國 立 交 通 大 學

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碩 士 論 文

微藻養殖生產油脂並利用微藻油脂產製生質柴油之研究

Study on the Lipid Production from Microalgal Cultures and Producing Biodiesel from the Microalgal Lipid

研 究 生：蔡 明 達

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中 華 民 國 九 十 八 年 七 月

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致　　謝

兩年的碩士班生活在實驗、課業與疑惑三部曲的交錯唱和下謝幕了，摻雜其中的汗水、淚水與歡笑如劃開暗夜的流星般在驚嘆中一瞬即逝，但此情此景卻如滾燙的烙印默默燒存在我腦海深處，不會忘記的是那一份份的感動與感激。最要感謝的是我的指導教授**林志生**老師，宛如嚴父的外表下我卻能體會那如慈母般柔軟的心，再多的教誨與叮嚀皆因恨鐵不成鋼，不僅教導我們實驗的技巧與思考能力，更身教言教的告訴我們面對人生應有的態度與精神，讓我更能了解翩然到來成功的勝利女神背後是奮力不懈與永不放棄的執著。雖然愚生駑頓與粗心，但我相信在社會上遭遇困難之時，猶言在耳的字句珠磯定能給我不同於此時的深層體會。

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于 2009 年交通大學

微藻養殖生產油脂並利用微藻油脂產製生質柴油之研究

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中 文 摘 要

近年來，全球面臨地球暖化危機與石化能源耗竭兩大危機。由於人類大量開採石化能源及發展工業化社會導致溫室氣體 CO_2 大量累積與石化燃料枯竭之嚴重問題。因故低污染的再生能源與 CO_2 減量之策略發展為世界各國所積極研究的議題。然而以海洋微藻培養之利用正是一項高效益的綠色能源開發方法。微藻為能行使光合作用之單細胞植物，能快速且大量生產植物生物質與累積大量油脂於微藻細胞中。有鑑於此，本研究之目的為以高脂海洋微藻利用廢氣 CO_2 培養以進行大量油脂生產並藉由轉酯化反應生成生質柴油之研究。在最佳培養狀況下所篩選海洋微藻生長效率可達一天微藻增生 2 至 3 倍。微藻培養時 *Chlorella* sp.與 *N. oculata* 之微藻內油脂從生長對數期至生長穩定期，培養狀態進行至氮源缺乏時，可由 12%與 21%分別提升至 21%與 50%。然而以半連續式微藻培養於光生物反應器，通入 2% CO_2 之半連續式微藻光生物反應器中，其生長能力與產脂率表現最佳，氮在不同濃度 CO_2 培養下（2 至 15%），高濃度 CO_2 對於微藻的生長與產脂量能保持穩定並不受高濃度 CO_2 的抑制。然而微藻也能利用不同的有機碳源在混營或異營脂培養狀態下進行生長與油脂堆積。本研究指出，以蔗糖行異營培養之 *N. oculata* 其油脂累積雖可達 54%，但其生物質產量有降低的情形，而在以蔗糖進行混營之培養下油脂產量可提昇至每公升 0.284 公克。因此微藻培養時可分為兩個階段：增值階段及肥育階段，先利用最適生長環境來快速培養增加微藻的細胞濃度，再將其轉入低硝酸鹽或以蔗糖混營培養環境中肥育，即可有效率的大量產脂。

本研究中，微藻油脂轉酯化生成生質柴油因其轉化方式可分為化學製程以及生物製程兩種，以最佳油醇莫耳比下，酸化學催化反應與微生物脂解酶催化反應可分別達到 88% 與 82%。酵素催化之轉化率雖佳，但其成本高且反應時間過長，在工業上多以快速簡便之化學催化轉酯化反應達到快速、低成本且高產率的目的。本研究成果證明由藻類油脂製成生質柴油作為再生燃料具可行性與未來性，且在藻類培養過程中能有效的降低環境中的溫室氣體之危害並能提供快速且大量生物油脂之生產以提供生成生質柴油。

關鍵字： 微藻、小球藻、擬球藻、微藻油脂、生質柴油、轉酯化反應

Study on the lipid production from microalgal cultures and producing biodiesel from the microalgal lipid

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Abstract

In recent years, people over the world face some acute problem with regard to global warming and energy crisis. Humans exploited the fossil energy and developed the industry and civilization well in past hundred years to result in the environmental problems, green-house gas emission rising, and petrochemical fuel exhausting. Photosynthetic organism, microalgae, can use solar energy efficiently to combine water with CO_2 to produce biomass. Microalgae can not only produce biomass but accumulate lipid in microalgal cells. Lipids from microalgae can be extracted and converted to biodiesel fuel by transesterification.

In the study, the biomass and lipid productivity of *Chlorella* sp. and *Nannochloropsis oculata* were evaluated in the different conditions of culture in the closed photobioreactors. Results showed that the lipid accumulation of *Chlorella* sp. and *N. oculata* from logarithmic phase to stationary phase were significantly increased from 12% to 24% and 21% to 50%, respectively. In the semiconscious culture of *Chlorella* sp. and *N. oculata*, the total lipid productivity was 0.143 and 0.142 (g/L/d), respectively although the cultures were daily replaced half of broth. The results showed that *Chlorella* sp. and *N. oculata* were potential candidates for biomass and lipid production by semicontinuous cultures. The comparison of lipid productivities in the semicontinuous systems in which the culture broth were replaced at an interval of 24 h (one-day replacement) or 72 h (three-day replacement) was performed.

The result indicated the total lipid yield in the semicontinuous culture operated by one-day replacement was more efficient.

Moreover, different carbon sources supplied in the culture could make distinct growing ability and lipid accumulation. The results showed the cultivation of *N. oculata* using sucrose as carbon source in mixotrophic growth gave the highest maximum biomass (0.80 g/L) and the lipid productivity was up to 0.284 (g/L). Although the lipid contents from the heterotrophic cultivations with sucrose could increase to 54%, the biomass productivity decreased in the heterotrophic cultures. Therefore, the recovery of microalgal cells from semicontinuous cultures can transfer to mixotrophic cultivation for higher lipid production. The fast analysis of lipid contents of live microalgal cells by Nile red staining under fluorescence was established. The linear regression of fluoresce intensity of lipid content was established to measure the lipid contents of microalgae.

The typical transesterification method used in this study was chemical and enzymatic processes. The results indicated the efficiency of transesterification by acid catalyst could approach 88% and the efficiency by lipase-catalyzed transesterification could reach 82% by the optimal oil/methanol molar ratio. The high cost and long reaction time of enzymatic processes may be not convenient for usage. In industrial and commercial application, the chemical catalyst is common for transesterification to reach the purposes of low-cost and fast reaction rate. Therefore, the fast and suitable transesterification method in this study is acid-catalytic process.

Keywords: Microalgae, *Chlorella* sp., *N. oculata*, Microalgal lipid, Biodiesel, Transesterification

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I. Literature Review

1-1 Global warming and energy shortage

Past years, the industrial revolution brought with it rapid economical development and a great improvement in our standard of living. As a result, it has also become an enormous burden on nature. The problem of global warming contributed by greenhouse gas is receiving great attention by the worldwide people.

Greenhouse gases are those gaseous constituents of the atmosphere, both natural and anthropogenic, those absorb and emit radiation at specific wavelengths within the spectrum of thermal infrared radiation emitted by the Earth's surface, and then this property causes the greenhouse effect. Greenhouse gases are essential to maintaining the current temperature of the Earth. Planet Earth is habitable because of its location relative to the sun and because of the natural greenhouse effect of its atmosphere [Karl, 2003]. Earth's most abundant greenhouse gases are water vapor, carbon dioxide (CO_2), methane (CH_4), nitrous oxide (NO_x), ozone (O_3), and chlorofluorocarbons. The most important greenhouse gases are water vapor and carbon dioxide [Treut et al., 2007]. Human activities intensify the blanketing effect through the release of greenhouse gases. For instance, the amount of carbon dioxide in the atmosphere has increased by about 31% from the 1961 to 1990, and this increase is known to be due to human activities, primarily the combustion of fossil fuels and removal of forests. In the researches, the level of carbon dioxide in pre-industrial period is 280 parts per million by volume (ppmv), and the current level of carbon dioxide increased to more than 370 ppmv today. Thus, humankind has dramatically altered the chemical composition of the global atmosphere with substantial implications for climate. **Figure 1-1** shows the amount of CO_2 is still elevating as an average increasing rate in these years and the global average surface heating approximates that of the increases of carbon dioxide [Houghton et al., 2001; Karl and

Trenberth, 2003]. The greenhouse gases trap outgoing radiation from the Earth to space, creating a warming of the planet.

The burning of fossil fuels has especially emitted large amounts of CO_2, into the atmosphere, which is causing a problem known as greenhouse effect and global warming [Michiki, 1995]. Over ten times more CO_2 is fixed by plants into biomass, and annually released by decomposers and food chains, than is emitted to the atmosphere due to the burning of fossil fuels. Human activity is already directly and indirectly affecting almost half of the terrestrial biological C cycle. Management of even a small fraction of the biological C cycle would make a major contribution to mitigation of this greenhouse gas [Hughes and Benemann, 1997].

CO_2 is responsible for well over half of the total warming potential of all greenhouse gases. Globally about 20 billion tons of fossil CO_2 are emitted each year from the burning of fossil fuels, and another 2 to 8 billion tons are released through human-mediated oxidation of the biosphere: intensive agriculture, deforestation and other unsustainable practices.

Cumulatively, net biosphere CO_2 emissions due to human activities over the past century have rivaled emissions from fossil CO_2 sources. Indeed, human activities are currently impacting a large fraction, approaching half, of the total annual terrestrial primary biological productivity of our planet, estimated at 500 billion tons of CO_2 fixed annually [Vitousek, 1994; Hughes and Benemann, 1997].

To solve this international problem, some researches of biological CO_2 fixation and utilization have been carried out to develop those technologies in which CO_2 is fixed by utilizing microorganisms such as bacteria and microalgae and converting it into useful substances [Michiki, 1995]. Electric power generation is responsible for roughly one third of fossil CO_2 emissions. Direct CO_2 mitigation processes are those that reduce fossil CO_2

emissions from specific power plants. Direct biological CO_2 mitigation processes include the cultivation of microalgae on flue-gas or captured CO_2, and the co-firing of wood with fossil fuels.

Another international problem is energy crisis which was also caused may be over-consumption of fossil energy. Majority of the world energy needs are supplied through petrochemical sources, coal and natural gases, with the exception of hydroelectricity and nuclear energy, of all, these sources are finite and at current usage rates will be consumed shortly [Srivastava and Prasad, 2000; Meher, 2006]. Diesel fuels have an essential function in the industrial economy of a developing country and used for transport of industrial and agricultural goods and operation of diesel tractor and pump sets in agricultural sector. Economic growth is always accompanied by commensurate increase in the transport. The high energy demand in the industrialized world as well as in the domestic sector and pollution problems caused due to the widespread use of fossil fuels make it increasingly necessary to develop the renewable energy sources of limitless duration and smaller environmental impact than the traditional one. Global consumption of petrodiesel raised in an average of 16.1 million tons/year among 1990 and 2003 [IEA, 2006]. If the tendency keeps on, the worldwide demand for petrodiesel will increase to 1383 million tons/year by 2050. The global supply of petroleum is finite and expected to peak between 5 and 30 years from 2008 [Pahl, 2005; Pin Koh, 2007], after which demand will inevitably outstrip production. This has stimulated recent interest in alternative sources for petroleum-based fuels. The alternative fuel must be technically feasible, economically competitive, environmentally acceptable, and readily available. One possible alternative to fossil fuel is the use of oils of plant origin like vegetable oils, tree borne oil seeds and microalgal oils for biodiesel production. This alternative diesel fuel can be termed as Meher et al. reported [2006].

1-2 Microalgae

Microalgae are diverse group of prokaryotic and eukaryotic photosynthetic microorganisms that grow rapidly due to their simple structure. It is estimated that the biomass productivity of microalgae could be 50 times more than that of switch grass, which is the fastest growing terrestrial plant [Demirbas, 2006; Nakamura, 2006]. They are single- cellular photosynthetic microorganisms that convert sunlight, water and carbon dioxide to microalgal biomass.

The primary producers of oxygen and consumer of CO_2 in aquatic environments are algae, especially planktonic microalgae. Microalgae are microscopic in size and grow in liquid culture, nutrients can be maintained at or near optimal conditions potentially providing the benefits of well-controlling. Microalgae can perform continuous productivity similar to microbial fermentation. From these points of view, the study aims to search and screen microalgae from the natural sources like oceans and lakes, and to establish the most appropriate culture conditions and then evaluate the photosynthesizing capabilities thereof. However, many microalgae are exceedingly rich in oil [Chisti, 2007; Banerjee, 2002], which can be converted to many products such as renewable fuels using existing technology. The utilization fields of microalgae can be categorized as follows [Michiki, 1995].

1. Energy-generating substances such as hydrocarbons, hydrogen, and methanol.

2. Foods and chemicals such as proteins, oils and fats, sterols, carbohydrates, sugar, alcohols.

3. Other chemicals colorants, perfumes, vitamins, physiologically-active substances. Elementary techniques including separation and purification have been studied and evaluated.

These characteristics of microalgae are potential for green-house gas reducing and production of renewable fuels. In our studies, we studied equipment containing a production

system in which lipids and biodiesel from microalgae are separated and purified.

1-3 Microalgae for reducing pollution and renewable fuels

Photosynthetic microorganisms, microalgae, hold the key to realize the effective system of CO_2 fixation and utilization. Particularly, about one half of global photosynthesis and oxygen production is accomplished by marine microalgae. They play an important role in CO_2 recycling through photosynthesis, which is similar to higher plants in O_2-evolved systems (PSI and PSII system).

Furthermore, technology development of microalgae culture that makes it possible to convert the formations resulting from photosynthesis into energy substances such as fuel oil. The study also seeks to develop technologies that separate and refine a variety of useful substances [Michiki, 1995]. Microalgae are sunlight-driven cell factories that convert carbon dioxide to potential biofuels, foods, feeds and high-value bioactives [Metting and Pyne,1986; Schwartz, 1990; Kay, 1991; Shimizu, 1996,2003; Borowitzka, 1999; Ghirardi et al., 2000; Akkerman et al., 2002; Banerjee et al., 2002; Melis, 2002; Lorenz and Cysewski, 2003; Metzger and Largeau, 2005; Singh et al., 2005; Spolaore et al., 2006; Walter et al., 2005; Chisti, 2007].

Notwithstanding, microalgae can provide several different types of renewable biofuels. These include methane produced by anaerobic digestion of the algal biomass [Spolaore et al., 2006; Chisti, 2007]; biodiesel derived from microalgal oil [Roessler et al., 1994; Sawayama et al., 1995; Dunahay et al., 1996; Sheehan et al., 1998; Banerjee et al., 2002; Gavrilescu and Chisti, 2005; Chisti, 2007] and photo-biologically produced biohydrogen [Ghirardi et al., 2000; Akkerman et al., 2002; Melis, 2002; Fedorov et al., 2005; Kapdan and Kargi, 2006; Chisti, 2007]. Using microalgae as a source of fuel is not a new idea [Chisti, 1980-1981;

Nagle and Lemke,1990; Sawayama et al., 1995; Chisti, 2007], but it is now being paid much attention because of the escalating price and exhausting of petroleum and, more significantly, the emerging concern about global warming that is associated with burning fossil fuels [Gavrilescu and Chisti, 2005].

1-4 Microalgae cultivation

Grow ability, chemical composition of microalgae, lipid content and lipid composition of microalgae are influenced by environmental conditions, such as culture medium, light, temperature, carbon dioxide and so on [Richmond, 1986; Tomaselli et al., 1988, James et al., 1989; Oliveira et al., 1999; Renaud et al., 2002]. Producing microalgal biomass is generally more expensive than growing crops. Photosynthetic growth requires light, carbon source, water and inorganic salts. Temperature must remain generally within 20 to 30°C. To minimize expense, biodiesel production must rely on freely available sunlight, despite daily and seasonal variations in light levels.

1-4-1 Growth medium

Growth medium must provide the inorganic elements that constitute the algal cell. Essential elements include nitrogen (N), phosphorus (P), iron and in some cases silicon. Minimal nutritional requirements can be estimated using the approximate molecular formula of the microalgal biomass, that is $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$. This formula is based on data presented by Grobbelaar [2004]. Sea water supplemented with commercial nitrate and phosphate fertilizers and a few other micronutrients is commonly used for growing marine microalgae [Molina Grima et al., 1999]. Growth media are generally inexpensive. Nutrients such as phosphorus must be supplied in significant excess because the phosphates added complex with metal ions, therefore, not all the added phosphorus is bio-available.

Silicon is specifically used for the growth of diatoms which utilize this compound for production of an external shell. Micronutrients consist of various trace metals and the vitamins thiamin (B_1), cyanocobalamin (B_{12}) and sometimes biotin. Two enrichment media that have been used extensively and are suitable for the growth of most algae are the Walne medium and the Guillard's f/2 medium. Commercially available nutrient solutions may reduce preparation labor. The complexity and cost of the above culture media often excludes their use for large-scale culture operations. And there are alternative suitable enrichment medium for mass production of microalgae in large-scale extensive systems contain only the most essential nutrients and are composed of agriculture-grade rather than laboratory-grade fertilizers.

1-4-2 Nitrogen contents

Organisms use carbon and nitrogen for the important nutrient source of energy and construction of cell structure. Other than carbon, nitrogen is quantitatively the most element in algal nutrition. Nitrogen supply is essential for preparation of algal culture medium for most microalgae. Microalgae are usually able to use nitrate, nitrite, ammonia, or other organic nitrogen sources such as urea. In practical, the preferred nitrogen source is ammonia or urea, either of which is economically more favorable than nitrate or nitrite, which is more expensive.

The growth capacity is usually the same with these nitrogen sources. The average nitrogen requirement for many green algae is approximately 5-10% of the dry weight or 5-50mM [Becker, 1994]. Nevertheless, nitrogen amounts are considerably variable, since the nitrogen content in the culture medium can be operated to produce nitrogen-limitation algal cells. Nitrogen limitation affects photosynthesis by reducing the efficiency of energy collection due to loss of chlorophyll a and increases in non-photochemically active carotenoid pigments [Berges et al., 1996]. Nitrogen deficiency will retard the growth rate of algal cells

but may cause accumulation of numerous carbon compounds, i.e. polysaccharides or lipids. A low supply of nitrogen may result in a low respiration rate and an increase in the lipid reserves in most microalgae [Becker, 1994].

The major nonpolar lipids reported in algae are the triglycerides and hydrocarbons, and the major polar lipids classes include phospholipid, cardiolipin, diphosphatidylglycerol and glycolipids [Volkman et al., 1989 Dembittsky et al., 1991, Behrens and Kyle, 1996]. It is widely known that the main storage lipid class, triglycerides, accumulates in response to exhaust of nitrogen supply and during the stationary phase of a batch culture in most species [Richardson et al., 1969; Gordillo et al., 1998]. At the same time, the amounts of nitrogen supply may make difference in the fatty acid compositions of total lipid production and the length of fatty acid chains [Makrides et al., 1995; Regnault et al., 1995].

1-4-3 Light

Solar light is the energy source of the algal culture system for photosynthetic metabolism and is one of the major factors to determine the efficiency of the whole system. There are some environmental factors affect the lipid composition and growth of algae such as temperature, irradiation, and nutrient status [Thompson et al.., 1996]. Light drives photosynthesis reaction and in this regard intensity, spectral quality and photoperiod need to be considered. Light intensity plays an important role, but the requirements vary greatly with the culture depth and density of the microalgal culture. However, light intensities that were adequate or optimal for growth in the log phase to declining growth phase can become stressful in stationary phase and lead to a condition known as photo-inhibition. It is important that while the measured light intensity within the culture will decrease with increasing biomass if the incident illumination is maintained relatively high and then a large proportion of cells may become stressed, and the photo-inhibited culture can be pushed into the death phase. This is especially the case if the culture is also nutrient stressed. It is

preferable for many species to halve or further reduce the incident light intensity when cultures enter stationary phase to avoid photo-inhibition.

At higher depths and cell concentrations, the light intensity must be rose up to penetrate through the culture medium. For example, 1000 lux is suitable for Erlenmeyer flasks, and 5000-10000 lux light is necessary for larger volumes. On the one hand, there are several studies of culturing *Botryococcus brauni* as well as *Chlorella* sp. *by* continuous light, and the other hand some studies are applying dark and light photoperiod for growing *Chlorella* sp. [Hogetsu and Miyachi, 1970; Maeda et al, 1995; Sawayama et al., 1995; Allard and Templier, 2000; Sato et al., 2003; Achitouv et al., 2004; Miao and Wu, 2006]. In order to complement and utilize the solar light at the maximum efficiency, many important studies of algae culture aim to develop a highly-efficient light collection device that minimizes the light loss due to reflection and absorption in the visible light area directly influencing the CO_2 fixation capability of microorganisms [Michiki, 1995].

1-4-4 Temperature

The optimal growth temperature for phytoplankton cultures is generally between 20 and 24°C, although this may vary with the elements of the culture medium, the species and strain diversity. Microalgae do not have the ability to regulate their internal temperature, so the temperature may in turn affect the microalgae growth and substrate utilization rate [Esener et al., 1981]. Most species of cultured microalgae generally grow in appropriate temperatures between 16-27°C. Temperatures lower than 16°C will decrease the growth rate, whereas those higher than 35°C are lethal for a number of species. If necessary, microalgal cultures can be cooled by a water flow of cooling system over the surface on the culture vessel or by controlling the air temperature with refrigerated air - conditioning units.

The influences of temperature on the biomass composition, nutrient requirement, nature of

metabolism and the metabolic reaction rate were well-known [Pirt, 1971; Novak, 1974; Mayo and Noike, 1996]. High growth temperature leads to different production of lipids and carbohydrates [Tomaselli et al., 1988; Oliveira et al., 1999; Renaud et al., 2002]. However, other studies have found that the response of microalgal composition to high and low growth temperatures varies from species to species. High growth temperature has been associated with increases in protein content and decreases in carbohydrate, and lipid in some species[Thompson et al., 1992; Renaud et al.,1995], but these workers found no overall trend in gross biochemical composition for all species under study. We known temperature is a major effect on the types of fatty acids produced by microalgae. In some microalgal species, decreasing of growth temperature can bring about increasing of the unsaturated to saturated fatty acids [Ackman et al.,1968; Satu and Murata, 1980; Mortensen et al.,1988; Thompson et al., 1992; Oliveira et al., 1999; Renaud et al., 2002]. However, the response to growth temperature varies from species to species, with no overall consistent relationship between temperatures and chemical composition.

However, for the large scale of outdoor culturing system, microalgal culture could be heated over 40°C by sunshine in some extremely hot places. Microalgal growth is highly inhibited at high temperature, so there are many studies for searching the microalgae good in thermo-stability and productivity at high temperature for special cultivation.

1-4-5 Carbon dioxide

Microalgae need inorganic carbons to perform photosynthesis. Both CO_2 and HCO_3^- are potential sources of carbon for photosynthesis in microalgae. While CO_2 can diffuse through the microalgal cell membrane, HCO_3^- needs specific transport mechanisms to enter the algal cell [Raven and Johnston, 1991; Beer, 1994; Moroney and Somanchi, 1999]. **Figure 1-2** shows the model for CO_2 concentration in eukaryotic microalgae. Microalgal cultures, in contrast to conventional agriculture, can not be supplied with carbon by simple

diffusion from the air. The natural CO_2 concentration in air (0.03%) is too low to sustain optimal growth and high productivity. Microalgae growing in fresh water with low salinity and at nearly neutral pH must be supplied with additional carbon to ensure satisfactory growth. Although intensive mixing may increase the entrance coefficient for CO_2 from the air into the culture, in most microalgal cultures, additional carbon mainly in the form of CO_2-enriched air is supplied [Becker, 1994]. There is also studies showing the utilization of organic carbon for heterotrophic or mixotrophic cultures.

CO_2 addition furthermore buffers the water against pH changes as a result of the CO_2/HCO_3^- balance. High CO_2 aeration will result in pH decreasing of culture solution. High CO_2 concentration aeration may be a harmful effect on the microalgal cells growth, but increasing of microalgal cell density could promote the growth capacity of microalgae in the cultures aerated with higher CO_2 concentrations [Chiu et al., 2008]. Therefore, the adequate amount of CO_2 supplied must not be inhibitory effects on microalgal cell growth inhibition; otherwise lipid content will decrease. It was reported that the carbon assimilation of lipid synthesis was decreased with decrease of pH [Yung and Mudd, 1966, Chiu et al., 2008]. Higher CO_2 aeration may cause the decrease of lipid content, but the increase of biomass may improve the lipid productivity.

1-4-6 pH value

The pH value in the medium in algal system is known to influence the biomass regulation, ion transport system and metabolic rate [Guffanti et al., 1984; Mayo and Noike, 1994]. So controlling pH in culture medium will be one of the most critical factors affecting biomass and lipid production of microalgae. The pH value of the culture medium may be a simpler, indirect method for determining the degree of the cell growth of microalgae because the pH gradually rises as bicarbonate added to the culture medium is dissolved to produce CO_2, which releases OH^- during cultivation [Richmond and Grobbelaar, 1986; Kim et al., 2007].

However, pH of algal cultures can be affected by several factors such as medium composition, buffering capacity of the medium, CO_2 dissolution efficiency, temperature (influences solubility of chemical compounds) and metabolic activity of the algal cells. Almost pH value of the medium in algal culture is usually neutral or slightly acidic values. The main reason is to avoid precipitation of microalgal cells and several major elements. Furthermore, different microalgal species vary greatly in their response to the pH and different microalgal strains show their own clear dependency on the pH of the growth in culture medium [Becker, 1994].

Increase of pH value during cultivation mainly caused by the depletion of the anions NO^{3+} and the formation of CO_2 as well as releasing of OH^- ions in the medium. When the pH value increases above 9, it may make the precipitation of several calcium salts, i.e. carbonates, phosphates and sulfates, following deficiencies of nutrients, growth retardations and even algal flocculation which is induced by the precipitating elements[Becker, 1994]. Therefore, controlling stable and suitable pH value in the broth must be necessary for healthy cultures and optimal productivity of microalgal cultivation.

1-4-7 Growth phase

There are 5 reasonably well defined phases of algal growth in batch cultures. The characterized phases are including lag phase, exponential phase, phase of declining growth rate, stationary phase, and death phase [Fogg and Thake, 1987]. Each phase is described in **Figure 1-3**.

Lag phase, the condition of the inoculums has a strong bearing on the duration of this period [Spencer, 1954]. A lag phase may also occur if the inoculum is transferred from one set of growth conditions to another. An inoculum taken from a healthy exponentially growing culture is unlikely to have any lag phase when transferred to fresh medium under similar growth conditions of light, temperature and salinity. In general the length of the lag

phase will be proportional to the length of time the inoculum has been in phases 3-5. Log phase, the cell density of culture during which numbers increase exponentially and which is represented by a part of the growth curve that is a straight line segment if the logarithm of numbers is plotted against time. The growth rate of a microalgal population is a measure of the increase in biomass over time and it is determined from the log phase. The duration of log (exponential) phase in cultures depends upon the size of the inoculum, the growth rate and the capacity of the medium and culturing conditions to support algal growth. Declining of growth phase normally occurs in cultures when either a specific requirement for cell division is limiting or something else is inhibiting reproduction. In the phase of growth biomass is often very high and exhaustion of a nutrient, limiting carbon dioxide or light limitation becomes the primary causes of declining growth. Light limitation at high density cultures occurs when the cells absorb most of the incoming irradiation and individual cells shade each other called self-shading effect. In stationary phase, cultures reach a relatively constant cell density, and cells may undergo dramatic biochemical changes within a matter of hours. The nature of the changes depends upon the growth limiting factor. Nitrogen limitation may result in the reduction in protein content and relative or absolute changes in lipid and carbohydrate content. Light limitation will result in increasing pigment content of most species and shifts in fatty acid composition. In the final phase, death phase, water quality deteriorates and nutrients are exhaust to a level incapable of sustaining growth and survival. Cell density decreases rapidly and the culture eventually collapses.

In the life cycle of algae, growth (in cell number or biomass) continues slowly for some time in a medium completely depleted of nitrogen, producing cells of high lipid content. When nitrogen depletion progresses, chlorophyll and protein contents decrease and carbohydrate content increases, followed by increases in lipid content [Iwamoto and Sugimoto, 1958].

The gross biochemical composition of microalgae was affected more by the growth phase than by the nitrogen source. Protein was higher during exponential growth (about 37-45% AFDW, ash free dry weight) but the pattern changed as the cultures aged, and lipids were the main algal constituents on all nitrogen sources in the late stationary phase. In all cultures, the relative abundance of neutral lipid increased in the late stationary phase along with a proportional reduction in phospholipids, whereas galactolipids only slightly changed during the growth of the cultures. Total fatty acid content was affected by nitrogen source and growth phase. Maximal PUFA (polyunsaturated fatty acid) values were obtained at the early stationary phase and decreased throughout the stationary phase [Fidalgo et al.,1998].

1-5 Microalgae cultures techniques

Photosynthetic microorganisms play an influential role in the conversion of solar energy into chemical energy. Photosynthetic conversion is an efficient and alternative process used in several industrial fields. This use of algal biomass is an important consideration for industrial applications of microalgal cultures. With advances in processing technology, algal biomass has come to be a possible source of fuels, fine chemicals, and pharmaceuticals. So, developing a large-scale method for the cultivation of microalgae have been performed using many different kinds of cultivation systems for providing alternatives to fermentation, agriculture, fuel products [Matsunaga et al., 2005; Chisti, 2007]. The microalgal culture systems have been constructed well in many different systems. The development of efficient large-scale culture systems is necessary for algal mass production and the industrial applications of microalgae. These open culture systems are the simplest method of algal cultivation and offer advantages in low construction cost and ease of operation [Torzillo et al., 2003].

Large-scale culture have been constructed and classified as open and closed systems. Open culture systems are uncovered cultures such as unstirred open ponds, raceway ponds, circular ponds, paddle-wheel pond and so on [Richmond, 1992; Chaumont, 1993]. The open culture systems require large surface areas and shallow depth (12-15 cm) to improve light penetration efficiency [Matsunaga et al., 2005]. In open pond systems, any cooling is achieved only by evaporation. Swung temperature is dependent on a diurnal cycle and seasons. Evaporative culture medium loss can be significant because of uncovered cultures and significant water losses to atmosphere, open pond systems low utilization efficiently of carbon dioxide than closed photobioreactors. The biomass productivity remains low because open pond systems can't be sustained in a stable condition. Furthermore, open ponds are poorly mixed and cannot keep an optically dark zone. However, the biggest problem in open culture systems is contamination by unwanted algal species and other microorganisms. Production of microalgal biomass for making biodiesel fuels has been extensively evaluated in raceway ponds in studies sponsored by the United States Department of Energy. Open ponds are considered to be less expensive than photobioreactors, because they cost less to build and operate. Although raceways are low-cost, they have low biomass productivity compared with photobioreactors [Sheehan et al., 1998; Chisti, 2007].

Closed systems, photobioreactors have been expected to overcome the problems of open culture systems, and also several types of photobioreactors have been created such as helix tubular reactors, plane tubular reactors, laminar reactors, column reactors and so on. Photobioreactors permit essentially single-species culture of microalgae for prolonged durations of growth. Photobioreactors have been successfully used for producing large quantities of microalgal biomass until now [Molina Grima et al., 1999; Pulz, 2001; Carvalho et al., 2006]. The advantages of closed photobioreactors are (1) facilitating maintenance of mono-algal cultures by preventing them from contamination; (2) reducing water evaporation

and the subsequent increase of salinity in the culture medium; (3) resulting in higher productivity with greater cell densities, reducing harvesting costs; and (4) applicable to various microalgal species under favorable and controllable culture conditions. The production yield of algal biomass cultures depends on the light path length to each cell, and therefore the increase of surface area-to-volume ratio (SA: VOL) is an important factor for efficient light utilization in photobioreactors. Consequently, light is a limitation factor for mass culture productivity, and high productivity of cell biomass at harvest potentially can elicit a low production cost. However, the closed systems do have some disadvantages. Oxygen concentrations resulting from active photosynthesis can be raised because of the high biomass, and therefore high concentration of dissolved oxygen in combination with intense sunlight by the high SA: VOL result in photo-inhibition and photo-oxidative damage to algal cells [Brown, 2002; Matsunaga et al., 2005; Chisti, 2007]. So degasser systems sometimes are required. Photobioreactors also require cooling systems during daylight hours and temperature control at night is also useful.

Open pond systems have lower productivity of algal biomass, require larger land areas for cultivation, and involve higher land costs. By contrast, closed culture systems can reach high-density culture and decrease large land costs. However, high surface area-to-volume ratio required for collecting a sufficient amount of solar energy, and therefore operating costs are higher than for open systems. Furthermore, suitable culture systems should be chosen according to the target products and available environmental conditions.

1-6 Mixotrophic and heterotrophic culture of microalgae

Autotrophs are organisms that produce complex organic compounds from simple inorganic molecules and external sources of energy, such as light or chemical reactions of inorganic

compounds. Most microalgae are autotroph organisms and also called photoautotrophs because they can perform photosynthesis by utilizing inorganic carbon, CO_2. However, heterotrophs are organisms that require organic substrates to get carbon for growth and development and mixotrophs are organisms obtain electrons from inorganic electron sources, but uses organic matters as carbon sources. So mixotrophic organisms can derive metabolic energy both from photosynthesis and from external energy sources. Some microalgae can be cultivated in heterotrophic or mixotrophic cultures.

A sufficient supply of carbon is vital important for successful cultivation since about 50% of the algal biomass consists of carbon [Sánchez Mirón et al., 2003]. Carbon can be supplied as an inorganic substrate in the form of CO_2, as is the case for most photoautotrophic forms. However, microalgae take up organic molecules as a primary source of nutrition in heterotrophic growth and may also obtain nutrition by combining photoautotrophic and heterotrophic mechanisms in mixotrophic growth. In heterotrophic and mixotrophic cultures, organic carbon sources are mainly sugars or acetate [Laliberté and de la Noüe, 1993; Becker, 1994]. Some algal species are applied to heterotrophic growth culture using organic carbon as their energy and carbon source. These species can be grown in conventional fermenters, and heterotrophs can be grown to much higher cell densities than phototrophs, resulting in high productivity because light is not required [Behrens and Kyle, 1996].

The economic feasibility of microalgal mass culture for biodiesel production greatly depends on the high biomass productivity and considerable lipid yields. However, the high cost of oil feedstock is the main reason to the high cost of biodiesel and makes the obstacle to its broader commercialization [Wright, 2006; Li et al., 2007]. There are some researches make efforts on minimizing oil feedstock costs [Al-widyan and Al-shyoukh, 2002; Antolin et al., 2002; Zhang et al., 2003; Noureddini et al., 2005; Dhaermadi et al., 2006; Li et al., 2007]. Heterotrophic culture may not only overcome nearly all of the limitations of the phototrophic

growth systems but also provide a cost-effective, large-scale alternative method of cultivation for some microalgae that utilize organic carbon substances as their sole carbon and energy sources. This mode of growth can apply to low cost culture which eliminates the requirement for light and offers the possibility of greatly increasing microalgal cell concentration and volumetric productivity in batch and fed-batch systems [Anderson et al., 1978; Boswell et al., 1992]. Li et al. [2007] reported that heterotrophic *Chlorella* grown in commercial large-scale bioreactors could reach a high biomass concentration and high lipid content. However, the heterotrophic or mixotrophic growth of microalgae may change the fatty acid composition significantly depending on the growth mode [Constantopoulus, 1970; Regnault et al., 1995; Behrens and Kyle, 1996]. For example, Regnaul et al. [1995] reported the photosynthetic culture of *Euglenophyceue* contain mostly C14-C22 with C16 and C18 dominating , while grown organisms in heterotrophic cultures contain largely 14:0, 16:0 and 18:l.

1-7 Acetyl-coenzyme A carboxylase of microalgae

Acetyl-coenzyme A carboxylase (ACCase) is a biotin-containing enzyme that catalyzes the the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA. This reaction is believed to be a key regulatory step in fatty acid biosynthesis in animals, bacteria, and plants, especially including microalgae [Roessler and Ohlrogge, 1993]. Two partial reactions are involved in this process. First is carboxylation of the enzyme-bound biotin molecule and second is transfer of the carboxyl group to acetyl-CoA.

The rate of fatty acid synthesis in plants appears to be dependent on the activity of ACCase. ACCase enzyme has been receiving an increasing amount of attention as a target for manipulation via genetic engineering for the purpose of increasing the lipid production

capabilities of various organisms because ACCase plays an important role in controlling the rate of fatty acid biosynthesis.

1-8 Extraction method of microalgae

High oil-contents microalgae can be used to produce biodiesel, which is chemically modified natural lipid and biodiesel form microalgae is emerging as an exciting new option for diesel engines. The actual production of the biofuel from the microalgae requires the extraction of the natural microalgal oils in the first, and then the formation of useable fuel from oil. Apart from culture affecting factors, ex. nitrogen source, light, and temperature, lipid extraction method can also significantly affect lipid yield [Grima et al., 1994; Lee et al., 1998; Rodriguez-Ruiz et al., 1998; Shen et al., 2009].

Typical microalgae lipid extraction usually follows two steps: cell disruption and solvent extraction. Several lipid extraction methods work differently on different algae species because various microalgae species have large variations in algal cell shape, cell size, cell wall structure and characteristics of algal lipids. Lee et al. [1998] reported the effects of five cell disruption methods along with five solvent systems on lipid extraction of *Botryococcus brauni*. The five cell disruption methods are French press, sonication, homogenization, bead-beater and lyophilization. For examples of press and sonication methods, expression press method is the simplest way to extract oil but works best based on algal species. Expression or expeller press method is essentially a large press used to compress the algae bodies making the natural oils to seep out and the microalgae must first be dried. And further, the other method, ultra-sonication, is the use of an ultrasonic reactor to extract the natural algal oils. Ultrasonic reactor sends ultrasonic waves through the microalgae which create cavitation bubbles. When cavitation bubbles break around and destroy the cell walls

of the microalgae, the shock waves produced collapse the cell wall releasing the intracellular contents. After all, the use of cell disruption usually couples with chemical extraction to reach optimum extraction efficiency.

Besides, the five disruption methods connect with five solvent systems, i.e. chloroform/methanol, hexane/isopropanol, dichloroethane/methanol, dichloroethane/ethnol and acetone/dichloromethane. However, different algal strains are suitable for own specific cell disruption/ solvent system. Some researches showed that bead-beater followed by solvent system of chloroform/methanol (2:1, v/v) was the most effective method for *Botryococcus brauni* and the dichloroethane/methanol and dichloroethane/ethanol systems were successful on lipid extraction of green alga *Cladofora* [Damyanova et al., 1992; Shen et al., 2009]. Solvent extraction is easy-manipulation and low capital investment for equipment, but the major disadvantages are that many of the chemicals have the potential to be harmful, non-continuous process with waiting periods and requirement of more manual labor. Therefore, the various lipid extraction of microalgae are developing for discover more effective method for optimal extraction technique of microalgal lipid. The different common lipid extraction methods applying to microalgae contain soxhlet extraction, supercritical fluid extraction, enzymatic extraction, osmotic shock extraction and so on.

1-9 Biodiesel

Because the prices for crude oil rose up dramatically and the concerns of global warming grew, people are searching for alternatives to petroleum-based energy sources actively. [Pacala and Socolow, 2004; Ragauskas et al., 2006; Tilman et al., 2006; Pin Koh , 2007]. Biodiesel fuel is a diesel-equivalent, renewable fuel derived from biological feedstocks (i.e., usually agricultural crops used as raw materials for biodiesel production). Biodiesel is

gaining more and more importance as an attractive fuel due to the depleting fossil fuel resources. A global shift in consumption trends from petroleum-based diesel (petrodiesel) to biodiesel could reduce the world's dependence on a finite supply of fossil fuels.

Presently, Western Europe (Germany, France, Italy, Austria, and Spain) makes efforts in biodiesel and contributes over 90% of global biodiesel production (global production: 2.8 million tons/year). Meeting the estimated global biodiesel demand in 2050 would require an almost 100-fold increase from current production capacities [Pahl, 2005; Pin Koh, 2007]. The world's largest consumer of petrodiesel-United States [IEA 2006]-strives for reducing its dependence on petroleum through its Energy Conservation Reauthorization Act of 1998, in which federal agencies are mandated to meet fuel credit requirements by either purchasing alternative-fueled vehicles or using motor fuel containing at least 20% biodiesel by volume [U.S. Department of Energy, 2001].

1-10 Biomass for biodiesel

Biodiesel generally falls under the category of biomass which refers to renewable organic matter such as energy crops, crop residues, wood municipal and animal wastes that are used to produce energy [Pahl, 2005]. More specifically, biofuels, a subcategory of biomass includes three energy-crop-derived liquid fuels: ethanol (grain alcohol), methanol (wood alcohol), and biodiesel. The plant oils usually contain free fatty acids, phospholipids, sterols, water, odorants and other impurities. Because of these, the oil cannot be used as fuel directly. To overcome these problems the oil requires slight chemical modification mainly transesterification, pyrolysis and emulsification. Among these, the transesterification is the key and foremost important step to produce the cleaner and environmentally safe fuel from vegetable oils. Pure biodiesel does not contain any petroleum diesel or fissile duel of any

sort.

Technically, biodiesel is fatty acid alkyl ester which can be easily made through a simple chemical process form virtually any vegetable oil, such as soy, corn, rapeseed, cottonseed, peanut, sunflower, avocado, mustard seed and so on. Actually, biodiesel can also be made from recycling cooking oil and animal fats. However, there have been more and more promising experiments with the use of microalgae as a biodiesel feedstock. And the chemical process of making biodiesel is so simple that biodiesel can be made by virtually anyone, although the chemicals required (usually lye and methanol) are hazardous and need to be handled with extreme caution.

1-11 Biodiesel characteristic

Biodiesel is an alternative source of energy that is replacing the use of gasoline and diesel. The emergence of biodiesel fuel substitutes has led to several studies on their properties. Biodiesel is similar to petroleum diesel fuel in many chemical and physical properties. Engine performance and the fuel consumption were favorable that make it better substitute to diesel fuel [Bajpai and Tyagi, 2006]. Biodiesel is 100% renewable and being plant based. The advantages of biodiesel as diesel fuel are liquid nature portability, ready availability, renewability, sustainable, higher biodegradable, higher combustion efficiency, higher cetane number, non-toxic, lower sulfur and aromatic content, non-polluting and environmentally safe [Ma and Hanna, 1999; Mudge and Pereira, 1999; Speidel et al., 2000; Zhang et al., 2003; Knothe et al., 2006]. Main advantages of biodiesel given in the literature include domestic origin, reducing the dependency on imported petroleum, biodegradability, high flash point and inherent lubricity in the neat form [Mittelbach and Remschmidt,2004; Knothe et al., 2005]. By the by, the higher oxygen content of biodiesel than diesel improves the

combustion efficiency due to the increase of the homogeneity of oxygen with the fuel during combustion and decreases its oxidation potential.

Biodiesel is the only alternative fuel so that low concentration biodiesel-diesel blends run on conventional unmodified engines. It can be stored anywhere where petroleum diesel fuel is stored. The risks of handling, transporting and storing biodiesel are much lower than petrodiesel. Biodiesel is safe and easy to handle and transport because it is as biodegradable and has a high flash point compared to petroleum diesel fuel. Biodiesel can be used alone or mixed in any ratio with petroleum diesel fuel. The most common blend is a mix of 20% biodiesel with 80% petroleum diesel, or B20 under recent scientific investigations.

In view of environmental considerations, biodiesel is considered as a "carbon neutral" fuel because all the carbon dioxide released during biodiesel consumption had been sequestered from the atmosphere for the growth of vegetable oil crops [Barnwal et al., 2005]. Studies have shown that the combustion of 1 liter of diesel fuel leads to the emission of about 2.6 kg of CO_2 against 1 kg of CO_2/liter of biodiesel [Tickell, 1999]. The combustion of biodiesel compared to diesel has been reported to emit lesser pollutants, such as SO_2, soot, CO, hydrocarbons (HC) and polyaromatic hydrocarbons (PAHs), and aromatics. Combustion of biodiesel alone provides over a 90% reduction in total unburned hydrocarbons, and a 75-90% reduction in polycyclic aromatic hydrocarbons (PAHs). Biodiesel provides a slight increase or decrease in nitrogen oxides depending on engine family and testing procedures. The average of pollutants emission from diesel engines using the biodiesel mixtures relative to the standard diesel is shown in **Figure 1-4** [Morris et al., 2003]. Biodiesel contains virtually trace amount of sulfur and the sulfur content of petrodiesel is 20-50 times those of biodiesels, so SO_2 emissions are reduced in direct proportion to the petrodiesel replacement. Emissions of all pollutants except NOx appear to decrease when biodiesel is used. The fact that NOx emissions increase with increasing biodiesel concentration could be a detriment in areas that

are out of attainment for ozone. The use of blends of biodiesel and diesel oil is preferred in engines, in order to avoid some problems related to the decrease of power and to the increase of NOx emissions (a contributing factor in the localized formation of smog and ozone) with increasing content of pure biodiesel in the blend [Schumacher et al., 1996].

1-12 Method of biodiesel production

Current oil used in producing biodiesel almost consists of triglycerides which contain three fatty acid molecules and a glycerol skeleton. Three molecules of fatty acid are esterified with a molecule of glycerol to form a triglyceride (TG) molecule [Chisti, 2007]. For the purpose of making biodiesel, the algal or vegetable oil is reacted with methanol and catalysts (alkaline, acid or enzyme). This reaction is known as transesterification or alcoholysis, and the products in this reaction are glycerol and fatty acid methyl esters, which is namely biodiesel [Srivastava and Prasad, 2000; Fukuda et al., 2001; Chisti, 2007].

Transesterification reaction requires 3 mol of alcohol for each mole of triglyceride to produce 1 mol of glycerol and 3 mol of methyl ester (**Figure 1-5A**). There are three steps in this reaction, the first one is diglycerides formation, and then diglycerides are converted to monoglycerides and eventually to glycerol (**Figure 1-5B**) [Meher, 2006]. The glycerol layer settles down at the bottom of the reaction vessel. Diglycerides and monoglycerides are the intermediates in this process.

The step wise reactions are reversible and a little excess of alcohol is used to shift the equilibrium towards the formation of esters. In presence of excess alcohol, the foreword reaction is pseudo-first order and the reverse reaction is found to be second order. It was also observed that transesterification is faster when catalyzed by alkali [Freedman et al., 1986].

1-12-1 Catalyst type and concentration in the transesterification

Catalysts used for the transesterification of triglycerides are classified as alkali, acid, enzyme or heterogeneous catalysts. The alkali catalysts are sodium hydroxide (NaOH), sodium methoxide (NaOMe), potassium hydroxide (KOH), potassium methoxide (KOMe) are more effective and usual for use [Ma and Hanna, 1999; Meher et al., 2006]. Some researches have investigated the inhibitory effect of base-catalyzed transesterification of triglycerides containing substantial amount of free fatty acid. Free fatty acids will react with the alkaline catalyst which is added for the reaction and give rise to soap, as a result of which, one part of the catalyst is neutralized and is therefore no longer enough for transesterification reaction. The soap consumes the catalyst and reduces the catalytic efficiency, as well as causing an increase in viscosity, the formation of gels, and difficulty in achieving separation of glycerol [Ma and Hanna, 1999].These high FFA content oils/fats are processed with an immiscible basic glycerol phase so as to neutralize the free fatty acids and cause them to pass over into the glycerol phase by means of monovalent alcohols [Turck, 2002; Meher et al., 2006]. Many studies stressed on the importance of oils being dry and free fatty acids and also suggested that the free fatty acid content of the refined oil should be as low as possible below 0.5% and the ester yields were significantly reduced if the reactants did not meet these requirements; sodium hydroxide or sodium methoxide reacted with moisture and carbon dioxide in the air which are diminishing their effectiveness[Freedman et al., 1984; Ma and Hanna, 1999; Feuge and Grose, 1949].

So when the oil has high free fatty acid content, high acid value and more water content, acid catalyzed transesterification is suitable to solve these problems even though the transesterification by acid catalysis is much slower than that by alkali catalysis [Ma and Hanna, 1999; Srivastava and Prasad, 2000; Fukuda, 2001; Freedman, 1984]. The most common acids to use are sulfuric acid (H_2SO_4), phosphoric acid (H_3PO_4), hydrochloric acid

(HCl) or organic sulfonic acid (R-SO_3H). Report said that it was necessary to perform transesterification under an acidic condition when the oil component was a low grade material such as sulphur olive oil [Aksoy et al., 1988]. In general, the ethyl esters of monounsaturated or short-chain fatty acids with 2% sulfuric acid should make good alternative fuels [Klopfenstein and Walker, 1983].

As a catalyst in the process of alkaline transesterification, mostly sodium hydroxide and potassium hydroxide have been used, both in concentration from 0.4 to 2% w/w of oil. In the meanwhile, there are studies showed acid catalyzed transesterification with waste vegetable oil. The reaction was conducted at four different catalyst concentrations, 0.5, 1.0, 1.5 and 2.25 M HCl in presence of 100% excess alcohol and the result was compared with H_2SO_4 in the range of 1.5- 2.25 M concentration [Mohamad and Ali, 2002].

Although chemical transesterification using an alkaline catalysis process gives high conversion levels of triglycerides to their corresponding methyl esters in short reaction times, the reaction has several drawbacks: it is energy intensive, recovery of glycerol is difficult, the acidic or alkaline catalyst has to be removed from the product, alkaline waste water require treatment, and free fatty acid and water interfere the base-catalyzed reaction. Enzymatic catalysts like lipases are able to effectively catalyze the transesterification of triglycerides in either aqueous or non-aqueous systems, which can overcome the problems mentioned above. **Figure 1-6** shows the flow chart of comparison with alkali- and lipase- catalysis process. The latter process is much simpler since recovery of un-reacted methanol and wastewater treatments are unnecessary. In addition, only a simple concentration is required to recover glycerol [Fukuda, 2001]. Furthermore, both extracellular and intracellular lipases are also able to effectively catalyze the transesterification of triglycerides in either aqueous or non-aqueous systems and as shown in **Table 1-1** [Fukuda, 2001; Meher et al., 2006]. In particular, it should be noted that the by-product, glycerol, can be easily recovered without

any complex process, and also that free fatty acids contained in waste oils and fats can be completely converted to methyl esters. On the other hand, in general the production cost of a lipase catalyst is significantly greater than that of chemical catalysts. By the way, lipase may cause serious degradation of lipase activity in the presence of a high concentration of methanol and the by-product; glycerol will suppress the transesterification reaction. There is a novel operation with stepwise addition of methanol has been developed and the usage of solvent-tolerant lipase can solve the negative effect of methanol [Kaieda et al., 1999; Shimada et al., 1999; Samukawa et al., 2000; Watanabe et al., 2000; Fukuda et al., 2001; Kaieda et al., 2001].

1-12-2 Alcohol type and effect of alcohol molar ratio in transesterification

Methanol and ethanol are most commonly used for transesterification of vegetable oils and fats but other alcohols can also be used. It is reported that yield of alcohol esters was the highest with methanol, as methanol is the shortest chain alcohol and is more reactive to oil with the added advantage of alkali catalysts being easily soluble in methanol [Bajpai and Tyagi, 2006].

The molar ratio of alcohol to triglyceride is one of the most important factors to affect the yield of fatty acid acyl ester. The theoretic chemical ratio for transesterification requires three moles of alcohol and one mole of triglyceride to yield three moles of fatty acid alkyl esters and one mole of glycerol.

However, transesterification is an equilibrium reaction in which a large excess of alcohol is required to drive the reaction to the ester yielding. For maximum conversion to the ester, a molar ratio of 6:1 should be used. The molar ratio has no effect on acid, peroxide, saponification and iodine value of methyl esters [Fukuda et al., 2001; Tomasevic and Marinkovic, 2003; Meher et al., 2006]. But, the high molar ratio of alcohol to oil interferes

with the separation of glycerol because there is an increase in solubility. When glycerol remains in biodiesel, it helps drive the reaction equilibrium to back to the left, lowering the yield of esters. Study showed the effect of molar ratios (from 1:1 to 6:1) on ester conversion with vegetable oils. Soybean, sunflower, peanut and cotton seed oils behaved similarly, with the highest conversion being achieved at a 6:1 molar ratio by basic catalysts [Freedman et al., 1984].

1-13 Biodiesel source and fatty acid composition

Biodiesel is an eco-friendly, alternative diesel fuel prepared from domestic renewable resources i.e. vegetable oils (edible or non-edible) and animal fats, that runs in diesel engines-cars, buses, trucks, construction equipment, boats, generators, and oil home heating units. Biodiesel is produced mainly from plant and animal oils, but few or even not from microalgae. This is likely to change as several companies are attempting to commercialize microalgal biodiesel. Biodiesel is a proven fuel to use easily and safely. Technology for producing and using biodiesel has been known for more than 50 years [Knothe et al., 1997; Fukuda et al., 2001; Barnwal and Sharma, 2005; Demirbas, 2005; Van Gerpen, 2005; Felizardo et al., 2006; Kulkarni and Dalai, 2006; Meher et al., 2006; Chisti, 2007]. More than 350 oil-bearing crops were identified, among which only soybean, palm, sunflower, safflower, cottonseed, rapeseed and peanut oils are considered as potential alternative fuels for diesel engines [Goering et al., 1982; Pryor et al., 1982]. Even though vegetable oils are a renewable and potentially inexhaustible source of energy with energy content close to diesel fuel, extensive use of vegetable oils may cause other significant problems such as starvation in developing countries. The vegetable oil fuels were not acceptable because they were more expensive than petroleum fuels. Other biolipids can be used to produce biodiesel. There are waste vegetable oil, animal fats including tallow, lard and yellow grease and

non-edible oils such as *Jatropha*, neem oil, castor oil, tall oil, etc.

In the United States, biodiesel is produced mainly from soybeans because Soybeans are commonly used in the United States for food products. Other sources of commercial biodiesel include canola oil, animal fat, palm oil, corn oil, waste cooking oil [Felizardo et al., 2006; Kulkarni and Dalai, 2006; Chisti, 2007], and *Jatropha* oil [Barnwal and Sharma, 2005; Chisti, 2007]. In Malaysia and Indonesia palm oil is used as a significant biodiesel source. In Europe, rapeseed is the most common base oil used in biodiesel production. In India and southeast Asia, the *Jatropha* tree is used as a significant fuel source [Demirbas, 2007].

However, biodiesel production from various vegetable oils and sources in different countries is given in **Table 1-2** [Venkataraman, 2004; Bajpai and Tyagi, 2006; Demirbas, 2007]. There have been various sources of biodiesel being discovered and developed. In the meanwhile, many scientists have studied the fatty acid composition of biodiesel obtained from different sources, some of those are given in **Table 1-3 [**Volkmann et al., 1898; Applewhite , 1980; Gunstone et al.,1994; Zhukova and Aizdaicher, 1995; Pratoomyot et al.,2005; Bajpai and Tyagi, 2006].

1-14 Biodiesel from microalgae

Alga is a novel source to produce biodiesel. Algae can grow practically in every place where there is enough sunshine and some algae can grow in saline water. The most significant difference of algal oil is in the yield and hence its biodiesel yield. According to some estimates, the yield (per acre) of oil from algae is over 200 times the yield from the best-performing plant/vegetable oils [Sheehan et al., 1998]. Microalgae are the fastest growing photosynthesizing organisms. They can complete an entire growing cycle every few days. Microalgae commonly double their biomass within 24 h. Biomass doubling times

during exponential growth are commonly as short as 3.5 h. Approximately 46 ton of oil/hectare/year can be produced from diatom algae. Different algae species produce different amounts of oil [Demirbas, 2007] and common algae produce up to 20% - 50% oil by dried weight. However, oil content in microalgae can even exceed 80% by weight of dry biomass [Metting, 1996; Spolaore et al., 2006]. Depending on species, microalgae produce many different kinds of lipids, hydrocarbons and other complex oils [Banerjee et al., 2002; Metzger and Largeau, 2005; Guschina and Harwood, 2006]. Not all algal oils are satisfactory for making biodiesel, but suitable oils occur commonly.

United States is the main region to consume the most fossil fuels than other countries and the usage of petroleum is approximate 25% in the world. 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 energy plants, waste cooking oil and animal fat cannot realistically satisfy this demand. For example, meeting only half the existing U.S. transport fuel needs by biodiesel would require unsustainably large cultivation areas for major oil crops. The comparison with different energy plants in oil productivity is shown in **Table 1-4** [Pahl, 2005; Chisti, 2007]. 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. Apparently, oil crops can not significantly contribute to replacing petroleum derived liquid fuels in the foreseeable future. If microalgae are used to produce biodiesel, only 1% and 2.5% of the total U.S. cropping area can be sufficient for producing algal biomass that satisfies 50% of the transport fuel needs of U.S. in **Table 1-4** [Pahl, 2005; Chisti, 2007]. Although, the production of algae to harvest oil for biodiesel has not been undertaken on a commercial scale, working feasibility studies have been conducted to arrive at the above number.

1-15 CO_2 Fixation and producing biodiesel using microalgal cultures

Atmospheric CO_2 has increased from 280 to 370 ppmv in the last 200 years and is responsible for about 50% enhancement in the greenhouse effect [Karube et al., 1992]. Annual anthropogenic emissions of CO_2 are estimated to be about 2×10^{10} tons, primarily from combustion of fossil fuels in association with an increasing population and industrialization. Recently, many attempts have been made to reduce atmospheric CO_2. The possible application of biological CO_2 fixation method to reduce anthropogenic CO_2 emission has been studied. Microalgal photosynthesis has increasingly received attention as a means of reducing the emission of CO_2 into the atmosphere and producing industrially valuable compounds, i.e. biodiesel [Chang and Yang, 2003]. For development of atmospheric CO_2 fixation systems using microalgae, efficient photobioreactors and microalgal strains that can fix large quantities of CO_2 are required. Microalgae that can survive and grow under extreme conditions will be required for direct CO_2 fixation. Many performed studies showed that direct biological utilization of CO_2 in emission gases from coal-fired power plants, steel plants and cement plants that produce large quantities of CO_2 and NOx by microalgae [Matsunaga et al., 2005].

Typical power plant flue gases have carbon dioxide levels ranging from 10%-15%. At the typical carbon dioxide percentages, microalgae show no signs of significant growth inhibition. However, studies have shown that the growth of microalgae respond better to increased carbon dioxide concentrations, distinct form low-density microalgae (on a biomass basis) exposed to only CO_2 from ambient air [Maeda et al., 1995; Brown, 1996]. In phototrophic microalgal cultures, all carbon of biomass is almost derived from carbon dioxide. Producing 100 t of algal biomass fixes roughly 183 tons of carbon dioxide. Carbon dioxide must be fed continually during daylight hours. Feeding controlled in response to signals from pH sensors minimizes loss of carbon dioxide and pH variations [Chisti, 2007].

Biodiesel production can potentially use some of the carbon dioxide that is released in power plants by burning fossil fuels [Sawayama et al., 1995; Yun et al., 1997]. This carbon dioxide is often available at little or no cost. Ideally, microalgal biodiesel would be carbon neutral, as all the power needed for producing and processing the algae would come from biodiesel itself and from methane produced by anaerobic digestion of biomass residue left behind after the oils has been extracted. Although microalgal biodiesel can be carbon neutral, it will not result in any net reduction in carbon dioxide that is accumulating as a consequence of burning of fossil fuels.

However, the specific objective of this research is using microalgae as good candidates for biodiesel production by utilization of waste green-house gas CO_2. **Figure 1-7** shows the flow chart of object of application in microalgae culture. Screened microalgae, *Chlorella* sp. NCTU-2 and *Nannochloropsis oculata* NCTU-3 are cultivated to obtain optimal microalgae growth condition and maximal lipid content. Grow capability and lipid contents of microalgae are affected by several environmental conditions, such as nitrogen concentration, culture age, CO_2 concentration, light intensity and so on. Moreover, the lipid of microalgal cells is extracted for analysis of lipid contents and biodiesel production.

II. Materials and Methods

2-1 Microalgal strains

These microalgae of *Chlorella* sp., *Nannochloropsis oculata*, *Skeletonema costatum*, *Isochrysis galbana* and *Tetraselmis chui* were obtained from Taiwan Fisheries Research Institute (Tung-Kang, Taiwan). The figures of these microalgae stains are showed in **Figure 2-1**. At first, these microalgae were screened at the same condition and provision to choose the high-growth and rich-lipid microalgae as the candidates to this study. In our subsequent results showed the *Chlorella* sp. and *N. oculata* had the potential for the research of biodiesel production and waste-gas reduction.

***Chlorella* sp.:** The species of *Chlorella* sp. was isolated in Taiwan but unidentified, nevertheless, the partial sequence of 18S rRNA (599 bp) of the *Chlorella* sp. has been amplified and sequenced for species identification within the research. The result of sequence alignment was performed by NCBI nucleotide blast [Wu *et al*., 2001]. The blast result provides an evidence to prove the *Chlorella* sp. used in our research is identified as several *Chlorella* sp. strain, such as KAS001, KAS005, KAS007, KAS012, MBIC10088, MDL5-18 and SAG 211-18.

***Nannochloropsis oculata*:** The microalga, *N. oculata* NCTU-3, was screened for its potential ability of growth and biomass production at National Chiao Tung University, Taiwan.

2-2 Microalgae inoculums

The steps of microalgal inoculums, initially, a stock culture of *Chlorella sp.* or *N. oculata* NCTU-3 (approximately 1×10^5 cells/mL) was incubated in an Erlenmeyer flask containing

800 mL working volume of modified f/2 medium at 26 ± 1°C and illuminated at 300 μmol/m^2/sec. After Six days culture, the microalgal cells were harvested by centrifugation at 3,000 × *g* for 5 min. After this step, the pelleted cells were re-suspended in 50 mL fresh modified f/2 medium. The density of cells in the culture was then measured and these microalgal cells were arranged for the further experiments.

2-3 Culture medium and nutrients

The microalgae were cultured in modified f/2 medium in artificial sea water which has the following composition (per liter): 29.23 g NaCl, 1.105 g KCl, 11.09 g $MgSO_4 \cdot 7H_2O$, 1.21 g Tris-base, 1.83 g $CaCl_2 \cdot 2H_2O$, 0.25 g $NaHCO_3$, and 3.0 mL of trace metal solution [Guillard, 1962; Guillard, 1975]. The trace elemental solution (per liter) contains chemicals in **Table 2-1**.

2-4 Microalgae cryopreservation

The microalgae in the early stationary phase (approximately 4 × 10^7 cells/mL) form the cultivation culture were obtained and centrifuged to get an approximately ten-fold higher cell density. Then the prepared 2.2 M glycerol was gradually added to an equal volume of freshly concentrated microalgal cell suspension in 1.5 mL cryotubes. After waiting for 20 minutes for the acclimatization of these samples at room temperature, the cryotubes were put into 4, -30, -80°C by turns in every 30 min. Lastly, cryotubes contained microalgae were transferred to freeze in liquid N_2 for preservation.

2-5 Experimental system with photobioreactors

The *N. oculata* NCTU-3 was cultured in a cylindrical glass photobioreactor (30 cm length, 7 cm diameter) with 800 mL of working volume placed at 26 ± 1°C under continuous, cool white, fluorescent lights. The setup of photobioreactor for microalgal culture system was described in the previous research [Chiu *et al*., 2008]. Light intensity was approximately 300 μmol/m^2/sec at the surface of photobioreactors. Gas provided as different concentrations of CO_2 mixed with ambient air was prepared with a volumetric percentage of CO_2 and filtered (0.22 μm) to give CO_2 concentrations of 2, 5, 10, or 15%. The microalgal cultures were aerated continuously with gas provided via bubbling from the bottom of reactor with an aeration rate of 200 mL/min (i.e., 0.25 vvm, volume gas per volume broth per min). A pre-cultured *N. oculata* NCTU-3 was inoculated in cylindrical glass photobioreactor in 800 mL culture volume at an initial biomass concentration (calculated dried weight of microalgal cells per liter, g/L) of 0.01 g/L (approximate 7×10^5 cells/mL) as a batch culture. Different concentrations of CO_2 aeration were mixed with air and pure CO_2, and adjusted by gas flow meter (Dwyer Instruments, Inc., Michigan, IN, USA) to give a flow rate of 0.25 vvm (volume gas per volume media per min).

2-6 Microalgal cell counting and biomass

A direct microscopic count was performed with Brightline Hemocytometer (BOECO, Hamburg, Germany) and a Nikon Eclipse TS100 inverted metallurgical microscope (Nikon Corporation, Tokyo, Japan). Cell density (cells/mL) was measured by an Ultrospec 3300 pro UV/Visible spectrophotometer (Amersham Biosciences, Cambridge, UK) at the absorbance of 682 nm (A_{682}). Each sample was diluted to give an absorbance in the range 0.1-1.0 if optical density was greater than 1.0. Microalgal dry weight per liter (g/L) was measured according to the method previously reported [American Public Health Association, 1998]. Microalgal cells were collected by centrifugation and washed twice with deionized

water. Microalgal pellet was dried at 105°C for 16 hr for dry weight measurement [Takagi *et al*., 2006].

2-7 Growth rate of Microalgae

A regression equation of the cell density and dry weight per liter of culture was obtained by a spectrophotometric method [Guillard, 1973; Chiu et al., 2008]. Biomass was calculated from microalgal biomass produced per liter (g/L). Specific growth rate (μ, 1/d) was calculated as follows:

$$\mu = \frac{\ln(W_f / W_o)}{\Delta t}$$

where *Wf* and W*0* were the final and initial biomass concentration, respectively. △*t* was the cultivation time in day [Ono and Cuello, 2007].

2-8 Measurement of nitrogen concentration in culture

Consumption of nutrients in microalgal culture was monitored by the determination of medium nitrate content [Tonon et al., 2002]. Nitrate concentration was determined according as the method reported by Collos et al. [1999]. A sample form cultures was centrifuged at 3,000 × *g* for 5 min. Take out the supernatant solution and measure the absorbance of supernatant was at 220 nm by UV-visible spectrometer. The standard curve was determined and calculated from authentic sodium nitrate at concentrations from 0 to 440 μM.

2-9 Lipid analysis

2-9-1 Measurement of lipid content by lipid extraction

To extract the lipid from the microalgae, the collected microalgal sample from the cultures were centrifuged at 3,000 × *g* for 15 min. The cells were washed with deionized water twice, and then obtained microalgal dry biomass by lyophilizing the samples. About weighted 30 mg sample was precipitated in methanol/chloroform in the ratio of 2:1 (v/v), following that sonicated for 1 hr. Chloroform and 1% NaCl were added to give the mixture to a ratio of methanol, chloroform, and water of 2:2:1. The mixture was centrifuged at 1,000 × *g* for 10 min and the chloroform phase was collected. Chloroform was removed under vacuum in a rotary evaporator to eliminate the organic solvent [Takagi *et al*., 2006]. The remaining from the evaporation was weighed as microalgal lipid. The lipid content (%) is calculated by the formula: weight of lipid (g)/weight of sample (g) ×100%.

2-9-2 Saponification and esterification for GC analysis

Lyophilized microalgal cells 0.1 g were mixed with 8 ml of 0.5N KOH in methanol and sonicated for 3 min. Firstly saponification, the mixture was heated to 100 °C for 15 min and then cooled to room temperature. And then, 8 mL of 0.7N HCl in methanol and 14% (v/v) BF3/CH_3OH were added to the mixture and heated to 100 °C for 15 min for esterification. After cooling to room temperature, 2 ml of a saturated solution of NaCl was added for washing and emulsification. The fatty acid methyl esters formed were extracted by using of n-hexane. The upper hexane layer was separated by centrifugation and analyzed for fatty acid methyl esters [Su et al., 2007].

2-9-3 GC analyzer

Fatty acid methyl esters were analyzed by standard gas chromatography (HP5890 series II) coupling with mass spectrometer (VG TRIO-2000). GC capillary column (DB-5MS, 30m,

0.25 μm, and 0.25 mm) was suitable for the analysis of fatty acid methyl esters and a flame ionization detector (FID). Helium with a flow rate of 1.5 mL/min was used as the carrier gas. Temperature was programmed from 70°C to 170°C at 30°C/min and thereafter to 220°C at 2°C/min. Injector and detector were maintained at 220°C and 300°C, respectively. Fatty acid methyl ester (or lipids) contents were determined from their peak areas. The data presented are the average of three estimates.

2-9-4 Measurement of lipid content by fluorescent spectrometry

For fast determination of lipid content, a fluorescent spectrometric method was applied. In the method, the microalgal cells were stained with Nile Red (Sigma, St. Louis, MO, USA) followed the protocol reported by de la Jara *et al*. [2003]. In brief, 1 mL of 1×10^6 cells suspension was added 50 μL of Nile Red in acetone working solution as a concentration of 0.1 mg/mL for lipid staining. The mixture was gently inverted for mixing and incubated at 37°C in darkness for 10 min. In the detection, the fluorometer with a 480 nm excitation filter and a 580 nm emission filter was used. Non-stained cells were used as an auto-florescence control. The relative florescence intensity of Nile Red was calculated as florescence intensity of Nile Red stained subtracted auto-florescence intensity signal [Lee et al., 1998; Liu et al., 2008]. The following equation of the correlation curve indicated fluorescent intensity of Nile Red staining *vs.* lipid content measured by gravimetric method.

$$y = 1.680x + 5.827 \qquad R^2 = 0.994 \ (p < 0.001)$$

The value y is total lipid content determined by gravimetric method. The value x is the relative arbitrary unit obtained Nile Red fluorescent spectrometric method.

2-10 Setup of semicontinuous culture system

Before the *N. oculata* NCTU-3 cultures applied to the semicontinuous system aerated with various CO_2 concentrations, the microalga was grown in a batch culture and aerated with air. When the cell density in the batch culture reached to about 1×10^7 cells/mL, the culture was changed into the aeration of 2% CO_2. After 4-6 days cultivation aerated with 2% CO_2, cell density of the culture reached up to about 1×10^8 cells/mL. The culture was then replaced half of broth with fresh medium each day and performed for 3 days. After that, the culture was also replaced half of broth with fresh medium at the forth day and aerated with 2, 5, 10, and 15% CO_2. After 4 days culture, the sampling time was at 6,12, and 24 hr everyday and the culture was replaced half of broth with fresh medium daily. This following method is published by Chiu et al. [2009].

2-11 Autotrophic, heterotrophic, and mixotrophic growth in microalgal cultures

The initial inoculum was prepared with 2×10^7 cells/mL of *N. oculata* incubated in an Erlenmeyer cylindrical flask (22 cm length, 7 cm diameter) containing 800 mL working volume of modified f/2 medium and incubated at 26 ± 1°C. In heterotrophic growth, cultures in the reactor were supplemented with each different kind of carbon source and aerated with filtered (0.22 μm) ambient air continuously. Whereas, for the mixotrophic growth, cultures were added with each different kind of carbon source and aerated with the filtered ambient air mixing with CO_2 that give the CO_2 concentrations of 2%. The cultures under photoautotrophic condition which represented the control for these studies were not supplemented with any organic carbon source in the medium. All the cultures for all of the growth conditions were aerated continuously at a rate of 200 mL/min (i.e., 0.25 vvm).

2-12 Effect of organic carbon on the growth

For the purpose to test the growth of *N. oculata* on different organic carbon sources, experiment was carried out in the dark (heterotrophic condition) and in the light (mixotrophic condition) with the presence of four organic carbon substrates, which were glucose, citric acid, sucrose and sodium acetate in the same concentration (10 mM) [Bouarab *et al*., 2004], and with 2% CO_2 aeration. The cultures were incubated at 26 ± 1°C under continuous, cool white, fluorescent light which is 300 μmol/m^2/s at the surface of the cultures for mixotrophic condition and photoautotrophic condition. For the heterotrophic condition, the reactors were wrapped with aluminum foil to maintain in the total darkness.

All organic nutrients were prepared in water sterilized by filtration through a 0.22 μm Millipore filter. The growth of *N. oculata* was measured according to a standard curve between cell density and absorbance at 682 nm.

2-13 Transesterification of microalgal oil

The reaction of transesterification by three types of catalysts: acid, base, and enzyme (lipase). The acid catalyst is 1.5% H_2SO_4 and the base catalyst NaOH is also 1.5%. The highest oil/methanol molar ratio in acid catalysis and alkali catalysis is 1:30 and 1:6. The transesterification reactions of acid and alkali catalysis are in 60°C for 1 hour by sonication. And in the transesterification of enzyme, catalysis is 10% *Rhizopus oryzae* to oil weight. The reaction performs in 30°C for 72 hour by 150 oscillations/min.

2-14 RNA extraction of microalgae

Freezing microalgae in liquid nitrogen or -80°C was grind with liquid nitrogen in a

pre-cooled mortar and pestle to a fine powder. Added 10 ml extraction buffer in 1g microalgae, mixed vigorously in the room temperature at least 30 min and extracted with 1 volume of chloroform/isoamyl alcohol (24/1) for 3 minute. After that, centrifuged at 12,000g and room temperature for 20 min, and then recovered the aqueous phase (upper phase). 0.2 - 0.3 volumes of ethanol were added to mix gently and extracted with 1 volume of chloroform for 3 minute. And then, centrifuged at 12,000 g and room temperature for 20 min and recovered the upper phase and added 0.25 volume of 12 M LiCl with mercaptoethanol to 1% final concentration to mix vigorously and place at -20° overnight. Centrifuged at 12,000g and 4°C for 1 hour, carefully removed the aqueous phase and inverted the tube on some absorbent paper. Dissolved the pellet in 1 ml of TE on ice and extracted with phenol/chloroform (50/50, pH 4.3). And then, precipitated the RNA from the aqueous phase by adding 0.1 volumes of 3M sodium acetate and 2.5 volumes of ethanol and placed it at -20°C for at least 6 hours before centrifuging for 20 minutes in 10,000g. Finally, washed the pellet 80% ethanol and re-suspended the RNA in TE or ultrapure water.

2-15 ACCase expression

N. oculata was cultured for ACCase (Acetyl-coenzyme A carboxylase) expression. The microalgal total RNA was extracted and then determine the cDNA via reverse transcription. PCR amplification was used to obtain 241-base pairs ACCase-encoding fragments. The following oligonucleotides were used in this study as primers for DNA amplification of ACCase. Forward primer (PR1) is TTTATGGGGGGAAGTATGGGCTC. The reverse primer (PR2) is CCAACAACAGGTGGTGTAACTGC (all sequences are written 5'→3'). The PCR reaction mixture 25μL included of 50 ng DNA, 0.1μM concentration of each primer species, 10 mM Tris-C1 (pH 8.3), 50 mM KCl, 1 mM MgCl, 0.2 mM dNTPs, and 1 unit of *Taq* DNA polymerase. The following thermal cycle was used Step 1, 94℃ for 5

min; Step 2, 94 ℃ for 1 min; Step 3,45℃ for 2 min; Step 4, 2℃/s to 72℃; Step 5, repeat steps 2 to 4 for 30 times total; and Step 6,72℃ for 8 min.

However, the ACCase expression is compared with the expression of actin as an internal control. The following oligonucleotides were used as primers for actin (all sequences are written 5'→3'); forward primer (PR3) is GACGCAGATCATGTTTGAGACCTTC and the reverse primer (PR4) is GACATCAAGGAGAAGCTGGGC.

2-16 Parameter analyses

Sample pH was directly determined with an ISFET pH meter KS723 (Shindengen Electric Mfg Co. Ltd., Tokyo, Japan) and the pH meter was calibrated daily using pH 4 and 7 solutions.

The CO_2 concentration in airstreams, CO_2 (g), was measured using a Guardian Plus Infra-Red CO_2 Monitor D-500 (Edinburgh Instruments Ltd, Livingston, UK).

Light intensity was measured from the light-attached surface of the photobioreactor using a Basic Quantum Meter (Spectrum Technologies, Inc., Plainfield, IL, USA).

2-17 Statistics

All values are expressed as mean ± standard deviation (SD). A Student's t test was used to evaluate differences between groups of discrete variables. A value of $p < 0.05$ was considered statistically significant.

III. Results and Discussion

3-1 Growth ability and lipid content of four potential strains

Microalgae strains of *Chlorella* sp., *N. oculata*, *Skeletonema costatum*, *Isochrysis galbana* and *Tetraselmis chui* were acquired from Taiwan Fisheries Research Institute. The cell density of these four strains was measured by optical density A_{682}. The linear regression of cell density and optical density was established firstly for comparing growth ability in the four strains (only showed *Chlorella* sp. and *N. oculata* in this study). **Figure 3-1** shows the growth ability of *Chlorella* sp., *N. oculata*, *Skeletonema costatum*, *Isochrysis galbana* and *Tetraselmis chui*. *Chlorella* sp. and *N. oculata* exhibited the potential for fast growth capability. Then these four algal strains were examined on lipid content for potential of biodiesel production (**Figure 3-2**). As the same to the growth ability, *Chlorella* sp. and *N. oculata* are richer in lipid content than other algal species. *Nannochloropsis oculata* contained the highest lipid contents of 35% and *Chlorella* sp. had the secondary rich lipid contents of 17%. However, lipid contents of *Skeletonema costatum*, *Isochrysis galbana* and *Tetraselmis chui* are only about 9-10%.

3-2 Cultivation with CO_2 and lipid composition

The potential microalgal species *Chlorella* sp. and *N. oculata* were cultured with aeration of simulated waste green-house gas CO_2. The cultures were aerated with 10% CO_2 for carbon source of essential photosynthesis. The growth of *Chlorella* sp. and *N. oculata* cultures with CO_2 aeration was rapider than cultures only with air aeration after 3 days (**Figure 3-3**). However, *Chlorella* sp. go into stationary phase in the fourth day and *N. oculata* kept growth ability.

Lipid composition of *Chlorella* sp. and *N. oculata* were analysis by Gas chromatography (**Figure 3-4** and **Table 3-1**). The length of fatty acid chain plays an important role to decide the characteristics of biodiesel such as pour point, boiling point and so on. C12:0~C18:0 and C18:1, C18:2 and C18:3 are usual fatty acid for biodiesel contents. In lipid composition of *Chlorella* sp. and *N. oculata*, C16:0 are 32.3% and 12.0%, respectively. Therefore, the algal oil of *Chlorella* sp. may have higher melting point suitable for usage in the torrid areas. In the results, the C18:1 and C18:3 could not be separated by GC in our methods. C18:2 of *Chlorella* sp. and *N. oculata* are 20.7% and 31.6%, respectively. Both C18:2 are higher than 12% that fits the definition by European legislation, but if the linolenic acids (18:3) are very abundant, the C18:3 fatty acids may require additional treatment of catalytic hydrogenation or the use in mixture with a biodiesel richer in saturated fatty acids [Converti et al., 2009].

3-3 Measurement of cell density and biomass

Cell density and biomass were measured more easily by optical density than by direct counting of cells or by cell dry weight [Rocha et al., 2003]. Therefore, relationships between optical density and cell density, as well as optical density and cell dry weight of *Chlorella* sp. and *N. oculata* were established by linear regression firstly (**Figure 3-5** and **Figure 3-6**). Both cell density ($R^2 = 0.997$; $p < 0.001$) and biomass ($R^2 = 0.991$; $p < 0.001$) can be precisely predicted by optical density. Therefore, the values of optical density were used to calculate the related biomass of *Chlorella* sp. and *N. oculata* NCTU-3 in each experiment according the equations established in this study.

3-4 Effect of CO_2 on biomass and lipid production

3-4-1 Growth of microalgae aerated with different CO_2 Concentration

To investigate the effect of CO_2 concentration on growth, *Chlorella* sp. and *N. oculata* in batch culture was incubated for 4-8 days at 26 ± 1℃ and 300 μmol/m^2 s and aerated with different concentrations of CO_2 at 0.25 vvm. The initial inoculum (approximate 8 × 10^5 cells/mL) was cultured and aerated with air (CO_2 concentration is approximate 0.03%), 2%, 5%, 10%, and 15% CO_2. Cultures were sampled when a stationary phase of growth was reached or a microalgal growth was significantly inhibited. Specific growth rate was calculated from the logarithmic growth phase over 1-2 days batch culture in each experiment.

Figure 3-7 shows the microalgal growth of *Chlorella* sp. and *N. oculata* aerated with different CO_2 concentrations. After the 6-8 days, the cells grew up to plateau stage and the biomass of *Chlorella* sp. in air, 2% and 5% CO_2 aeration were 0.537 ± 0.016 g/L, 1.211 ± 0.031 g/L, and 0.062 ± 0.027 g/L and. Furthermore, the biomass of *N. oculata* in air and 2% were 0.268 ± 0.022 and 1.277 ± 0.043 g/L, respectively. At the aeration of 2% CO_2, *Chlorella* sp. grew most rapidly at the specific growth rate of 0.492 1/d than aeration of air (specific growth rate is 0.230 1/d) and the specific growth rate markedly fell to be 0.127 $d^{-1}$when the cultures were aerated with 5% CO_2. The growth of *Chlorella* sp. at 10% and 15% CO_2 aeration was almost completely inhibited, so the specific growth rates were not available. Similarity, the *N. oculata* culture aerated with 2% CO_2 showed an optimal growth potential and the specific growth rate in the 2% CO_2 and air aerated cultures were 0.571 1/d and 0.194 1/d. Whereas, the growth of *N. oculata* aerated with 5%, 10%, and 15% CO_2 were completely inhibited.

When the microalgal cultures aerated with 2% CO_2, not only the biomass was greatly produced but also the specific growth rate was enhanced compared with those in the culture aerated with air. This result was confirmed by Hu and Gao [2003] and Chiu et al. [2009]. They indicated that microalga, *Nannochloropsis* sp., grew well in an enriched CO_2 aeration compared with air aeration. It may due to enough carbon sources for microalgal growth

without carbon source limitation. The significant inhibition of high CO_2 aeration, 5-15%, was also confirmed by the reports that the concentration of CO_2 aeration above 5% could be harmful to microalgal cells and inhibit the microalgal growth [Silva and Pirt, 1984; Sung et al., 1999; Chang and Yang, 2003; de Morais and Costa, 2007a, b; Chiu et al., 2009].

3-4-2 Effect of CO_2 on cell growth in microalgal semicontinuous cultivation

For the study of lipid accumulation in response to higher CO_2 aeration, the microalgal cells pre-adapted to CO_2 were applied. In the experiment, microalgal cells were pre-adapted to 2% CO_2 before the microalga was inoculated into the semicontinuous cultures.

Therefore, the semicontinuous culture was carried out in two stages. A batch culture had an initial high cell density of inoculum (approximate 8×10^6 cells/mL) firstly. At 2% CO_2, cell density was allowed to increase until it reached an optical density (A_{682}) over 5 (the cell density was about 1×10^8 cells/mL), which occurred after 6-8 days of incubation. After that, the semicontinuous system was operated and half of the culture broth was replaced with fresh modified f/2 medium each day and the culture was incubated with 2%, 5%, 10%, and 15% CO_2 aeration. These semicontinuous cultures aerated with different CO_2 concentrations were operated for 24 days and the growth of microalgae was stable by each day replacement and was maintained at logarithmic growth potential. The growth of *Chlorella* sp. and *N. oculata* in the semicontinuous culture was constantly similar at 2%, 5%, 10%, and 15% CO_2. The average specific growth rate and biomass of *Chlorella* sp. and *N. oculata*, respectively, were 0.58-0.66 1/d, 0.76-0.87 g/L and 0.683-0.733 1/d, 0.745-0.928 g/L after 8 days of incubation at 2-15% CO_2 aeration. *N. oculata* showed slight great growth potential than *Chlorella* sp. These results show that a high concentration of CO_2 (10-15%) may directly introduce to a high-density *Chlorella* sp. and *N. oculata* culture in the semicontinuous photobioreactor system. Although, high CO_2 aeration (5-15%) may be a harmful effect on the microalgal cells growth as shown in **Figure 3-7**, increasing the inoculated cell density and

pre-adapting to 2% CO_2 culture could promote the growth capacity of microalga in the cultures aerated with higher CO_2 concentrations.

In the results of semicontinuous culture of microalgae, the high CO_2 concentration did not cause harmful effects on microalgae, indicating that the CO_2 can be as carbon source for the growth of a variety of photosynthetic microalgae at high-density culture. An initial high-density cultures were adapted to 2% CO_2 may overcome environment stress and drastic harmful effects on microalgal cell growth induced by higher CO_2 (10-15%) aeration. Selection of the mutant of *Chlorella* sp. represents one approach to elevating CO_2 tolerance of microalgae [Chang and Yang, 2003]. However, growth and cell density in the cultures aerated with high levels of CO_2 are still limited in the application of these mutants. Chang and Yang [2003] have isolated *Chlorella* strains NTU-H15 and NTU-H25 and found that the greatest biomass produced by each strain at 5% CO_2 was 0.28 g/L d. The other mutant, *Chlorella* strain KR-1, showed a potential biomass of 1.1 g/L/d at 10% CO_2 [Sung et al., 1999]. However, increasing the cell density in the cultures or pre-adapting cells in a low concentration of CO_2 are alternative approaches to increase CO_2 tolerance of microalgae without effects on microalgal growth [Lee et al., 2002; Yun et al., 1997]. In our semicontinuous photobioreactors, *Chlorella* sp. and *N. oculata* cells that were pre-adapted to 2% CO_2 not only grew into a high-density microalgal culture but also grew fast at 10% or 15% CO_2. Our results confirmed these previous studies and provided a useful system that can be applied to conversion of CO_2 into biomass.

3-4-3 Effect of CO_2 on lipid and biomass production in semicontinuous cultures

Lipid and biomass productivity in the semicontinuous *Chlorella* sp. and *N. oculata* cultures were determined before the culture broth was replaced each day. **Table 3-2** and **Table 3-3** summarize the results, lipid and biomass productivity, collected from the single photobioreactor cultures under different CO_2 aeration. As a daily 50% culture broth

replaced in the 800 mL semicontinuous photobioreactor aerated with 2, 5, 10, and 15% CO_2, the total biomass productivity and lipid productivity per day (400 mL of waste broth was recovered for measurement) of *Chlorella* sp. in each photobioreactor was 0.421, 0.404, 0.366, and 0.361 g/L/d, and 0.143, 0.130, 0.124, and 0.121 g/L/d, respectively. Moreover, the total biomass productivity and lipid productivity per day of *N. oculata* culture in each photobioreactor was 0.480, 0.441, 0.398, 0.372 g/L/d, and 0.142, 0.113, 0.097, 0.084 g/L/d, respectively. The total biomass productivity and lipid productivity of *Chlorella* sp. cultures were similar to *N. oculata*. In the result, *Chlorella* sp. and *N. oculata* are potential candidates for biomass and lipid production by semicontinuous cultures.

However, in the single semicontinuous culture, both of lipid and biomass productivity decreased when the aerated CO_2 concentration was increased (**Table 3-2** and **Table 3-3**). Lipid content in *Chlorella* sp. and *N. oculata* cultured at 2, 5, 10, and 15% CO_2 were very similar (approximately 32-34% and 22-29% of dry weight). In the semicontinuous culture, the optimum condition for biomass productivity was at 2% CO_2 aeration and lipid content was not apparently affected even by high CO_2 aeration. Biomass productivity of *Chlorella* sp. and *N. oculata* at 15% CO_2 aeration were 68% and 77% of that at 2% CO_2 aeration. Nevertheless, our results still show the potential growth of microalgal *Chlorella* sp. and *N. oculata* for lipid and biomass productivity in the semicontinuous system even the cells were cultivated in the condition aerated with 15% CO_2.

It is reported that the lipid content was increasing associated with the increasing CO_2 concentration of aeration in *Chlorella fusca* and *Phaeodactylum tricornutum* cultures [Dickson et al., 1969; Yongmanitchai and Ward, 1991]. The data in our studies showed an inverted result may due to different microalgal species, growth condition, and medium content [Hu and Gao, 2006]. Our results show that the pH of *N. oculata* cultures with 2%, 5%, 10%, and 15% CO_2 aeration was maintained at pH 7.8, 7.7, 7.3, and 7.0, respectively.

Yung and Mudd [1966] reported that the carbon assimilation of lipid synthesis was decreased with decrease of pH. This may be possibly because the higher pH having higher available bicarbonate for carboxylation of lipid synthesis. This inference supports the result that lipid accumulated in microalgae may be mainly affected by pH and lipid content of the microalgal cultures was decreased with decrease of broth pH.

3-5 Lipid content of microalgae at different growth phases and nitrate limitation

The microalgal cells from logarithmic, early stationary phase and stationary phase were collected to measure lipid content and supernatant from the collected samples was also obtained for determining the nitrate content in broth. To investigate the effect of nitrogen concentration on growth, *Chlorella* sp. and *N. oculata* in batch culture was incubated for 8-9 days at 26 ± 1℃ and 300 μmol/m^2 s and aerated with 10% of CO_2 at 0.25 vvm. The nitrate concentration was measured by optical density at A_{220}. The standard curves and equations of optical density at A_{220} to the nitrate concentration were introduced in **Figure 3-8**.

Figure 3-9 and **Figure 3-10** shows that the lipid accumulation in microalgal cells was associated with growth phases and nitrate concentration. The lipid content of *Chlorella* sp. cells at logarithmic, early stationary phase and stationary phase was 12, 17 and 24%. And the lipid content of *N. oculata* NCTU-3 cells at logarithmic, early stationary phase and stationary phase was 21, 34 and 50%, respectively. This result indicated that lipid accumulation increases as microalgae approach into stationary phase. The decreased nitrate content in the broth of microalgal culture from logarithmic phase to stationary phase was along with the growth of microalgal cells. It is hinted that the *Chlorella* sp. and *N. oculata* culture from logarithmic phase to stationary phase would accompany with the nitrate

depletion. Roessler [1988] reported that the nutrient deficiency induced an increase in the rate of lipid synthesis in a diatom, *Cyclotella cryptica*, and resulted in lipid accumulation in the cells. It is also indicated that lipid accumulation is related to nitrogen depletion as a nutrient deficiency [Roessler et al., 1994; Takagi et al., 2000]. Report showed the knowledge that illumination of photosynthetic tissue stimulates nitrate reduction and lipid synthesis. That lipid synthesis is stimulated under nitrogen starvation indicate that photosynthetically produced reluctant may be used either for lipid synthesis or the several steps of nitrate reduction [Yung and Mudd, 1966]. The result is confirmed by these previous reports that the microalgae, *Chlorella* sp. and *N. oculata* NCTU-3, shows the metabolic effect of nitrogen depletion related to the increasing lipid accumulation.

3-6 Comparison of productive efficiencies in semicontinuous system with different culture approaches

The comparison of productive efficiencies in the semicontinuous systems in which the culture broth were replaced at an interval of 24 h (one-day replacement) or 72 h (three-day replacement) was performed. In the systems, approximate 0.4 g/L of *N. oculata* NCTU-3 cells was inoculated and the microalgal cultures were replaced half (for one-day replacement) or three fifth (for three-day replacement) of broth with fresh medium in the semicontinuous system after the cultures was aerated with 2% CO_2. **Figure 3-11** shows the stable growth profiles of *N. oculata* NCTU-3 cultured with one-day and three-day replacement. In the cultures, the broth was replaced at logarithmic phase in one-day replacement and replaced before the cells reached to early stationary phase in three day replacement. The growth profiles of both one-day and three day replacement cultures were stable over 12 days cultivation. **Table 3-4** shows the biomass and lipid productivity of *N. oculata* NCTU-3 cells in the semicontinuous culture system with one-day and three-day replacement. The total

volume of replaced broth was 4800 mL in one-day replacement and only 1,920 mL in three-day replacement over 12 d. The lipid content of microalga in the three-day replacement was significantly higher than that in the one-day replacement culture (41.2% vs. 30.7%). However, the total biomass and total lipid yield in the three-day replacement culture were only 24% and 32% compared with those in the one-day replacement culture, respectively. It means that the culture broth being daily replacement could be more efficient not only for biomass production but also for lipid yield. In conclusion, the total biomass and lipid yield in the semicontinuous culture operated by one-day replacement were more efficient compared with those in three-day replacement, although the *N. oculata* NCTU-3 cells in the three-day replacement could increase lipid accumulation because of nutrition-deficient effect.

3-7 Biomass and lipid production of microalgae in mixotrophic and heterotrophic cultivation with various carbon sources

3-7-1 Effects of organic carbons on the growth of N. oculata

The influence of four organic carbon sources, glucose, citric acid, sucrose and sodium acetate (10 mM), on the growth of *N. oculata* in mixotrophic and heterotrophic cultivations compared with photoautotrophic cultures was represented in **Figure 3-12**. The initial inoculum of cell density was 2×10^7 cells/mL in cultivating batch culture. *N. oculata* was incapable of heterotrophic growth on the supplementation of those carbon substrates (**Figure 3-12**). The cell densities obtained in the heterotrophic cultivations with sodium acetate, citric acid, glucose and sucrose on the fifth day were 2.0, 2.2, 2.3 and 2.5×10^7 cells/mL, respectively. Whereas, the cell density for the control culture of heterotrophic cultivations on the fifth day was only 2.9×10^7 cells/mL. The growth capabilities in all of the in heterotrophic cultivations with different carbon sources as well as the control culture of

heterotrophic cultivation (without any carbon source supplementation) were not efficient. This indicated the growth of this *N. oculata* in this study did not grow well in dark condition. The study of Vazhappilly and Chen [1998] showed *N. oculata* UTEX LB 2164 grew not well in heterotrophic growth when glucose and acetate was used as the sole carbon and energy source.

Compared with the cell densities of photoautotrophic culture (7.7×10^7 cells/mL), the cell densities of the cultures supplemented with sodium acetate, citric acid, glucose and sucrose on the fifth day reached 9.2, 10.4, 11.2 and 12.9×10^7 cells/mL in the mixotrophic cultivations, respectively (**Figure 3-12**). Overall, organic carbon supply enhanced the growth capacities of *N. oculata* in the mixotrophic cultivation compared with those in the heterotrophic cultivation. The result shows that cultivation with sucrose in the mixotrophic condition obtained the best growth capacity, and the cell density was 5.2-fold higher than heterotrophic cultivation with sucrose. Sucrose gave the highest cell density; this might be the reason that the sucrose is metabolized to glucose and fructose in one equivalent each in microalgal cells and fructose might be use as the carbon source. Glucose and fructose were used as organic carbon substrates in the cultures of microalgae such as a blue green alga, cyanobacterium *Anabaena variabilis* [Pearce and Carr 1969; Valiente et al. 1992].

However, the growth capability of mixotrophic culture with acetate and citrate was lower than the other organic carbon source. The same as our result, wood et al. [1999] indicated that the *Nannochloropsis* strains seemed to be particularly sensitive to acetate inhibition. However, some researches showed *Nannochloropsis* sp. can cultivate in mixotrophic culture with supply of acetate but it cannot uptake glucose [Hu and Gao 2003]. And in *Nannochloropsis* strain investigated by Liang et al. [2009], acetate utilization especially when nitrogen was limited, provided higher lipid content compared with those from growth on glucose. Consequently, various organic carbon sources may make different effects in

different species even in *Nannochloropsis* strains.

3-7-2 Effects of inorganic and organic carbons on the growth of N. oculata

In **Figure 3-13**, the photoautotrophic culture of *N. oculata* cultured with 2% aeration of CO_2 is slightly slower than the culture with the source of sucrose (11.1×10^7 cells/mL *vs.* 12.9×10^7 cells/mL), and approximately same as the culture with glucose (11.2×10^7 cells ml^{-1}), but higher than those with the supplementation of sodium acetate and citric acid in mixotrophic condition and the control culture of mixotrophic cultivations. Carbon sources are necessary to provide the carbon skeletons and energy for cell growth [Wen and Chen 2003]. In this study, cultivation of *N. oculata* using sucrose as carbon substrate in mixotrophic growth gave the highest growth capability. The synergistic effect of light and the organic carbon is essential for high ATP production and biomass productivity in mixotrophic culture [Cid et al., 1992; Yang et al., 2000]. Mixotrophic growth doesn't require high light intensities, and consequently could reduce the energy costs of microalgal cultures and photobioreactors [Read et al., 1989; Fernández Sevilla et al., 2004].

3-7-3 Lipid contents and production in N. oculata cultures with different carbon sources

Lipid production of *N. oculata* cultures in mixotrophic and heterotrophic cultivations as well as the control cultures in those two cultivations were illustrated in **Figure 3-14**. Lipid contents of the mixotrophic cultivations supplemented with sodium acetate, citric acid, glucose and sucrose were 39, 37, 37 and 36%, respectively. Whereas, the lipid contents of the heterotrophic cultivations with sucrose, citric acid, sodium acetate and glucose were 54, 29, 25 and 20%. Besides, lipid contents produced in the control culture of mixotrophic cultivations was only 13% and in the control culture of heterotrophic cultivations was only 11%. Among these mixotrophic and heterotrophic growths, the cultivation with sucrose provided the highest lipid content in the heterotrophic cultivation. This suggested that the

energy produced in the cultivation with sucrose in heterotrophic growth was used for the production of lipid compounds. The report of Xu et al. [2004] also showed the heterotrophic *Nannochloropsis* sp. growth gave the highest production of lipid (42.7%) compared to mixotrophic growth (38.7%) and photoautotrophic (38.1%).

With the supplement of the organic carbon sources in mixotrophic condition, the biomass of the cultures supplemented with sodium acetate, citric acid, glucose and sucrose were 0.538 and 0.641, 0.690 and 0.798 g/L, whereas lipid production in the cultures supplemented with those carbon substrates were 0.208, 0.240, 0.255 and 0.284 g/L, respectively (**Table 3-5**). Compared with the results from those different organic carbon sources, it showed that with the supplementation of sucrose, it produced the highest lipid production. However, biomass of the culture with 2% CO_2 under photoautotrophic growth was moderate with the dry-weight of 0.681 g/L and the lipid production was the lowest, 0.192 g/L compared with other mixotrophic cultivations. Results show lipid production of mixotrophic cultures with different organic carbon was higher than photoautotrophic culture. Generally, accumulation of triacylglycerols has been associated with the cessation of cellular division at the onset of stationary phase in aged cultures of *N. oculata* [Hodgson et al., 1991]. However, carbon metabolism produced sugars that provide carbon skeletons for other metabolic reactions such as lipid production. The assimilation of organic carbon sources could increase the lipid contents of the cells and relative abundance of neutral lipids. So, microalgal cells in the mixotrophic cultures could assimilate organic carbon sources to produce energy for lipid synthesis leading to the weakened dependence on light [Bouarab et al. 2004].

3-8 Measurement of microalgae by fluorescence

Figure 3-15A shows the image of lipid contents stained by Nile red of *Nannochloropsis*

oculata cells under confocal microscope. Nile red, a lipid-soluble fluorescent dye, has been frequently employed to evaluate the lipid content of animal cells and microorganisms, such as mammalian cells, bacterium, yeasts and microalgae [Elsey et al., 2007]. The image of fluoresce indicated the oil drops of microalgae were dispersed on the periphery of cell bodies. The image also showed the amounts of oil drops could reach to 4~5 in single microalgal cell body due to accumulation of lipid contents. These lipid drops in cells after Nile red staining would emit fluoresce of yellow color, and the intensity of fluoresce can be measured for analysis of lipid contents.

The linear regression ($R^2 > 0.99$; $p < 0.001$) was established by the relationship between the intensity of fluoresce and lipid contents of *Nannochloropsis oculata* (**Figure 3-15B**). Nile red method can be used as a sensitive, quantitative, and high throughput method for screening of cellular neutral lipid content in green algae as well as other classes of algae [Chen et al., 2009]. The conventional method for lipid determination involves solvent extraction and gravimetric determination [Bligh and Dyer, 1959]. Further quantification of neutral lipids requires the separation of the crude extractions and quantification of the lipid fractions by thin-layer chromatography (TLC), HPLC or gas chromatography (GC) [Eltgroth et al., 2005]. The procedure used for lipid analysis must ensure complete extraction and at the same time avoid decomposition or oxidation of the lipid constituents. The conventional method is that it is time- consuming and labor-intensive, making it difficult to screen large numbers of algae. The in situ quantitative determination of cellular neutral lipid content using a Nile red method can provide a fast and convenient tool for microalgae screening. The microalgae don't need to be dried for determination of lipid contents. And the cell density of sample for Nile red staining only as low as about 1×10^6 cells/mL can be detected on lipid contents [de la Jara et al., 2003].

3-9 Comparison between ACCase expression and lipid contents

Acetyl-coenzyme A carboxylase (ACCase) is a biotin-containing enzyme that catalyzes the the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA. This enzyme is believed to be a key regulatory enzyme for fatty acid biosynthesis microalgae. **Figure 3-16** showed the lipid contents in log, early stationary and stationary phase compared the ACCase expression in microalgal cell in different growth phase. In the result, the lipid contents of log, early stationary and stationary phase were 22, 37, and 43%. As well as in ACCase expression increased along with growth phase. Therefore, the expression of ACCase are relative to the lipid biosynthesis and may control the accumulation of neutral lipid in microalgal cells.

3-10 Transesterification by different catalyst types and methanol molar ratio

The transesterification of lipid dependent on catalysts are classified to chemical and biological (enzymatic) processes. Chemical process of transesterification provides high conversion levels of triglycerides to their corresponding methyl esters in short reaction times. But the chemical process has some disadvantage: it is energy intensive, recovery of glycerol is difficult, the acidic or alkaline catalyst has to be removed from the product, alkaline wastewater requires treatment, and free fatty acids and water interfere with the reaction [Fukuda et al., 2001]. However, these problems won't happen in enzymatic process by lipase catalyst and the by-product, glycerol, can be easily recovered without any complex process. The drawbacks of enzymatic process were long reaction time, low methanol tolerance, high cost of enzyme purification and instability of recovered lipase.

In this study, the conversion rate of enzymatic transesterification could reach about 82% (**Figure 3-17A**). The conversion rate of base and acid catalysts in theoretic oil/methanol

molar ratio 1:3 were only 21% and 58%. A research showed the optimal oil/methanol molar ratio of alkali- and acid-catalysts in transesterification was 1:6 and 1:30 [Freedman et al. 1986]. **Figure 3-17B** shows the conversion rate of transesterification by acid-, alkali- and enzymatic-catalysts in their own highest oil/methanol molar ratio were 88, 46 and 82%. The efficiency of acid catalysts was higher that might due to the low neutral lipid or high amount of free fatty acid in the lipid. Another reason was the un-purified mixtures in lipid interfere with the base catalysts to decrease the alkali-catalyzed transesterification reaction. Although the conversion rate of enzymatic process was good enough, the high cost and long reaction time may be not convenient and suitable for usage. In industrial and commercial application, the chemical catalyst is common for transesterification to reach the purposes of low-cost and fast reaction rate. In addition, the excess methanol concentration used for improving transesterification efficiency will be recovered for reuse of organic solvents in industrial application.

3-11 Biodiesel applied to the biodiesel car

In the study, a biodiesel production machine was created for 5L of batch production. The reaction tank was constructed by stainless steel and used ultrasonic device for improving transesterification efficiency. The wash tank and distillation tank were connected with the reaction tank. The distillation tank can perform methanol recovery and water removal. **Figure 3-18A** shows the biodiesel from *Nannochloropsis oculata*. Biodiesel production form transesterifcation in the machine was supplied to the biodiesel car (**Figure 3-18B**). Our biodiesel car was added with mixed diesel/biodiesel for testing. The highest diesel/biodiesel mixed ratio added in the car was B70 and the running mileage of biodiesel usage is over 1000 kilometers.

IV. Conclusions

The industrial revolution in the past years brought with not only economical developments but also great pollution of green-house gas, especially CO_2. The problem of global warming is worse caused by high atmospheric CO_2 concentration. However, the international problem, energy crisis, concerned people all over the world. Therefore, a novel research of microalgae has been developed to solve these serious problems. The microalgae are perfect candidates because of fast growth and high lipid contents.

In the studies, *Chlorella* sp. and *N. oculata* have high growth ability and are rich in lipid contents than *Skeletonema costatum*, *Isochrysis galbana* and *Tetraselmis chui*. *Chlorella* sp. and *N. oculata* can be cultivated in the batch cultures aerated with CO_2. However, the higher CO_2 concentration would be harmful and inhibitory to microalgal culture. When the microalgal cultures aerated with 2% CO_2 could improve the great biomass production and specific growth rate was enhanced.

The microalgae pre-adapting to 2% CO_2 cultured in a semicontinuous system with a high cell density of inoculum could grow well in the system aerated with higher CO_2 concentration (5-15% CO_2). Growth, biomass productivity and lipid productivity with high CO_2 aeration remained constant in the photobioreactor. However, increasing biomass production and lipid accumulation would not be followed as the cultures aerated with higher CO_2. The highest biomass and lipid production in *Chlorella* sp. and *N. oculata* are 0.422 and 0.480 g/L/d, and 0.143 and 0.142 g/L/d, respectively. The results show the semicontinuous culture of *Chlorella* sp. and *N. oculata* had good performance of biomass and lipid productivity even though the lipid contents of *Chlorella* sp. was higher in the condition of cultivation. Achieving the optimal condition for a long-term biomass and lipid yield in the semicontinuous system, the microalga could be cultured with 2% CO_2 aeration in one-day

replacement operation. Nevertheless, the results also show the lipid accumulation of *N. oculata* NCTU-3 and *Chlorella* sp. NCTU-2 could be increased from logarithmic growth phase to stationary growth phase according to consumption of nitrogen source. In addition, the intensity of ACCase expression in microalgal cells was relative to the accumulation of lipid contents form log phase to stationary phase.

The supply of organic carbon sources of microalgal mixotrophic and heterotrophic cultures were evaluated in the studies. Mixotrophic and heterotrophic cultures can decrease the cost of lipid production of microalgae with dependence of high light intensity. Results show high growth capability and lipid production were provided by mixotrophic cultures than photoautotrophic and heterotrophic cultures. However, *N. oculata* was incapable of heterotrophic growth on the supplementation of those carbon substrates, but the accumulation of lipid was high in heterotrophic cultures in study. With the supplement of the organic carbon sources in mixotrophic condition, the biomass of the cultures supplemented with sodium acetate, citric acid, glucose and sucrose were 0.538 and 0.641, 0.690 and 0.798 g/L, whereas lipid production in the cultures supplemented with those carbon substrates were 0.208, 0.240, 0.255 and 0.284 g/L, respectively. The supply of sucrose provided the highest biomass and lipid production in mixotrophic and heterotrophic cultures. Maybe, the recovery of microalgal cells form semicontinuous cultures can transfer to heterotrophic cultivation for higher lipid accumulation.

The fast analysis of lipid contents of live microalgal cells was investigated in this research. The application of Nile red staining under fluorescence in lipid determination was performed for situ quantitative measurement of cellular neutral lipid content. The linear regression ($R^2 > 0.99$; $p < 0.001$) of intensity of fluoresce and lipid content was established in microalgae *Nannochloropsis oculata*. Nile red staining under fluoresce can provide convenient method for microalgae screening and the cell density of sample for Nile red staining only as low as

about 1×10^6 cells/mL can be detected on lipid contents.

The transesterification of lipid by different-type catalysts and optimal lipid/alcohol molar ratio were examined in the study. The conversion rate of base, acid and enzymatic catalysts in theoretic oil/methanol molar ratio 1:3 were 21%, 58% and 82%. However, rising of oil/methanol molar ratio to 1:6 in alkali-catalysts could provide the highest conversion rate of biodiesel. The conversion rate of transesterification by acid-, alkali- and enzymatic-catalysts in their own highest oil/methanol molar ratio were 88, 46 and 82% Acid-catalysts can provide higher conversion levels of lipid to their corresponding methyl esters in short reaction times rather than enzymatic catalysts. In industrial and commercial application, the chemical catalyst is common for transesterification and the excess methanol concentration used for improving transesterification efficiency will be recovered for reuse of organic solvents in industrial application.

Consequently, semiconscious of microalgal cultures have the potential for fixation of waste gas, CO_2 and high quantity of biomass and lipid production for alternative biofuels. At the typical carbon dioxide percentages of power plant (10-15%), the microalgae can not only survival, but also perform perfect growth ability and great quantity of lipid production. The lipid compositions of *Chlorella* sp. and *N. oculata* mainly are C16:0, C18:0, C18:1 and C18:2, the main mixtures of biodiesel. As well, the lipid can be converted to biodiesel by acid-catalytic transesterification and the reaction in optimal condition can provide fast, economic and efficiency performance for industrial biodiesel production.

V. References

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Table 1-1. Comparison between alkali-catalysis and lipase-catalysis methods for biodiesel fuel production.

	Alkali-catalysis process	**Lipase-catalysis process**
Reaction temperature	60-70°C	30-40 °C
Free fatty acids in raw materials	Saponified products	Methyl esters
Water in raw materials	Interference with the reaction	No influence
Yield of methyl esters	Normal	Higher
Recovery of glycerol	Difficult	Easy
Purification of methyl esters	Repeated washing	None
Production cost of catalyst	Cheap	Relatively expensive

Enzymatic catalysts like lipases are able to effectively catalyze the transesterification of triglycerides in either aqueous or non-aqueous systems, which can overcome the problems of chemical transesterificaiton [Fukuda, 2001; Meher et al., 2006].

Table 1-2. Production of Biodiesel in Different Countries.

Country	Major feedstock of biodiesel
USA	soybean (mustard is under study)
Brazil	Soybean
Europe	rapeseed oil (＞80％), sunflower oil
Germany	rapeseed oil
Spain	linseed, olive oil
France	sunflower oil
Italy	sunflower oil
Australia	animals fat, beef tallow, rapeseed oil
Canada	vegetable oil, animals fat
Ireland	animals fat, beef tallow
Indonesia	palm oil
Malaysia	palm oil
India	*Jatropha*
China	*Jatropha*, guang pi

Biodiesel production from various vegetable oils and sources are different in different countries [Venkataraman, 2004; Bajpai and Tyagi, 2006; Demirbas, 2007].

Table 1-3. Fatty acid composition of biodiesel obtained from different sources.

Oil or Fat	Fatty acid composition (wt %)							
	12:0	**14:0**	**16:0**	**18:0**	**18:1**	**18:2**	**18:3**	**others**
Canola	--	--	4-5	1-2	55-63	20-31	9-10	(22:1) 1-2
Coconut	44-51	13-18.5	7.7-10.5	1-3	5-8.2	1.0-2.6	--	--
Corn	--	--	7-13	2.5-3	30.5-43	39-52	1	(20-1) 0-2
Cottonseed	--	0.8-1.5	22-24	2.6-5	19	50-52.5	--	--
Linseed	--		6	3.2-4	13-37	5-23	26-60	--
Olive	--	1.3	7-18.3	1.4-3.3	55.5-84.5	4-19	--	--
Palm	--	0.6-2.4	32-46.3	4-6.3	37-53	6-12	--	(20:0) 0.3
Peanut	--	0.5	6-12.5	2.5-6	37-61	13-41	--	(22:1) 1
Rapeseed	--	1.5	1-4.7	1-3.5	13-38	9.5-22	1-10	(22:1) 40-64
Safflower		--	6.4-7.0	2.4-29	9.7-13.8	75.3-80.5	--	--
Safflower (high oleic)	--	--	4-8	2.3-8	73.6-79	11-19	--	--
Sesame	--	--	7.2-9.2	5.8-7.7	35-46	35-48	--	--
Soybean	--	--	2.3-11	2.4-6	22-30.8	49-53	2-10.5	--
Sunflower	--	--	3.5-6.5	1.3-5.6	14-43	44-68.7	--	(22:0) 0.6
Tallow (beef)	--	3-6	25-37	14-29	26-50	1-2.5	--	--
Microalgae	--	--	9-18	0-5	2-8	5-10	10-38	--

There have been various sources of biodiesel discovering and developing, in the meanwhile, many scientists have studied the fatty acid composition of biodiesel obtained from different sources [Volkmann et al., 1898; Applewhite , 1980; Gunstone et al.,1994; Zhukova and Aizdaicher, 1995; Pratoomyot et al.,2005; Bajpai and Tyagi, 2006].

Table 1-4. Oil productivity of different energy plants.

Oil-Producing Crops	Oil production (L/ha/years)	Relative oil productivity	Land area needed (M ha) [a]	Percent of existing US cropping area [a]
Oil Palm	5950	5	45	24
Coconut	2689	2.26	99	54
Jatropha	1892	1.59	140	77
Rapeseed/Canola	1190	1	223	122
Peanut	1057	0.89	259	144
Sunflower	954	0.8	287	159
Safflower	776	0.65	353	196
Mustard	571	0.48	480	267
Soybean	446	0.38	594	326
Corn(Maize)	172	0.14	1540	846
Microalgae[b]	136,900	115.04	2	1.1
Microalgae[c]	58,700	49.33	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.

Oil crops can not significantly contribute to replacing petroleum derived liquid fuels in the foreseeable future. If microalgae are used to produce biodiesel, only 1% and 2.5% of the total U.S. cropping area can be sufficient for producing algal biomass that satisfies 50% of the transport fuel needs of U.S. [Pahl, 2005; Chisti, 2007].

Table 2-1. Table of trace elemental solution of f/2 culture medium.

Chemical	Trace elemental solution (per L)
$NaNO_3$	75 g
$NaH_2PO_4 \cdot H_2O$	5 g
$Na_2 \cdot EDTA$	4.36 g
$FeCl_3 \cdot 6H_2O$	3.16 g
$MnCl_2 \cdot 4H_2O$	180 mg
$CoCl_2 \cdot 6H_2O$	10 mg
$CuSO_4 \cdot 5H_2O$	10 mg
$ZnSO_4 \cdot 7H_2O$	23 mg
Na_2MoO_4	6 mg
vitamin B_1	100 mg
vitamin B_{12}	0.5 mg
Biotin	0.5 mg

Table 3-1. Lipid components of *Chlorella* sp. and *N. oculata*

Fatty acid components	In all lipid (%)	
	N. oculata	***Chlorella* sp.**
C14:0	--	1.1%
C16:0	12.0%	32.3%
C16:1	2.6%	5.3%
C16:2	10.9%	3.5%
C16:3	13.8%	7.0%
C18:0	0.4%	2.8%
C18:1 & C18:3	27.9%	21.5%
C18:2	31.6%	20.7%
C20:0	--	1.6%
Others	0.8%	4.2%

Table 3-2. Recovery of lipid and biomass productivity of the *Chlorella* sp. as waste broth in the semicontinuous photobioreactor under different concentrations of CO_2 aeration.

CO_2 aeration	**Biomass productivity (g/L/d)**	**Lipid productivity (g/L/d)**	**Percentage of lipid content (%)**
2%	0.422 ± 0.061	0.143 ± 0.020	35% ± 1.1%
5%	0.393 ± 0.040	0.130 ± 0.011	34.9% ± 1.8%
10%	0.366 ± 0.089	0.124 ± 0.029	34% ± 2.3%
15%	0.361 ± 0.057	0.121 ± 0.007	33.6% ± 2.0%

The culture volume in the photobioreactor is 800 mL. Daily waste broth was 400 mL. Each data indicates the mean ± SD, which were measured daily from Day 1 to Day 8

Table 3-3. Daily recovery of biomass and lipid of *N. oculata* NCTU-3 cultured in the semicontinuous system aerated with different concentrations of CO_2.

CO_2 aeration	**Biomass productivity (g/L/d)**	**Lipid productivity (g/L/d)**	**Percentage of lipid content (%)**
2%	0.480 ± 0.029	0.142 ± 0.049	29.7 ± 2.0
5%	0.441 ± 0.044	0.113 ± 0.035	26.2 ± 1.9
10%	0.398 ± 0.069	0.097 ± 0.026	24.6 ± 1.7
15%	0.372 ± 0.022	0.084 ± 0.021	22.7 ± 1.9

The culture volume in the photobioreactor is 800 mL. Daily waste broth was 400 mL. Each data indicates the mean ± SD, which were measured daily from Day 1 to Day 8.

Table 3-4. Biomass and lipid productivity of *N. oculata* NCTU-3 cultured in the semicontinuous system aerated with 2% CO_2 under the treatment of one-day and three-day replacement.

Culture [a]	Biomass productivity (cell dry weight, g/L/d)	Lipid productivity (g/L/d))	Percentage of lipid content (%)
One-day replacement	0.497 ± 0.032	0.151 ± 0.021	30.7 ± 2.4
Three-day replacement	0.296 ± 0.009	0.121 ± 0.035	41.2 ± 1.9

[a] The semicontinuous cultures were replaced half (for one-day) or three fifth (for three-day) of broth with fresh medium at interval of 24 and 72 hours, respectively. The total biomass and lipid productivity were measured from the total replaced broth divided by day. The total replaced broth volume was 4,800 mL in one-day replacement and only 1,920 mL in three-day replacement over 12 days. Each data indicates the mean ± SD.

Table 3-5. Biomass and lipid production of the *N. oculata* cultutre supplemented with different organic carbons under mixotrophic condition and photoautrophic culture with 2% CO_2 aeration.

Carbon source	Biomass productivity (g/L)	Lipid productivity (g/L)	Lipid content (%)
Sodium acetate (10 mM)	0.538 ± 0.043	0.208 ± 0.022	38.72 ± 1.55
Citric acid (10 mM)	0.641 ± 0.057	0.240 ± 0.025	37.38 ± 1.10
Glucose (10 mM)	0.690 ± 0.062	0.255 ± 0.031	36.98 ± 2.11
Sucrose (10 mM)	0.798 ± 0.065	0.284 ± 0.026	35.54 ± 0.56
2% CO_2 (0.25 vvm)	0.681 ± 0.070	0.192 ± 0.035	28.19 ± 2.65

Each data indicates the mean ± SD from three experiments.

Lipid production = biomass × lipid content

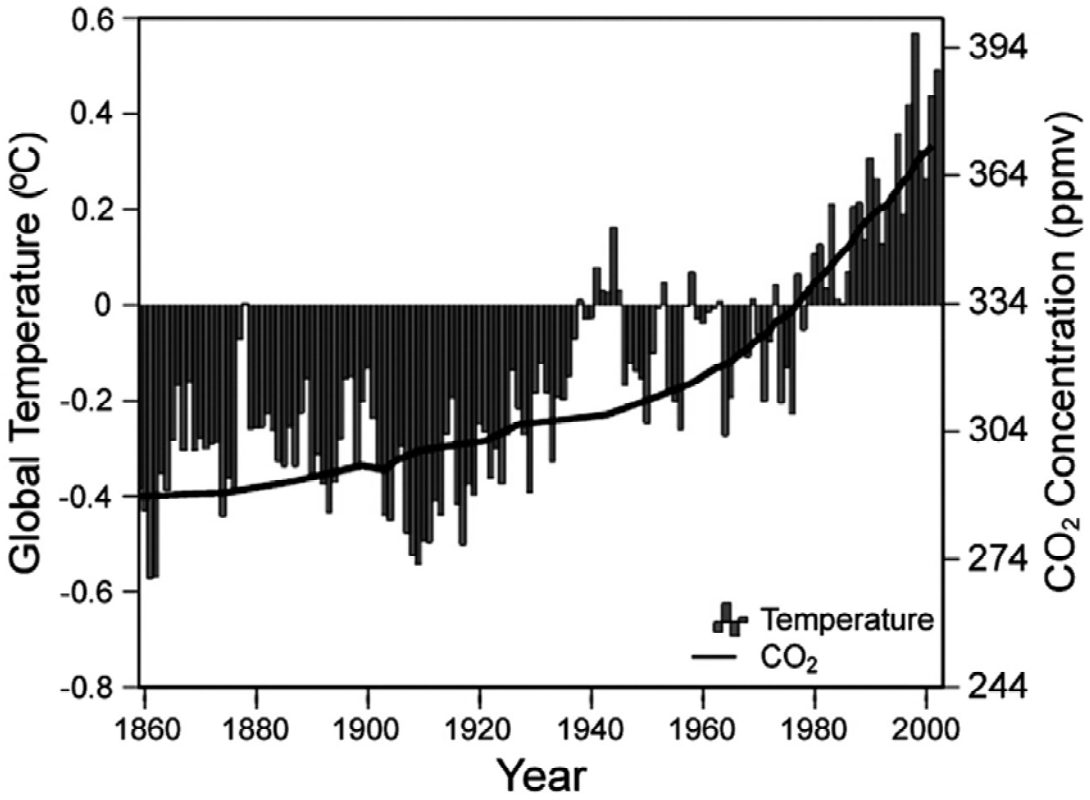


Figure 1-1. The relationship of carbon dioxide concentration elevating and global temperature. Time series of departures from the 1961 to 1990 base period for an annual mean global temperature of 14.0°C (bars) and for a carbon dioxide mean of 334 ppmv (solid curve) during the base period. The global average surface heating approximates that of carbon dioxide increases, because of the cancellation of aerosols and other greenhouse gas effects, but this does not apply regionally [Karl and Trenberth, 2003].

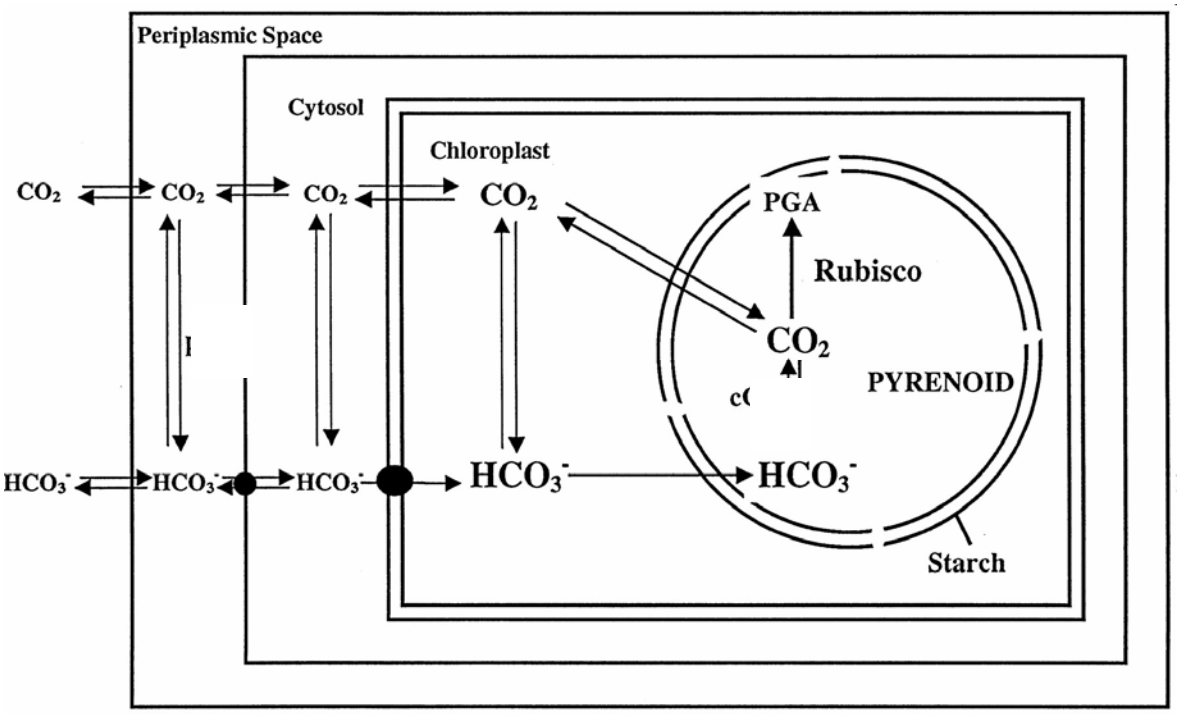


Figure 1-2. A model for CO_2 concentration in eukaryotic microalgae. The font sizes of CO_2 and HCO_3^- indicate the relative concentrations of these inorganic carbon species [Moroney and Somanchi, 1999].

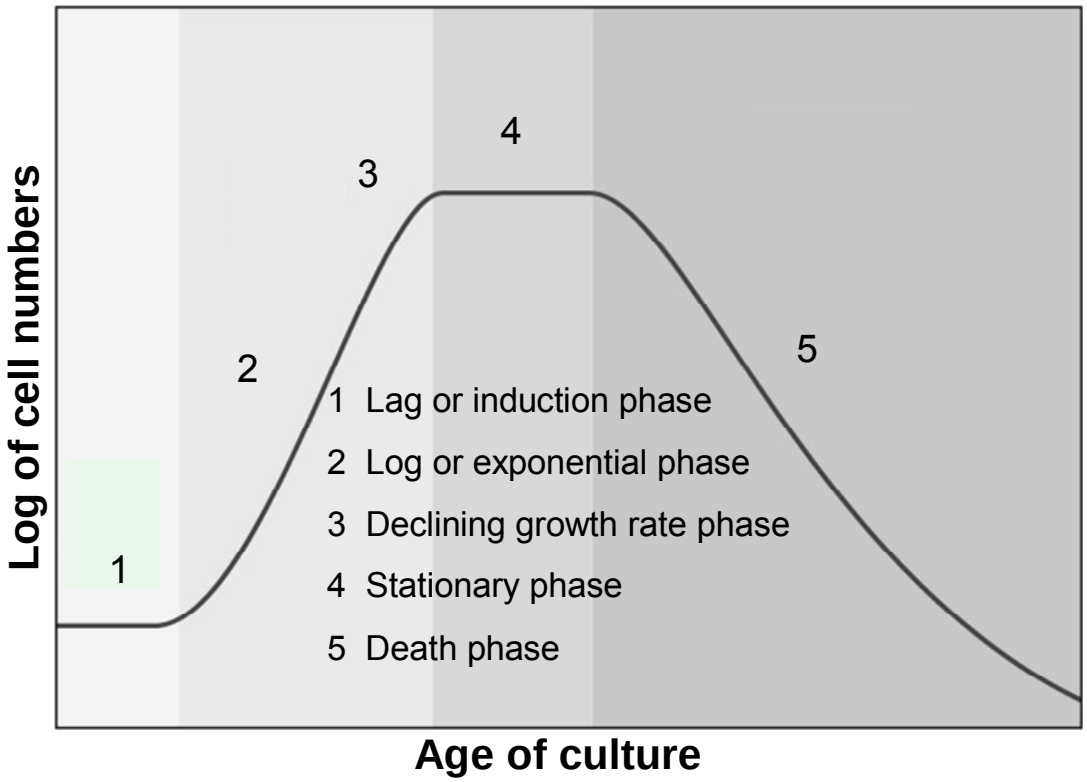


Figure 1-3. Growth phases of microalgae cultures. The growth curve of microalgae was observed and the lag, log, stationary and decline phases were identified.

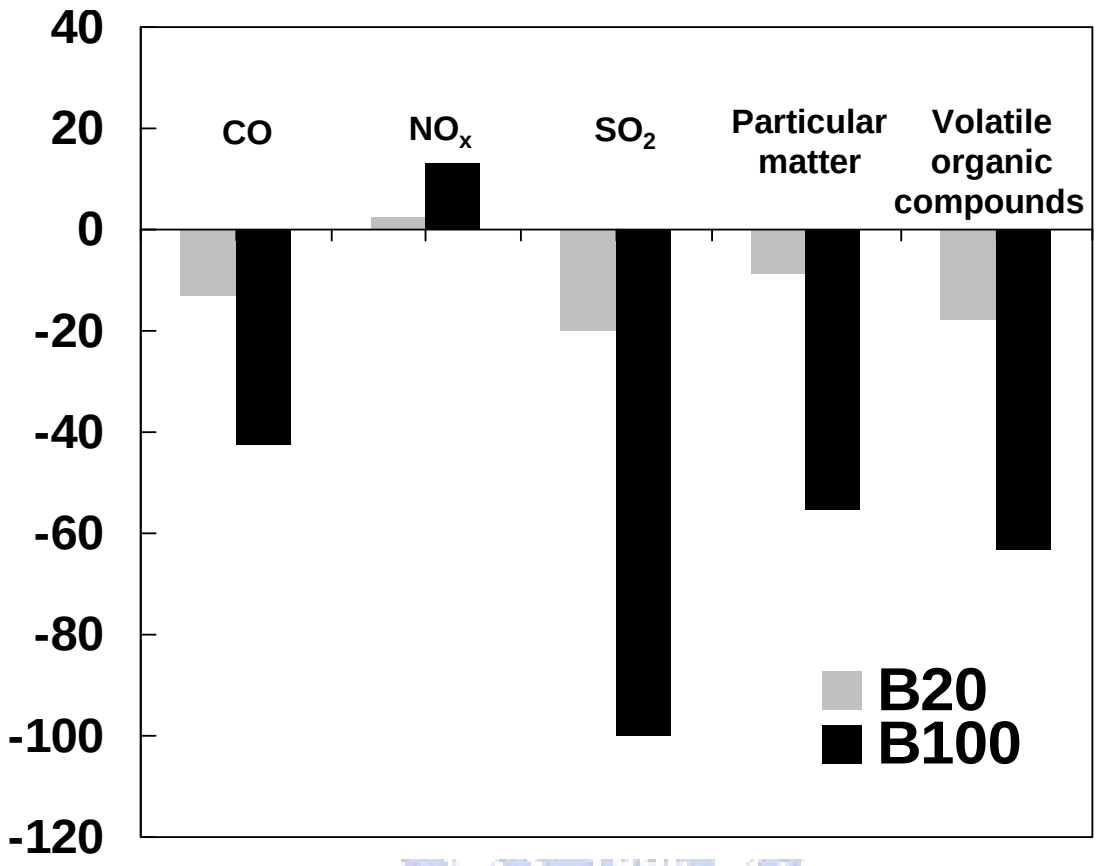
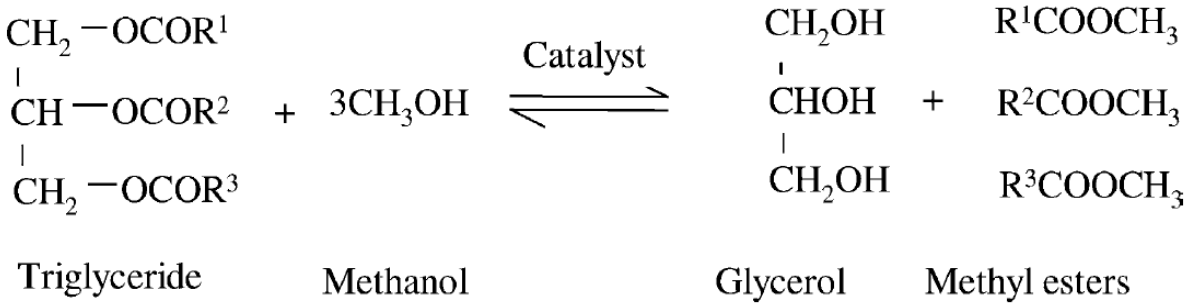


Figure 1-4. Average changes in mass emissions from diesel engines using the biodiesel mixtures relative to the standard diesel fuel (%). The average of pollutants emission from diesel engines using the biodiesel mixtures relative to the standard diesel is different. The combustion of biodiesel compared to diesel has been reported to emit lesser pollutants, such as SO_2, CO, hydrocarbons (HC) and so on [Morris et al., 2003].

A



B

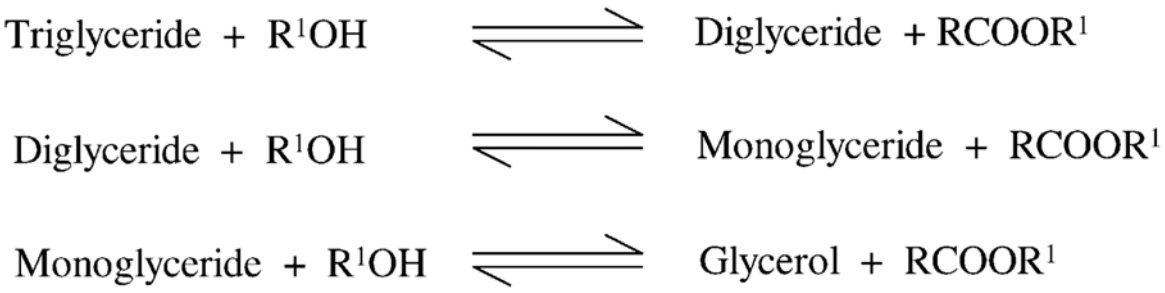


Figure 1-5. Transesterification reaction of general equation and mechanism. A, general equation for transesterification of triglycerides**.** B, the mechanism of transesterification. Transesterification reaction requires 3 mol of alcohol for each mole of triglyceride to produce 1 mol of glycerol and 3 mol of methyl esters. First step is diglycerides formation, and then diglycerides are converted to monoglycerides and eventually to glycerol [Meher et al., 2006].

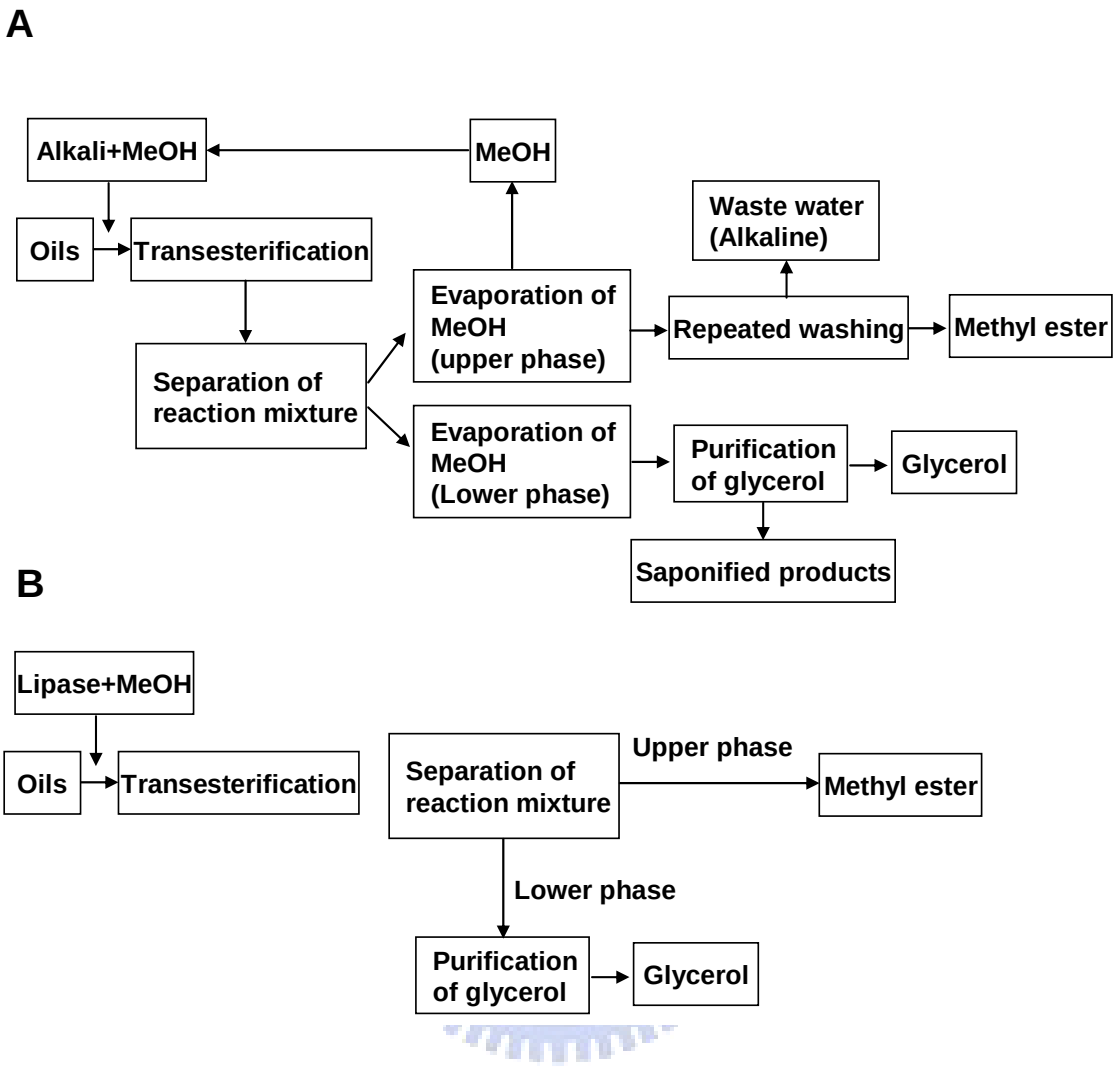


Figure 1-6. Flow diagrams comparing biodiesel production using the alkali- (A) and lipase-catalysis (B) processes. The enzymatic process is simpler since recovery of un-reacted methanol and wastewater treatments are unnecessary [Fukuda, 2001].

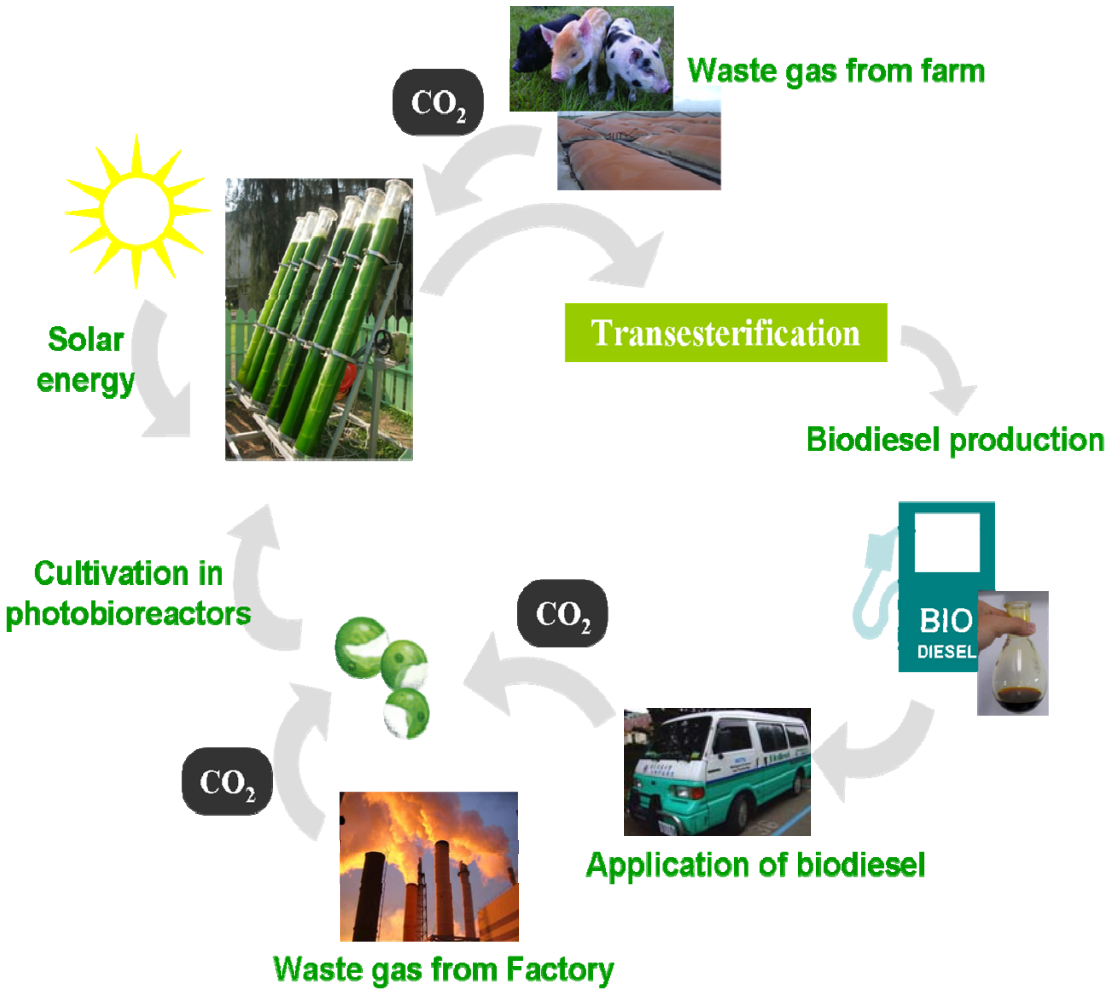


Figure 1-7. The strategy of culturing microalgae in the photobioreactors for the application to mitigate greenhouse gas and develop substitute energy. Screened microalgae, *Chlorella* sp. NCTU-2 and *N. oculata* NCTU-3 are cultivated to obtain optimal microalgae growth condition and maximal lipid content by waste gas. The lipid of microalgal cells is extracted for analysis of lipid contents and biodiesel production.

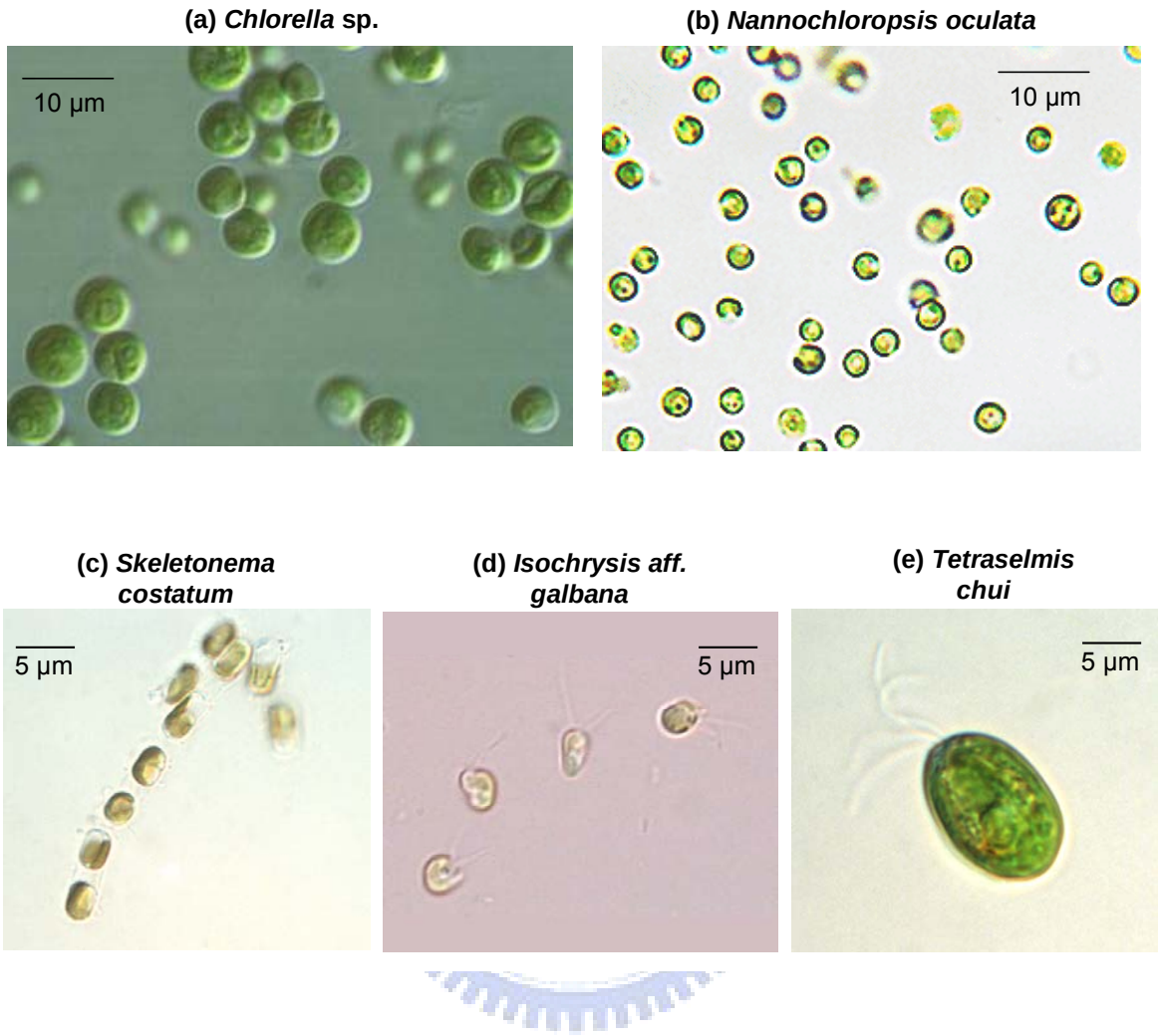


Figure 2-1. The photograph of five different microalgae stains. The five microalgal strains are (a.) *Chlorella* sp., (b.) *Nannochloropsis oculata*, (c.) *Skeletonema costatum*, (d.) *Isochrysis galbana* and (e.) *Tetraselmis chui*.

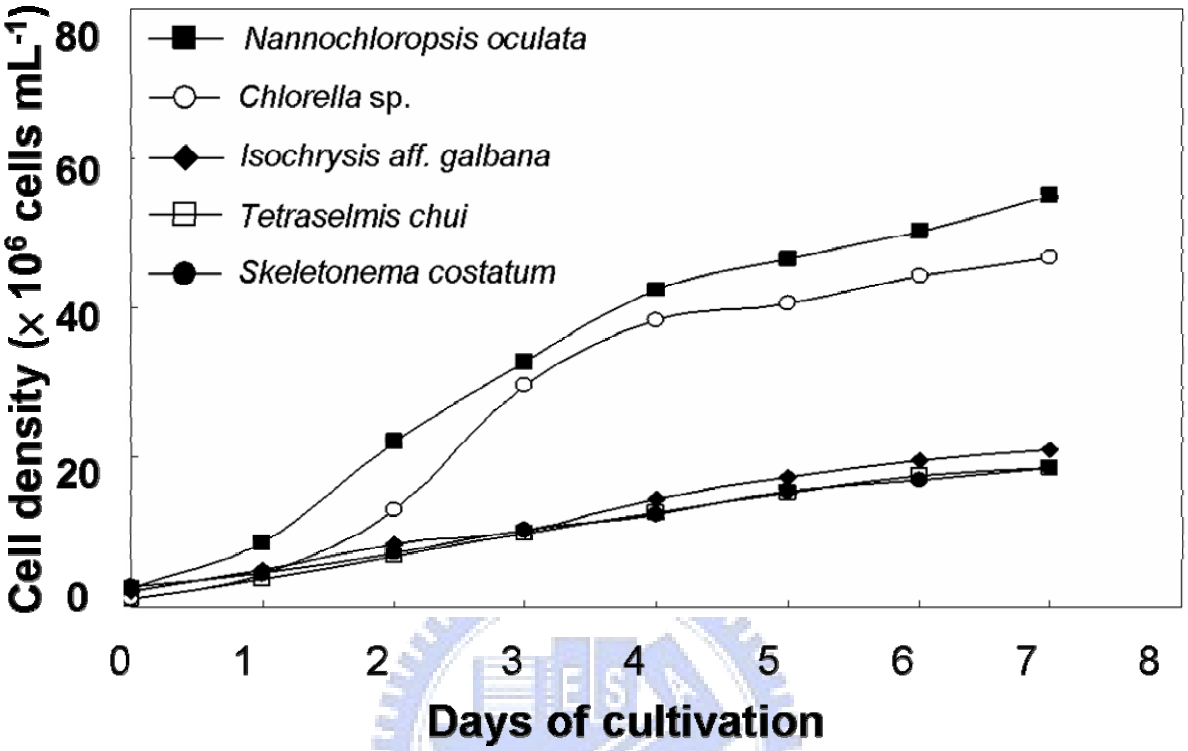


Figure 3-1. The comparison with different microalgal strains cultures in growth. Figure shows the growth curves of *Chlorella* sp. (●), *Nannochloropsis oculata.* (○), *Skeletonema costatum* (■), *Isochrysis aff. galbana* (□), *Tetraselmis chui* (△) with 8 days of cultivation. Optical density at A_{682} is regarded as the reference parameter of growth.

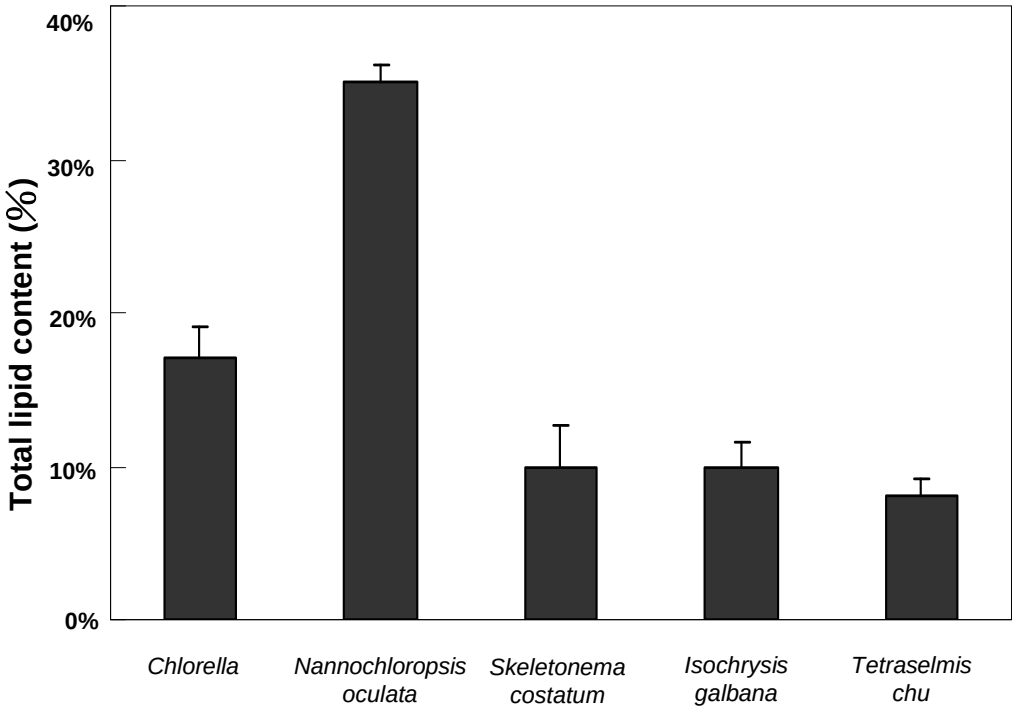


Figure 3-2. The comparison with different microalgal strains cultures in lipid contents. Figure shows the difference in percentage of lipid content between four microalgal strains: *Chlorella* sp., *Nannochloropsis oculata*, *Skeletonema costatum*, *Isochrysis aff. galbana*, *Tetraselmis chui*.

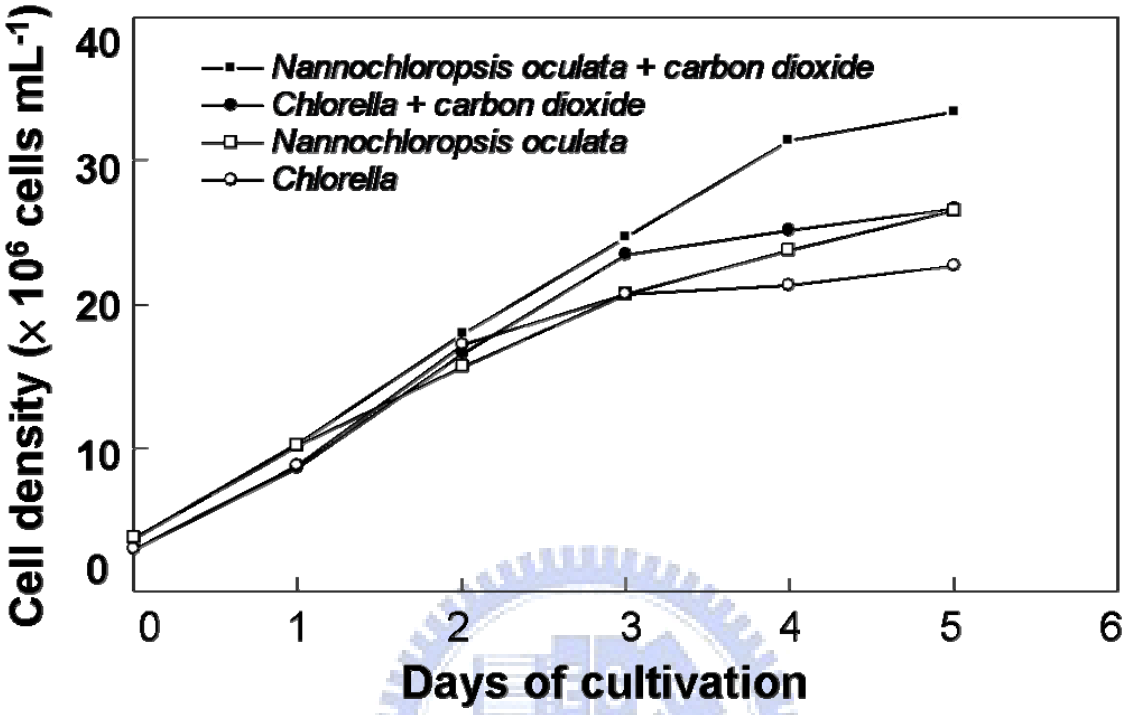


Figure 3-3. The comparison with *Chlorella* sp. and *N. oculata* cultures by aeration of CO_2 in growth. Figure shows the growth curves of *Chlorella* sp. (○), *Chlorella* sp. with 10% CO_2 (●), *Nannochloropsis oculata.* (□), *Nannochloropsis oculata.* with 10% CO_2 (■), in 5 days of cultivation. Optical density at A_{682} is regarded as the reference parameter of growth.

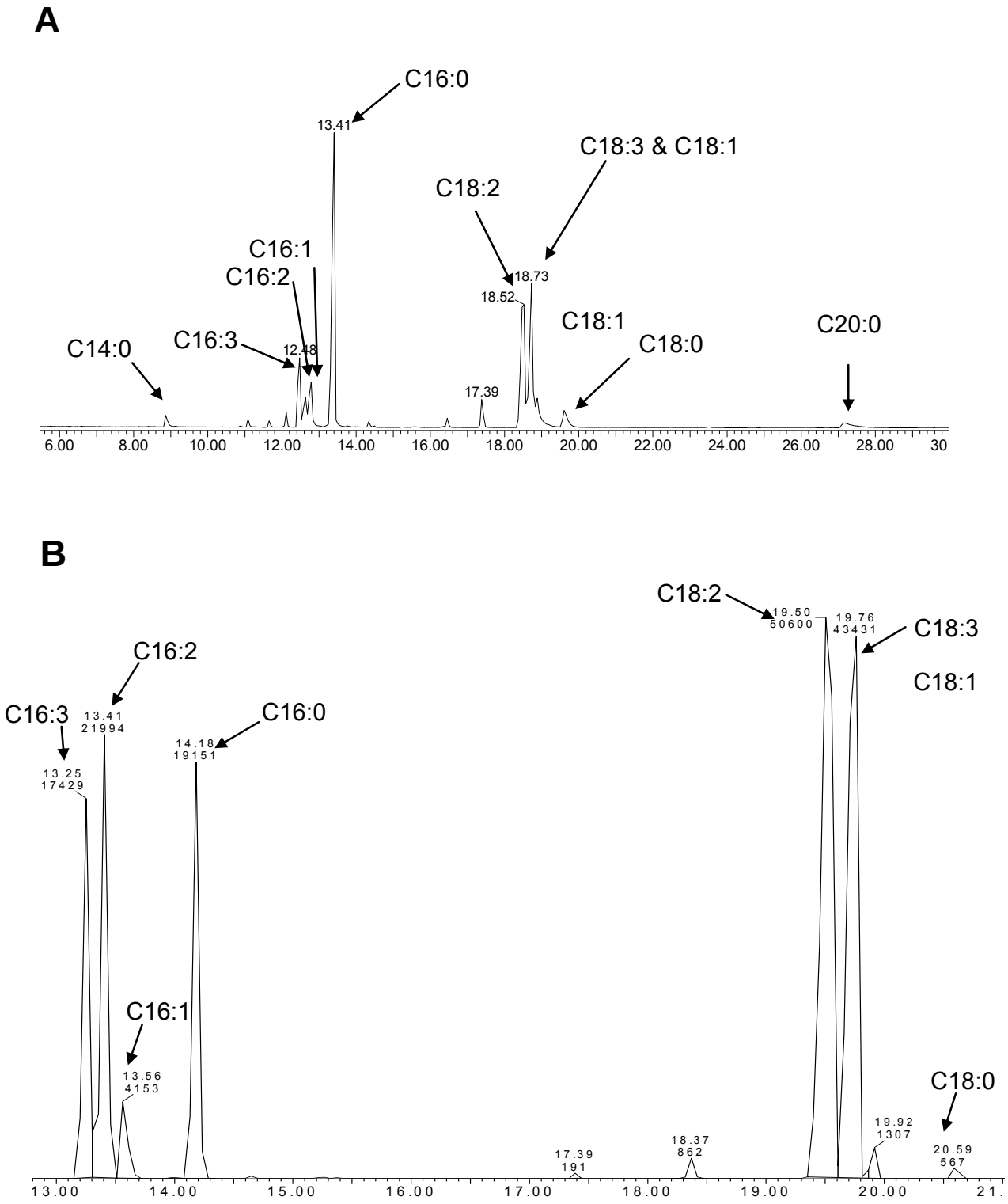


Figure 3-4. The gas chromatography patent of *Chlorella* sp. (A) and *N. oculata* (B). The length of fatty acid chain plays an important role to decide the characteristics of biodiesel such as pour point, boiling point and so on. C12:0~C18:0 and C18:1, C18:2, C18:3 are usual fatty acid for biodiesel contents.

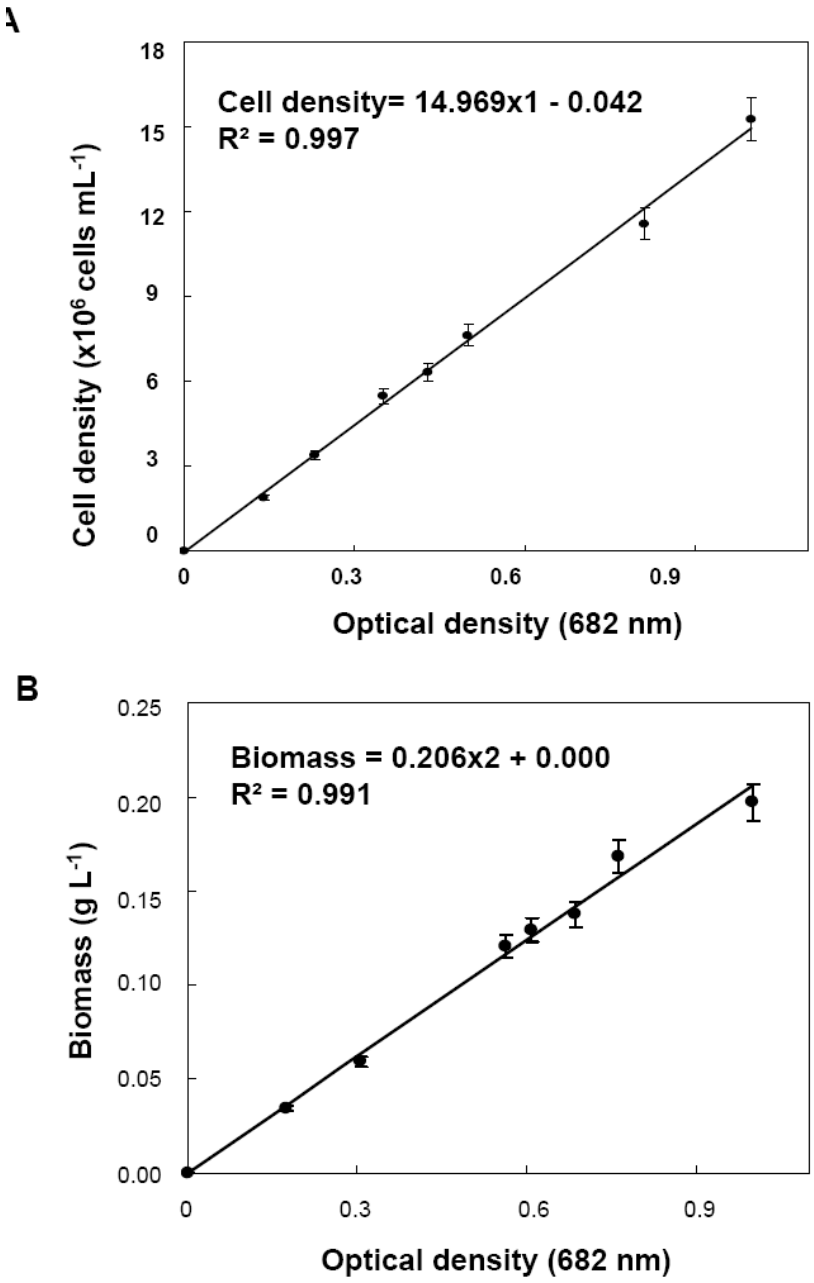


Figure 3-5. Calibration curves and equations of optical density at A_{682} to the cell density (A) and biomass concentration (B) in *Chlorella* sp. cultures. Relationships between optical density and cell density, as well as optical density and cell dry weight of *Chlorella* sp. were established by linear regression.

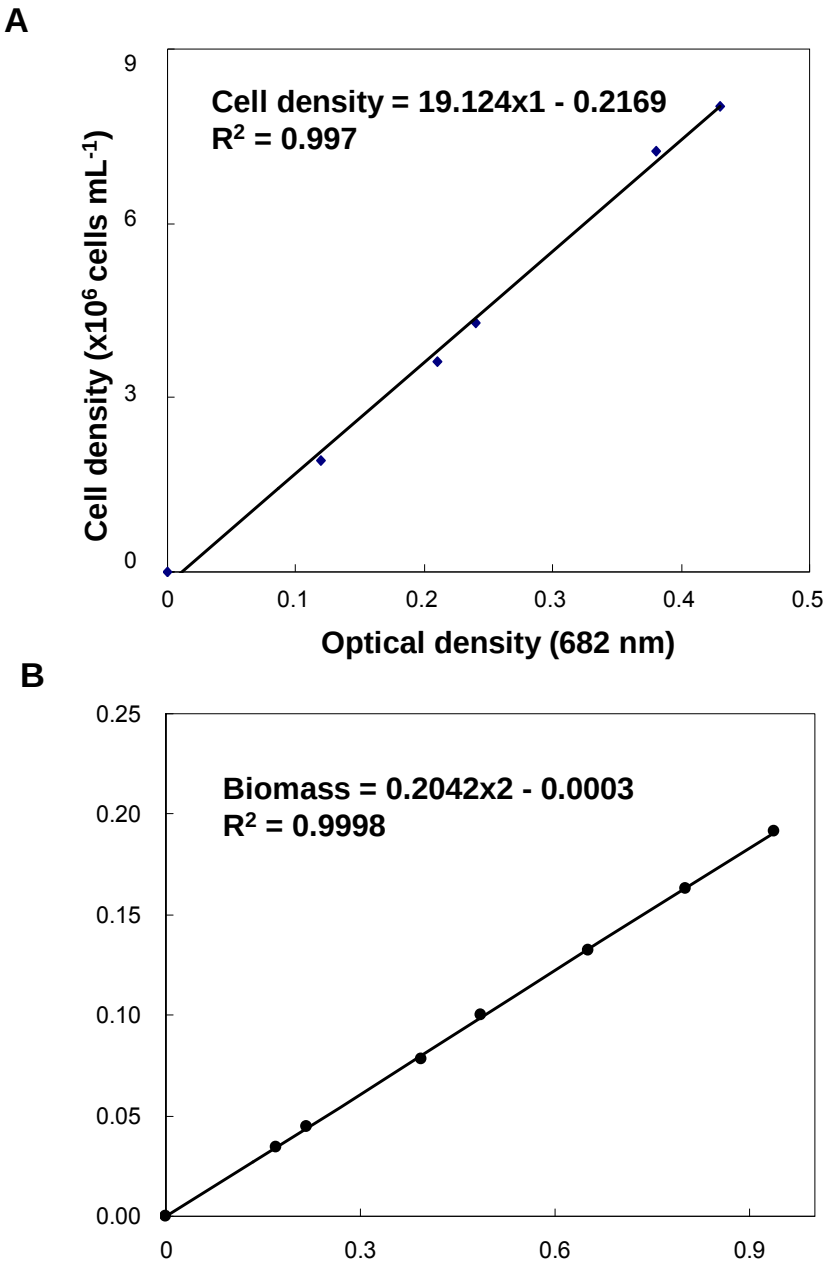


Figure 3-6. Calibration curves and equations of optical density at A_{682} to the cell density (A) and biomass concentration (B) in *N. oculata* cultures. Relationships between optical density and cell density, as well as optical density and cell dry weight of *N. oculata* were established by linear regression.

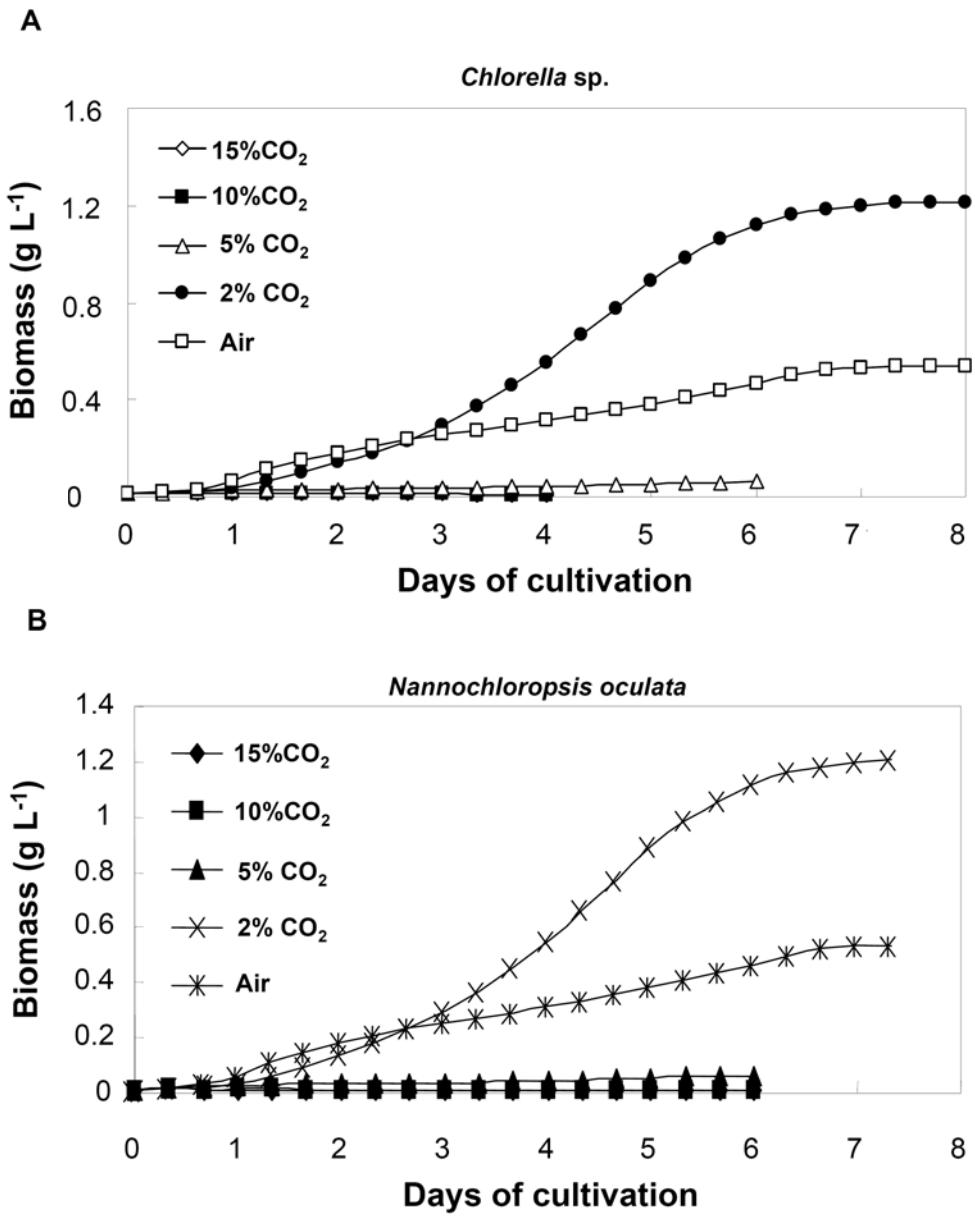


Figure 3-7. Effects of different concentrations of CO_2 aeration on the growth of (A) *Chlorella* sp. and (B) *Nannochloropsis oculata.* **A**, shows the growth curve of *Chlorella sp.* inoculated at low-density cells (8×10^5 cells/mL in an 800-mL cultivation) with different CO_2 concentration.. **B**, shows the growth curve of *Nannochloropsis oculata* inoculated also at 8×10^6 cells/mL density in an 800-mL cultivation. All experiments were carried out in triplicate. The cultures were illuminated at 300 μmol/m^2/s and bubbled with a flow rate of 0.25 vvm airstreams at 26 ± 1°C.

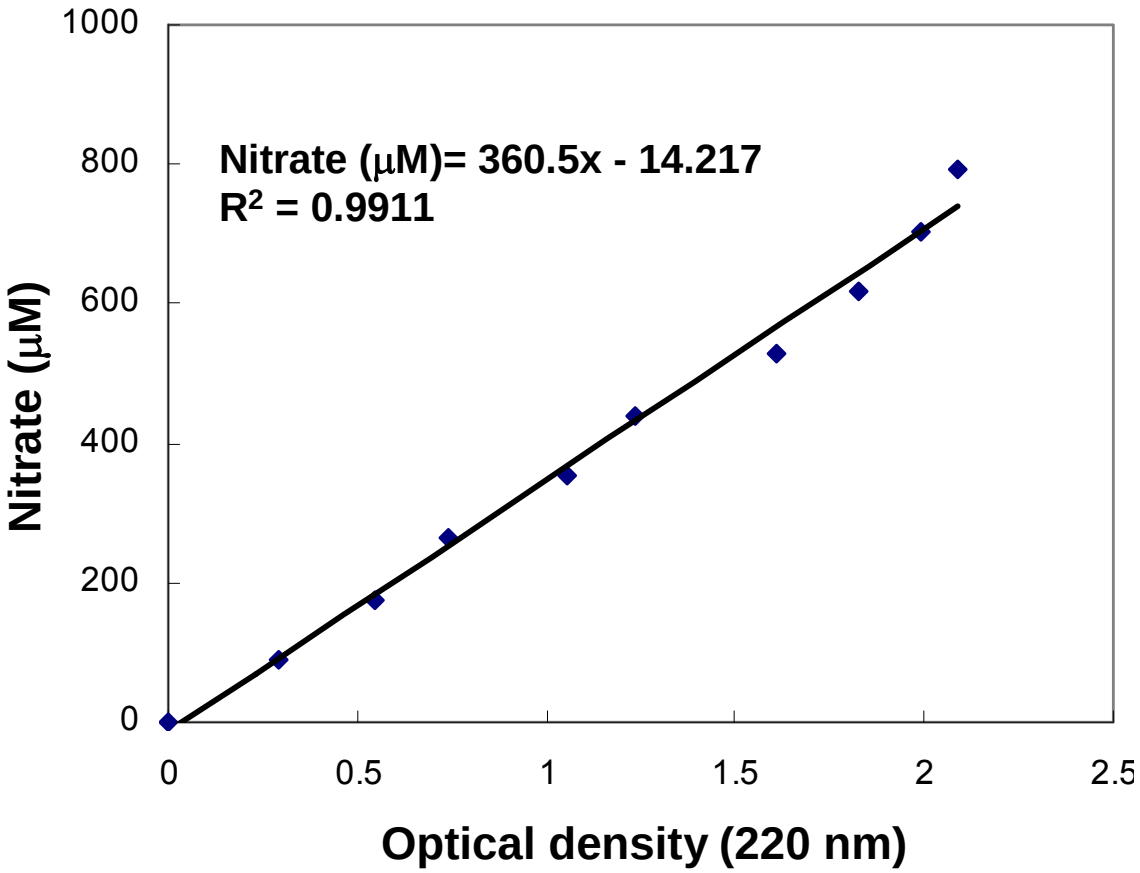


Figure 3-8. Calibration curves and equations of optical density at A_{220} to the nitrate concentration in microalgal cultures. The nitrate concentration was measured by optical density at A_{220}. The standard curves and equations of optical density at A_{220} to the nitrate concentration were established by linear regression.

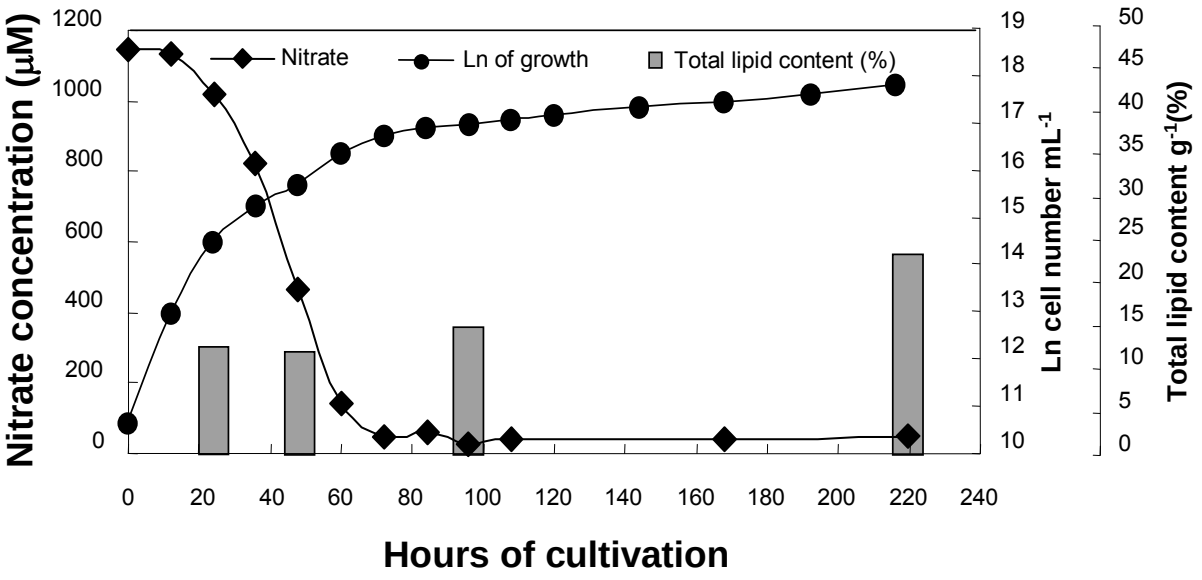


Figure 3-9. Growth and lipid content of *Chlorella* sp. culture with nitrate consumption. The culture cultivated under an illumination at 300 μmol/m^2/s and bubbled with a flow rate of 0.25 vvm airstreams (10% CO_2) at 26 ± 1℃. The cultivations were continuously operated for about 10 days. Monitor the relationships between nitrate consumption, growth and lipid contents. The lipid contents of *Chlorella* sp. in log phase were about 12%. When the culture stepped into stationary phase, the accumulation of lipid contents increased from 12% to 24%.

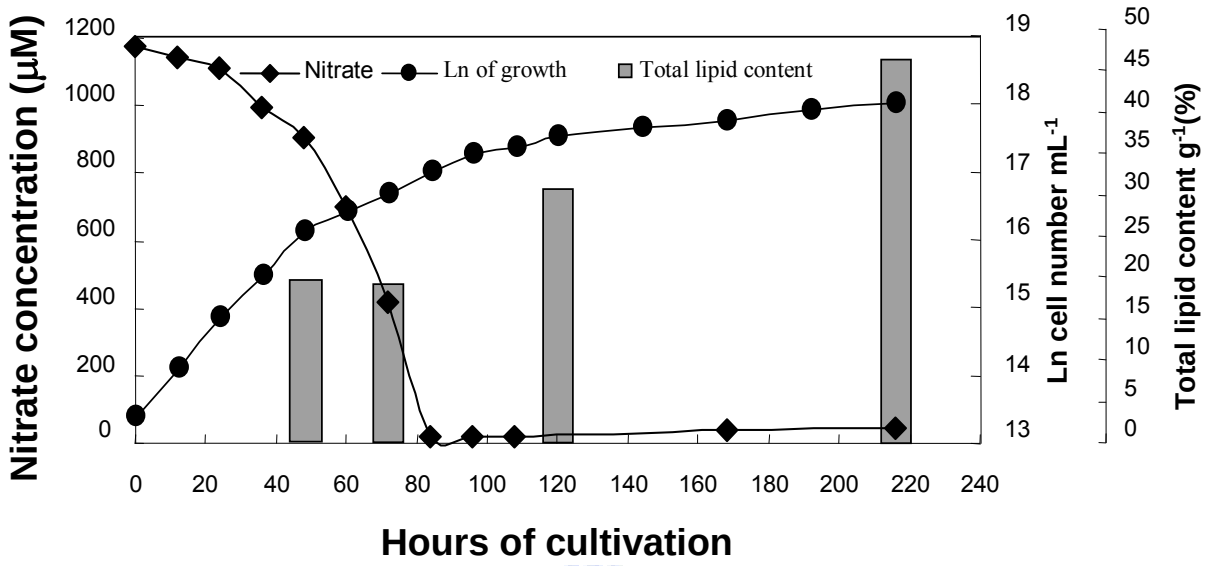


Figure 3-10. Growth and lipid content of *Nannochloropsis oculata* culture with nitrate consumption. The culture cultivated under an illumination at 300 μmol/m^2/s and bubbled with a flow rate of 0.25 vvm airstreams (10% CO_2) at 26 ± 1℃. The cultivations were continuously operated for about 10 days. Monitor the relationships between nitrate consumption, growth and lipid contents. The lipid contents of *Nannochloropsis oculata* in log phase were about 19%. When the culture stepped into stationary phase, the accumulation of lipid contents increased from 30% to 47%. The lipid contents of *Nannochloropsis oculata* in not only log phase, but also stationary phase were higher than *Chlorella* sp.

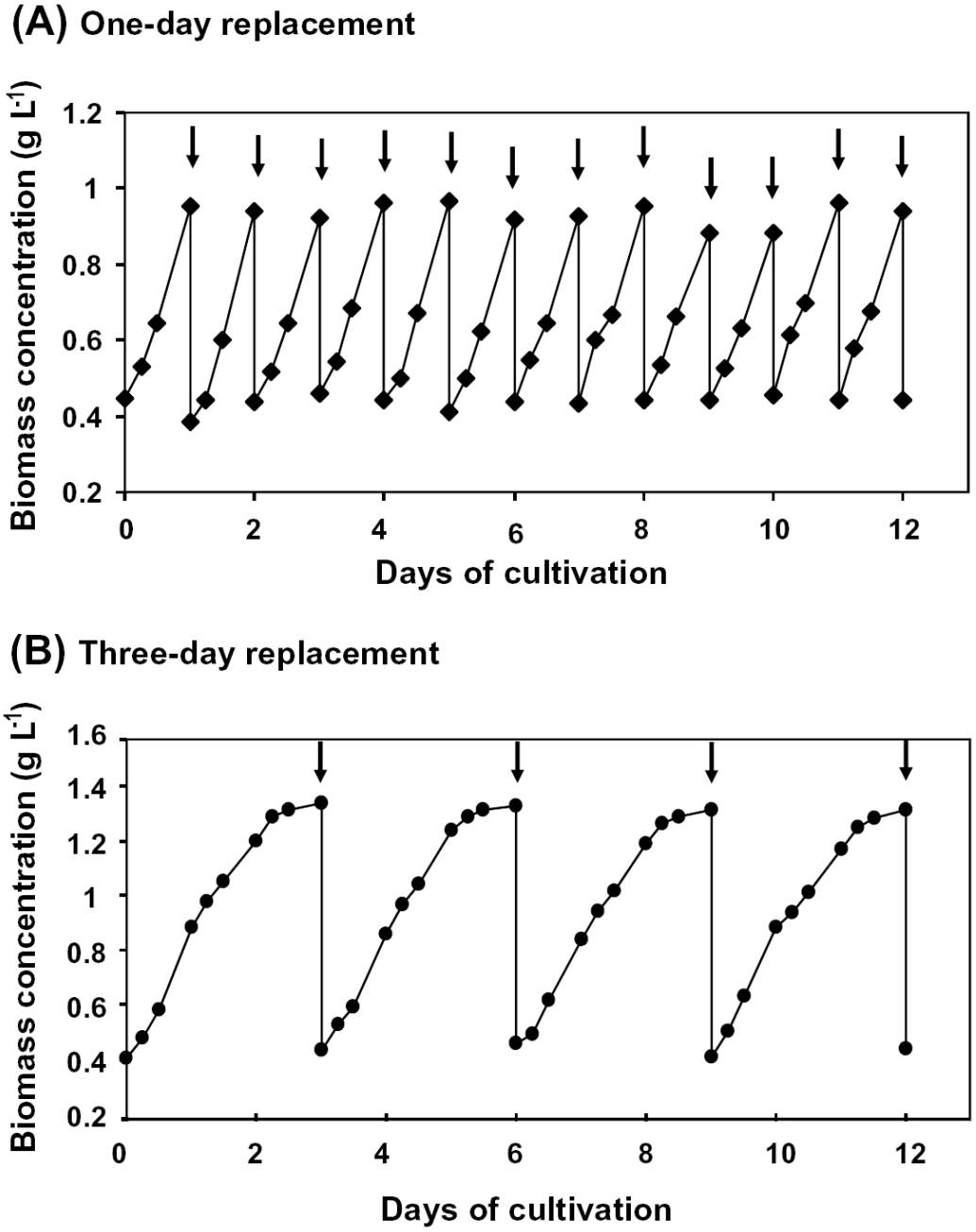


Figure 3-11. Growth profiles of N. oculata NCTU-3 cultured in the semicontinuous system with 2% CO_2 aeration and operated by one-day and three-day replacements. In the cultures, approximate 0.4 g/L of microalgal cells was inoculated and cultivated under an illumination at 300 μmol/m^2/s and bubbled with a flow rate of 0.25 vvm airstreams at 26 ± 1℃. The cultivations were continuously operated for 12 d. Amount of half and three fifth of cultured broth was replaced with the fresh modified f/2 medium at interval of 24 h (one-day replacement; half broth was replaced each day) and 72 h (three-day replacement; three fifth broth was replaced every 3 d), respectively. The arrows indicate the time when the cultured broth was removed and fresh medium was added.

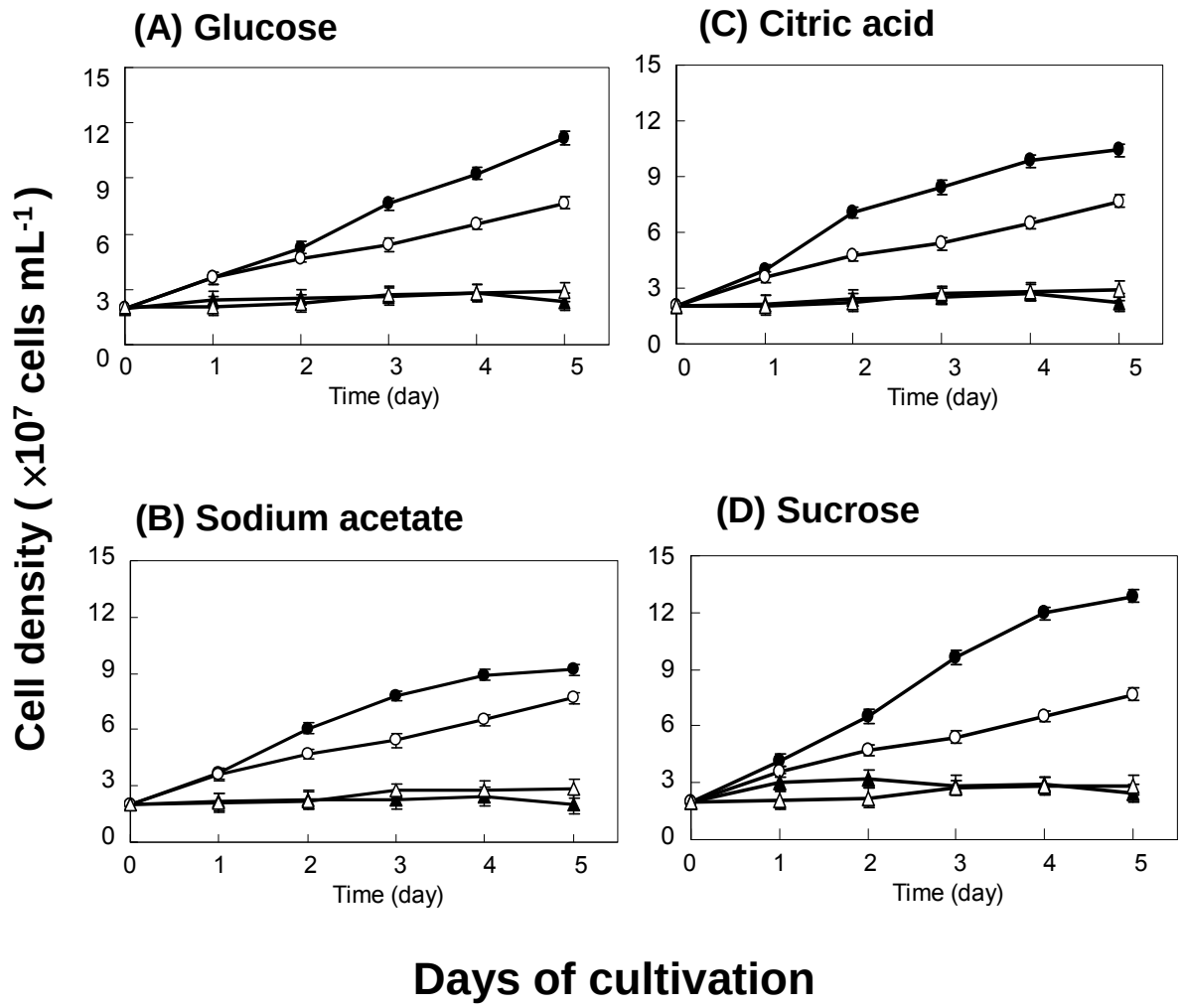


Figure 3-12. The growth curves of the *N. oculata* cultures with aeration of the ambient air supplemented with (A) sodium acetate, (B) citric acid, (C) glucose and (D) sucrose in mixotrophic (closed circles), heterotrophic (closed triangles) cultivations. The control culture of mixotrophic (open circles) and the control culture of heterotrophic (open triangles) cultivations. The cultures cultivated under mixotrophic conditions and the control culture of mixotrophic cultivations were provided with continuous, cool white, fluorescent light which the light intensity of 300 μmol/m^2/s at the surface of the cultures. Each point of the curves was averaged from three independent readings.

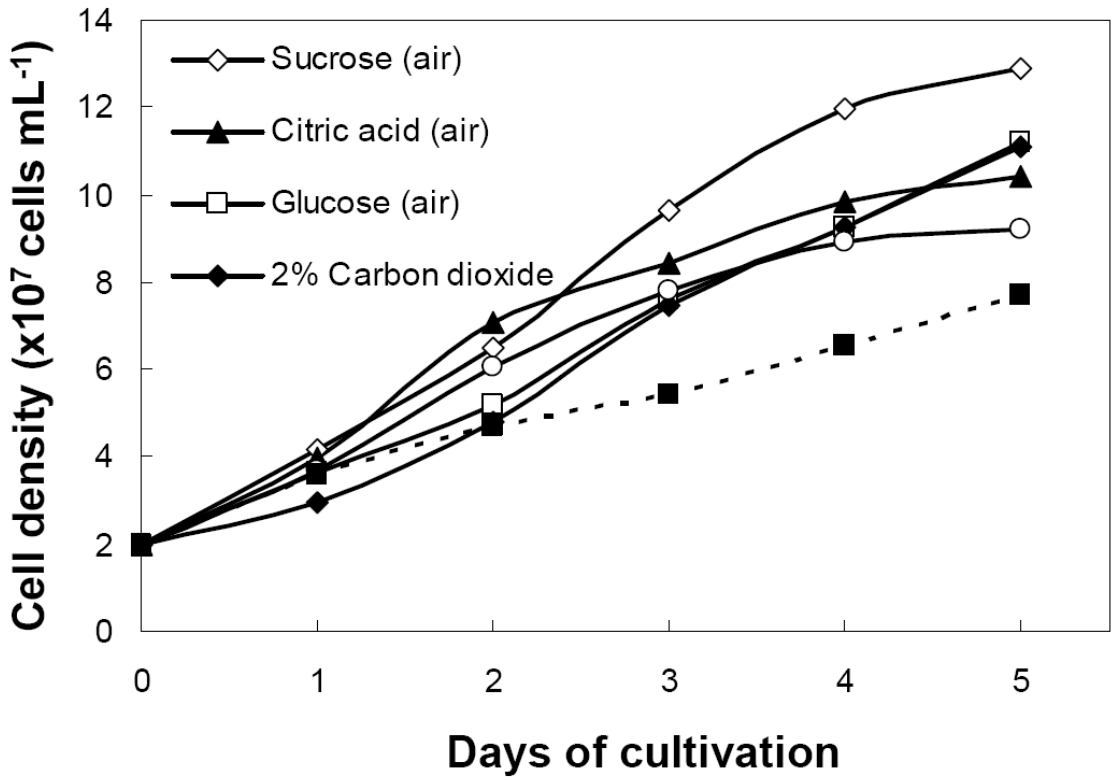


Figure 3-13. The growth curves of the *N. oculata* cultures with aeration of the ambient air supplemented with glucose, sodium acetate, citric acid and sucrose in mixotrophic condition. The control cultures of mixotrophic cultivations cultivated with air without supplementation of any carbon substrate and photoautotrophic culture with 2% CO_2. All the cultures cultivated were provided with continuous, cool white, fluorescent light which the light intensity of 300 μmol/ m^2 /s at the surface of the cultures.

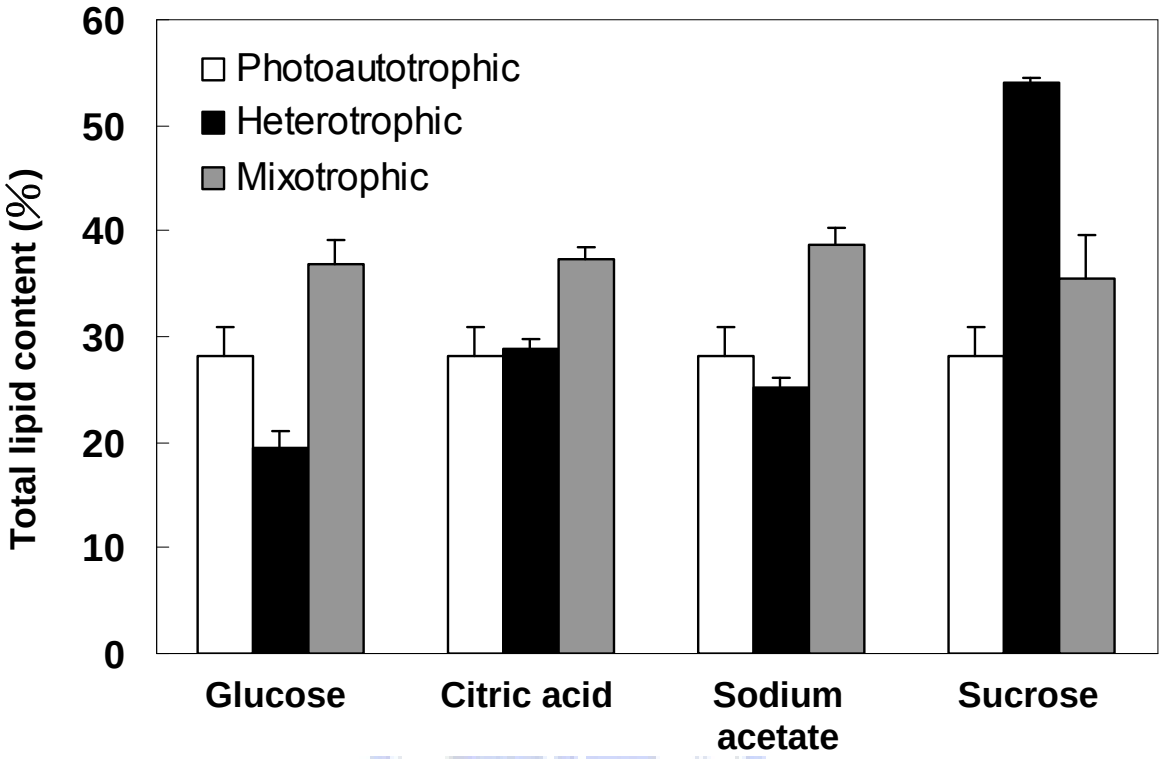


Figure 3-14. Effects of different carbon sources on lipid content of *N. oculata* compared with the control cultures of mixotrophic and heterotrophic cultivations. The cultures were supplemented with sodium acetate, citric acid, glucose and sucrose, respectively in the same concentration (10 mM) for the mixotrophic and heterotrophic cultivations with the aeration of air. All the mixotrophic cultures and the control culture of mixotrophic cultivations were provided with continuous, cool white, fluorescent light which the light intensity of 300 μmol/ m^2 /s at the surface of the cultures. Each data was averaged from three independent readings.

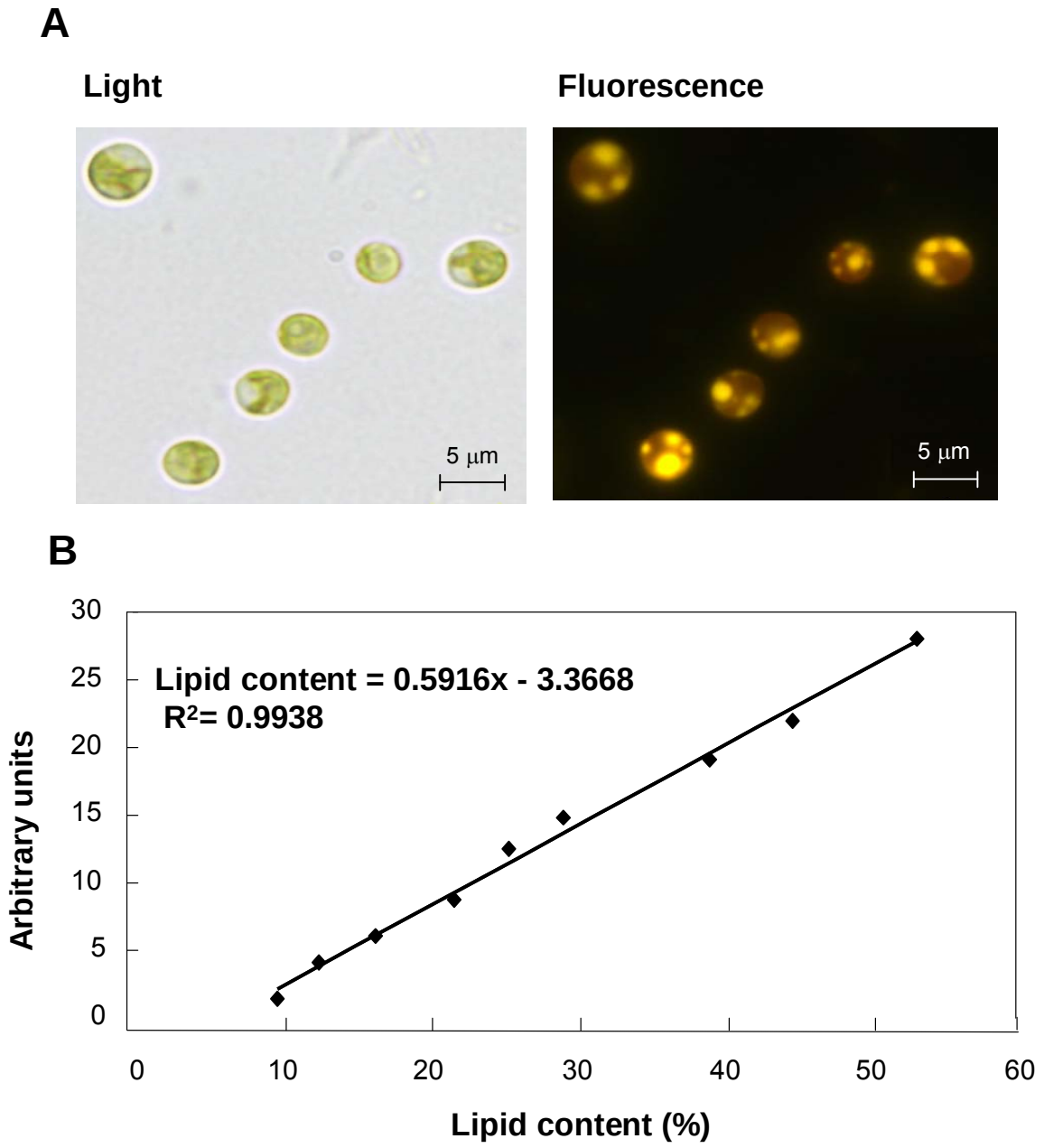


Figure 3-15. **Images of Nile Red stained *N. oculata* NCTU-3 cells and the calibration curve of Nile Red fluorescent intensity vs. lipid content.** A, shows the light and fluorescent images of N. oculata NCTU-3 cells stained with Nile Red. B, shows the calibration curves and equations of Nile Red fluorescent intensity vs. lipid content (%). Y axis shows the relative fluorescent intensity as arbitrary units. The calibration curve, correlative equation and R^2 value of each correlation were indicated. Each point was averaged from three independent measures.

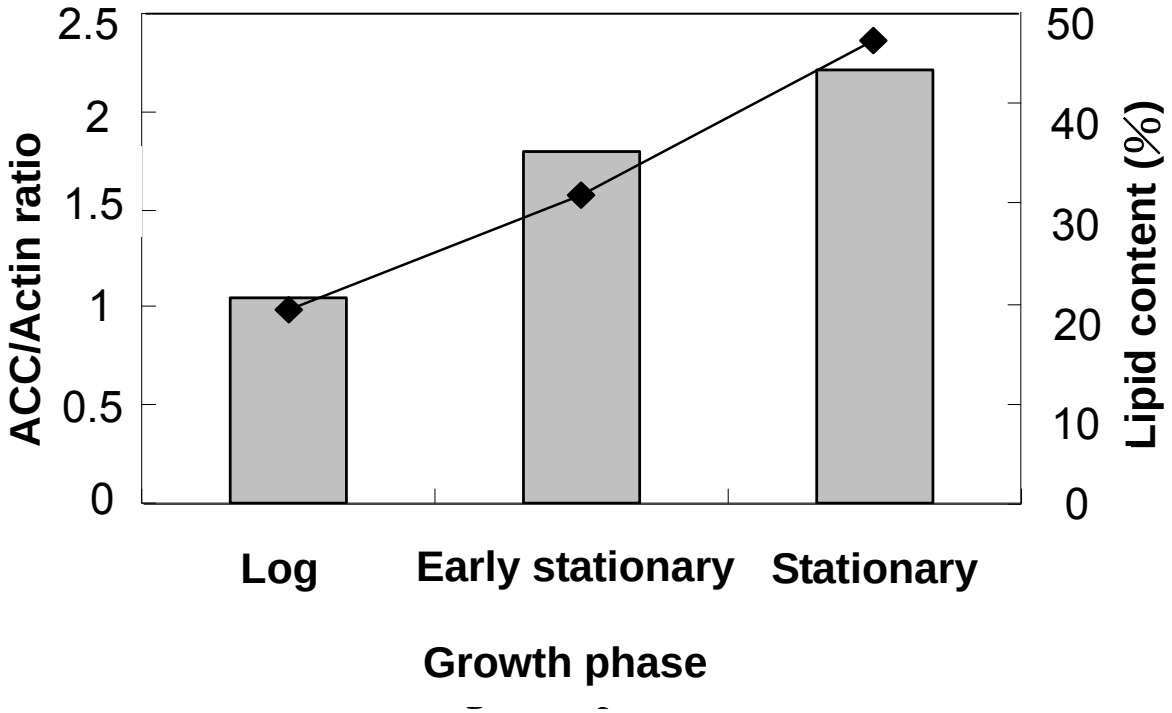


Figure 3-16. ACC expression compare with lipid content. *N. oculata* was cultured for ACCase (Acetyl-coenzyme A carboxylase) expression. The following oligonucleotides were used in this study as primers for DNA amplification of ACCase. Forward primer (PR1) is TTTATGGGGGGAAGTATGGGCTC. The reverse primer (PR2) is CCAACAACAGGTGGTGTAACTGC (all sequences are written 5'→3'). The ACCase expression is compared with the expression of actin as an internal control.

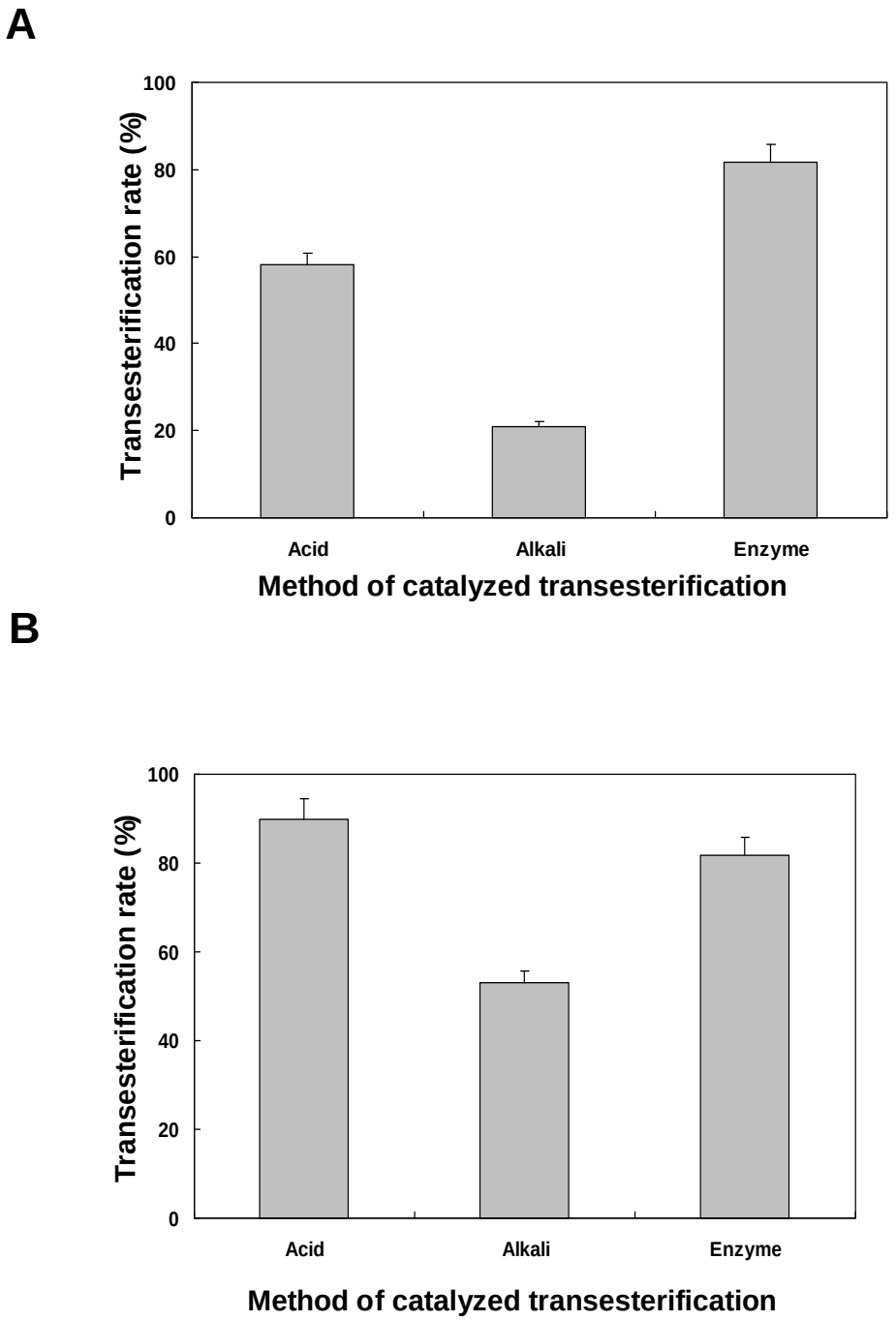


Figure 3-17. The reaction of transesterification by three types of catalysts: acid, base, and enzyme (lipase). A, shows the transesterification rate in the theoretical oil/methanol molar ratio by different catalysts. B, shows the transesterification rate in the highest oil/methanol molar ratio by different catalysts. The acid catalyst is 1.5% H_2SO_4 and the base catalyst NaOH is also 1.5%. The highest oil/methanol molar ratio in acid catalysis and alkali catalysis is 1:30 and 1:6. The transesterification of acid and alkali catalysis is in 60°C by sonication. And the transesterification of enzyme catalysis is in 30°C for 72 hour by 150 oscillations/min.

A

B

Figure 3-18. Figure of microalgal oil and biodiesel car. A, shows the microalgal oil form *N. oculata*. B, shows the biodiesel car of our laboratory. The highest diesel/biodiesel mixed ratio added in the car was B70 and the mileage by biodiesel is over 1,000 kilometers.