d	Reference	Note
Chlorococcum littorale	40	30	N/A	1.0	(Iwasaki et al., 1998, Murakami and Ikenouchi, 1997)	
Chlorella kessleri	18	30	0.087	0.163*	(de Morais and Costa, 2007b)	
Chlorella sp. UK001	15	35	N/A	>1	(Murakami and Ikenouchi, 1997)	
Chlorella vulgaris	15		N/A	0.624	(Yun et al., 1997)	Artificial Wastewater
Chlorella vulgaris	air	25	0.040	0.075*	(Scragg et al., 2002)	Watanabe's medium
Chlorella vulgaris	air	25	0.024	0.045*	(Scragg et al., 2002)	Low-N medium
Chlorella sp.	40	42	N/A	1.0	(Sakai et al., 1995)	
Dunaliella	3	27	0.17	0.313*	(Kishimoto et al., 1994)	High salinity, ß-carotene
Haematococcus pluvialis	16-34	20	0.076	0.143	(Huntley and Redalje, 2007)	Commercial scale, outdoor
Scenedesmus obliquus	air	-	0.009	0.016	(Gomez-Villa et al., 2005)	Wastewater, outdoor, winter
Scenedesmus obliquus	air	-	0.016	0.031	(Gomez-Villa et al., 2005)	Wastewater, outdoor, summer
Botryococcus braunii	-	25-30	1.1	>1.0	(Murakami and Ikenouchi, 1997)	Accumulating Hydrocarbon
Scenedesmus obliquus	18	30	0.14	0.26	(de Morais and Costa, 2007a)	
† *Spirulina sp.*	12	30	0.22	0.413*	(de Morais and Costa, 2007a)	

* Calculated from the biomass productivity according to equation, CO_2 Fixation Rate (P_{co2}) = 1.88×Biomass Productivity (P), which is derived from the typical molecular formula of microalgal biomass, $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$ (Chisti, 2007).

† All species except *Spirulina sp.*, which is a prokaryotic cyanobacteria (*Cyanophyceae*) species, are eukaryotic green algae (*Chlorophyta*) species (NCBI website).

- Not specified or not controlled

Nitrogen Sources

While some microalgal species could fix molecular nitrogen for cell growth, nitrate, ammonia and urea (or combinations of them) are the most common nitrogen sources for microalgae (Lourenco et al., 1998). Worth mentioning is that ammonium is the chemical form of nitrogen most readily taken up and assimilated by phytoplankton. Unlike nitrate, it does not require reduction prior to being assimilated into amino acids. However, research indicated that ammonium at high concentration has toxic effects on microalgal growth (Lourenco et al., 2002). Besides, ammonia may escape into the atmosphere, causing environmental and economic concerns.

A cheap source of nitrogen would be wastewater or secondary wastewater, which contains large quantities of different forms of nitrogen sources. However, the use of wastewater for microalgal cultivation may cause contamination problems and/or complicate downstream processing and therefore should be used with precautions.

For all eukaryotic algae, the only forms of inorganic nitrogen that are directly assimilable are nitrate (NO_3^-), nitrite (NO_2^-), and ammonium (NH_4^+). The more highly oxidized form, nitrate, is the most thermodynamically stable form in oxidized aquatic environments, and hence is the predominant form of fixed nitrogen in aquatic ecosystems, though not necessarily the most readily available form. Following translocation across the plasmalemma (which is an energy-dependent process), the assimilation of NO_3^- requires chemical reduction to NH_4^+ (Laura Barsanti et al, 2006). This process is mediated by two enzymes, nitrate reductase and nitrite reductase, and the overall stoichiometry for the reduction of nitrate to ammonium can be written as:

$$NO_3^- + 8e^- + 10H^+ \longrightarrow NH_4^+ + 3H_2O \quad (2.1)$$

Afterwards, ammonium is incorporated into amino acids by sequential actions.

It would be necessary for the microalgae to have a high calorific value if they are used for biofuel production through the aforementioned biomass conversion processes. Microalgae grown under normal conditions have been shown to have calorific values between 18 and 21 KJ/g (Illman et al., 2000), which can be improved by optimizing cultivation conditions. For instance, studies have shown that the calorific values of microalgae biomass could be enhanced by cultivation in nitrogen-limiting condition, the calorific value of *C. vulgaris* biomass, which was 18 KJ/g grown in nitrogen sufficient medium, was found to be increased to 23 KJ/g grown in low nitrogen medium and the calorific value of *C. emersonii* grown in low nitrogen medium was found to be 29 KJ/g. Although this calorific value of 29 KJ/g is somewhat lower than that of diesel, which is 43 KJ/g, *Chlorella* biomass is still regarded as suitable for use as diesel replacements (Illman et al., 2000).

The major components of microalgae biomass are protein, carbohydrate and lipids. Lipids are the most desirable component from the energetic point of view. The cells with a higher lipid content and lower carbohydrates and proteins have elevated calorific value and produce higher yields of oil when processed via, for instance, biomass liquefaction (Scragg et al., 2002, Illman et al., 2000, Ginzburg, 1993, Yamaberi et al., 1998, Tornabene et al., 1983). Several microalgal strains have been

reported to have the ability to accumulate large quantities of lipids. Nitrogen limitation was observed to lead to the increase of the lipid content in some chlorella strains such as *C. emersonii* (63%), *C. minutissima* (56%) , C. *vulgaris* (57.9%), *C. luteoviridis* (28.8%), *C. capsulata* (11.4%), and *C. pyrenoidosa* (29.2%) (Illman et al., 2000). An oil-rich microalgal species, *Neochloris oleoabundans* (Kawata et al., 1998), was reported that under nitrogen deficient conditions, accumulated 35-54% lipids of its cell dry weight and triglycerides comprised 80% of the total lipids (Tornabene et al., 1983). It was also observed (Yamaberi et al., 1998) that the triglycerides accumulated in *Nannochloris sp.* cells could be 2.2 times that in the cells in nitrogen sufficient cultures. It is recommended that the intracellular triglyceride content could be increased by prolonging the cultivation period during the stationary phase after nitrogen depletion. Table 2.3 summarizes a few microalgal strains that have been studied for lipid productivity.

Table 2.3 Lipid productivity of some microalgal species (Li , et al, 2008)

Species	P_{DCW} (g/l)/d†	Lipid (%)	TAG (%)	T (°C)	P_{lipid} (mg/l·d)	Refs
Parietochloris incise	(7)/14		43-77	25	17.9	(Solovchenko et al., 2008)
Parietochloris incise	(6.3)/38			26	31.5	(Cheng-Wu et al., 2002)
Nannochloris sp. UTEX LB1999	(2.7)/12	34.0	18.8	28	76.5	(Yamaberi et al., 1998)
Nannochloris sp. UTEX LB1999	(2.16)/	50.9	47.6			(Takagi et al., 2000)
Chlorella protothecoides *	(16.8)/8	57.8	NA		1214	(Xiong et al., 2008)
Chlorella emersonii	(1.11)/14	63	NA		50	(Illman et al., 2000)
Chlorella	(0.46)/14	57	NA		18.7	(Illman et al., 2000)
Chlorella vulgaris	(0.52)/14	40	NA		14.9	(Illman et al., 2000)
Chlorella vulgaris	NA	56.6	NA	20	NA	(Liu et al., 2008)
Dunaliella	(0.5)/10	67	NA	28	33.5	(Takagi et al., 2006)
Neochloris oleoabundans	NA	35-54	80	28	NA	(Tornabene et al., 1983)
Neochloris oleoabundans	(2.4)/6	34	NA	34	134	Current Study

* Heterotrophic cultivation

† Final biomass concentration with cultivation time

However, there is a dilemma in the fact that high-lipid and high caloric cells are usually produced in a stressed state, which is associated with reduced cell division (Ratledge, 2002). Biomass yield and overall lipid/energy productivity will therefore be compromised as a result. For instance, studies on *Chlorella vulgaris* and *C. emersonii* grown in a 230 L pumped tubular photo-bioreactor in Watanabe's medium and a low nitrogen medium (Scragg et al., 2002) showed that, the low nitrogen

medium induced higher lipid accumulation in both algae with calorific value increased and the highest calorific value was obtained with *C. vulgaris* (28 kJ/g) grown in the low nitrogen medium. However, the biomass productivity was 24 mg DCW g/l·d in the low nitrogen medium, only slightly more than half of that obtained with Watanabe's medium (40 mg DCW g/l·d). The overall energy recovery was lower with the low nitrogen medium than with the Watanabe's medium. It is important to find the balance between producing high caloric value cells and maintaining high biomass productivity by optimizing nitrogen in medium for the growth of microalgae.

Phosphorus Sources

Phosphorus is another element that has significant relevance to the cell growth and metabolism of microalgae. It is one of the essential elements comprising DNA, RNA, ATP and cell membrane materials, etc. It is worth noting that, as a constituent element of ATP, phosphorus is essential to the cellular processes related to energy transfer (e.g., photophosphorylation). On another relevant notion, photosynthesis requires large amounts of proteins (notably Rubisco) and the proteins are synthesized by phosphorus-rich ribosomes (Agren, 2004).

As aforementioned, phosphorus-containing ATP/ADP are essential for photophosphorylation. As a consequence, limitation of growth by phosphate starvation may have a severe impact on various aspects of microalgal metabolism, including photosynthesis and lipid accumulation.

Phosphorus is preferentially assimilated as inorganic phosphates in the form of $H_2PO_4^-$ and HPO_4^{2-} (Gauthier and Turpin, 1997, Martinez et al., 1999). It has been

pointed out that phosphates may form complexes or precipitations with some metal ions and not all the added phosphorus is bioavailable (Yun et al., 1997). Therefore, phosphorus may need to be supplied in excess.

Phosphorus can be removed by two different mechanisms, biological assimilation preferentially taking place during the biomass growth phase, while chemical precipitation (abiotic process) predominated when biomass concentration reached a threshold value (Wang et al., 2008).

Algae use three different bio-processes to transform P into high energy organic compounds: phosphorylation at the substrate level, oxidative phosphorylation, and photophosphorylation (Martinez Sancho et al., 1997).

The general reaction can be represented by:

$$ADP + P_i \xrightarrow{Energy} ATP$$

In addition, photosynthetic carbon assimilation was responsible for pH increase in the medium that can induce insoluble phosphates to precipitate, thus considerably increasing the fraction of total phosphorus removed.

Other elements

Sulfur, iron, magnesium and other elements are also indispensable for the growth of microalgae. Sulfur is an essential component of cysteine and methionine. In the absence of sulfur, protein biosynthesis is impeded and the photosynthetic system PSII repair cycle is blocked (Zhang et al., 2002). Magnesium is required for nitrogenase activity using a creatine phosphate/kinase/ATP generating system as one of its roles in cell metabolism. Iron is involved in electron flow from H_2O to $NADP^+$ (Roden and

Zachara, 1996). Some trace metals play key roles in (non-cyclic) photosynthetic electron transport (Raven et al., 1999). For instance, manganese is essential for O_2 evolution and calcium has an important role in the thylakoid lumen in facilitating H_2O dehydrogenation and O_2 evolution.

2.2.2 Culture parameters

The most important parameters regulating algal growth by means of photosynthesis, in addition to the previously discussed nutritional factors, are light, pH, turbulence, salinity, and temperature. The range of optimal conditions as well as the toleratable range of operating conditions are species specific and various factors may be interdependent (Laura Barsanti, 2006).

Temperature

In general, the cultivation temperature should ideally be as close as possible to the temperature at which the organisms were collected (polar regions <10 °C, temperate regions 10-25 °C, and tropical regions >25 °C). Some species of microalgae tolerate temperatures above 30 °C, which could be ideal candidate for CO_2 mitigation of flue gas. An intermediate value of 18-25 °C is most often employed.

Temperature simultaneously influences three competing cellular processes of microalgae, photosynthesis, photo-respiration, and endogenous metabolism. The overall effect of temperature on cell growth in a particular temperature range depends on the net result of these competing processes. Temperature may also influence the production of metabolites as a result of its influence on the metabolism of microalgae.

Light

Light is the source of energy for the autotrophic growth of microalgae. Light intensity, spectral quality, and photoperiod need to be considered to optimize a microalgal farming process. Light intensity plays an important role, however, it depends greatly on the depth and the cell density of the algal culture: at higher depths and cell concentrations the light intensity must be increased to penetrate through the culture. Precautions should be taken as too high light intensity (e.g. direct sunlight, small container close to artificial light) may result in photo-inhibition. The most commonly employed light intensities range between 100 and 200$\mu E/m^2 \cdot s$, which corresponds to about 5-10% of full daylight (2000 $\mu E/m^2 \cdot s$). Moreover, overheating due to both natural and artificial illumination should be avoided. Some microalgal species do not grow well under constant illumination hence a light/dark (LD) cycle may be required (maximum 16:8 LD, usually 14:10 or 12:12).

Light as the energy source is often the principal limiting factor in microalgal cultivation. When the light intensity is below the light saturation point, the rate of photosynthesis is directly proportional to light intensity in a typical scenario. In most microalgae, photosynthesis is saturated at about 30% of the total terrestrial solar radiation, i.e. 1,700–2,000 $\mu E/m^2 \cdot s$ (Kirk1994). It should be noted that light inhibition might occur when light intensity goes beyond certain point. Photo inhibition could be reversible or irreversible, depending on the light stress and the length of time the microalgae are exposed to such a stress. It was reported that microalgae could adjust, within certain range, to a higher light intensity through a process called photo

adaptation.

Photosynthesis is the process photosynthetic species, including microalgae, use to capture light energy to produce glucose from CO_2. Light energy is converted in the photosynthetic process to chemical bonding energy stored in cell materials (biomass). Photosynthesis involves two major reaction sequences: the light-dependent reactions (the light reactions) and the light-independent reactions (the dark reactions). In the light reactions, light energy is captured and converted to energy currency, NADPH and ATP. The dark reaction involves a sequence of reactions that fix and reduce inorganic carbon utilizing the ATP and NADPH generated in the light reaction. As shown in equation 2.2, the overall result of photosynthesis is that carbon is converted from CO_2 to carbohydrates, $[CH_2O]n$, using light energy. The carbohydrates are subsequently converted to other cell materials for cell growth and cell maintenance.

$$n\,CO_2 + nH_2O + \text{light} \xrightarrow[\text{Chlorophyll } a]{} (CH_2O)_n + n\,O_2 \qquad (2.2)$$

It is worth mentioning that the natural rhythm of light/dark cycle has impact on microalgal farming designed for solar energy capturing and CO_2 sequestration. Due to the lack of light energy, microalgae would have to conduct respiration, which consumes glucose and/or other cell materials and oxygen and release CO_2 to produce bioenergy (ATP) for the maintenance of cell viability. As a result, significant loss of biomass (up to 30%) could be observed as a result of the respiration in the nighttime. The respiration during nighttime should therefore be minimized.

Considerable efforts have gone into the design of photobioreactors that would maximize photosynthetic efficiency. It was noted the highest photosynthetic efficiencies recorded to date have been achieved in open ponds and raceways exposed to full sunlight of up to 2000 μmol quanta /m^2·s. There is considerable scope for design of process improvements that would further enhance this result. For example, exposure to photon flux density (PFD) of 8000 μmol quanta /m^2·s yields an improvement of 50% in productivity compared to that in full sunlight. One example of a process improvement would be the removal of auto-inhibitory growth factors by medium replacement, which can lead to a four-fold enhancement in area productivity. This particular method could be accomplished by the two-stage cultivation process, in which culture medium from the photobioreactor is significantly diluted upon transfer to the second-stage open-pond batch culture (Huntley and Redalje, 2007).

pH

The pH range for most cultured algal species is 7 - 9. Some species have pH optima in more acid or basic ranges. For instance, the optimal pH of the cyanobacterium *Spirulina platensis* is in the range of 8.0 - 9.0. It is crucial to maintain culture pH in the optimal range as complete culture collapse due to the disruption of many cellular processes can result from failure to maintain an acceptable pH. In the case of high-density algal culture in controlled systems using air enriched with carbon dioxide (pure CO_2 or high CO_2 flue gases), the balance between the mass transfer of CO_2 from gas phase to liquid phase and the consumption of CO_2 by algal cells will determine the dissolved CO_2 concentration in the culture, which may be the

determinant factor of the culture pH in some scenarios.

Mixing

Microalgae live in their natural habitats at a density of 10^3 cells/ml and at distances of more than 1,000 μm between cells. However, in high-cell-density microalgal cultures, the cell density could be as high as up to 10^9 cells/ml, creating a microenvironment that differs drastically from what the nature provides and is not optimal for cell growth and productivity. Mixing is therefore necessary to facilitate mass and heat transfer between the microenvironment surrounding individual cell as well as the bulk and the environment to improve the microenvironment for individual cells for optimal cell growth and productivity. More specifically, mixing of microalgal cultures is necessary because of the following reasons: 1) to prevent sedimentation of the algae; 2) to ensure that all cells of the population are equally exposed to light and nutrients; 3) to facilitate heat transfer and avoid thermal stratification; 4) to improve gas exchange between the culture medium and the air, which is critical for good CO_2 mass transfer and for avoiding oxygen toxicity. It should be noted that in the ocean cells seldom experience turbulence and hence mixing should be gentle.

2.3 CO_2 Bio-mitigation and biofuel production by microalgae

Biological CO_2 mitigation has attracted much attention as an alternative strategy because it leads to production of biomass energy in the process of CO_2 fixation through photosynthesis (Kondili and Kaldellis, 2007, Ragauskas et al., 2006) (Ragauskas et al., 2006). It was estimated (IPCC 1995) that biological mitigation options could offset 10-20% of projected fossil fuel emissions by 2050. Biological CO_2 mitigation can be carried out by plants and photosynthetic microorganisms. However, the potential for increased CO_2 capture in agriculture by plants has been estimated to contribute only 3-6% of fossil fuel emissions (Skjanes et al., 2007), largely due to the slow growth rates of conventional terrestrial plants. On the other hand, the microalgae-for-CO_2-mitigation strategy offers numerous advantages. Photosynthetic microorganisms in general have much higher growth rates, photosynthetic efficiency and CO_2 fixation abilities due to their vast surface to volume ratio and simple structure, compared to conventional forestry, agricultural, and aquatic plants (Chisti, 2007, Borowitzka, 1999). It has been reported that microalgae have the ability to fix CO_2 while capturing solar energy with an efficiency 10 to 50 times greater than that of terrestrial plants (Li et al., 2008b, Usui and Ikenouchi, 1997). It could completely recycle CO_2 since carbon dioxide is converted into the chemical energy via photosynthesis, which can be converted to fuels using existing technologies (Demirbas, 2004). In comparison, the chemical reaction-based CO_2 mitigation approaches, as discussed above, have disposal problems because both the captured CO_2 and the wasted absorbents need to be disposed of (Yeh et al., 2001,

Bonenfant et al., 2003). Chemical reaction-based CO_2 mitigation approaches are energy consuming and costly processes (Resnik et al., 2004, Lin et al., 2003) and the only economical incentive for CO_2 sequestrating using chemical reaction-based approach is the CO_2 credits to be generated under the Kyoto protocol. Very encouraging, CO_2 bio-mitigation via microalgal farming could potentially be made profitable from the production of biofuels and other novel bioproducts. Nevertheless, the large costs associated with microalgal farming are still the most significant obstacle toward the commercial implementation of this strategy and a few strategies could be employed to enhance its cost-effectiveness.

2.3.1 Biofuel Production

Biomass produced in the process of CO_2 fixation can be converted efficiently into biofuels for energy production. An estimate made in 2003 indicates that the costs of biofuel production are in general about 2.3 times more expensive than fossil fuels (Kondili and Kaldellis, 2007). There is no doubt that fast technology development and the soaring energy prices have improved and will improve the situation rapidly and biofuel production from microalgae is deemed to be the most promising biofuel production strategy (Li, 2008). A commercial-scale (2 ha) demonstration microalgal farming facility was operated consecutively for 4 years to produce *Haematococcus pluvialis* for biodiesel production (Huntley and Redalje, 2007). A two-stage strategy was employed for the operation. Closed photobioreactors were used for the first stage and open ponds for the second. Daily production of 1.9 kg dry biomass was achieved with a 25,000 l photobioreactor, corresponding to a biomass productivity of 0.076 g

/l·d, at a biomass concentration of 0.3 g/l. An annual averaged rate of microalgal oil production, which was equivalent to 420 GJ/ha·yr, was obtained. While the maximum production rate achieved with *Haematococcus pluvialis* was equivalent to 1014 GJ /ha·yr, it was predicted that a rate of 3200 GJ/ha·yr is feasible using fast-growing *Chlorella* species. This is a rate possible to replace the reliance on current fossil fuel usage equivalent to about 300 EJ/yr and eliminate fossil fuel emissions of CO_2 of about 6.5 Gigatons of Carbon (GtC) per year using only 7.3% of the surplus arable land projected to be available by 2050. It was also expected that other microalgal biodiesel processes such as the one being developed at the University of Utah would be cost-competitive with regular diesel by 2009 (Seefeldt, 2007). There is no doubt that global efforts from both the public and private sectors will be continued and accelerated in order to make biofuels from microalgae a practical replacement of fossil fuels in the near future.

There are several ways to convert microalgae biomass to biofuels, which can be classified into biochemical conversion, chemical reaction, direct combustion and thermochemical conversion (Fig. 2.1) (Demirbas, 2001, McKendry, 2002). More specifically, example processes belonging to biochemical conversion include anaerobic digestion for methane production and fermentation for ethanol production (Spolaore et al., 2006); an example chemical conversion process involves extraction of lipids accumulated in microalgae cells and conversion of the extracted lipid to biodiesel via a simple transesterification reaction (Chisti, 2007, Belarbi et al., 2000); and some example thermochemical conversion processes include pyrolysis

(Chiaramonti et al., 2007), gasification (Hirano et al., 1998) and liquefaction (Minowa and Sawayama, 1999). Energy stored in microalgal biomass could also be utilized via direct combustion or co-firing. Thermochemical conversion (Demirbas, 2004) is one of the most practical biomass conversion strategies.

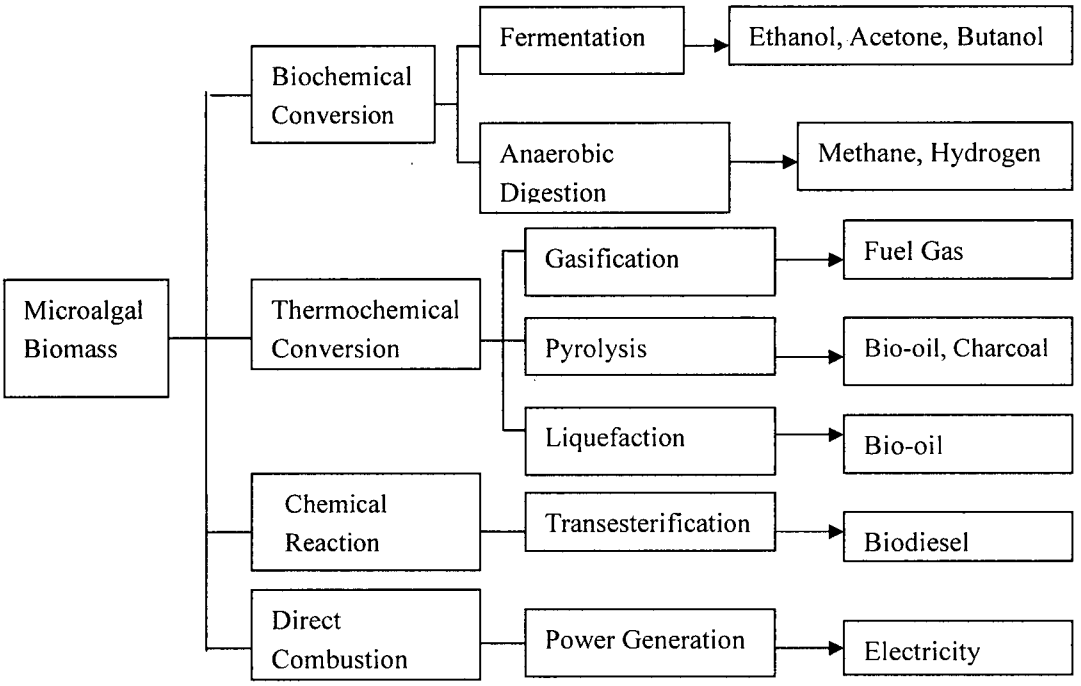

Figure 2.1 Energy production via microalgal biomass conversion using biochemical, thermochemical, chemical and direct combustion processes. (Wang, B, 2008)

Biodiesel

Biodiesel is produced by a mono-alcoholic transesterification process, in which triglycerides reacts with a mono-alcohol (most commonly methanol or ethanol) with the help of basic, acidic and enzymatic catalysts (Meher, Vidya Sagar et al. 2006; Demirbas 2007). It has similar combustion properties to diesel (Marchetti, Miguel et al. 2007) and has been produced commercially or in backyard facilities to fuel vehicles. Currently, biodiesel production relies on animal fats and plant oils. However, replacing all the transport fuel consumed in the United States with biodiesel will require 0.53 billion m^3 of biodiesel annually at the current rate of consumption. Oil crops, waste cooking oil and animal fat cannot realistically satisfy this demand. This is demonstrated in Table 2.4. Using the average oil yield per hectare from various crops, the cropping area needed to meet 50% of the U.S. transport fuel needs is calculated in column 3 (Table 2.4). In column 4 of Table 2.4, this area is expressed as a percentage of the total cropping area of the United States. If oil palm, a high-yielding oil crop can be grown, 24% of the total cropland will need to be devoted to its cultivation to meet only 50% of the transport fuel needs. Clearly, oil crops cannot replace petroleum derived liquid fuels in the foreseeable future. This scenario changes dramatically, if microalgae are used to produce biodiesel. Between 1 and 3% of the total U.S. cropping area would be sufficient for producing algal biomass that satisfies 50% of the transport fuel needs. The microalgal oil yields given in Table 2.4 are based on experimentally demonstrated biomass productivity in photobioreactors.

Table 2.4 Comparison of some sources of biodiesel (Chisti, 2007)

Crop	Oil yield (L/ha)	Land area Needed (M ha)[a]	Present of existing US cropping area[a]
Corn	172	1540	846
Soybean	446	594	326
Canola	1190	223	122
Jatropha	1892	140	77
Coconut	2689	99	54
Oil palm	5950	45	24
Microalgae[b]	136,900	2	1.1
Microalgae[a]	58,700	4.5	2.5

[a] For meeting 50% of all transport fuel needs of the United States.

[b] 70% oil (by wt) in biomass.

[c] 30% oil(by wt) in biomass.

In view of Table 2.4, microalgae appear to be the only source of biodiesel that has the potential to completely displace fossil biodiesel. Unlike other oil crops, microalgae grow extremely rapidly and many are exceedingly rich in oil. Microalgae commonly double their biomass within 24h. Biomass doubling times during exponential growth are commonly as short as 3.5h. Oil content in microalgae can exceed 80% by weight of dry biomass. Oil levels of 20-50% are quite common (Table 2.5). Oil productivity, that is the mass of oil produced per unit volume of the microalgal broth per day, depends on the algal growth rate and the oil content of the biomass. Microalgae with high oil productivities are desired for producing biodiesel.

Table 2.5 Oil content of some microalgae (Chisti, 2007)

Microalga	Oil content (% dry wt)
Botrycococcus braunii	25-75
Chlorella sp.	28-32
Crypthecodinium cohnii	20
Cylindrotheca sp.	16-37
Dunaliella primolecta	23
Isochrysis sp.	25-33
Monallanthus salina	>20
Nannochloris sp.	20-35
Nannochloropsis sp.	31-68
Neochloris oleoabundans	35-54
Nitzschia sp.	45-47
Phaeodactylum tricornutum	20-30
Schizochytrium sp.	50-77
Tetraselmis sueica	15-23

Oils suitable as the feedstock of biodiesel production are triglycerides, which consist of three fatty acid residuals and a glycerol residue. Transestrification, which produces fatty acid methyl esters (FAME, biodiesel) and glycerol, requires 3 mol of alcohol for each mole of triglyceride to produce 1 mol of glycerol and 3 mol of FAME. Industrial processes commonly use 6 mol of methanol for each mole of triglyceride to ensure that the reaction is driven in the direction of FAME formation and high conversion of triglycerides. The yield of FAME on the basis of triglycerides typically exceeds 98% (w/w).

Transesterification can be catalyzed by acids, alkalis or lipase enzymes. Alkali-catalyzed transesterification is about 4000 times faster than the acid catalyzed reaction and are commonly used as commercial catalysts. Nevertheless, alkali can form soup with free fatty acids, which exist in most commercial oils, especial in waste oils. Existence of soup drastically increases difficulties in downstream processing. A

common technique of deal with this problem is to use acid catalyze the esterification between free fatty acids and methanol and then use alkali to catalyze the transesterification reaction between triglycerides and methanol. It has been demonstrated that alkoxides such as sodium methoxide are even better catalysts than sodium hydroxide and are being increasingly used. Use of biocatalysts such as lipases offers important advantages, however, is not currently feasible because of the relatively high cost of the catalyst. Nevertheless, progress has been made in maintain the activity of lipase in heterogeneous transesterification for prolonged enzymatic reaction, which led to drastic reduction of production costs. It is expected bio-transesterification will probably gain popularity in the near future.

Bio-hydrogen

Hydrogen, a carbon-free renewable energy carrier undergoes combustion without the release of green house gasses, is perceived to be one of the major alternatives to mitigating the current global environmental challenges.

At present hydrogen is produced mainly from the reformation of natural gas via the water-gas shift reaction, which constitutes about 90%, while a limited amount is produced via other thermo-chemical processes and electrolysis of water. These pathways, however, are energy intensive, making them costly and with inherently high environmental impact compared to biological hydrogen production. Biohydrogen production, a renewable method of hydrogen production, is potentially cost-effective, sustainable, and of little or no environmental footprint. Often the main consideration for process selection is cost, operational flexibility, process efficiency, safety and

environmental concerns.

Microalgae, like higher plants, fix carbon dioxide to produce oxygen in the presence of light to produce glucose and biomass via in the process termed photosynthesis, which involves two photosynthetic centres: photosystem II (PS II) and photosystem I (PS I). Some species can utilize the same photosynthesis system for the generation of hydrogen gas rather than for carbon fixation. In the process of hydrogen production by the pathway of water photo-splitting, water splitting and O_2 evolving system is carried out by PSII and the reducing power for the reduction of proton to molecular hydrogen is generated by PSI (Das and Vezirolu, 2001). Since it was discovered by Garon et al. in early 1940s (Gaffron and Rubin, 1942) that the eukaryotic unicellular green algae, *Scenedesmus obliquus*, was able to evolve molecular hydrogen by means of a hydrogenase in the light under anaerobic conditions, a large number of different microorganisms including green algae, cyanobacteria, photosynthetic bacteria and fermentative bacteria have been found to have the capacity to produce hydrogen through different pathways.

Bioethanol

Bioethanol, and to a less extent biobutanol, has been being promoted as clean and renewable fuel that will reduce global warming and air pollution for decades. Production of ethanol via fermentation of sugars derived from corn, sweet potato, and sugar cane (e.g., molasses) is a proven technology and is currently the only means of commercial production of ethanol. This approach, however, competes for land and water resources with the food industry and is therefore not a sustainable means of

biofuel production from a long term perspective.

Extensive studies have been carried out on the utilization of liogcellusic biomass such as trees, grasses, corn stover, and wheat straw for bio-ethanol production.

Up to this point, not much study has been carried out in terms of using microalgae or microalgal biomass for ethanol production. However, it was reported that a few microalgal species could accumulate large quantities of starch as storage materials. The microalgal starch could be employed for ethanol production via saccharification and fermentation similar to ethanol production from corn. It is also possible to use the hybrid approach for ethanol production of ethanol, i.e., gasify microalgal biomass to produce syngas and then produce ethanol by fermentation.

Bio-oil and bio-syngas

When biomass is processed under high temperature in the absence of oxygen, products are produced as three phases: the vapour phase, the liquid phase, and the solid phase. The liquid phase is a complex mixture called bio-oil. Both bio-oils have been demonstrated to be suitable for power generation via both external combustion (e.g., steam cycles, organic Rankine cycles, and Stirling engines) and internal combustion (e.g., diesel engines and gas-turbine engines) or by co-firing with fossil diesel or natural gas (Czernik and Bridgwater, 2004, Bridgwater et al., 1999, Chiaramonti et al., 2007). Nevertheless, they have several undesirable features such as high oxygen content, low heat content, high viscosity at low temperature, and chemical instability (Czernik and Bridgwater, 2004, Bridgwater et al., 1999, Chiaramonti et al., 2007) that impede their use as quality transportation fuels.

Chiaramonti et al., 2007) that impede their use as quality transportation fuels. Production of high-grade transportation fuels from biomass has been demonstrated to be technically feasible by gasification and subsequent Fischer Tropsch synthesis (Petrus and Noordermeer, 2006, Lv et al., 2007). Recent work by a group in China has demonstrated that hydrogen gas can be derived reliably by steam-reforming bio-oil (Wang et al., 2008).

Most studies have so far focused on the use of conventional biomasses from forestry and agricultural sources (Demirbas, 2001). It was estimated that in year 2000, the majority of biomass energy was produced from wood and wood wastes (64%), followed by municipal solid waste (MSW) (24%), agricultural waste (5%) and landfill gases (5%) (Demirbas, 2001, Demirbas and industry, 2000). Recently, a few investigations have been carried out regarding the suitability of microalgal biomass for bio-oil production (Miao and Wu, 2004, Miao et al., 2004, Demirbas, 2006). It was shown that, in general, microalgae bio-oils have higher quality than bio-oil from wood (Demirbas, 2006).

2.3.2 Combination of microalgal cultivation with wastewater treatment

Microalgal CO_2 bio-mitigation could be made more economically cost-effective and environmentally sustainable, especially when it is combined with other processes such as wastewater treatment. The utilization of wastewater for microalgal cultivation will bring about remarkable advantages which includes: 1) microalgae have been shown to be efficient in nitrogen and phosphorus removal (Mallick, 2002) as well

wastewater treatment will significantly enhance the environmental benefit of this strategy; and 2) it will lead to savings in term of minimizing the use of chemicals such as sodium nitrate and potassium phosphorus as exogenous nutrients and 3) it will result in savings of the precious freshwater resources. Figure 2.2 depicts a conceptual flow chart for the complete "recycling" of CO_2 for solar energy capturing. This review strives to provide a systematic account of recent developments in the field of microalgal CO_2 bio-mitigation, with a focus on microalgal strains for the fixation of CO_2 from different sources, the combined CO_2 mitigation and biofuel production strategy, the combined wastewater treatment and CO_2 mitigation strategy, microalgal nutrition and cultivation, and microagal biomass harvesting.

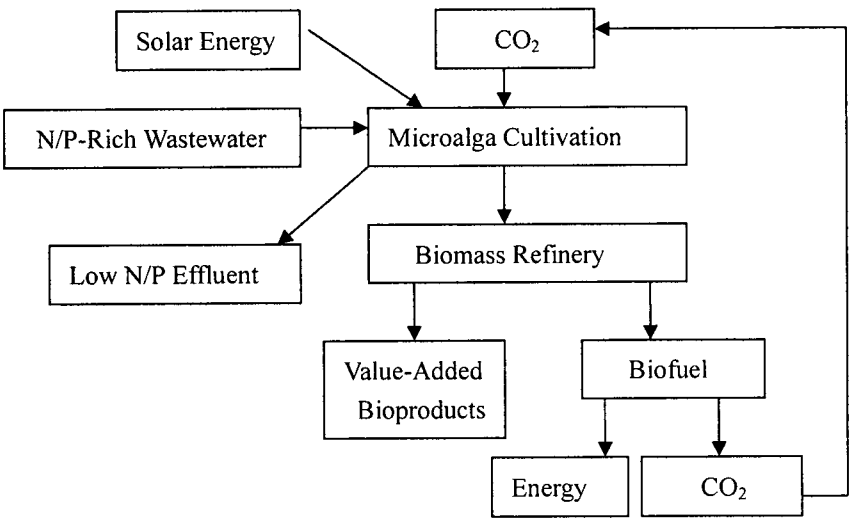

Figure 2.2 A conceptual microalgal system for combined biofuels production, CO_2 bio-mitigation and N/P removal from wastewater. Inputs: carbon source, CO_2; nitrogen and phosphorus sources, N/P-rich wastewater; energy source, solar energy. Outputs: Low N/P effluent, value-added bioproducts, and biofuels.

Combination of wastewater treatment and CO_2-fixation from microalgal biomass provides additional economic incentives due to the savings from chemicals (the nutrients) and the environment benefits (Mallick 2002), which include: 1) microalgae have been shown to be efficient in nitrogen and phosphorus removal (Mallick 2002) as well as in metal ion depletion, and combination of microalgal cultivation with wastewater treatment will significantly enhance the environmental benefit of this strategy; and 2) it will lead to savings in term of minimizing the use of chemicals such as sodium nitrate and potassium phosphorus as exogenous nutrients and 3) it will result in savings of the precious freshwater resources. Figure 2.2 depicts a conceptual flow chart for the complete "recycling" of CO_2 for solar energy capturing. Finally, enhance the primary production of oceans by means of ocean fertilization may provide a cost effective strategy of large scale CO_2 sequestrating that is efficient enough to cope with the global warming. The potential of wastewater treatment by microalgae have been investigated by a few researchers (Oswald 1973; Benemann, Koopman et al. 1977) with several microalgal strains tested for this purpose. For instance, *B. braunii* was shown to be able to remove nitrogen and phosphorus from secondarily treated wastewater (which has been eliminated the easily settled materials and organic materials) in a batch system and a continuous bioreactor system with hydrocarbon production. *Chlorella vulgaris* (Yun, Lee et al. 1997) was cultivated in wastewater discharged from a steel-making plant with the aim of developing an economically feasible system to remove ammonia from wastewater and CO_2 from flue gas simultaneously. CO_2 fixation and ammonia removal rates were estimated as

$CO_2/m^3 \cdot h$ (0.624 g/l·day) and 0.92 g NH_3 $/m^3 \cdot h$, respectively, when the alga was cultivated in wastewater supplemented with 46.0 g PO_4^{3-}/m^3 without pH control at 15% (v/v) CO_2.

Microalgal biomass production by outdoor cultivation of *Scenedesmus obliquus* in artificial wastewater under the winter and summer tropical conditions of Mazatlán, Sinaloa, Mexico has been reported (Gomez-Villa et al., 2005). The biomass concentrations were 26 an 43 mg/l after three days of cultivation in the winter and summer, respectively, corresponding to biomass productivities of 9 and 16 mg/l· day, which were equivalent to CO_2 fixation rates of 16.07 and 31.0 mg CO_2 /l· day when the aforementioned typical biomass molecular formula was adopted for the calculation. The final dissolved nitrogen concentrations were 53% of the initial value in winter and 21% in summer. Phosphorus was removed only during the day, with a total abatement of 45% in winter and 73% in summer.

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Chapter 3 Optimizing the lipid production of the green alga *Neochloris oleoabundans* using Box-Behnken experimental design in combination with factor grouping

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3.1 Abstract

A systematic approach involving factor grouping, factorial design and response surface analysis was demonstrated to be an effective method for studying the effects of a large number of nutrients on cell growth and lipid production of the green alga *Neochloris oleoabundans* for the purpose of medium optimization. Box-Behnken experimental design (BBD) was used for factorial experimental design and response surface method (RSM) was used for data analysis. The optimized medium obtained was able to support a lipid concentration of 641.4 mg lipid/l and a cell density of 2.54 g/l, which were 4.5-fold and 2.2-fold higher than that obtained with the basic medium, modified Bristol medium (MBM), respectively.

Keywords: microalgae, lipid, factorial design, nutrient grouping, Box-Behnken experimental design, response surface analysis, *Neochloris oleoabundans*

3.2 Introduction

Biodiesel is one of the most promising renewable transportation fuels that have achieved remarkable success worldwide and it is predictable that massive global demand on renewable energy will continue to drive the rapid growth of biodiesel production in an unprecedented scale (Courchesne et al., 2009). Development of sustainable and cost-effective alternative sources of lipids, the raw material of biodiesel production is therefore of great need. To this end, oil-rich microalgae have been demonstrated to be promising candidates. In fact, it might be the only practical lipid source for sustainable biodiesel production from a long-term perspective since production of lipids by agricultural crops is too slow due to the limited photosynthetic efficiency of terrestrial plants (Li et al., 2008a, Christi, 2007).

Several microalgal species have been investigated for lipid production. It was reported by Reitan et al. (Reitan et al.,1994) that *Chlorella emersonii*, *Chlorella minutissima*, and *Chlorella vulgaris* could accumulate lipids of up to 63%, 56%, and 57.9%, respectively under nitrogen starvation conditions. Yamaberi et al. (Yamaberi et al., 1998) reported *Dunaliella*, a marine alga, accumulated a high intracellular lipid content of 67% when the initial NaCl concentration in medium was 1.0 M (seawater equivalent to 0.5 M NaCl). *Neochloris Oleoabundans* was reported to be able to accumulate 35-54% lipids of its cell dry weight and triglycerides comprised 80% of the total lipids (Tornabene et al., 1983). Of particular relevance, a recent study carried out by our group demonstrated that *N. oleoabundans* was capable of producing lipids at a productivity of 0.133 g/l·d at 5 mM with a lipid cell content of 0.34 g/g and

a biomass productivity of 0.40 g/l·d (Li et al., 2008a), which were confirmed recently by another group of researchers using an airlift photobioreactor. Nevertheless, the primary challenge impeding commercial microalgal production remains the relatively high production costs of microalgal cultivation, which is expected to be overcome by extensive studies including medium optimization. Systematic studies on the effects of different nutritional and operational factors on cell growth and lipid biosynthesis of oil-rich microalgal strains are therefore of both scientific and practical relevance. Understanding of these effects is essential for formulation of optimal medium to enhance oil production and reduce production costs.

Most studies regarding the cultivation of microalgae have been carried out using single factor searching, which is based on investigating the effects of a single nutrient on the physiology of microalgae while fixing the composition of other nutrients. The single factor searching approach, although capable of providing valuable information, is in general tedious and of low efficiency. It is especially so for the optimization of microalgal media considering the large number of nutrients contained in a microalgal medium. It is therefore of value to develop a more efficient approach for microalgal medium optimization.

Statistic experimental design such as Box-Behnken experimental design (BBD), which allows the study of multiple factors and their interactions using workable number of factors, is getting popularity for medium optimization for different microbial systems(Bae and Shoda, 2005, Annadurai et al., 1999, Ramnani and Gupta, 2004). However, information regarding the application of this approach with

microalgae is scarce in the literature. Furthermore, the extraordinarily large number of nutrients involved in the medium of some microalgal species makes it difficult to handle even with statistical experimental designs. In this study, we employed an integrated approach to systematically study the effects of different nutrients on cell growth and lipid production of *N. oleoabundans*. This approach involved the grouping of different nutrients according to their effects on the physiology of microalgae, which were then treated as individual single factors in pre-studies and statistical investigations. The Box-Behnken experimental design (BBD) was used for statistical studies and five macroelements, i.e., nitrogen, phosphorus, magnesium, iron, and sulphur, were selected for studies using lipid productivity as the response. It was demonstrated that the grouping strategy, when combined with carefully designed BBD experiments, allowed high-efficiency systematic studies on the effects and co-effects of a large number of medium components.

3.3 Materials and Methods

3.3.1 *Microalgal strain and cultivation conditions*

Green alga *N. oleoabundans* was purchased from the UTEX Culture collection of Algae (UTEX, Texas). The alga culture was adapted to a cultivation temperature of 28±2°C before it was used in the optimization studies.

Experiments were conducted in 500 ml cylinder flasks with 400ml working volume. Agitation was provided by a magnetic stirrer. The culture was bubbled continuously with filtered air enriched with 5% CO_2 at a flowrate of 0.75 vvm. The

cultivation temperature was maintained at 28±2°C. Continuous illumination at 6000 lux was provided by 6×100w fluorescent lights, measured with a quantum meter (model QLS, Biospherical Instruments Inc., CA, USA). The initial biomass concentration for all cultures was controlled in the range of 0.2-0.22 (OD_{600nm}).

3.3.2 *Media*

Modified Bristol Medium, which was comprised of Bristol medium (Tam and Wong, 1996) plus 1 ml A_5 solution per 1000 ml medium, was used as the basic medium for this study. The Bristol medium consisted of the following components (g/ l): $NaNO_3$ (0.25), K_2HPO_4 (0.075), KH_2PO_4 (0.175) and $MgSO_4$ (0.037) , $FeCl_3$ (0.003), $CaCl_2.2H_2O$ (0.025), NaCl (0.025), and the A_5 solution is comprised of the following components (mg/l): EDTA-Fe (1,642.3), H_3BO_3 (2,860), $MnCl_2.4H_2O$ (1,810), $ZnSO_4.7H_2O$ (220), $CuSO_4.5H_2O$ (79), and $(NH_4)_6MO_7O_{24}.4H_2O$ (39). In the optimization studies, medium composition varied as indicated in the text to investigate the effects of different nutrients on cell growth and lipid production.

3.3.3 *Analytical methods*

Biomass concentration was determined turbidometrically at 600 nm using a spectrophotometer (GENESYS 10UV, Thermo Electron Co., USA). Samples were diluted to give an OD_{600} reading between 0.2 – 0.4 if applicable. The OD_{600} reading was then multiplied with a pre-determined conversion factor of 0.4 to obtain the dry cell weight (DCW g/l).

The chlorophyll *a* content of cells was determined as follows: 2 ml of algal suspension was centrifuged at 12000 rpm for 12 min and the supernatant was

discarded. The algae cells were washed with distilled water for three times and then suspended in 1 ml methanol and stored overnight at a temperature of 4°C. The suspension was then centrifuged at 12000 rpm for 12 min and the absorbance of the supernatant was measured at wavelengths 665 nm and 650 nm, respectively. The chlorophyll *a* concentration in the extract (supernatant) was calculated using the following equation (Aslan and Kapdan, 2006):

$$\text{Chlorophyll } a \text{ (mg/L)} = (16.5 \times A_{665}) - (8.3 \times A_{650}) \quad (1)$$

The lipid content of cells was determined using Soxhlet extraction. Algal biomass was first dried and grounded thoroughly in a laboratory oven at 90°C. The dried powder was placed in the thimble of a Soxhlet extractor and the lipid was extracted using 60 ml of ethyl ether for 12 h. Then the residue was dried and extracted again using approximately 60 ml ethyl ether. After extraction, ethyl ether was evaporated and lipid was determined gravimetrically. The lipid content was calculated by the following equation:

$$\text{Lipid content (\%)} = (\text{weight of lipid} / \text{weight of dried algal cells}) \times 100 \quad (2)$$

3.4 Factor grouping and experimental design

3.4.1 The pre-studies: effects of microelements and macronutrients on cell growth

This study was carried out in two stages, pre-studies and BBD optimization. In pre-studies, all 13 different salts used in the medium of *N. oleoabundans* cultivation were divided into two groups according to their concentrations in the medium: microelements and macronutrients. Microelements with the ratios fixed as that of the A5 solution and macronutrients with ratios fixed as that of BM medium, were treated as two independent factors. Effects of microelements and macronutrients on cell growth of *N. oleoabundans* were investigated by varying their compositions in cultivation media.

To investigate the effects of microelements, a volume of 1, 2, 3, 4, or 5 ml of A5 solution was added in 1000 ml medium to formulate media containing one to fivefold of microelements. Similarly, the composition of macronutrients in medium was varied between one to fivefold as that in MBM to investigate the effects of macronutrient on cell growth of *N. oleoabundans*.

3.4.2 The BBD experimental design

BBD design and RSM analysis were employed to elucidate the effects of five important macronutrient salts on cell growth and lipid production of *N. oleoabundans*. Factor grouping was used to reduce the number of factors to increase the efficiency of the optimization studies.

3.4.2.1 Factor selection and grouping

Nitrogen is the constituent element of a large number of essential cell materials (Lourenco et al., 2002) including proteins, nucleic acids, cell wall, pigments, etc. It has been well established that nitrogen-sufficiency promotes high growth rates at the price of lowered oil accumulation in cells, whereas nitrogen-deficiency reduced growth rate and resulted in high oil cell content. In fact, nitrogen is the most commonly used limiting-factor for the stimulation of lipid accumulation in microalgae (Courchesne et al., 2009, Li et al, 2008b, Wang et al., 2008). It is therefore selected as a single factor in the BBD experiments.

Phosphorus is another key macroelement that has significant relevance to virtually every metabolic process of cell growth (Agren, 2004). It is a constituent element of DNA, RNA, adenosine triphosphate (ATP), cell membrane materials, etc. ATP is essential to the cellular processes related to photosynthesis (e.g., photophosphorylation) and other energetic metabolisms (Martinez and Jimenez, 1999, Gauthier and Turpin, 1997), which are critical for cell growth and lipid production of microalgae. Phosphorus is preferentially assimilated as inorganic phosphates in the form of $H_2PO_4^-$ and HPO_4^{2-}, which could serve as the buffering agents for pH control as well. Phosphate, in the form of NaH_2PO_4/Na_2HPO_4 pair, was therefore selected as another factor for optimization.

Magnesium, sulphur and iron are the other three important macronutrients that have significant effects on microalgal physiology and metabolism. Of particular relevance, magnesium is a constituent element of chlorophyll molecule, which is the

key device of photosynthesis. Both iron and magnesium could regulate the uptake of other essential elements and enhance the production of lipids(Roden and Zachara, 1996). Furthermore, Magnesium is required for nitrogenase activity using a creatine phosphate/kinase/ATP generating system as one of its roles in cell metabolism and iron is involved in electron flow from H_2O to nicotinamide adenine dinucleotide phosphate (Raven, 1999). Sulfur is an essential component of cysteine and methionine. In the absence of sulfur, protein biosynthesis is impeded and the photosynthetic system PSII repair cycle is blocked (Zhang et al., 2002). These three elements are supplied by two salts, magnesium sulfate and ferrous chloride. These two salts (i.e., the three elements) are required in small quantities in comparison to that of N and P and both had influent in photosynthesis, therefore, they were grouped as a single factor to reduce the number of factors.

Accordingly, concentrations of $NaNO_3$, the KH_2PO_4/K_2HPO_4 pair at a fixed ratio of 17.5:7.5 w/w, and the $MgSO_4/FeCl_3$ pair at a fixed ratio of 37:3 w/w were chosen to be the three factors of BBD experiments and designated as X_1, X_2 and X_3, respectively (Table 3.1). The ratios of the KH_2PO_4/K_2HPO_4 pair and the $MgSO_4/FeCl_3$ pair followed that in the basic medium, MBM. The numbers shown in Table 3.1 were the total amounts of corresponding salt pairs.

Table 3.1 Coded and real concentration values of variables

Independent Variables(g/l)	Coded symbols	Levels		
		-1	0	1
$NaNO_3$	X_1	0.25	0.375	0.50
K_2HPO_4-KH_2PO_4 (0.43:1)	X_2	0.25	0.5	0.75
$MgSO_4$ –$FeCl_3$ (12:1)	X_3	0.04	0.08	0.12

3.4.2.2 Levels

Since the pre-study results showed that the optimal values of macronutrients is located between one to three times of that of the MBM medium (see discussion in later sections), one, two and three times of the concentration of a salt or a salt pair in MBM were set as levels -, 0 and + for the BBD experiments, respectively, however, for nitrogen source, one, 1.5, and twofold of nitrate was set as levels -, 0 and + due to our previous investigation of nitrogen effect of lipid accumulation (Li et al, 2008). (Table 3.1) The actual design of 15 experiments is given in Table 3.2. Computation was carried out using multiple regression analysis using the least squares method.

Table 3.2 Experimental design and results of dry weight (DCW) and lipid concentration

Series	$NaNO_3$	K_2HPO_4-KH_2PO_4 (0.43:1)	$MgSO_4$ –$FeCl_3$ (12:1)	DCW (g/l)		Lipid (mg/l)	
				Experiment	Predicted	Experiment	Predicted
1	0	0	0	2.21	2.25	499.2	496.0
2	0	-	-	1.71	1.57	122.7	90.0
3	-	-	0	1.86	1.88	354.7	349.1
4	-	+	0	1.59	1.48	407.0	316.2
5	0	0	0	2.31	2.25	488.1	495.9
6	+	0	+	2.38	2.25	202.6	158.5
7	-	0	-	1.12	1.23	97.8	142.0
8	-	0	+	1.65	1.57	289.9	321.0
9	+	+	0	1.79	1.69	173.4	163.8
10	+	0	-	0.94	1.03	36.1	4.3
11	0	0	0	2.23	2.25	500.67	496.0
12	0	-	+	1.95	2.00	420	398.3
13	0	+	+	1.79	1.88	217	222.3
14	+	-	0	2.12	2.17	133.6	195.9
15	0	+	-	0.95	0.79	189.2	197.9

3.4.2.3 Result analysis and data fitting

The BBD experimental results were fitted with the following second-order polynomial equation using a multiple regression technique:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (3)$$

Where Y is the predicted response (lipid concentration in this study, mg/l), β_0, β_i, β_{ii}, β_{ij} are constant coefficients, and x_i, x_j (i = 1-3; j =1-3, $i \neq j$) represent the independent variables (medium composition) in the form of coded values. The quality of fit of the second-order model equation was expressed by the coefficient of determination, R^2, and its statistical significance was determined by an F-test. 3D response surface analysis was conducted by keeping one independent variable at constant level, changing the other two independent variables. The computer software used was jmp (version 6.0, James M. Pleasants Co., Inc., USA)

3.5 Results and discussion

3.5.1 Significant factors affecting cell growth and lipid production

As shown in Fig. 3.1, varying the composition of microelements in the tested range did not result in significant difference in cell growth of *N. oleoabundans*, indicating that microelements were not significant factors to *N. oleoabundans* growth within the test range in this study. On the other hand, as shown in Fig. 3.2, notably improved cell growth was obtained when the macronutrient concentration increased up to threefold. However, further increase from threefold to fourfold did not produce substantial difference in cell growth. This result indicates that some macronutrients were deficient in the MBM and the optimal concentrations of these limiting

macronutrients most likely locate in the range of one to threefold of that in MBM. The lack of variation of cell growth in media containing threefold to fivefold of macronutrients in MBM indicates that they are not inhibitive in this range.

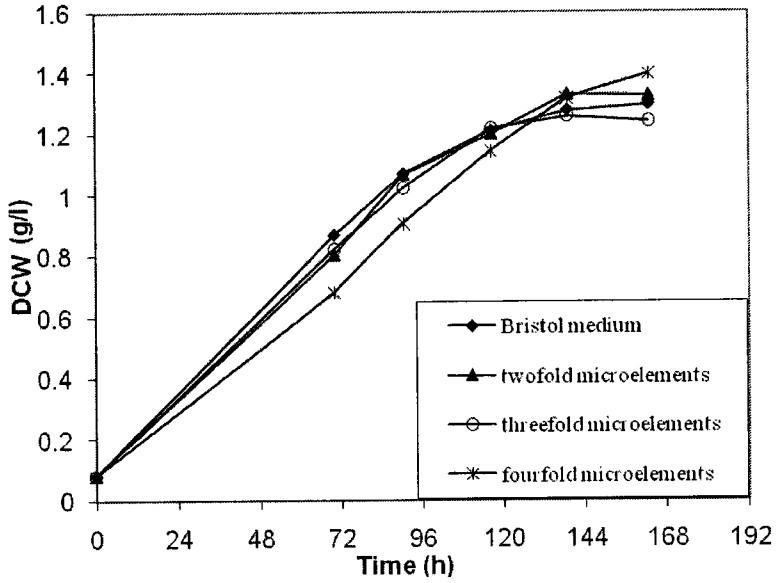


Figure 3.1 Cell growth of *N. oleoabundans* in media containing different strengths of microelements

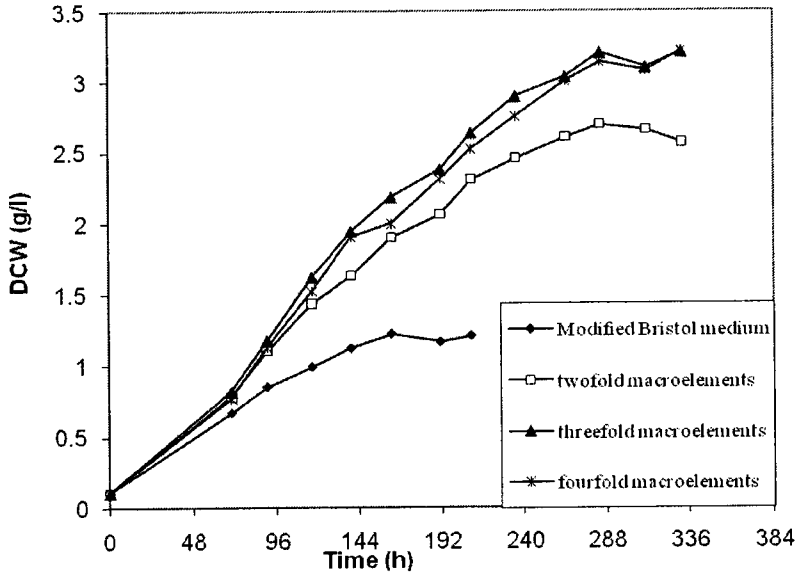


Figure 3.2 Cell growth of *N. oleoabundans* in media with varied macroelements concentrations

The collective factor "macronutrients", which has proven to have significant effects on cell growth of *N. oleoabundans*. It is therefore the logical next step to investigate the effects of the individual elements composing the macronutrients: N, P, S, Fe, and Mg. These five elements were supplemented in the form of five different salts, $NaNO_3$ for N, K_2HPO_4/KH_2PO_4 pair for P, $MgSO_4$ for Mg and S, and FeCl for Fe. As described in the Material and Methods section, BBD design and RSM analysis were used to study the effects of these five salts, which were treated as three independent collective factors: $NaNO_3$, the nitrogen source; K_2HPO_4/KH_2PO_4 pair at a fixed ratio of 7.5:17.5 (w/w), the phosphorus sources; and $MgSO_4/FeCl_3$ pair at a fixed ratio of 37:3(w/w), the source of sulphur, iron, and magnesium. The grouping of different nutrients allowed the reduction of the number of factors from five to three, which can be investigated using 15 experiments with the BBD design. The experimental design and the results of the 15 experiments are shown in Tables 3.1 and 3.2. The results were analyzed using the Analysis of Variance (ANOVA) method and the resulting parameters calculated based on dry cell weight (DCW) and lipid concentration as responses are presented in Tables 3.3 and 3.4, respectively. Substituting the coefficients (β_{ij}) presented in Tables 3.3 and 4 to Eq. 3 results in Eqs 4 and 5, respectively:

$$C_{CDW}(g/l) = 2.251 + 0.124X_1 - 0.225X_2 + 0.386X_3 - 0.018X_1X_2 + 0.223X_1X_3 + 0.165X_2X_3 - 0.242X_1^2 - 0.203X_2^2 - 0.486X_3^2 \quad (4)$$

$$C_{Lipid}(mg/l) = -75.518X_1 - 16.811X_2 + 83.18X_3 - 0.651X_1X_2 - 6.393X_1X_3 - 71.047X_2X_3 - 154.788X_1^2 - 84.399X_2^2 - 184.59X_3^2 + 495.98 \quad (5)$$

Table 3.3 Analysis of variance (ANOVA) for dry weight from BBD design

Source	Coefficient (β)	Standard error	t-Value	P-value*
Intercept	2.2507	0.086	26.16	<0.0001*
X_1	0.1237	0.0527	2.34	0.066
X_2	-0.2249	0.0576	-3.90	0.0114*
X_3	0.3865	0.0527	7.33	0.0007*
X_1X_2	-0.01759	0.079	-0.22	0.8327
X_1X_3	0.2233	0.0745	3.00	0.0302*
X_2X_3	0.1654	0.079	2.09	0.0905
X_1^2	-0.2423	0.0776	-3.12	0.0261*
X_2^2	-0.2032	0.088	-2.31	0.0689
X_3^2	-0.4856	0.0776	-6.26	0.0015*

Significant at P-value less than 0.05

Table 3.4 Analysis of variance (ANOVA) for lipid concentration from BBD design

Source	Coefficient (β)	Standard error	t-Value	P-value*
Intercept	495.98	35.3028	14.05	<0.0001*
X_1	-75.5185	21.6437	-3.49	0.0175*
X_2	-16.8108	23.6332	-0.71	0.5087
X_3	83.1804	21.6437	3.84	0.0121*
X_1X_2	-0.6512	32.4274	-0.02	0.9848
X_1X_3	-6.3925	30.5732	-0.21	0.8426
X_2X_3	-71.047	32.4274	-2.19	0.0800
X_1^2	-154.7875	31.8215	-4.86	0.0046*
X_2^2	-84.3989	36.0898	-2.34	0.0665
X_3^2	-184.59	31.8215	-5.80	0.0021*

Significant at P-value less than 0.05

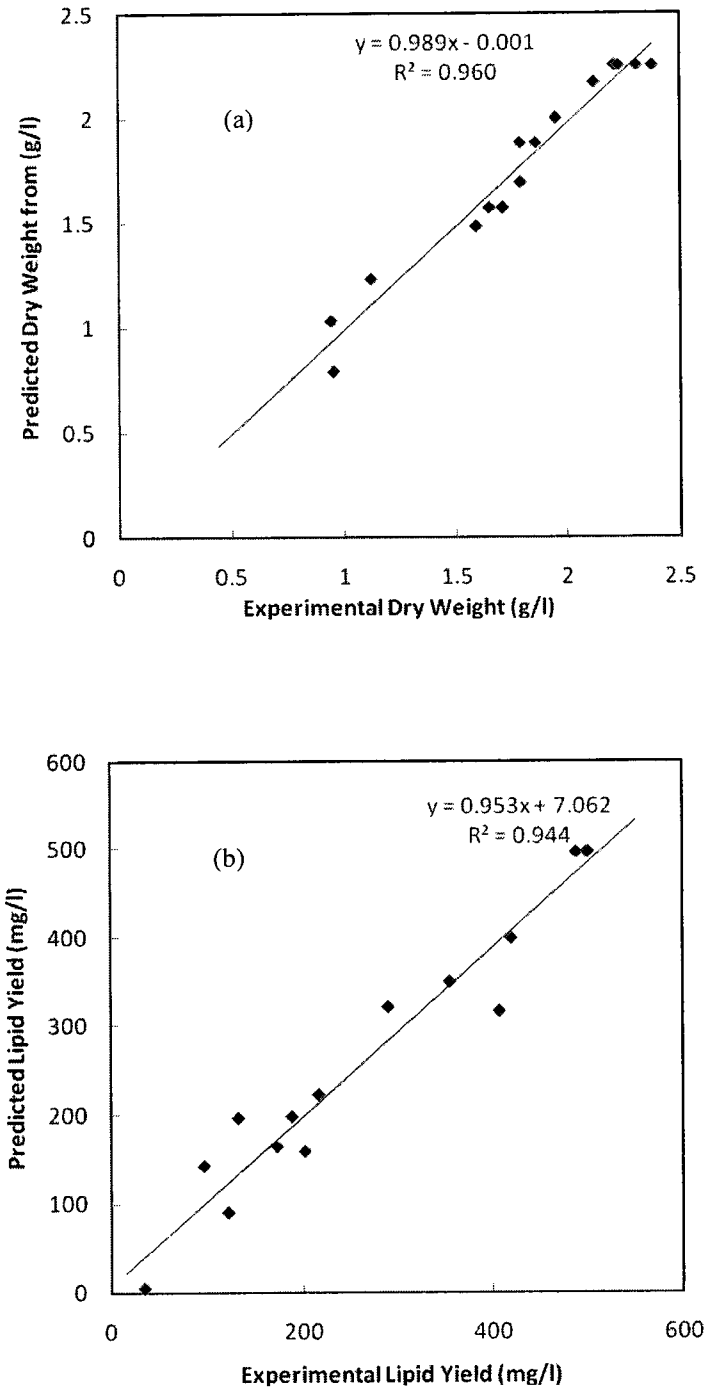
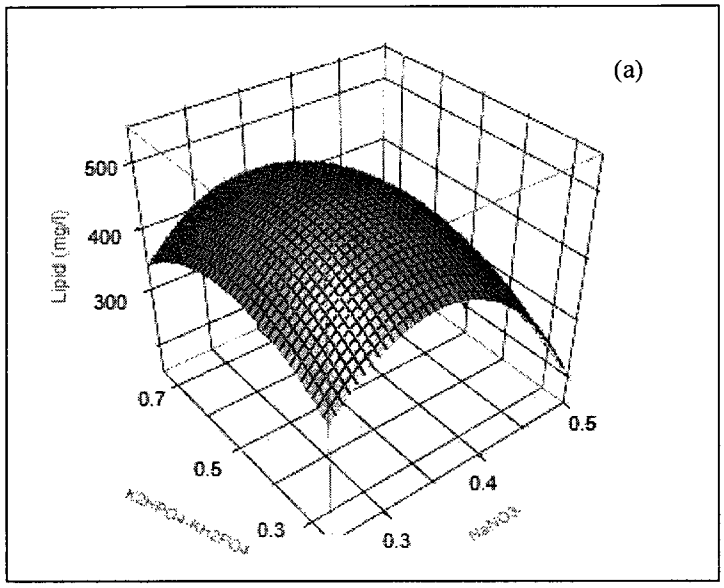


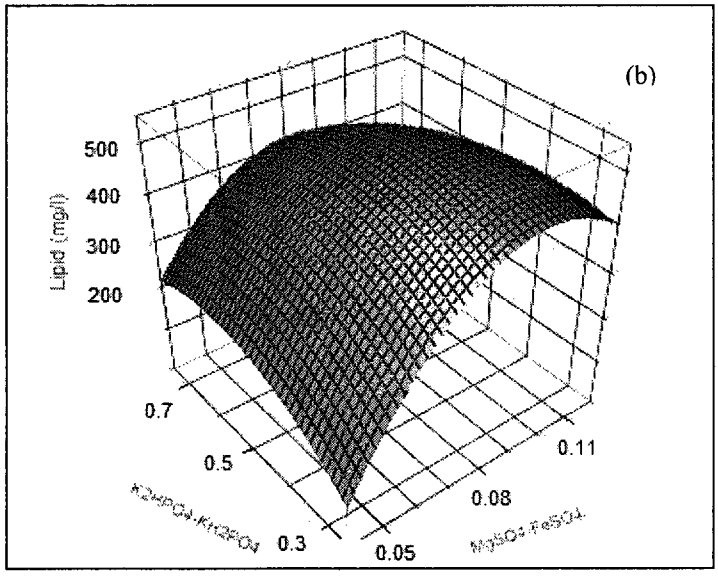
Figure 3.3 Comparison of experimental data and the predicted data: (a) dry cell weight (DCW) and (b) lipid concentration

Eqs 4 and 5 represent the quantitative effects of individual factors and their interactions on the response, C_{DCW} or C_{lipid}. The coefficients as shown in Tables 3.3 and 3.4 are related to the effects of these factors on DCW (Table 3.3) and lipid concentration (Table 3.4). The multiple correlation coefficients (R^2) of the regression equation for DCW and lipid concentration obtained from ANOVA were 0.96 and 0.95, respectively, indicating that these quadratic equations could adequately describe the relationships between the factors and the responses. The sufficiency of the models is also demonstrated by Figure 3.3, which shows good correlation between the experimental values of DCW and lipid concentration and corresponding values calculated using equation 4 and equation 5, respectively.

A model term is considered to be significant when the P-value is less than 0.05. As shown in Table 3.3, when DCW is used as the response, K_2HPO_4/KH_2PO_4 (X_2, P = 0.0114) and $MgSO_4/FeCl_3$ (X_3, P = 0.0007) are significant factors while the effects of $NaNO_3$ (X_1) on cell growth are less than significant in the rested range. Furthermore, the interaction between X_1 and X_3 shows significant effects on cell growth of *N. oleoabundans*. As shown in Tables 3.4, the concentration of $NaNO_3$ (X_1, P = 0.0175) and that of the $MgSO_4/FeCl_3$ pair (X_3, P = 0.0121) are significant factors for lipid production while the phosphate salt pair (X_2) showed less than significant effects.

Three-dimensional response surfaces were generated to visualize the combined effects on the lipid concentration, which are shown in Fig. 3.4. When the effect of two factors was plotted, the other factor was set at level zero as described in Table 3.1.


(a)
Lipid (mg/l)
500
400
300
0.7
0.5
0.3
0.3
0.4
0.5


(b)
Lipid (mg/l)
500
400
300
200
0.7
0.5
0.3
0.05
0.08
0.11

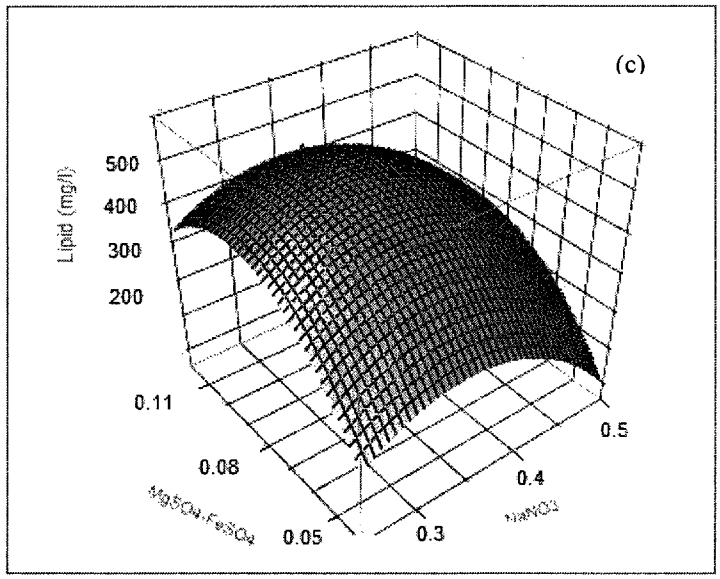


Figure 3.4 Response surface plot for lipid concentration (mg/l) (a) Combined effect of $NaNO_3$ and $K_2(HPO_4)_2$-KH_2PO_4 (b) Combined effect of $K_2(HPO_4)_2$-KH_2PO_4 and $MgSO_4$ –$FeCl_3$ (c) Combined effect of $NaNO_3$ and $MgSO_4$ –$FeCl_3$

Fig. 3.4a shows the effects of nitrate and phosphate on lipid production. Lipid concentration increases with an increase of nitrate at the low concentration range. However, it decreases slightly in the high nitrate concentration range. Apparently, there is an optimal value of sodium nitrate concentration for optimized lipid production. This observation is compatible with the results of our previous studies (Li et al., 2008b). On the other hand, no significant effect on lipid production was observed by varying the concentration of the phosphate pair within the tested range.

Fig. 3.4b depicts the interaction between phosphate salt pair (X_2) and the magnesium sulfate and ferrous chloride pair (X_3) when the sodium nitrate concentration is fixed at level 0 (0.375 g/l). At the low range of the $MgSO_4$/$FeCl_3$ pair

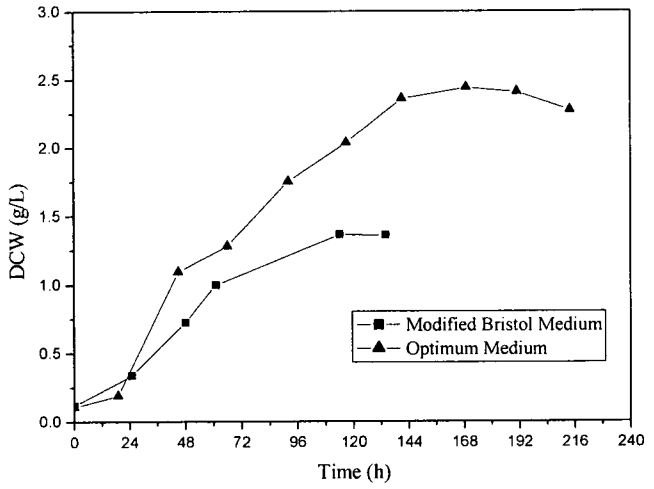
(X_3), lipid concentration increases rapidly with the increase of X_3. However, lipid concentration decreases slightly with the increase of X_3 after the optimal value. On the other hand, the effect of the phosphate salt pair is relatively moderate.

The effects of sodium nitrate (X_1) and $MgSO_4/FeCl_3$ pair (X_3) when X_2 is fixed at level zero are depicted in Fig. 3.4c. The optimum values of lipid correspond to approximately the coded zero level of each factor.

3.5.2 Optimal medium for lipid production of *N. oleoabundans*

Among the major cell components of microalgae, i.e., proteins, carbohydrates and lipids, lipids are the most desirable from an energetic point of view (Wang et al., 2008). The biomass with a higher lipid content and lower carbohydrate and protein contents have elevated calorific values and produce higher concentration of oil when processed via, for instance, biomass liquefaction (Ginzburg, 1993, Scragg et al., 2003, Illman et al., 2000). Microalgal oils are also regarded as the feedstock of future for biodiesel production. For this reason, we optimized the medium with respect to lipid concentration.

A maximum lipid concentration was predicted as 603 mg/l with the optimal medium under the investigated conditions (composition in g/l): $NaNO_3$ (0.35), K_2HPO_4 (0.138), KH_2PO_4 (0.322) $MgSO_4$ (0.0823), $FeCl_3$ (0.0068) and the rest the same as that in MBM. Verification of the optimal medium composition was carried out in duplicates. The time-course profiles of biomass concentration and the cell content of chlorophyll *a* are shown in Fig. 3.5.



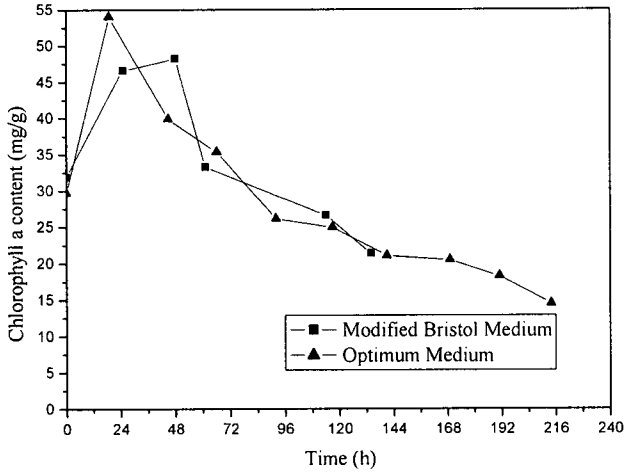


Table 3.5 Comparison of biomass productivity and lipid productivity of *N. oleoabundans* in Modified Bristol medium (basic medium) and the optimized medium

As shown in Fig. 3.5a, cell growth was significantly improved with the optimized medium in comparison to that obtained with the basic medium. The maximum biomass density in *N. oleoabundans* cultures was two times higher than that obtained with in the basic medium. As shown in Fig. 3.5b, the cell contents of chlorophyll *a* of cells growing in the two media showed similar trends of change: they

both increased significantly in the first day of cultivation and started to decrease from the second day or the third day of cultivation. The trend of chlorophyll *a* decreasing continued until the end of cultivation. The decrease of chlorophyll cell content was also observed in our previous studies and was explained by the fact that *N. oleoabundans* utilized chlorophyll as the internal nitrogen reservoir to support cell growth after the external nitrogen reservoir (i.e., sodium nitrate) was depleted from the culture (Li et al., 2008b).

As summarized in Table 3.4, significant improvements were obtained with the optimized medium when comparing with the basic medium, the MBM. The maximum biomass concentration obtained with the optimized medium was 2.54 g/l, more than twofold of that obtained with MBM (1.19 g/l). The lipid concentration was 635 mg/l, which was 4.5-fold of that obtained with MBM.

Table 3.5 Comparison of biomass productivity and lipid productivity of *N. oleoabundans* in Modified Bristol medium (basic medium) and the optimized medium

	Basic medium	Optimized medium
Biomass Concentration (g/l)	1.19	2.54
Lipid Cell Content (g lipids/g DCW)	0.12	0.25
Lipid Concentration (mg/l)	142.8	634.9

3.6 Conclusions

In this study, the BBD with response surface analysis was successfully employed for the optimization of medium in combination with a factor grouping approach. All the nutrients were first divided according to their concentration in medium into two groups: macronutrients and microelements, which were studied consequently as two individual factors. Several important macronutrients, including nitrogen, phosphorus, sulfate, magnesium, and iron, were then grouped into three groups, sodium nitrate as nitrogen source, phosphate salt pair as phosphorus source, and magnesium sulfate and ferrous chloride salt pair as the sources of sulphur, iron, and magnesium. The factor grouping approach made it possible to use a reasonable number of experiments to investigate the effects of a large number of nutrients, including 6 microelement-salts and 7 macronutrient-salts on the cell growth and lipid production of *N. oleoabundans*. This study demonstrates that the BBD design, when combined with careful factor selection and grouping, could be employed for efficient medium optimization.

It was determined that in the tested range, microelements have no significant effects on cell growth. Among the macronutrients in the tested range, it was determined the $NaNO_3$ has significant effects on lipid accumulation, and magnesium sulfate/ferrous chloride salt pair has significant effects on both cell growth and lipid accumulation. It was also determined that the interaction between $NaNO_3$ and the phosphate salt pair had significant effects on cell growth, even though the effect of $NaNO_3$ alone was not significant in the tested range.

An optimal medium was formulated according to the optimization results, which

is comprised of (g/l): $NaNO_3$ (0.35), K_2HPO_4 (0.138), KH_2PO_4 (0.322) $MgSO_4$ (0.0823), $FeCl_3$ (0.0068) and the rest the same as that in MBM.

A biomass concentration of 2.54 g DCW /l and a lipid concentration of 634.9 mg/l were obtained with the optimized medium, corresponding to 2.2-fold of cell growth and 4.5-fold of lipid concentration in comparison with that obtained with the basic medium, the MBM medium.

Acknowledgement

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Chapter 4 Removal of nitrate, ammonium and phosphate from artificial or municipal wastewater by *Neochloris oleoabundans*

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4.1 Abstract

The capacity of a *N. oleoabundans* strain for phosphorus (P) removal and nitrogen (N) removal was investigated using artificial wastewater and municipal wastewater effluents. It was demonstrated that the strain could achieve near-zero residual phosphate and nitrogen at the end of cultivation when artificial wastewater contained up to 140 mg $N\text{-}NO_3^-$/l and 47.0 mg $P\text{-}PO_4^{3-}$/l at rates of N and P removal of 27.5 mg $N\text{-}NO_3$/ l·d, and 9.4 mg $P\text{-}PO_4^{3-}$/l·d, respectively. No sign of inhibitive effect on cell growth of *N. oleoabundans* was observed when the effluent of a municipal wastewater treatment facility was used as the base for medium formulation. However, while it was observed that phosphorus content of the wastewater effluent was sufficient, the nitrogen content was insufficient and additional nitrogen source (i.e., $NaNO_3$) of 70 mg/l was required for optimal cell growth, CO_2 fixation, and phosphate removal.

Keywords: wastewater effluent, N/P removal, biofuel, microalgae, CO_2 sequestration

4.2 Introduction

Effluents generated from urban, agricultural and industrial wastewater treatment facilities require pre-treatment before disposal into river, lakes and oceans according to environment regulations. These effluents have been deprived of easily-settled--materials and organic materials by primary and secondary treatments, respectively. However, inorganic nitrogen (N) and phosphorus (P) contained in the effluents (i.e. the secondary effluents) can cause long-term environmental problems such as eutrophication of lakes and other water bodies and need to be removed by tertiary and higher order treatment processes (De la Noue et al., 1992).

The most widely used tertiary procedure for P and N removal has been physico-chemical dephosphatization and biological nitrification and denitrification up to now. (Guomin et al., 2009, Balamane-Zizi and Ait-Amar, 2009). The primary disadvantage of physico-chemical dephosphatization processes concerns the high operational costs and secondary pollution due to addition of chemicals for phosphate precipitation. As for biological nitrification and denitrification processes, intensive power consumption associated with the vigorous aeration required for these processes presents one of the largest challenges. It was estimated that a tertiary process aimed at complete removal of ammonium, nitrate and phosphate could be four times more expensive than the combination of primary and secondary treatments (De la Noue et al., 1992).

The very reason that makes the secondary effluents a potential environment hazard, i.e., they contain large quantities of inorganic nutrients such as nitrogen,

phosphorus and metal ions, allows them to be employed to reduce the cost of microalgal farming, which has been demonstrated to be one of the most promising strategies for recycling CO_2 to biofuels. Combining wastewater tertiary treatment with microalgal cultivation for biofuel production offers many advantages (Mallick, 2002), which include: 1) microalgae have been shown to be efficient in nitrogen and phosphorus removal (Mallick, 2002) as well as in metal ion depletion, and combination of microalga cultivation with wastewater treatment will significantly enhance the environmental benefit of this strategy; and 2) it will lead to savings in term of minimizing the use of chemicals such as sodium nitrate and potassium phosphorus as exogenous nutrients and 3) it will result in savings of the precious freshwater resources.

A few microalgal strains have been demonstrated to be efficient cellular photobioreactors for CO_2 sequestration, biofuel production, and/or wastewater treatment (Wang et al., 2008). Of particular interest, *N. oleoabundans* has been demonstrated to be a fast growing microalgal species that could accumulate large quantities of triglycerides (Tornabene et al., 1983). Our earlier studies have shown that this species could accumulate up to 40% lipid on dry biomass basis at a lipid productivity of 0.133 kg $m^{-3}d^{-1}$, making it a promising candidate for microalgal biodiesel production (Li et al., 2008b). These results were recently confirmed by a different group of researchers using airlift photobioreactor (Pruvost et al., 2009). It is therefore of interest to investigate the ability of this species to remove phosphorus and nitrogen from wastewater and the potential of using secondary wastewater effluents as

the base of medium formulation to harness the combined benefits of cost-effective CO_2 recycling for biofuel production, pollution reduction, and freshwater saving.

4.3 Materials and methods

4.3.1 Microalgae and Characteristics of secondary wastewaters

Microalgae

The green alga strain *N. oleoabundans* OU2 was adopted from a strain purchased from the UTEX Culture collection of Algae (UTEX, Texas).

Artificial wastewater and secondary municipal wastewater

The composition of artificial wastewater used in this study was: Artificial wastewater contained the following ingredients (mg/l): $MgSO_4$ (37), $FeCl_3$ (3), $CaCl_2.2H_2O$ (25), NaCl (0.025), $NaNO_3$ (850), KH_2PO_4 (75), K_2HPO_4 (175), EDTA-Fe (1.642), H_3BO_3 (2.860), $MnCl_2.4H_2O$ (1.810), $ZnSO_4.7H_2O$ (0.220), $CuSO_4.5H_2O$ (0.079), and $(NH_4)_6MO_7O_{24}.4H_2O$ (0.039). The concentration of $NaNO_3$, K_2HPO_4, and KH_2PO_4 might be varied as indicated in the text.

When applicable, secondary municipal wastewater effluent was provided by ROPEC wastewater-treatment plant (Ottawa, ON, Canada). Effluent was filtered by Whatman Grade GF/C Glass Microfiber Filters (1.2μm) to remove solids. The properties of the secondary municipal wastewater are listed in Table 4.1. Additional nitrogen source (i.e., $NaNO_3$) and phosphorus source (i.e., NaH_2PO_4 and Na_2HPO_4) might be included as indicated in the text.

Table 4.1 major compositions of secondary municipal wastewater

Composition	Concentration (mg/l)
$N\text{-}NH_4^+$	12.3
$N\text{-}NO_3^-$	10
$P\text{-}PO_4^{3-}$	3-6
COD	340-560

Algal cultivation

Experiments were conducted in an algal cultivation system described in a previous publication (Li et al., 2008a). Briefly, cultivation was carried out in 500 ml cylinder flasks with 400 ml working volume. Agitation was provided by a magnetic stirrer. The culture was bubbled continuously with air enriched with 5% CO_2 at a flowrate of 0.75 vvm. Cultivation bottles were located inside a cubic chamber which was equipped with 16 fluorescent lamps (tubes). Temperature inside the chamber was controlled by forced circulation of ambient air using two fans located on two sides of the chamber (Li et al., 2008a).

4.3.2 Analytical methods

Biomass measurement

Biomass concentration was determined turbidometrically at 600 nm using a spectrophotometer (GENESYS 10 UV, Thermo Electron Co., USA). Samples were diluted to give an OD_{600} reading between 0.2 – 0.4 if applicable. The OD_{600} reading was then multiplied with a pre-determined conversion factor of 0.4 to obtain the dry cell weight (DCW g/l).

Nitrate determination

The concentrations of nitrate in media and cultivation broths were determined with HPLC method (Jovanovic et al., 2007). The measurement was performed using an HPLC system (Agilent Technologies Inc., CA, USA) loaded with an Anion IC-PAK^{TM} column (50 mm × 4.6 mm, 10 μm particle size) (Waters, Millipore, USA). The flow rate of mobile phase was controlled at 1.2 ml/min and the UV detector set at 220 nm. The mobile phase (pH 8.5) was composed of borate buffer/gluconate concentrate, methanol, acetonitrile, and deionized water at a ratio of 2:12:12:74 (v/v/v/v). The borate buffer/gluconate concentrate was composed of 0.07 M sodium gluconate, 0.3 M H_3BO_3, 0.1 M $Na_2B_4O_7$ and 3.8 M glycerol in deionized water (100 ml). The inject volume was 50 μL.

Ammonium determination

Ammonia (NH_4^+) concentration was determined according to Standard Method 4500D (APHA 1992) using an Orion ammonia electrode (model 95-12) connected to a Fisher Accumet® model 750 pH/ion meter (Fisher Sci., Ottawa, ON).

Phosphate determination

PO_4^{3-} was determined by the colorimetric method of reduction with ascorbic acid. (APHA, 1992) All the analyses were made in the culture medium after separation of the cells by centrifugation.

Calculations

The rate of the removal of a substrate of interest, R_i, (i = phosphate-P, Nitrate-N, or Ammonia-N), is calculated by the following equation:

$$R_i = \frac{S_{0,\,i} - S_i}{t_{0,\,i} - t_i} \qquad (1)$$

where $S_{0,i}$ is the initial substrate concentration of substrate i, S_i the corresponding substrate concentration at time t.

4.4 Results

4.4.1 Effect of temperature on algae growth in artificial wastewater

As shown in Fig. 4.1, the growth profiles of *N. oleoabundans* were very similar to each other when temperature varied from 25 ℃ to 30℃, with a slightly higher biomass concentration of 3.0 g/l obtained at 30 ℃ at the end of the 240 h cultivation period. The biomass concentrations obtained in the same period were 2.8 and 2.5 g/l at 28 ℃ and 25 ℃, respectively. However, cell growth was significantly reduced when temperature increased to 32 ℃. As can be seen from Fig. 4.1, at 32 ℃, the stationary phase of microalgal cell growth started at approximately 96 h of cultivation and the maximum biomass concentration was 1.5 g/l, only 50% of what was obtained at 30 ℃. It is worth mentioning that alga growth curves at all the four different temperatures were mostly linear before the stationary phase was reached. This is in consistence with our previous observations and the linear growth characteristic of the photoautotrophic growth of microalgae was due to light limitation when cell density reached certain level (Li et al., 2008a).

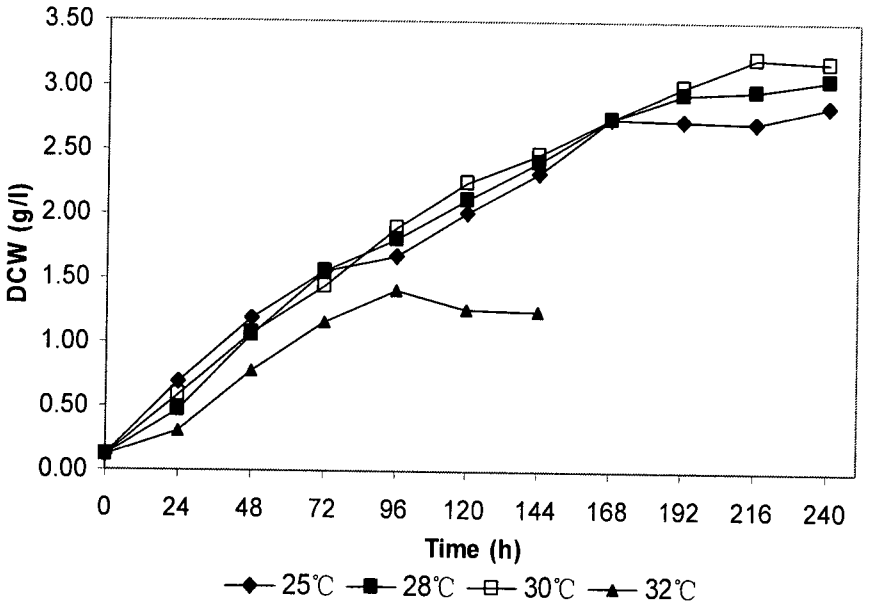


Figure 4.1 Growth Curve and dry cell weight of *N. oleoabundans* with different temperatures

4.4.2 Effect of initial nitrate concentration, phosphate concentration, and N:P ratio in artificial wastewater on cell growth, and N/P removal of *N. oleoabundans*

Some industrial and agricultural wastewaters contain high concentration of inorganic nitrogen (e.g., nitrate and ammonium) and inorganic phosphorus compounds (e.g., phosphates). For instance, piggery wastewaters typically contain total phosphorus of 50-1500 mg P/l and total nitrogen of 300-4000 mg N/l. (De la Noue et al., 1992). They represent a group of high strength wastewaters which require special attention in tertiary treatment. On the other hand, they may also be utilized as low-cost sources of inorganic nitrogen, phosphate and metal ions for alga cultivation. It is therefore of interest to study cell growth, nitrogen removal, and phosphate

removal in artificial wastewaters of varied strengths, i.e., containing different concentrations of nitrate and phosphate.

According to our previous study (Li et al., 2008a), nitrate was the favorite nitrogen source among ammonia, nitrate and urea. Coincidently, nitrate is also one of the major organic nitrogen compounds in wastewater effluents. To study the effect of nitrate concentration on cell growth and N removal of *N. oleoabundans*, artificial wastewater containing 45, 70, 140, 218 mg $N\text{-}NO_3^-$ /l, which corresponded to N:P ratios of 0.42, 0.65, 1.33, and 2.02, respectively, were used. A relatively high phosphate concentration of 108 mg/l $P\text{-}PO_4^{3-}$, which exceeded the phosphate requirement of this strain, was used to avoid phosphate limitation. The results are shown in Fig. 4.2 and Table 4.2.

As shown in Fig. 4.2a, the cell growth of *N. oleoabundans* improved significantly from 1.6 g/l to 3.0 g/l when sodium nitrate concentration increased from 45 to 140 mg-N /l. However, cell growth slightly decreased when sodium nitrate concentration further increased from 140 to 218 mg $N\text{-}NO_3^-$/l, indicating that 140 mg $N\text{-}NO_3^-$/l was optimal for cell growth in the tested range and that 218 mg $N\text{-}NO_3^-$/l was inhibitive to cell growth of *N. oleoabundans* under the specified conditions. On the other hand, as shown in Fig. 4.2b, *N. oleoabundans* has a large nitrate removal capacity, which was not adversely affected by the initial sodium nitrate concentration of up to 218 mg $N\text{-}NO_3^-$/l.

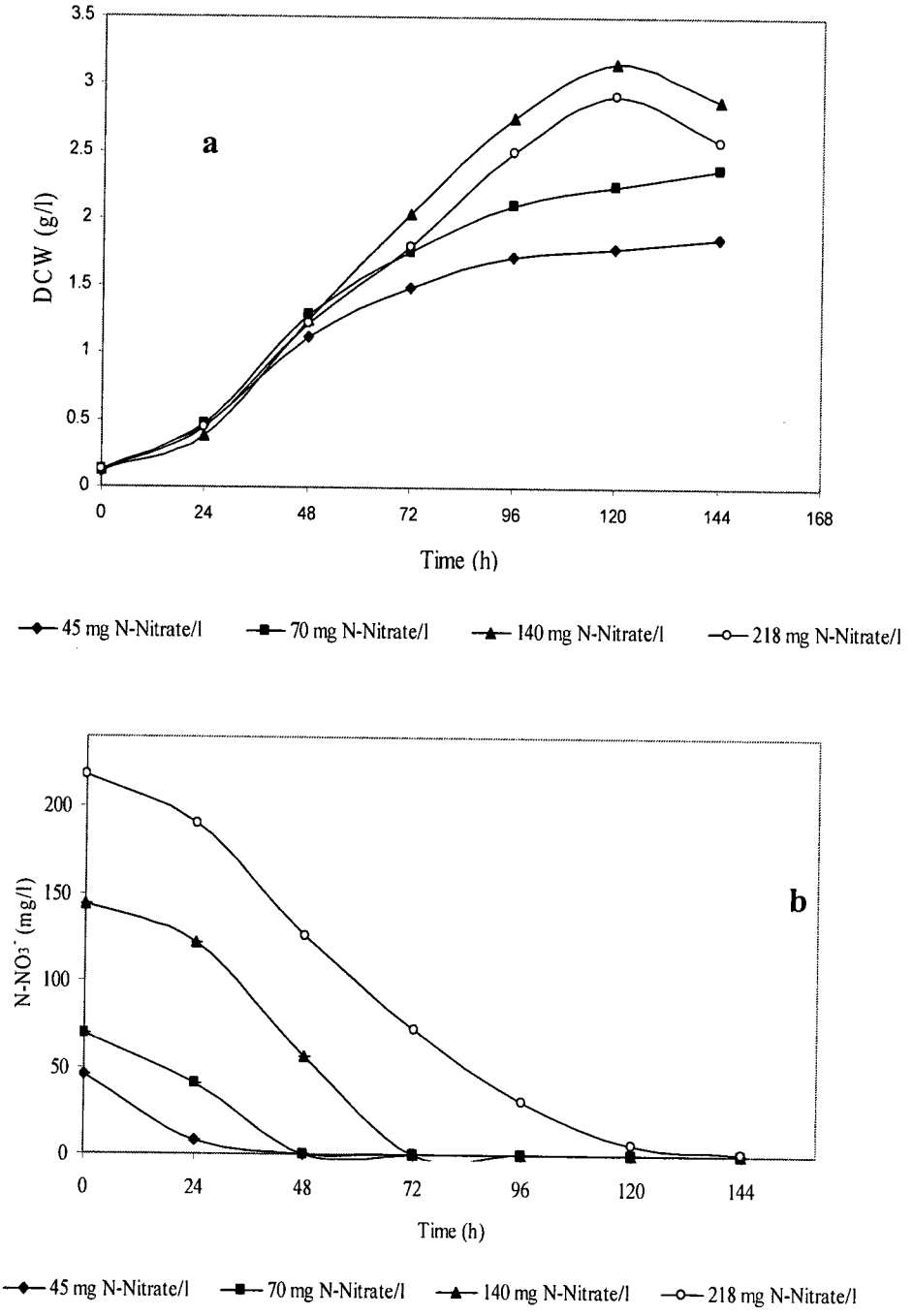


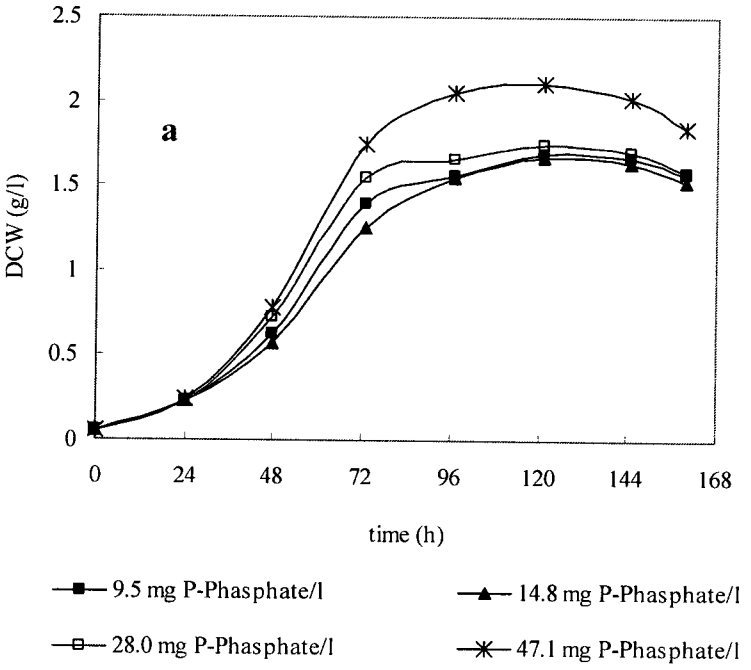
Figure 4.2 Effects of initial nitrate concentration on: a, cell growth, and b, N removal of *N. oleoabundans*.

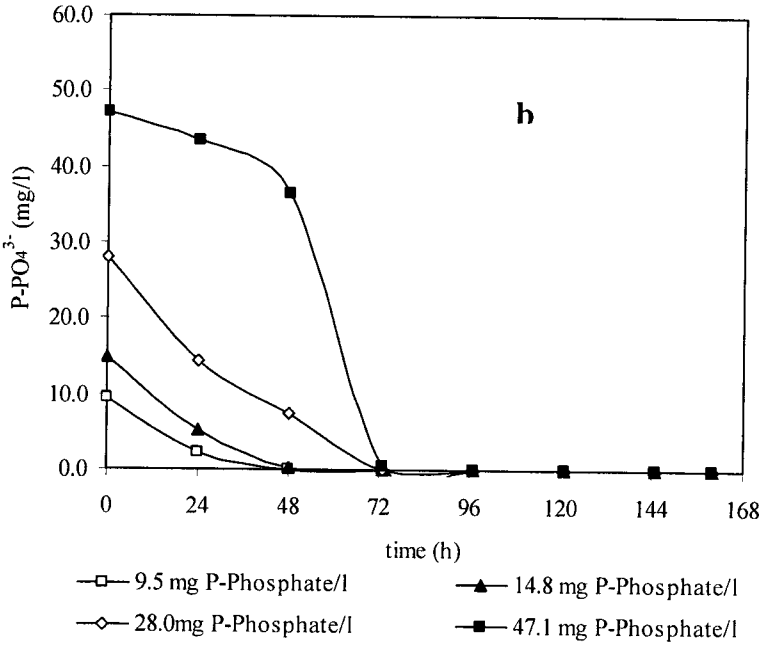
Table 4.2 cell growth and nitrogen removal with different initial nitrogen concentrations

Initial $NaNO_3$ (mg N/l)	N:P	Max. biomass conc. (g DCW/l)	Biomass productivity (g DCW/ l·d)	Residue $N\text{-}NO_3^-$ (mg /l)	Removal Rate (mg/l ·h)	Specific N Removal (mg N/g)
45	0.42	1.85	0.31	0	0.94	24.3
70	0.65	2.37	0.40	0	1.45	29.5
144	1.33	3.15	0.63	0	1.82	45.7
218	2.02	2.91	0.58	1.4	1.77	74.4

Artificial wastewaters containing 140 mg N-Nitrate/l sodium nitrate and varied phosphate concentrations of 5.3, 9.5, 14.8, 28, 47.0 mg $P\text{-}PO_4^{3-}$/l, which corresponded to N:P ratios of 14.7, 9.5, 5.0, and 3.0, respectively, were used to investigate the effects of phosphate concentration and N:P ratio on cell growth, nitrate removal, and phosphate removal when nitrate was supplied in excess. As shown in Fig. 4.3a, there was no significant difference in cell growth of *N. oleoabundans* when the initial phosphate concentration ($P\text{-}PO_4^{3-}$) increased from 5.3 to 14.8 mg/l. However, the growth rate slightly increased and DCW reached approximately 1.8 g/l when the initial $P\text{-}PO_4^{3-}$ was further increased to 28 mg/l and the maximum cell density increased noticeably to 2.1 mg/l at an initial $P\text{-}PO_4^{3-}$ concentration of 47.0 mg/l.

The $P\text{-}PO_4^{3-}$ and $N\text{-}NO_3^-$ concentration time course profiles at different initial $P\text{-}PO_4^{3-}$ concentration are shown in Fig. 4.3b & 3c, respectively. There were residual nitrate left in the cultures with initial P-phosphate at the low range of 9.5 - 28.0 mg $P\text{-}PO_4^{3-}$/ l. Zero residual nitrate was achieved when initial phosphate concentration increased to 47.0 mg P/ l.


a
DCW (g/l)
2.5
2
1.5
1
0.5
0
0
24
48
72
96
120
144
168
time (h)
9.5 mg P-Phasphate/l
14.8 mg P-Phasphate/l
28.0 mg P-Phasphate/l
47.1 mg P-Phasphate/l


b
P-PO4 3- (mg/l)
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
24
48
72
96
120
144
168
time (h)
9.5 mg P-Phosphate/l
14.8 mg P-Phosphate/l
28.0mg P-Phosphate/l
47.1 mg P-Phosphate/l

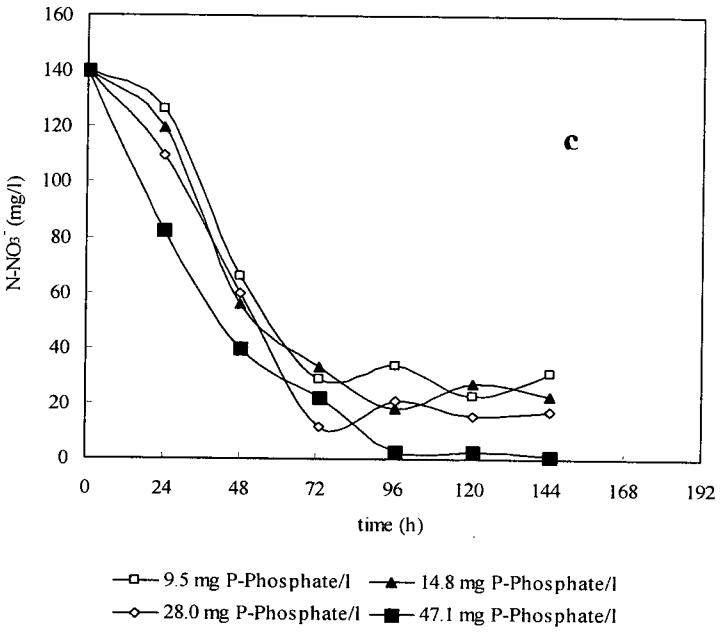


Figure 4.3 Effects of initial phosphate concentrations on: a, cell growth, b, phosphorus removal, and c, nitrogen removal

4.4.3 Cell growth in secondary municipal wastewater effluents

As shown in Table 4.1, the secondary municipal wastewater effluent contains relative low levels of inorganic nitrogen and phosphorus. It is of interest to test cultivation of *N. oleoabundans* in enriched municipal secondary effluent for two purposes 1) investigate the feasibility of using secondary municipal wastewater effluent to replace freshwater for microalga cultivation; and 2) using the enriched secondary effluent to represent the scenario when a source of secondary effluent containing corresponding high levels of inorganic nitrogen and phosphate is available.

Growth curves of *N. oleoabundans* in secondary municipal wastewater effluent enriched with 0 to 105 mg $N\text{-}NO_3^-$ /l were shown in Fig. 4.4 (a). Cell growth in original secondary municipal wastewater containing no additional nitrate ceased at approximately 96 hours after inoculation with a maximum cell density of 0.6 g/l. The

best cell growth was obtained in secondary municipal wastewater with addition of 70 mg/l $N\text{-}NO_3^-$ with a maximum cell density of 2.0 g/l. Increasing nitrogen to 105 mg/l $N\text{-}NO_3^-$ did not lead to higher growth rate or higher maximum cell density. Zero residual $P\text{-}PO_4^{3-}$ and zero residual nitrate were achieved in all tests (data not shown).

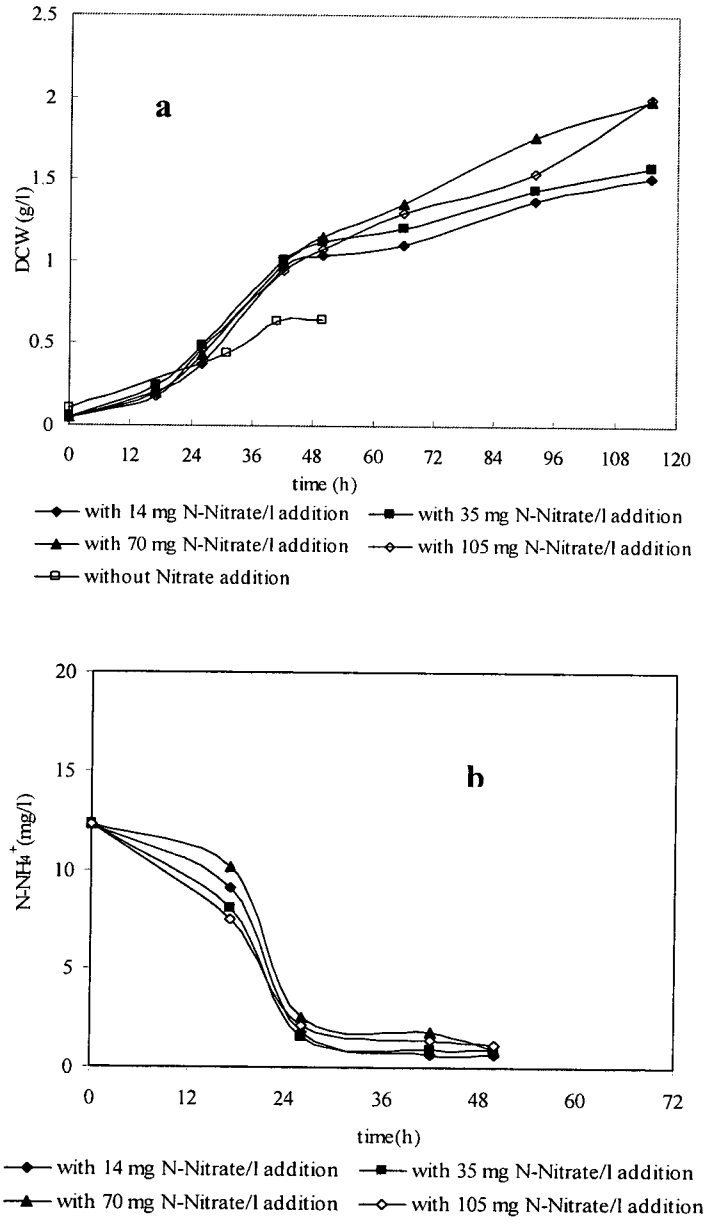


Figure 4.4 Cell growth (a) and $N\text{-}NH_4^+$ removal (b) in secondary municipal wastewater with addition of nitrate

The consumption of ammonium, which was contained in the secondary wastewater effluent, was shown in Fig. 4.4 (b). The ammonium profiles of all the four different nitrate additions show similar trends and the majority of the approximately 12.3 mg $N\text{-}NH_4^+$ /l contained in the secondary effluent (and therefore the medium) was consumed in the first 24 hours, leaving residual ammonium in the broth ranging from 0.5 to 0.8 mg $N\text{-}NH_4^+$ /l. Although significant effects on cell growth were observed with the varied amounts of sodium nitrate added into the medium, the rate of ammonium consumption/removal was very similar in all four cultures at approximately 0.52 mg $N\text{-}NH_4^+$ /h.

4.5 Discussion

4.5.1 Effect of temperature on the cell growth of *N. Oleoabundans*

It was observed that the optimal growth temperature of *N. oleoabundans* was between 25 and 30 °C and significant inhibition to cell growth was observed at 32 °C. A close exam of the growth profiles as shown in Fig. 4.1 reveals that the varied biomass concentrations at different cultivation temperatures was due to two reasons: 1) lower growth rate at 32 °C, and 2) the greatly reduced length of cell growth period, which decreased from between 172 and 216 hours in the optimal temperature range (i.e., 25 – 30 °C) to a drastically shortened period of 96 hours when the temperature was maintained at a unfavorable value of 32 °C. The drastic effect of a small temperature deviation from its optimal range on the cell metabolism of microalgae observed in this study is not unique. For instance, it was reported that an increase in

the temperature to above 28 °C will originate in *Phaeodactylum tricornutum* a sudden drop in the concentration of proteins and PUFAs due to the caused metabolic stress (Bitaube Perez et al., 2008).

4.5.2 Effects of nitrate, phosphate concentration and N:P ratio

The effects of nitrate concentration on cell growth when phosphate was supplied in excess, which are shown in Fig. 4.2a, are reasonable. Nitrogen is an essential element of cells required for the biosynthesis of a large number of cell components including proteins, nucleic acids (RNA and DNA) and photosynthetic pigment (e.g., chlorophyll). Consequently, nitrate limiting could significantly reduce cell growth rate and maximum biomass concentration achievable at the end of cultivation. On the other hand, high concentration of sodium nitrate in the medium (e.g. 218 mg $N\text{-}NO_3^-$ in this study) may cause substance inhibition. Furthermore, nitrogen removal is associated with the cell growth of *N. oleoabundans*. Logically, when the nitrogen source in medium was not sufficient to support good cell growth, the rate of nitrogen removal would be adversely affected. This is reflected by the data shown in Figs. 4.2b & 5 and Table 4.2, which indicate that the rate of $N\text{-}NO_3^-$ removal increased rapidly from 0.9 to 1.4 mg $l^{-1}h^{-1}$ when the initial $NaNO_3$ concentration increased from 45 to 70 mg/l and further increased to approximately 1.8 mg/l·h when initial $NaNO_3$ concentration increased to 140 mg N-Nitrate/l. However, increasing initial $NaNO_3$ beyond 140 mg N-Nitrate/l up to 218 mg N-Nitrate/l did not result in increase of the rate of nitrate removal. It is worth mentioning that, as shown in Table 4.2, zero residual nitrate level was achieved in *N. oleoabundans* cultures containing up to 140

mg N-Nitrate/l corresponding N:P ratios ranging from 0.42 to 1.33. However, 1.4 mg N-Nitrate/l residual nitrate existed at the end of the cultivation when 218 mg N-Nitrate/l was included in the medium (N:P ratio 2.02).

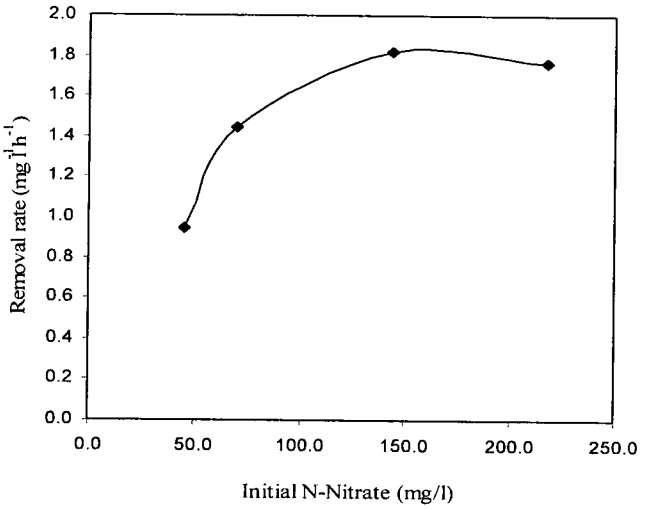


Figure 4.5 $N\text{-}NO_3^-$ removal rate at different initial N-Nitrate concentration

It is also interesting to notice that, as shown in Table 4.2, the specific nitrate removal increased from 24.3 to 74.4 mg N-nitrate/g DCW when the initial nitrate concentration increased from 45.0 mg N-Nitrate/l to 218.0 mg N-Nitrate/l. This is because *N. oleoabundans*, as other microalgae (Leonardos and Geider, 2004), could have very different stoichiometry when growing in media with different N:P ratios, leading to cells of drastically different biochemical composition. More specific to *N. oleoabundans*, it was observed in our earlier studies that a strain of this species could accumulate varied quantities of N-rich photosynthetic pigment, chlorophyll, depending on the availability of nitrogen source in media (Li et al., 2008).

Phosphorus, which is required in the cellular processes such as energy transfer

and nucleic-acid synthesis, is another principal element required for growth of algae but is required at a much lower level than nitrogen. It is uptaken by cells preferentially from inorganic phosphorus in the forms of $H_2PO_4^-$ and HPO_4^{2-}, which are also the primary forms of inorganic phosphate in secondary wastewater effluents. As shown in Fig. 4.3a, cell growth was nearly independent of initial phosphate concentration when it was in the range of 5.3 to 28 mg $P\text{-}PO_4^{3-}$/l, which seems to suggest that phosphate was not limiting in this range under the specified conditions. However, the final biomass concentration in culture was enhanced by 15% from 1.8 g/l to 2.1 g/l, when initial $P\text{-}PO_4^{3-}$ (HPO_4^{2-}-$H_2PO_4^-$) increased from 28 to 47.0 mg/l, probably owing to the buffering capacity of phosphate, which helped stabilize culture pH (data not shown).

As shown in Fig. 4.6, the rate of $P\text{-}PO_4^{3-}$ removal observed a nearly linear relationship to initial $P\text{-}PO_4^{3-}$ concentration, which increased from 0.2 to 0.6 mg P $l^{-1}h^{-1}$ when the initial $P\text{-}PO_4^{3-}$ concentration increased from 4.0 to 47.0 P mg/l. Furthermore, as shown in Fig. 4.3b, zero residual phosphate was achieved for all initial phosphate concentrations ranging from 5.3 to 47.0 mg/l. It is also shown in Fig. 4.3b that the phosphate profile of 47.0 mg/l initial phosphate is distinctively different from the rest, observing a sudden decrease from a relatively high level of approximately 37.0 mg/l at 48 hours to zero at 72 hours. It should be pointed out that the culture pH typically increased from 6.8 -7.2 in the early growth phase to 8.5 – 9.0 in the later growth phase (data not reported), corresponding to the fast phosphorus removal shown in Fig. 4.3b. We hypothesize that the phosphate removal mechanism for the cultures containing high initial phosphate concentration of 47.0 mg/l was

achieved by the combination of abiotic precipitation owing to the elevated culture pH and biological assimilation of phosphate by microalgal cells. The phenomenon of abiotic phosphate removal, which refers to chemical precipitation of phosphate phosphorus due to elevation of culture pH to a threshold value caused by cellular metabolism of microalgal cells, has been observed to be significant when biomass concentration reaches a high level (Wang et al., 2008)(Laliberte et al., 1997).

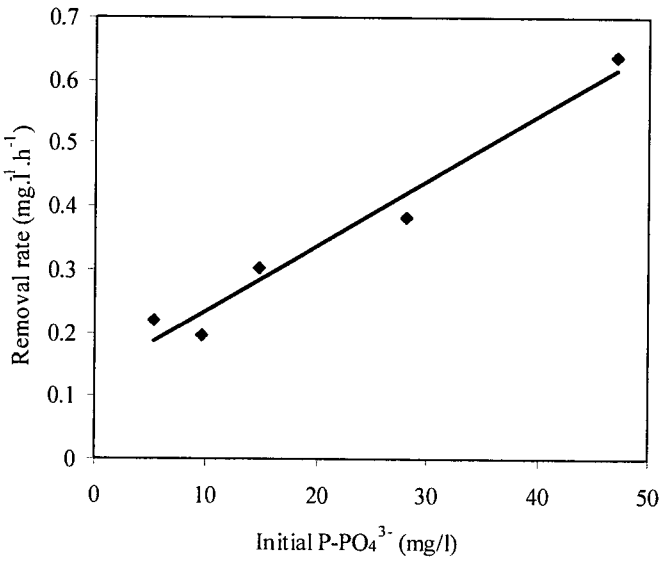


Figure 4.6 Rate of phosphorus removal at different initial $P\text{-}PO_4^{3-}$ concentration

Table 4.3 summarizes the N removal, P removal and cell growth of *N. oleoadundans* in artificial wastewater containing 140 mg $N\text{-}NO_3^-$ /l and varied amount of P-phosphate, which corresponded to N:P ratios in the range of 3.0-14.7. It was evident from the data that the level of residual nitrate in culture at the end of cultivation, which reflects the completion of nitrogen removal, decreased remarkably with the decrease of N:P ratio, from 31.2 to 1.2 mg $N\text{-}NO_3^-$ /l when N:P ratio

decreased from 31.2 to 3.0. Comparing these result with that shown in Table 4.2, it seems to be evident that complete nitrogen removal was possible only at relatively low N:P ratio of close to 2.0. On the other hand, the residual phosphate in culture, which reflects the completion of P removal, was relatively independent of N:P ratio in the tested range, possibly owing to two reasons: 1) nitrogen and other nutrients were in excess in the tested range and 2) the existence of the aforementioned abiotic phosphate removal mechanism, i.e., chemical precipitation of phosphate as a result of elevated culture pH.

Table 4.3 nitrogen and phosphorus removal capacity and efficiency with 140 mg/l initial nitrogen and various initial phosphate concentrations

Initial			Final		N,P removal rate		Removal efficiency		Biomass
$N\text{-}NO_3^-$ (mg/l)	$P\text{-}PO_4^-$ (mg/l)	N:P ratio	N (mg/l)	P (mg/l)	N (mg/l·d)	P (mg/l·d)	N (%)	P (%)	Dry cell weight (g/l)
140	9.5	14.7	31.2	0	23.5	1.9	78	100	1.69
140	14.8	9.5	22.7	0	23	3	84	100	1.7
140	28	5	17.3	0	24.9	5.6	88	100	1.75
140	47	3	1.2	0.03	27.5	9.4	99	100	2.1

4.5.3 Cell growth and N/P removal in municipal secondary effluents

The relatively poor cell growth of *N. oleoabundans* in the secondary municipal wastewater effluent without nitrogen enrichment in comparison to those with enrichment, as shown in Fig. 4.4, indicates that nitrogen in the effluent was not sufficient to support good algal growth for biofuel production and/or CO_2

sequestration. Consequently, it needs to be enriched by adding appropriate amount of heterogeneous nitrogen sources such as sodium nitrate.

Judging from data shown in Fig. 4.4, for the specific municipal effluent used in this study, which contained approximately 12.3 mg N- NH_4^+ /l, The addition of 70 mg N/l sodium nitrate yielded the best cell growth, resulting in a mximum biomass concentration of approximately 1.9 g DCW/l, approximately 3 times of that obtained with basic secondary effluent without nitrate enrichment (0.68 g DCW/l). Further increase nitrate addition up to 105 mg N/l did not lead to significant improvement in cell growth in comparison to that obtained at 70 mg N/l. Meanwhile, results indicate that phosphate (Fig. 4.3b), nitrate (Fig.4.3c) and ammonium (Fig. 4.4 b) could be completely depleted when the concentrations of these compounds were controlled at an appropriate level.

Fig. 4.4 b shows that most of the ammonium existing in the municipal wastewater, which was approximately 12.3 mg N-NH_4^+ /l, could be removed quickly within the first 24 hours. The ammonium profile in the *N. oleoabundans* culture in the secondary municipal wastewater enriched with different levels of sodium nitrate was practically independent of the sodium nitrate enrichment. In other words, the presence of nitrate didn't affect the ammonium removal rate. This is reasonable because ammonium is typically uptaken by microalgae before nitrate is assimilated. The reason being that nitrate is more oxidized than ammonium and needs to be reduced to ammonium before it could be integrated into cellar molecules such as amino acids. The reduction of nitrate to ammonium is an energy consuming process. In other word,

the ammonium consumption by *N. oleoabundans* was likely to be independent of nitrate concentration in the tested range because nitrate was not assimilated before ammonium depletion, which occurred at approximately 24 hours of cultivation.

4.5.4 Potentials of *N. oleoabundans* for combined wastewater treatment and biofuel production

The capacity of *N. oleoabundans* to reduce the residual nitrate and phosphate level in culture at the end of cultivation to zero, even when the initial phosphate and nitrate content of the artificial wastewater or enrich municipal secondary effluent were at relatively high level, implies that it could be utilized for N/P removal from high strength secondary wastewater effluents to produce tertiary effluents satisfying environmental regulations. According to the strength of particular secondary effluents, appropriate dilution may be required for the best results. In case lipids are the target product (e.g., for microalgal biodiesel production), nitrogen (nitrate and ammonium) concentration should be controlled at such a level to allow the maximum lipid productivity (Li et al., 2008a).

The nitrogen and phosphorus removal efficiencies of *N. oleoabundans* varied depending not only on the initial nitrogen concentration but also phosphorus concentration. With 140 mg/l initial $N\text{-}NO_3^-$ and 47.0 mg/l initial $P\text{-}PO_4^{3-}$, a maximum biomass concentration of 2.1 DCW g/l was obtained. The nitrogen and phosphorus removal rates were 27.5 mg/l·d and 9.4 mg/l·d, respectively, under the specified conditions. As shown in Table 4.4, these results are very competitive to literature data reported for other microalgal species.

As summarized in Table 4.4, nitrogen and phosphorus removal efficiencies and biomass productivities of different microalgal species varied significantly from each other. For instance, *Botryococcus braunii* was shown to be able to remove nitrogen and phosphorus from secondary effluent in both batch system and continuous bioreactor systems while producing large quantities of hydrocarbons. The culture was able to consume completely up to 510 $N\text{-}NO_3^-$ mg/l and around 29 mg $P\text{-}PO_4^{3-}$/l within 6 days of batch operation (An et al., 2003). *Chlorella vulgaris* was demonstrated to be able to remove 7.7 mg/l initial PO_4-P concentration with 78% efficiency. The main reason for low removal efficiency was hypothetically attributed to light limitation at high nutrient contents (Aslan and Kapdan, 2006). It has also been reported that the removal rates of nitrogen $N\text{-}NO_3^-$ and phosphorus $P\text{-}PO_4^{3-}$ by *Spirulina platensis* were 3.24-4.06 mg/l·d, 0.312-0.623 mg/l·d, individually. (Lodi et al., 2003) and the removal rates of *C.pyrenoidosa* were reported as 3.4 mg N/l·d and 10.7 mg P/l·d (Tam, 1994). Nitrogen (ammonium and nitrate) and phosphate were removed after 50 to 75 h of growth of *PhormidiumBohneri* in domestic wastewater with phosphorus addition, and biomass productivities of 23-57 mg/l·d, combined with rates of ammonium and phosphate removal up to 20 mg/l·d.

In comparison to the literature data shown in Table 4.4, *N. oleoabundans* demonstrated showed very good performance in the biomass productivity, rate of CO_2 fixation, rate of nitrogen removal and rate of phosphorus removal, with all these parameters only second to that of *B. braunii* as reported by Aslan and Kapdan (Aslan and Kapdan, 2006). In comparison to *B. braunii*, which produces large molecular

weight hydrocarbons as its primary lipid content, *N. oleoabundans* has the unique advantage of producing triglycerides, which are suitable for microalgal biodiesel production.

Table 4.4 nitrogen removal rate, phosphorus removal rate and biomass productivities of some strains

Strains	$R_{N\text{-}NO3}^-$ (mg/l·d)	$R_{P\text{-}PO4}^{3-}$ (mg/l·d)	Biomass Productivities (mg/l·d)	R_{CO2}* (mg/l·d)	Ref
Scenedesmus obliquus	27.4	11.8	84	157.9	(Martnez et al., 2000)
Botrycoccus braunii	510	29	584	1097.9	(An et al., 2003)
C.vulgaris	7	1	N/A		(Aslan and Kapdan, 2006)
C.pyrenoidosa	3.4	10.7	N/A		(Tam, 1994)
Spirulina platensis	3.24-4.06	0.312-0.623	34.7-42.4	72.5	(Lodi et al., 2003)
PhormidiumBohneri	20	4	23-57	75.2	(Laliberte et al., 1997)
Neochloris leoabundans	27.5	9.4	350	658	

* Calculated from the biomass productivity according to equation, CO_2 Fixation Rate (P_{co2}) = 1.88×Biomass Productivity (P), which is derived from the typical molecular formula of microalgal biomass, $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$ (Chisti, 2007).

For high strength wastewaters, dilution would be necessary to adjust the initial nitrogen and phosphorus concentrations to the optimal values, which were 140 mg/l and 47.0 mg/l, respectively, in the investigated artificial wastewaters.

The results that municipal secondary effluent enriched with appropriate amount of sodium nitrate (70 mg $N\text{-}NO_3^-$/l in this study) led to decent growth of *N. oleoabundans* up to 1.9 g DCW/l implies that this strain could potentially be cultivated in wastewater effluent for biofuel production. The ability of this strain to

achieve zero residual nitraogen and zero residual phosphate, when the N:P ratio was controlled at the right range, suggests that it could be a promising candidate for efficient N/P removal from low N/P effluent to generate effluent satisfying more strict environmental regulations, which are expected to come in the future.

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Chapter 5: Conclusions

Microalgae have been demonstrated to be a promising feedstock for biofuels production, and among them, green alga *Neochloris oleoabundans* is a good candidate since this strain was able to accumulate high lipid content with high growth rate. According to previous study, medium components could affect lipid accumulation of cells and algal growth, and sufficient nutrients lead to high growth rate with low lipid content of cells.

Bristol medium is a typical basic medium for green algal growth. It was shown that macronutrients such as $NaNO_3$, K_2HPO_4/KH_2PO_4 and $MgSO_4/FeCl_3$, has significant effect on algal growth while micronutrients were not significant factors. Box-Behnken experimental design (BBD) and response surface method (RSM) were used for factorial experimental design and data analysis.

Significant factors affecting cell growth and lipid yield were investigated. K_2HPO_4/KH_2PO_4 (X_2, P = 0.0114) and $MgSO_4/FeCl_3$ (X_3, P = 0.0007) are significant factors while the effects of $NaNO_3$ (X_1) on cell growth are less than significant in the rested range when DCW is used as the response. The concentration of $NaNO_3$ (X_1, P = 0.0175) and that of the $MgSO_4/FeCl_3$ pair (X_3, P = 0.0121) significant factors for lipid production while the phosphate salt pair (X_2) showed less than significant effects and three-dimensional response surfaces were generated to visualize the combined effects on the lipid yield.

Predicted optimal medium is comprised of (g/l) $NaNO_3$ (0.35), K_2HPO_4 (0.138), KH_2PO_4 (0.322) $MgSO_4$ (0.0823), $FeCl_3$ (0.0068). Significant improvements were

obtained with the optimized medium when comparing with the basic medium. The maximum biomass concentration of *N. oleoabundans* obtained with the optimized medium was 2.54 g/l, more than twofold of that obtained with basic medium (1.19 g/l). The lipid yield was 635 mg/l, which was 4.5-fold of that obtained with basic medium. Green alga *N. oleoabundans* is a potential strain for removal of nitrogen and phosphorus in the secondary wastewater due to its high growth rate. The effect of temperature in the range of 25 ℃ to 32 ℃ on algal growth was investigated and the optimal temperature range was between 28 ℃ to 30 ℃.

Synthetic medium was used to investigate the effect of nitrogen and phosphorus on algal growth and N/P removal in high strength wastewater. According to our previous study (Li et al., 2008a), Nitrogen was found to be the favourable nitrogen source. For this research, it was shown that 140 mg $N\text{-}NO_3^-$/l was sufficient for the growth of *N. oleoabundans*. *N. oleoabundans* has a large nitrate removal capacity of 140-218 mg $N\text{-}NO_3^-$/l with excess present of phosphorus, indicating that *N. oleoabundans* has a potential to remove nitrate in wastewater which contains high concentration of nitrate, such as piggery wastewater. The maximum $N\text{-}NO_3^-$ removal rate by *N. oleoabundans* was 1.8 mg $l^{-1}h^{-1}$.

Cell growth were nearly independent of initial phosphate concentration when it was in the range of 5.3 to 28mg $P\text{-}PO_4^{3-}$/l, however, growth enhanced by 15% when initial $P\text{-}PO_4^{3-}$(HPO_4^{2-}-$H_2PO_4^-$) increased to 47 mg/l. Meanwhile, 47 mg $P\text{-}PO_4^{3-}$/l was able to be removed at optimal nitrogen concentration. $P\text{-}PO_4^{3-}$ removal rate increased with increasing of initial phosphorus concentration with a linear relationship

and the maximum value was 0.63 mg/l·h.

Overall, nitrogen and phosphorus removal efficiencies vary depending on not only the initial nitrogen concentration but also phosphorus concentration. With 140 mg/l initial $N\text{-}NO_3^-$ and 47.1 mg/l initial $P\text{-}PO_4^{3-}$ could not only result in maximum dry cell weight 2.1 g/l, but also achieve 99% nitrogen, phosphorus removal efficiency, and nitrogen and phosphorus removal rate were 27.5 mg/l·d and 9.4 mg/l·d respectively.

For typical municipal wastewater which has low concentration of nitrogen and phosphorus (usually lower than 10 mg/l), additions of 70 mg/l N-Nitrogen was suggested to enhance biomass concentration. Nitrogen and phosphorous at these concentration range are able to be removed completely according to nitrogen and phosphorus capacity, besides, Ammonium could also be removed quite quickly, and the present of nitrate didn't affect the ammonium remove rate.