# รายงานวิจัยฉบับสมบูรณ์

# โครงการ การคัดแยกและการเพาะเลี้ยงสาหร่ายขนาดเล็กที่มีน้ำมันสูงในน้ำทิ้งโรงงานอุตสาหกรรมเพื่อการบำบัดน้ำทิ้งและการผลิตไบโอดีเซล

# Screening and Cultivation of Oleaginous Microalgae in Wastewater from Industrial Plant for Wastewater Treatment and Biodiesel Production

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กรกฎาคม 2554

สัญญาเลขที่ MRG5280211

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย  สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

## กิตติกรรมประกาศ

คณะผู้วิจัยขอขอบคุณสำนักงานคณะกรรมการการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัยที่ให้การสนับสนุนภายใต้ทุนพัฒนาศักยภาพในการทำงานวิจัยของอาจารย์รุ่นใหม่ เป็นเวลา 2 ปี ทำให้คณะผู้วิจัยสามารถนำความคิดสู่การปฏิบัติซึ่งช่วยให้เห็นผลที่เป็นรูปธรรม และเกิดการเรียนรู้ที่มีคุณค่ายิ่งต่อการพัฒนางานวิจัยของประเทศ

## บทคัดย่อ


**รหัสโครงการ:** **MRG5280211**

**ชื่อโครงการ:** **การคัดแยกและการเพาะเลี้ยงสาหร่ายขนาดเล็กที่มีน้ำมันสูงในน้ำทิ้งโรงงานอุตสาหกรรมเพื่อการบำบัดน้ำทิ้งและการผลิตไบโอดีเซล**



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**ระยะเวลาโครงการ:** **2 ปี**



งานวิจัยนี้ได้คัดแยกสาหร่ายขนาดเล็กสีเขียวที่มีลักษณะคล้ายสายพันธุ์ *Botryococcus* 4 ไอโซเลต (SK, TRG, PSU และ KB) จากทะเลสาบและแหล่งน้ำจืดในภาคใต้ของประเทศไทย โดยใช้วิธีแยกด้วยหลอดแก้วขนาดเล็กปราศจากเชื้อ (sterile micropipette washing method) ผลการเปรียบเทียบการเจริญเติบโตและการผลิตน้ำมันของสาหร่ายขนาดเล็กทั้ง 4 ไอโซเลต เมื่อนำไปเลี้ยงในอาหารเหลวสูตร modified Chu 13 ที่พีเอช 6.7 อุณหภูมิ 25 องศาเซลเซียส ภายใต้ความเข้มแสง 33 μmol.photon.$m^{-2}$ $s^{-1}$ ช่วงสว่างสลับช่วงมืด 16:8 ชั่วโมง เป็นเวลา 20 วัน พบว่าสายพันธุ์ KB มีอัตราการเจริญเติบโตเร็วที่สุด (0.223 ต่อวัน) รองลงมา คือ สายพันธุ์ TRG (0.182 ต่อวัน) และ SK (0.0135 ต่อวัน) ในขณะที่สายพันธุ์ PSU มีอัตราการเจริญเติบโตช้าที่สุด (0.061 ต่อวัน) และพบว่าปริมาณน้ำมันที่ได้จากสาหร่ายสายพันธุ์ KB, TRG, SK และ PSU ภายใต้การเพาะเลี้ยงในสภาวะที่มีแหล่งไนโตรเจน (nitrogen-rich condition) เท่ากับร้อยละ 20, 26, 17 และ 5 ของน้ำหนักแห้ง ตามลำดับ จากการศึกษาสภาวะทางกายภาพ-เคมี ได้แก่ สภาวะที่มีการจำกัดแหล่งไนโตรเจน (nitrogen-limitation), ความเข้มข้นของเกลือ, ความเข้มแสง และความเข้มข้นของธาตุเหล็ก เพื่อศึกษาความสามารถในการผลิตน้ำมันของสาหร่ายทั้ง 4 สายพันธุ์ พบว่าการเพาะเลี้ยงสาหร่ายในสภาวะที่มีการจำกัดแหล่งไนโตรเจนร่วมกับการให้ความเข้มแสงในระดับปานกลางจะสามารถเพิ่มการผลิตน้ำมันของทุกสายพันธุ์ให้สูงขึ้นได้ นอกจากนี้ยังพบว่าความเข้มข้นของธาตุเหล็กที่สูงยังสามารถเพิ่มการสะสมน้ำมันของสาหร่ายให้สูงขึ้นได้อีกด้วย โดยพบว่าภายใต้สภาวะดังกล่าวสายพันธุ์ TRG จะให้การผลิตน้ำมันสูงที่สุด คิดเป็นร้อยละ 35.9

เพื่อเป็นการลดต้นทุนการผลิตน้ำมัน งานวิจัยนี้ได้ศึกษาการรวมการผลิตน้ำมันและบำบัดน้ำเสียโดยใช้สาหร่ายสายพันธุ์ TRG ที่มีความสามารถในการผลิตน้ำมันได้สูงสุดจากการทดลองแรก โดยทำการเพาะเลี้ยงสาหร่ายสายพันธุ์ TRG ในน้ำเสียจากโรงงานแปรรูปอาหารทะเลที่ผ่านการบำบัดขั้นที่สองมาแล้ว ผลการทดลองพบว่าสาหร่ายสายพันธุ์ TRG ให้การเจริญเติบโตและการผลิตน้ำมันได้ดีที่สุดเมื่อเลี้ยงในน้ำเสียที่ไม่ผ่านการเจือจางร่วมกับการให้

ก๊าซคาร์บอนไดออกไซด์ที่ความเข้มข้นร้อยละ 2.0 และยังพบว่าสาหร่ายสายพันธุ์ TRG ยังมีประสิทธิภาพในการลดไนเตรตเริ่มต้นในน้ำเสียได้ถึงร้อยละ 82

นอกจากนี้งานวิจัยนี้ได้ศึกษาการเพาะเลี้ยงสาหร่ายสายพันธุ์ TRG ในสภาวะที่มีการสังเคราะห์แสงเพียงอย่างเดียว (photoautotroph), สภาวะที่มีการให้แหล่งคาร์บอนอินทรีย์แต่ไม่ให้แสง (heretotroph) และสภาวะที่มีการสังเคราะห์แสงร่วมกับการให้แหล่งคาร์บอนอินทรีย์ (mixotroph) โดยใช้กลูโคสและกากน้ำตาลเป็นแหล่งคาร์บอนอินทรีย์ ผลการทดลองพบว่าสาหร่ายสายพันธุ์ TRG สามารถใช้กลูโคสและกากน้ำตาลในการเจริญเติบโตได้ดีภายใต้สภาวะที่มีการสังเคราะห์แสงร่วมกับการให้แหล่งคาร์บอนอินทรีย์ โดยให้น้ำหนักเซลล์แห้งและปริมาณน้ำมันสูงสุดที่ 245 มิลลิกรัมต่อลิตร และร้อยละ 37.4 ตามลำดับ เมื่อใช้กลูโคสความเข้มข้น 10 กรัมต่อลิตร

ในการศึกษาการเพาะเลี้ยงสาหร่ายสายพันธุ์ TRG ในถังปฏิกรณ์ชีวภาพแบบให้แสงที่มีการกวน (stirred tank photobioreactor) โดยเปรียบเทียบรูปแบบการเพาะเลี้ยงแบบกะและแบบกึ่งต่อเนื่อง พบว่าในการเพาะเลี้ยงแบบกะให้น้ำหนักเซลล์แห้งสูงสุดที่ 267 มิลลิกรัมต่อลิตร และให้ปริมาณน้ำมันร้อยละ 30.9 ในขณะที่การเพาะเลี้ยงแบบกึ่งต่อเนื่องจะให้น้ำหนักเซลล์แห้งสูงสุดถึง 508 มิลลิกรัมต่อลิตร และให้ปริมาณน้ำมันร้อยละ 32.2 ในวันที่ 21 ของการเพาะเลี้ยง นอกจากนี้ยังมีการขยายขนาดการทดลองเพาะเลี้ยงสาหร่ายสายพันธุ์ TRG กลางแจ้งโดยใช้ถังปฏิกรณ์ชีวภาพระบบปิดแบบท่อให้แสง (closed tubular photobioreactor) ขนาดความจุ 20 ลิตร ผลการทดลองพบว่าสาหร่ายเจริญเติบโตได้น้อย โดยให้น้ำหนักเซลล์แห้งสูงสุดเพียง 157 มิลลิกรัมต่อลิตร และให้ปริมาณน้ำมันเพียงร้อยละ 26 ซึ่งสาเหตุน่าจะเกิดจากความเข้มแสงและอุณหภูมิที่สูงเกินไปในช่วงเวลากลางวัน

**คำหลัก:** ไบโอดีเซล ไขมัน สาหร่ายขนาดเล็ก การบำบัด น้ำทิ้ง

## ABSTRACT

**Project Code:** **MRG5280211**

**Project Title:** **Screening and Cultivation of Oleaginous Microalgae in wastewater from Industrial Plant for Wastewater Treatment and Biodiesel Production**

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**Project Period:** **2 years**

Four *Botryococcus*-like microalgae (SK, TRG, PSU and KB) were isolated from lakes and freshwater ponds in southern Thailand using sterile micropipette washing method. The growth and lipid content of four strains were compared in modified Chu 13 medium at pH 6.7, 25°C under 16:8 light and dark cycles with light intensity of 33 μmol.photon.$m^{-2}s^{-1}$ for 20 days. The KB strain grew fastest (0.223 $d^{-1}$) followed by TRG (0.182 $d^{-1}$) and SK (0.135 $d^{-1}$), whereas the growth of PSU strain was slowest (0.061 $d^{-1}$). The lipid content obtained from KB, TRG, SK and PSU strains under nitrogen-rich condition were 20%, 26%, 17% and 5% of total algal dry weight, respectively. To improve lipid production in these algae, the physio-chemical trails including nitrogen limitation, high of salt concentration, light intensity and iron concentrations were performed. The combined trials of nitrogen limitation, moderate light intensity were preferred for lipid accumulation in all strains. High iron concentrations also improved considerable lipid accumulation. The highest amount of hydrocarbon content 35.9% was found in TRG strain.

Coupling of lipid production and wastewater treatment using microalgae is a promising approach for economical production of lipid. A lipid-rich green microalga TRG strain was cultivated in secondarily pretreated effluent from seafood processing plant. The cell growth and its lipid content were enhanced when using the undiluted effluent supplemented with 2.0% $CO_2$. In addition to lipid production, TRG strain also efficiently removed nitrate in the effluent more than 82%.

Green microalga TRG strain was grown under photoautotrophic, heterotrophic and mixotrophic culture conditions using glucose and molasses as organic carbon source. The results showed that TRG strain grew well in the presence of glucose and molasses under mixotrophic culture. The highest dry cell weight of 245 mg/L and lipid content of 37.4% were achieved under mixotrophic condition using glucose concentration of 1%.

The batch and semi-continuous cultures of TRG strain were performed in a stirred tank photobioreactor. The highest dry cell weight of 267 $mg.L^{-1}$ and lipid content of 30.9% were obtained in the batch culture. In semi-continuous culture, the higher dry cell weight of 508 mg/L and lipid content of 32.2% were achieved at day 21 of cultivation. Furthermore, outdoor cultivation of TRG strain was attempted in a 20 L closed tubular photobioreactor. Unfortunately, TRG strain had a low dry cell weight of 157 $mg.L^{-1}$ and low lipid content of 26%. This could be due to the exposure to high light intensity and temperature factors that had significantly affect to the content of biomass and caused lasting depression in the lipid content.

**Keywords:** Biodiesel, Lipid, Microalgae, Treatment, Wastewater

## EXECUTIVE SUMMARY

Nowadays, the fuels derived from fossil such as petroleum, natural gas and coal are decreasing. And the price of petroleum is getting higher and higher. It is also known that emissions from the combustion of these fuels are the principal causes of global warming. Therefore, a renewable and non-toxic fuel, biodiesel fuel has received considerable attention in recent years. Unlike fossil reserves, different regions of the world have their own oil resources that could be exploited for biodiesel production. Biodiesel has been produced from a variety of sources including refined and crude oils, used cooking oils, which are extracted from vegetable and animal including oils from some oleaginous microalgae. Microalgae can accumulate oils that can be cracked to form a liquid fuel. Since microalgae are fast-growing plants and can accumulate fuel oils in a large amount, it was reported that the productivity of fuel oils from microalgae was higher than other oil-plants. Moreover, microalgae ponds have been utilized for the treatment of wastewaters, with its mainly providing dissolved oxygen for bacterial decomposition of the organic wastewater. Microalgae could also efficiently use nitrates, ammonia, and phosphates in wastewater for their growth and improve the quality of polluted water. Thus, the cultivation of oil-producing and wastewater-treating microalgae might be suitable to biological treatment of wastewater and economical production of fuel oil. And the algae cultivation could promote effective use of natural resources and be environmental friendly, because algae could process photosynthesis and use $CO_2$ for their growth. In this study, the oleaginous microalgae *Botryococcus* sp. were isolated and grown in wastewater from the industrial plant. Four microalga *Botryococcus* sp. (SK, TRG, PSU and KB) were isolated from lake and reservoir pond. The combined trials of nitrogen limitation, moderate light intensity were preferred for lipid accumulation in all strains. High iron concentrations also improved considerable lipid accumulation. The highest amount of lipid content 35.9% found in TRG strain and 30.2%, 28.4% and 14.7% of total algal dry weight in KB, SK and PSU strains, respectively. Palmitic and oleic acid were dominant fatty acid in lipid of isolated microalgae. The conditions for cultivating isolated TRG strain in effluent from seafood processing plant were optimized. Undiluted effluent with 2.0% $CO_2$ was optimum for growth and lipid production of TRG strains. Besides the lipid accumulating ability, TRG strain also removed 80% of nitrate in the effluent. TRG strain could grow under photoautotrophic, heterotrophic and mixotrophic culture conditions with glucose and molasses as carbon source. The highest dry cell weight of 245 $mg.L^{-1}$ and lipid content of 37.4% were achieved under mixotrophic condition using glucose concentration of 10 $g.L^{-1}$. TRG strain also grew well when 15 $g.L^{-1}$ molasses was used instead of glucose. Furthermore, the cultivation of TRG strain was scaled up in photobioreactors. The batch culture in a stirred tank photobioreactor gave the highest dry cell weight of 267 $mg.L^{-1}$ and lipid content of 30.9%. In semi-continuous culture, the higher dry cell weight of 508 $mg.L^{-1}$ and lipid content of 32.2% were achieved at day 21 of cultivation. Outdoor cultivation of TRG strain was also performed in a 20 L closed tubular photobioreactor. TRG strain gave a lower dry cell weight of 157 $mg.L^{-1}$ and lower lipid content of 26%.

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# CHAPTER 1

# INTRODUCTION

## Introduction

The need to develop alternative fuels to those derived from fossil fuels is clear-cut. Not only are fossil fuels limited resources but also burning those produces carbon dioxide, which is one of the causes of global warming. There are a number of alternative sources of energy such as hydroelectric, nuclear, wave, and wind power and biological materials. The advantages of biological sources of energy are that they are renewable, biodegradable, produce fewer emissions and do not contribute to the increase in carbon dioxide in the atmosphere. Biological materials can produce energy by burning of biomass (wood and grasses), the production of biogas (methane), the generation of hydrogen, the production of ethanol by fermentation, and the use of plant-derived oils (Scragg *et al*., 2003).

Fuels from the pyrolysis of biomass have lower sulphur and nitrogen content than do fossil fuels such as coal and petroleum (Miao and Wu, 2004). They are therefore cleaner, causing less pollution. And so, microalgae can be used in a number of ways for the production of energy. Microalgae can be digested to produce methane. They can produce hydrogen photosynthetically, and some algae accumulate oils that can be cracked to form a liquid fuel. Microalgae appear to be the only source of biodiesel that has the potential to completely displace fossil diesel. Unlike other oil crops, microalgae grow extremely rapidly and many are exceedingly rich in oil. Doubling times of microalgae are shorter than other crops. Oil content in microalgae can exceed 80% by weight of dry biomass. Moreover, the yields of microalgae are higher than 10 times those for land plants so much less land is needed (Chisti, 2007).

*Botryococcus* is a microalga that is regarded as a potential source of renewable fuel because of its ability to produce large amounts of lipid. Especially *Botryococcus braunii*, its matrix consists mainly of botryococcenes, about 75% of dry cell weight, which have potential as a hydrocarbon fuel (Sawaddiraksa, 2006). Furthermore, it was also reported that the algae grown in $CO_2$-enriched air can yield oil that can be converted into biodiesel. Such an approach can contribute to solving two major problems: air pollution resulting from $CO_2$ evolution, and future crises due to a shortage of energy sources (Chisti, 2007).

Seafood industry wastewater contains a large amount of organic matter and nitrogenous compounds, and they contain highly concentrated pollutants, including suspended solids, organics and nutrients. These may deteriorate the quality of the aquatic environments into which they are discharged. Wastewater treatment by microalgal culture has several major advantages: it rests on the principles of natural ecosystems and is therefore not environmentally hazardous; it causes no secondary pollution, as long as the biomass produced is reused; and it allows efficient recycling of nutrients (An *et al*., 2003). The important advantages of microalgae are that they can grow photosynthetically so that no carbon source is required for growth and any carbon dioxide released on combustion will be fixed by their photoautotrophic ability with the aim of contributing to a reduction in greenhouse gases and global warming (Wikipedia, 2008a). Moreover, cultivation of microalgae with pretreated wastewater could alleviate the economic problems by its ability to use inorganic nutrients, mainly nitrogen and phosphorus in wastewater. In addition, the use of algae in purification facilities to eliminate nutrients continues to be studied widely (Craggs *et al*., 1995; Martinez *et al*., 1999; Converti *et al*., 2006). The microalgae are highly effective not only at using the inorganic nitrogen and phosphorus for growth but also at purifying the waste by producing oxygen and removing heavy metals and xenobiotic substances (Martinez *et al*., 1999). Incorporation of algal systems into conventional wastewater treatment offers the combined advantages of treating the wastewaters and simultaneously producing biomass, which can be applied to the production of biofuel.

For primary wastewater treatment the settled and floating materials are removed. However, wastewater may still contain high levels of the nutrients nitrogen and phosphorus when it is discharged in secondary treatment. It was reported that secondary effluent of seafood processing plant contains high levels of nitrogen source (Sohsalam *et al*., 2008). On the one hand, these organic and inorganic nutrients need to be removed, and on the other hand they are suitable and cost-effective for microalgal cultivation. Microlagae can uptake nutrient nitrogen into the cell and also stripping ammonia through elevated pH. In addition, the process has no carbon requirement for nitrogen removal, which is attractive for the treatment of secondary effluent (Aslan and Kapdan, 2006).

Today, the most common procedure for cultivation of microalgae is autotrophic growth. Because all microalgae are photosynthetic, and many microalgae are especially efficient solar energy convertors, microalgae are cultivated in illuminated environments naturally or artificially. Under autotrophic cultivation, the cells harvest light energy and use $CO_2$ as a carbon source. The introduction of sufficient natural or artificial light to allow massive growth and dense populations is the main objective and a limiting factor of the cultivation: the more light, up to a limit for the species (Yang *et al*., 2000; Suh and Lee, 2003). Therefore, as practiced with other microbial communities producing economic products, open ponds that mimic natural environments of microalgae are the most common option for mass cultivation (Tredici, 2004). A feasible alternative for photoautotrophic cultures, but restricted to a few microalgal species, is the use of their heterotrophic growth capacity in the absence of light, replacing the fixation of atmospheric $CO_2$ of autotrophic cultures with organic carbon sources dissolved in the culture media. Heterotrophic is defined as the use of organic compounds for growth. Heterotrophic are organisms whose substrate and energy needs are derived from organic compounds synthesized by other organisms. The basic culture medium composition for heterotrophic cultures is similar to the autotrophic culture with the sole exception of adding an organic carbon (Tsavalos and Day, 1994). Mixotrophic growth regime is a variant of the heterotrophic growth regime, where $CO_2$ and organic carbon are simultaneously assimilated and both respiratory and photosynthetic metabolism operates concurrently (Lee, 2004). Some microalgal species are not truly mixotrophs, but have the ability of switching between photoautohic and heterotrophic metabolisms, depending on environmental conditions (Perez-Garcia *et al*., 2011)

The cultivation of microalgae is used for the production of valuable chemical compounds, including natural pigments, biofuels and dietary supplements. In spite of the progresses in cultivation techniques and the design of high efficiency photobioreactors, biomass productivity is still low and more research is needed to develop large-scale processes for the production of these microorganisms. In several species of microalga including of *Botryococcus* photosynthesis is the main carbon-fixation route, but during the light phase of cultivation *Botryococcus* can combine autotrophic photosynthesis and heterotrophic assimilation of organic compounds in a process known as mixotrophy (Perez-Garcia *et al*., 2011; Andrade and Costa, 2007). Photosynthetic fixation of inorganic carbon is influenced by light intensity while the heterotrophic assimilation of carbon is influenced by the availability of organic carbon (Zhang *et al*., 1999).

The commercial exploitation of photosynthetic microalgae is based on typical large, outdoor open ponds and closed tubular photobioreactors occupying vast land extensions. These systems although make use of natural sunlight for the production of energy fixed chemically (Apt and Behrens, 1999; Borowitzka, 1999), they present a great disadvantage, however, cellular self-shading hinders light availability, severely limiting biomass production and the low biomass concentrations reached decrease efficient harvesting of the cells. Strategies to improve the efficient use of light or eliminate its requirement by cells and so reduce the cost of microalgal biomass production, involve mixotrophic, photoautotrophic or heterotrophic growth of algae. Especially, mixotrophic and photoautotrophic allow microalgal cells to synthesise simultaneously compounds characteristic of both heterotrophic and photosynthetic metabolisms at high production rates (Ogbonna *et al*., 2000). Moreover, some researchers indicated that heterotrophic growth of microalgae gave high biomass production and induced high accumulation of lipid in microalgal cells (Dote *et al,* 1994; Minola *et al*, 1995; Miao and Wu, 2006).

The objective of this study focused on isolation of *Botryococcus*-like green microalgae from lakes and freshwater ponds in southern Thailand and its possibility for lipid production. The effect of nitrogen limitation and its combination with salinity, light intensity and iron concentration trials on growth and lipid production by 4 isolated microalgal strains were investigated. The biodiesel production from lipid of isolated microalgae was performed. The most suitable microalga for lipid production was cultivated in secondarily pretreated effluent from seafood processing plant. To enhance lipid production by this selected strain, the cultivation under photoautotrophic, heterotrophic and mixotrophic conditions was evaluated. Furthermore, batch and semi-continuous cultures of the microalga were performed in a 2 L stirred tank photobioreactor. In addition, the cultivation of the microalga in a 20 L outdoor closed tubular photobioreactor was also attempted.

**Objectives of Study**

1. To screen lipid producing-*Botryococcus*-like green microalga from lakes and reservoir ponds in southern Thailand.
2. To evaluate the effect of physio-chemical environments on lipid production by isolated strains and select the most suitable one.
3. To optimize condition for cultivation of selected microalga in pretreated effluent from seafood processing plant.
4. To perform batch and semi-continuous cultures of selected microalga in a photobioreactor for lipid production.

# Literature Review

## 1. Microalgae as a source of lipid and fatty acid

Algae are a very diverse group of organisms and are known to produce unique and unusual compounds (Harwood and Jones, 1989). As in other organisms, lipids and fatty acids are basic cellular constituents that can serve both as structural components of the cell and as a storage product for the cell. Algae have not been as extensively characterized with respect to their lipid and fatty acid content as other microorganisms, but the available information nonetheless suggests that they can provide a novel source of unusual and interesting lipids and fatty acids (Behrens and Kyle, 1996).

Algae contain many of the major lipid classes found in other organisms. The major nonpolar lipids reported in algae are the triacylglycerols and hydrocarbons, and the major polar lipids classes include phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylglycerol, cardiolipin, diphosphatidylglycerol and glycolipids (Cranwell *et al*., 1988, 1989; Dembitsky *et al*., 1991). Some species of algae are reported to produce large quantities of lipid, primarily in the form of large droplets of triacylglycerol in the cytoplasm. However, algae are metabolically very flexible and their lipid content can change significantly depending on the growth conditions used (Grima *et al*., 1996; Regnault *et al.,* 1995).

Depending on species, microalgae produce many different kinds of lipids, hydrocarbons and other complex oils (Banerjee *et al*., 2002). Not all algal oils are satisfactory for making biodiesel, but suitable oils occur commonly. Oil content in microalgae can exceed 80% by weight of dry biomass. Oil levels of 20–50% are quite common as shown in Table 1 (Chisti, 2007).

Table 1: Oil content of some microalgae

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

Source: Chisti (2007)

**2. *Botryococcus braunii***

*B. braunii* (Bb) is a green, pyramid shaped colloidal microalgae of the order Chlorococcales (class Chlorophyceae, as shown in Table 2) that is of potentially great importance in the field of biotechnology. Colonies held together by a lipid biofilm matrix (as shown in Figure 1) can be found in temperate or tropical oligotrophic lakes and estuaries, and will bloom when in the presence of elevated levels of dissolved inorganic phosphorus. The species is notable for its ability to produce high amounts of hydrocarbons, especially oils in the form of triterpenes that are typically around 30-40 % of their dry weight (Metzger and Largeau, 2005).

*B. braunii* is classified into A, B and L races depending on the type of hydrocarbons synthesized (Table 3 and Figure 2). Race-A produces $C_{23}$–$C_{33}$ odd numbered n-alkadienes, mono-, tri-, tetra-, and pentaenes, which are derived from fatty acids (Metzger and Largeau, 2005). These linear olefins can constitute up to 61% of the dry cell mass of the green active state colonies. The L race produces a single tetraterpene hydrocarbon known as lycopadiene ($C_{40}H_{78}$) and it constitutes up to 2–8% of the dry biomass. The B race produces polyunsaturated and branched $C_{30}$–$C_{37}$ terpenoid hydrocarbons referred to as polymethylated botryococcenes. These compounds are promising as a renewable energy source as they accumulate to very high levels (26–86% on dry weight) in the algae (Dayananda *et al*., 2007a). The biosynthetic pathways of lipid in *B. braunii* is shown in Figure 3.

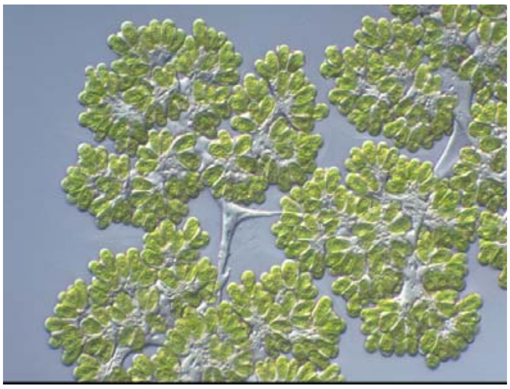

Figure 1 : Colonies of *Botryococcus braunii*
Source: Wikipedia (2008b)

Table 2 : Scientific classification

| | |
|---|---|
| Kingdom | Plantae |
| Division | Chlorophyta |
| Class | Chorophyceae |
| Order | Chorococcales |
| Family | Dictyosphaeriaceae |
| Genus | Botryococcus |
| Species | *B. braunii* |

Source: Wikipedia (2008b)

Table 3: Distinctive features of races of *B. braunii*

| ***B. braunii*** | | | |
|---|---|---|---|
| | Race-A | Race-B | Race-L |
| Nature of hydrocarbon | $C_{25}$–$C_{31}$ odd numbered n-alkadienes/trienes | Botryococcenes (triterpenes) $C_nH_{2n-10}$, n = 30–37 | Lycopadienes (tetra-Terpene) $C_{40}H_{78}$ |
| Colony color in stationary phase | Pale yellow or green | Orange-reddish or orange-brownish due to accumulation of carotenoids | |
| Long chain alkenyl phenols | Present | Absent | Absent |
| Nature of biopolymers | Very long aliphatic chains cross-linked by ether bridges and bearing fatty esters | | Tetraterpenoid cross-linked by ether bridges |

Source : Banerjee *et al*. (2002)

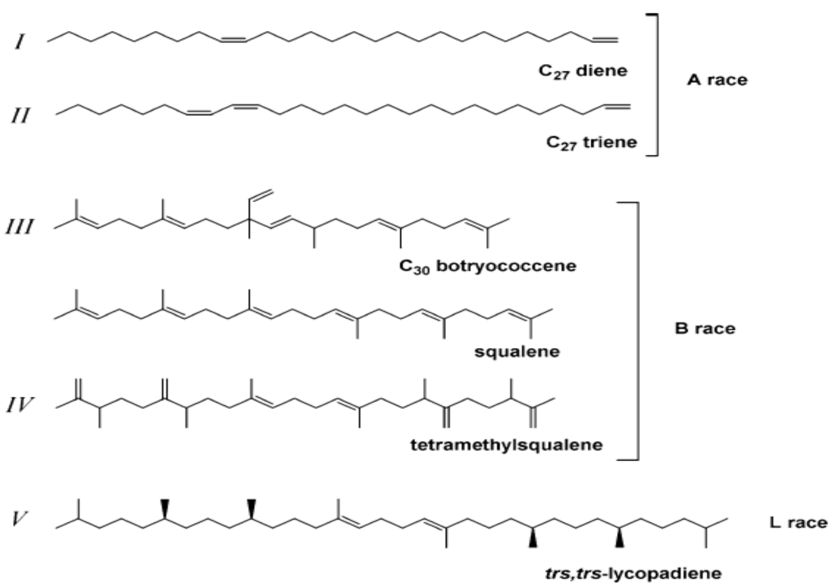


Figure 2: Types of hydrocarbons produced by the three chemical races of *B. braunii*

Source : Metzger and Largeau (2005)

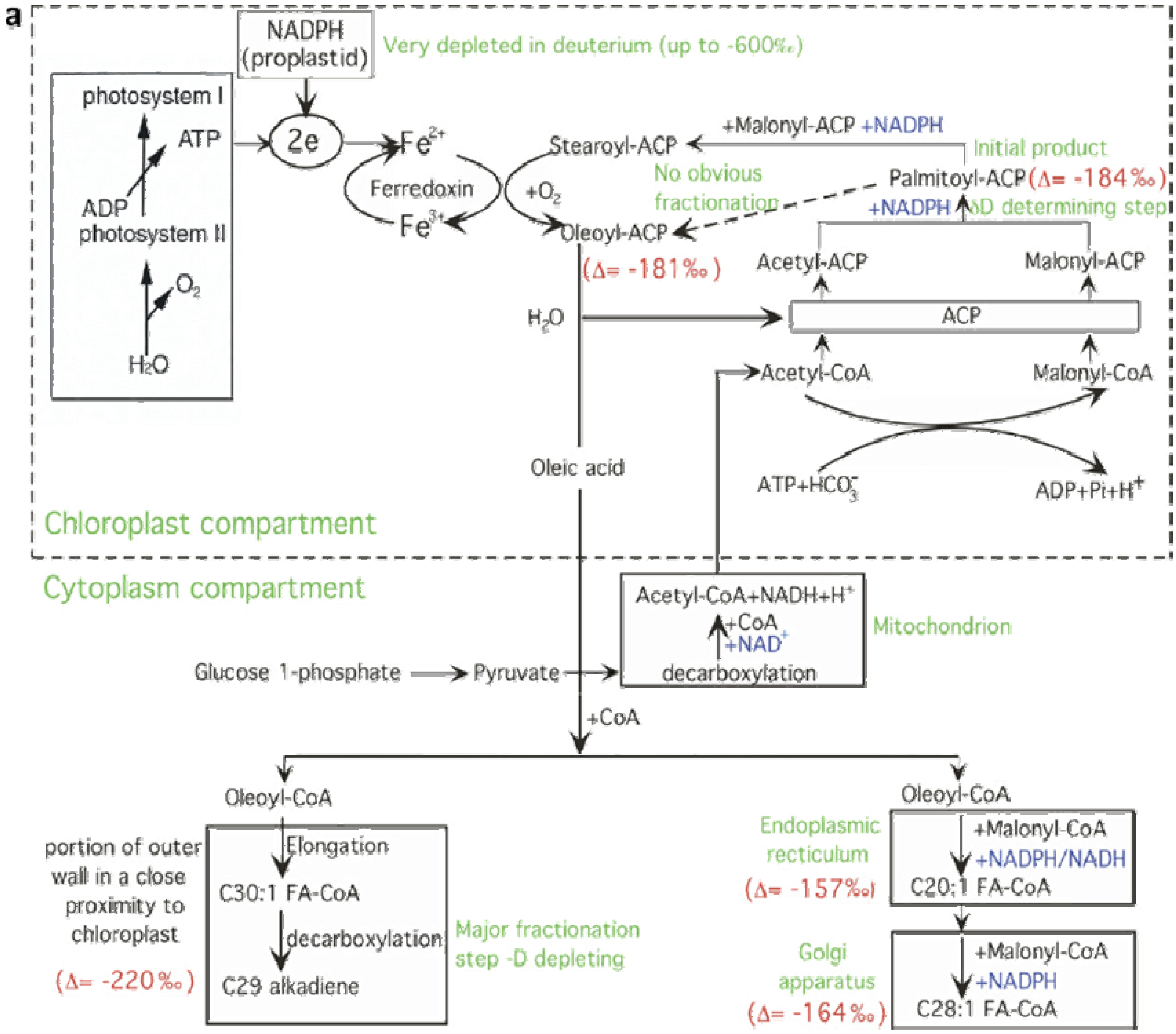


Figure 3A : Lipid biosynthetic pathways in *B. braunii*.

(IPP : isopentenyl pyrophosphate; DMAPP: dimethylallyl diphosphate; GPP: geranyl diphosphate; GGPP: geranylgeranyl diphosphate; FPP: farnesyl diphosphate and PSPP: presqualene diphosphate)

Source : Zhang and Sachs (2007)

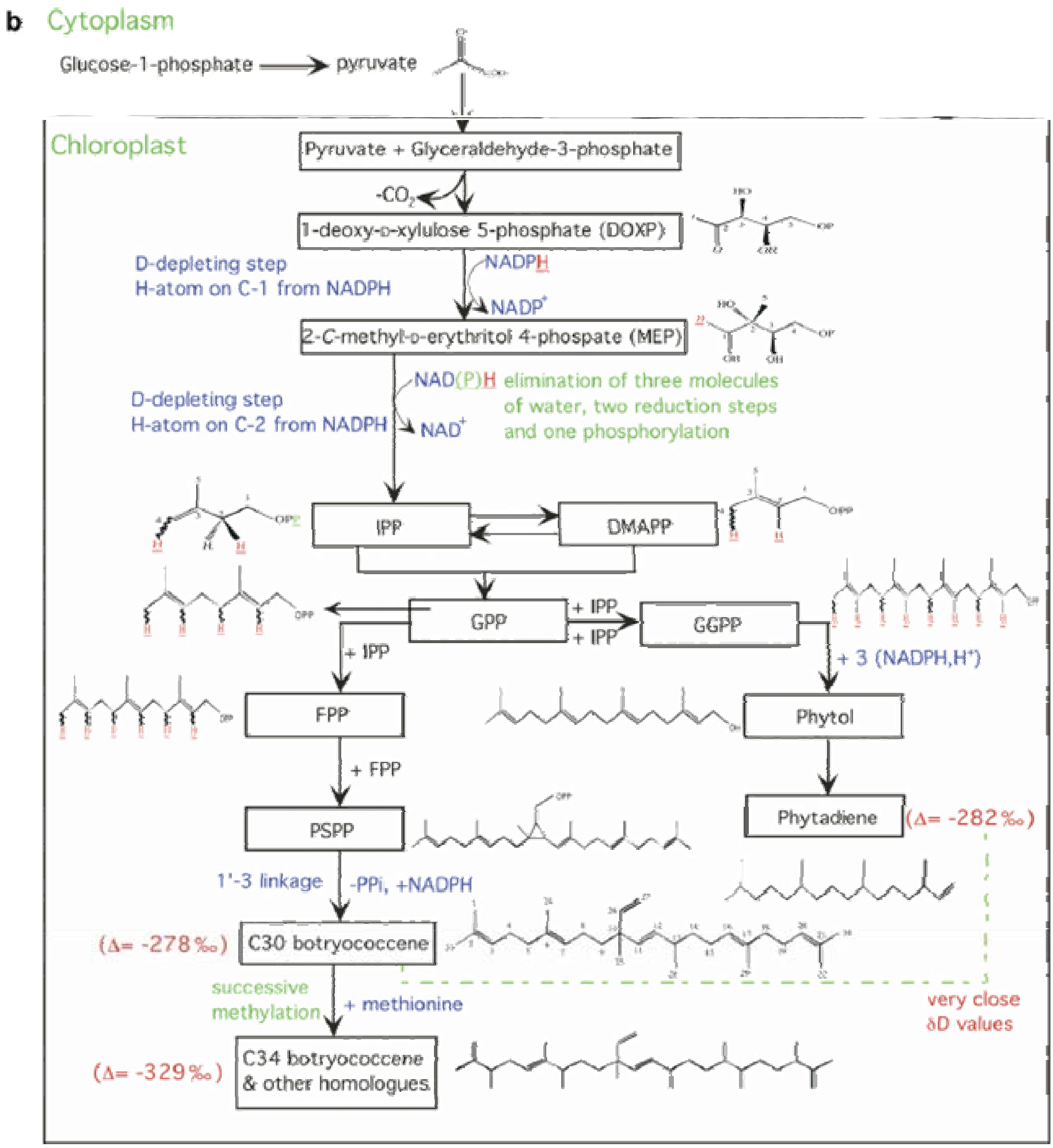


Figure 3 B: Lipid biosynthetic pathways in *B. braunii*.

(IPP : isopentenyl pyrophosphate; DMAPP: dimethylallyl diphosphate; GPP: geranyl diphosphate; GGPP: geranylgeranyl diphosphate; FPP: farnesyl diphosphate and PSPP: presqualene diphosphate)

Source : Zhang and Sachs (2007)

In Figure 3, (a) Fatty acids and alkadienes in the A race are produced using acetyl coenzyme-A as a precursor. (b) Botryococcenes and phytadiene in the B race are isoprenoid (i.e., branched) lipids synthesized using isopentenyl pyrophosphate as a precursor (IPP). The $C_{30}$ botryococcene is the precursor for the $C_{31}$–$C_{34}$ botryococcenes which are synthesized by methylation on positions 3, 7, 16 and/or 20 of the $C_{30}$ backbone The relative abundance of $C_{30}$ botryococcene in a population results from a balance between its production and its loss via methylation to form $C_{31}$–$C_{34}$ botryococcenes (Okada *et al*., 2004).

*B. braunii* is microalga widespread in freshwater and brackish lakes, reservoirs, ponds, or even ephemeral lakes situated in continental, temperate, alpine, or tropical zones (Metzger and Largeau, 2005).

Okada *et al*. (1995) isolated four new strains of *B. braunii* (Yamanaka, Yayoi, Kawaguchi-1 and Kawaguchi-2) from Japanese waters under defined conditions. Their hydrocarbon content and composition were analyzed and compared with those of the Darwin and Berkeley strains. The Yamanaka strain produced only alkadiences characteristic of the A race, whereas the others, the Yayoi, Kawaguchi-1 and -2 strains as well as the Darwin and Berkeley strains, produced botryococcenes peculiar to the B race. The hydrocarbon content of the Yamanaka strain was 16.1% dry weight and that of the B race strains ranged from 9.7-37.9%.

Kalacheva *et al*. (2002) compared lipid composition and hydrocarbon structure of two colonial green algae of the genus *Botryococcus*, i.e., a museum strain and a field sample collected for the first time from Lake Shira (Khakasia, Siberia). The dominant fraction in the museum strain was formed by polar lipids (up to 50% of the lipids) made up of fatty acids from $C_{12}$ to $C_{24}$ The *Botryococcus* sp. found in Lake Shira is characterized by a higher lipid content (<40% of the dry weight). Polar lipids, sterols, triacylglycerols, free fatty acids and hydrocarbons have been identified among lipids in the field sample. The main lipids in this sample were dienes and trienes (hydrocarbons<60% of total lipid). The chemo-taxonomic criteria allow us to unequivocally characterize the organism collected from Lake Shira as *B. braunii*, race A.

Dayananda *et al*. (2007b) collected microalga *B. braunii* from Bear Shola Falls at Kodaikanal, Tamil Nadu, India. Specimens were isolated, cultured and examined for its hydrocarbon content. Inter simple sequence repeats (ISSR) finger printing revealed strong genetic similarity among the authentic strain (*B. braunii* N-836) and the Indian isolated strain (*B. braunii* CFTRI-Bb1) from Kodaikanal. The type of hydrocarbons produced by the Kodaikanal isolated were analyzed and identified as saturated hydrocarbon in the range of $C_{21}$-$C_{33}$. Tetracosane and octacosane were found as the major components among the saturated hydrocarbons produced by this alga, constituting 17.6% and 14.8%, respectively. Hydrocarbon content of the organism was in range of 13-18% of its dry biomass.

## 3. Factors affecting growth and lipid production of microalgae

### 3.1 Nutrients

Much work has been done on establishing the optimal nutritional requirements and culture conditions for producing *B. braunii* hydrocarbons. The production of lipid in *B. braunii* appears to be growth associated, irrespective of the specific culture conditions and the nutrients used. Apparently, the observed slow growth rate of the alga is not a consequence of a limited supply of nutrients but the result of the substantial commitment by the cells to produce energetically expensive hydrocarbons. Like other microalgae, *B. braunii* culture requires water, light, $CO_2$, and inorganic nutrients. Culture productivity is affected by factors such as pH, $CO_2$, irradiance, salinity, and temperature.

#### 3.1.1 Carbon dioxide

With the biological approach, $CO_2$ is converted into algal biomass and then into value-added products such as proteins, vitamins, food, and oil (Jeong *et al*., 2003). It is believed that the majority of oil and natural gas originates from algae. Oil (petroleum) consists of liquid hydrocarbons which are compounds composed of carbon and hydrogen. At least 80% w/w of oil is carbon. Since the reports that microalgae can grow at $CO_2$ concentrations in excess of 5%, several researches attempted to cultivated microalga using difference concentration of $CO_2$.

Photosynthetic cultures of *B. braunii* require $CO_2$. Cultures aerated with 0.3% $CO_2$-enriched air have a much shorter mass doubling time (40 h) compared with 6 days for cultures supplied with ambient air. $CO_2$ enrichment favors the formation of lower botryococcenes ($C_{30}$–$C_{32}$), whereas cultures sparged with ambient air accumulate higher botryococcenes ($C_{33}$–$C_{34}$). Apparently, methylation steps leading from $C_{30}$ to $C_{31}$ and $C_{32}$ are faster in $CO_2$-enriched cultures than steps leading to $C_{33}$, $C_{34}$ and higher homologues. Although autotrophic, *B. braunii* utilizes exogenous carbon sources for improved growth and hydrocarbon production. Various carbon sources, including $C_1$–$C_6$ compounds and disaccharides (lactose, sucrose), have been screened in attempts to decrease the mass doubling time of the alga from ≥1 week to less than 2 to 3 days (Banerjee et al., 2002).

Dumrattana and Tansakul (2006) cultivated the hydrocarbon-rich alga, *Botryococcus braunii* in Modified Chu 13. Growth of *B. braunii* was studied by using batch culture under air-lift condition. The best growth of this alga was achieved under continuous illumination (24 hours), air-1% $CO_2$, rate of 7 l/min and the highest specific growth rate was 3.6 per day.

Dayananda *et al*. (2007b) reported photoautotrophic growth of selected *B. braunii* from Indian freshwater bodies for higher growth and hydrocarbon production. It was evident that 2% of $CO_2$ supplementation was found to be better growth and hydrocarbon production (0.75-0.86 g/l and 13-18%, respectively).

**3.1.2 Nitrogen**

Provision of biogenic elements, mainly nitrogen, is one of the main factors affecting algal metabolism. The change in the carbon/nitrogen ratio in a medium is known to result in a change in the directionality of metabolism. In many microalgae, an increase in this ratio results in an accumulation of neutral lipid. Although a deficiency of nitrogen favors lipid accumulation (Ben-Amotz *et al*., 1985), nitrogen is required for growth. Studies with nitrogen supplied as $NO_3^-$, $NO_2^-$, and $NH_3$ reveal that the primary factor regulating nitrogen metabolism in *B. braunii* is the nitrate uptake system. Nitrogen is generally supplied as nitrate salts. An initial $NO_3^-$ concentration of ≥0.2 kg/m$^3$ favors hydrocarbon production (Casadevall *et al*., 1983). With 1 kg/m$^3$ $KNO_3$, the hydrocarbon concentration after 30 days was 4.8 kg/m$^3$. About the same amount of hydrocarbon (4.5 kg/m$^3$) was obtained if the initial concentration of $KNO_3$ was 3 kg/m$^3$. This is because at high concentrations nitrate interferes with hydrocarbon production (Brenckman *et al*., 1989). When the cells are exposed to 5 mM $NH_4^+$ for 24 h, the nitrate reductase enzyme becomes inactive. The use of $NH_4^+$ as the nitrogen source causes the culture pH to decline to less than pH 4, and this is damaging to cells. This $NH_4^+$ related toxicity manifests itself in the late exponential phase of growth, and the damage it causes is irreversible (Lupi *et al*., 1994). Photosynthesis and hydrocarbon production are drastically diminished when *B. braunii* (B race) cells are exposed to $NH_3$.

The search for cheaper nitrogen sources like urea (Stanca and Popovici, 1996) or ammonium salts (Boussiba, 1989) is particularly attractive from the economic point of view. Sanchez-Luna *et al*. (2004) cultivated *Spirulina platensis* by fed-batch addition of urea as a nitrogen source. Continuous and pulse feeding regimes of this nitrogen source (time intervals of 24 h) were compared. The results shown that the intermittent addition of urea yielded results similar to those obtained by the continuous feeding and gave highest maximum cell concentration 1231±86 mg/L.

The influence of the media constituent potassium nitrate, magnesium sulphate, dihydrogen potassium phosphate and ferric citrate on growth and hydrocarbon production in *B. braunii* (SAG 30.81) was investigated using response surface methodology (RSM). Among the individual variables, potassium nitrate and ferric citrate exhibited marked effects on the response functions (yield of biomass and hydrocarbon production). The optimum concentrations of dihydrogen potassium phosphate, potassium nitrate, magnesium sulphate and ferric citrate were found to be 0.0195, 0.05, 0.02 and 0.0185 g/l, respectively, in the medium for 0.65 g/l biomass and 50.6% (w/w) hydrocarbon production (Dayananda *et al*., 2005).

Yang *et al*. (2004) studied the utilization of nitrite by *B. braunii* as a nitrogen source compared with nitrate. The results was found that nitrite at 2 mM did not affect the growth of *B. braunii* and served as the sole nitrogen source giving a minimum biomass doubling time of 4.2 d, which was equal to that of the culture using 4 mM nitrate as nitrogen source. With nitrite at 4 mM, after a lag phase of about 10 d, the alga grew quickly, reaching 1.1 g/1 at the end. Respective nitrite removals were 100% and 99.7%. There were few differences in the hydrocarbons produced using different nitrogen sources.

Dayananda *et al*. (2006) cultivated *B. braunii* under the influence of different nitrogen sources (sodium nitrate, potassium nitrate, ammonium nitrate, calcium nitrate and urea). Potassium nitrate was found to be the best source of nitrogen source for growth and hydrocarbon yield in *B. braunii*, and gave biomass yields 1.2 g/l with 30-35% (w/w) hydrocarbon yield.

Dayananda *et al*. (2007a) studied growth of *B. braunii* using different autotrophic media such as bold basal medium (BBM), and bold basal with ammonium carbonate (BBMa), BG11, modified Chu 13 medium. Among the different autotrophic media used, BG11 was found to be the best medium for biomass and hydrocarbon production, although *B. braunii* showed appreciable level of growth and biomass production in all the tested media. The culture of BG11 was found to be the best for growth (2.0 and 2.8 g/l of biomass was produced by the *B. braunii* strains SAG 30.81 and LB-572, respectively) and hydrocarbon production (46% and 33%, respectively, by SAG 30.81 and LB 572 strains on dry weight basis).

The enhancement of lipid storage during nitrogen-deficient conditions has been demonstrated in green algae. The effect of nitrogen limitation on the composition of intracellular lipids in the microalga *B. braunii* Kutz IPPAS H-252 was evaluated by Zhila *et al*. (2005). The results indicated that, under the condition of nitrogen limitation, the alga accumulated lipids as triacylglycerols and increase the content of saturated acid (up to 76.8%). Nutrient depletion, which is not necessarily followed by an immediate entry into a stationary phase, may also result in changes in biomass composition. During nutrient depleted growth phases, starch and other carbon and energy storage compounds may accumulate and constitute a major part of the biomass production in green algae (Zhila *et al*., 2005). In nitrogen depleted and carbon sufficient bacteria, accumulation of carbon and energy storage compounds may account for all the produced biomass. Depletion of nutrients, in particular the nitrogen source, also results in break-down of the photosynthetic apparatus, including the photosynthetic pigments (Eriksen and Riisgard, 2007). Moreover, a decrease in the concentration of chlorophyll a accompanied by increase in the content of carotenoids was noticed under the conditions of nitrogen limited (Zhila *et al*., 2005).

## 3.2 Environmental factors

### 3.2.1 Light and photoperiod

The major effect of light concerns photosynthesis in term of photoinhibition and photoinactivation. Impairment of photoautotrophic growth by supraoptimal intensities of light is a well-known phenomenon readily observed in the laboratory as well as in the field. The extent of such photoinhibition is known to depend on the incident irradiance as well as the spectral quality of light. The time of exposure to a given quantum flux area density is also an important factor governing the onset of photoinhibition. Adaptations to different quantum flux area densities on the biochemical and physiological level vary greatly.

Studies have attempted to identify the optimal irradiance levels for supporting growth and hydrocarbon production. Growth of *B. braunii*: the hydrocarbon-rich alga was studied by using batch culture in Modified Chu 13 medium. The alga was incubated at the temperature of 25°C with light density of 120 $\mu Em^{-2}s^{-1}$ and diurnal illumination cycle under 12 hours of light/ 12 hours of dark; 16 hours of light/8 hours of dark and continuous illumination (24 hours). The best growth of this alga was achieved under continuous illumination with highest specific growth rate of 3.6 per day and higher than that of the culture under diurnal light cycles (Dumrattana and Tansakul, 2006). Dayananda *et al*. (2007a) studied growth of *B. braunii* using autotrophic media, Bold Basal Medium (BBM) The culture maintained at 1.2±0.2 Klux light intensity at 25± 1°C with 16:8 h light and dark cycle; continuous light 24:0 h and continuous light 24:0 h with shaker at 90 rpm. The results shown that 16:8 h light and dark cycle has maximum influence for biomass and hydrocarbon production.

Qin (2005) examined the algal growth and lipid content of *B. braunii* (China strain 1) under various light and photoperiod in an attempt to obtain the optimal of light condition for the maximum biomass and hydrocarbon production. In the experiment of high light range, the 30 and 60 $W/m^2$ treatments were more suitable to algal culture than high light intensities. Total lipid content at 30 and 60 $W/m^2$ was significantly higher than that at other light intensity. Lipid content decreased sharply when light intensity increased from 60 to 150 $W/m^2$. However, lipid content did not differ when the light intensity was above 100 $W/m^2$ ($P>0.05$).

Vladislay *et al*. (1994) reported that photoperiod could be one of the factors that triggered hydrocarbon production, but the impact of photoperiod on the growth of *B. braunii* is not clear yet. Qin (2005) found that no significant difference in algal growth between 12 and 24 h light daily ($P>0.05$), but a low photoperiod of 4 and 8 h light could not sustain the algal growth. Algal cells under 24 h light daily showed brown colour at the end of cultivation compared with 12 h light. Therefore, they considered that the 12 h light and 12 h dark cycle is a better light regime for *B. braunii*, not only to stimulate algal growth in a long term, but also to run this project in an outdoor environment.

**3.2.2 pH**

The increase in algae biomass is influenced by pH. The variation of pH in the cultures of microalgae is occurred due to the solubilization and consumption of $CO_2$ and other substrates and the production of metabolites (Grima *et al*., 1999). The variation in pH affects the solubility and the bioavailability of nutrients, transport of substrates across the cytoplasmic membrane and enzyme activity and electron transport in photosynthesis and respiration (Carrion *et al*., 2001). In the some strain alga cultures, pH became more alkaline with increasing biomass, probably due to the formation of carbonic acid ($H_2CO_3$), which dissociates into bicarbonate ($HCO^{-}_3$) and hydrogen ions ($H^+$), the bicarbonate itself dissociating into $CO_2$ or carbonate ions ($CO^{2-}_3$) depending on the pH of the solution. Since $CO_2$ is removed by algal metabolism at a rate depending on their photosynthetic activity (which itself depends on available light) and the proximity of the algal trichomes, hydroxide ions ($OH^-$) are formed, and the pH becomes more alkaline (Richmond, 1986).

The pH of the culture medium is generally adjusted to between pH 7.4 and 7.6 before inoculation. A regular increase in pH is observed during active growth followed by a slight decline later (Casadevall *et al*., 1985). The increase in pH is partly due to the consumption of dissolved $CO_2$ for photosynthesis. Similar changes in pH are commonly observed in $CO_2$- enriched cultures during exponential growth.

Dayananda *et al*. (2006) studied the effect of pH for biomass and hydrocarbon production by *B. braunii* LB 572 and SAG 30.81. The biomass yields of *B. braunii* indicated at the organism can grow in the pH range of 6.0-8.5, although higher biomass, 0.77 and 1.9 g/l obtained, respectively for SAG 30.81 and LB 572 at pH 6.0.

The hydrocarbon content in the organism was not much affected by the pH, since hydrocarbon yields were in the range of 25-31% (w/w) and 38-41% (w/w), respectively for SAG 30.81 and LB 572. The results showed that the organism grew better in acidic to neutral pH than the alkaline. Maximum biomass was reported in the cultures of green alga grown at neutral pH whereas lowest in alkaline pH 9.0 (Benerjee *et al*., 2002)

**3.2.3 Temperature**

Temperature may have a significant influence on the chemical composition of microalgae. Temperature was one of the most important environmental factors influencing the fatty acid composition, and it was shown to influence cell concentration, cell metabolism, and nutritional needs of the cell and biomass composition (Jensen and Knutsen, 1993)**.**

Qin (2005) reported the effect of temperature for growth and hydrocarbon production by *B. braunii* China strain 1. The results shown that optimal temperature for the growth of China strain 1 was 23 °C, though many studies have shown that the optimal temperature for majority of *B. braunii* strains was about 25 °C. They found that *B. braunii* could tolerate 30 °C, but the algal growth was slowest and turned into the stationary phase in an early stage. Moreover, the optimum temperature for various strains of *B. braunii* could fluctuate from 20 to 28°C. It seemed that China strain 1 is a relatively cold-favoring strain. They also found that the biomass and lipid content of *B. braunii* declined when temperature increased.

The algal colour under high temperature was brown and most of cells inclined to flocculate. Moreover it was easy to find air bubbles on the surface when stirring the liquid. Compared with high temperature, the algal color under lower temperatures was fresh green and the intercellular particles were clearly observed (data not shown) (Qin, 2005).

**3.2.4 Salt concentration**

Both algae and land plants differ greatly in the ability to tolerate NaCl in the environment. In particular, shifts in salt content may change the phytoplankton communities according to the tolerance capability of the microalgae. Tolerance depends on specific metabolic adjustments, which allow the maintenance of photosynthetic performance under unfavorable conditions. Salt stress affects basic processes of photosynthesis. Several studies have shown that photosystem II (PSII) is a major target of increased $Na^+$, mainly due to the degradation of the D1 protein of the reaction center (RCII) and also due to alterations of the water oxidation complex (Sudhir and Murthy, 2004). Sudhir *et al*. (2005) have recently shown that NaCl also induces degradation of the CP47 inner antenna of PSII, leading to impair energy transfer to the PSII reaction center. NaCl-dependent changes in the light harvesting complex II and photosystem II coupling occur in the halotolerant green alga *Dunaliella salina* (Liu and Shen, 2006). In plants adapted to high salt conditions (halophytes), PSII presents interesting, unique features such as high tolerance to photoinhibition (Qiu *et al*., 2003) and increased thermostability (Lu *et al*., 2003; Wen *et al*., 2005).

*B. braunii* could not only survive in freshwater, but also adapt to large salinity variations (Derenne *et al*., 1992). In Qin (2005)'s study, the maximum biomass and lipid production were obtained in the 0.15 M NaCl medium. The biomass yield and the cellular composition of *B. braunii* race A are affected by the salinity (NaCl concentration) of the culture medium. A slight increase in the lipid content was observed as a result of increasing salinity until the salinity equaled that of seawater. Lower than normal content of protein, carbohydrates, and pigments were found in cells grown at 0.5 M NaCl (Ben-Amotz *et al*., 1985). Although the total lipid content of cells remained constant, the composition of lipids changed with NaCl concentration. In the lipid fraction the amount of hydrocarbons, acylglycerol, and phosphorus remained constant, but the hexose content (especially galactose) decreased. This was attributed to the consumption of UDPgalactose in the biosynthesis of osmoregulatory compounds. There was also a change in the fatty acid distribution in polar lipids, and the proportion of polyunsaturated fatty acids was higher than the saturated fatty acids when the salinity increased. The most important effect of saline stress was a proportional increase of α-laminaribose (o-β-D-glucopyranosyl-[1-3]- α-D-glucopyranose) content, which acts as an osmoprotectant. The concentration of α-laminaribose tends to be lower in the Gottingen strain than in the Austin strain of the alga, and this explains the higher salt tolerance of Austin strain. Although organic osmoregulatory compounds (e.g., glycerol, glycine) are known to occur in algae, the osmoprotectory activity of α-laminaribose was first reported in *B. braunii* (Vanquez and Bertha, 1991).

Qin (2005) demonstrates that *B. braunii* China 1 failed to grow if salinity was higher than 0.5 M of NaCl. High algal mortality occurred in 0.7 M of NaCl. The results showed that the amount of intercellular particles and cell division were obviously reduced when salinity was up to 0.5 M NaCl. Since, that increased saline stress does not result in lipid accumulation inside the cells. The phenomenon of flocculation in this experiment might be due to the death of cells or to some liberation of substance from the cells for protection.

A moderate increase in salinity (<0.25 M of NaCl) could stimulate algal growth and lipid content. A recent study has shown that the lipid composition, especially the polar lipids, can be changed by salinity. Therefore, the salinity manipulation may be used as a tool to yield algal biomass containing desired lipid composition (Vazquez-Duhalt and Arredondo-Vego, 1991).

Rao *et al*. (2007) reported that growth of *B. braunii* (race 'A') and production of its constituents via, hydrocarbon, carbohydrate, fatty acid, and carotenoids were influenced by different levels of salinity. Under salinity at 34 mM and 85 mM, 1.7–2.25-fold increase in the relative proportion of palmitic acid and two fold increases in oleic acid were observed. The increase in biomass yields and changes in other constituents indicated the influence of salinity and the organism's adaptability to the tested levels of salinity (17mM to 85mM). However, Hart *et al*. (1991) showed the reduced growth at higher salinities due to decrease in photosynthetic rate.

### 3.2.5 Metal ion

The role of Fe nutrition in phytoplankton has attracted much attention. The availability of Fe for microbial uptake appears to be an important variable in determining the stability and composition of aquatic ecosystems. Phytoplankton, like other living organisms, exhibits a specific nutritional requirement for Fe (Keshtacher-Liebson *et al*., 1995). Fe is essential for $NO_3^-$ utilization, chlorophyll biosynthesis, and numerous other cellular functions in phytoplankton.

In recent years, the function of iron in microalgae growth has been noted by many investigators. On the basis of fluorescence measurements taken over 12 years, Behrenfeld *et al*. (2006) demonstrated that iron had a key function in regulating phytoplankton biomass in both HNLC (high nitrate low-chlorophyll) and oligotrophic waters near the Equator and further south. However, whether the ‘‘bioavailable’’ iron deficiency is one of the main factors limiting algal biomass productivity in batch culture conditions is, to the best of our knowledge, unknown. And some biochemical components such as lipids in response to iron have not been well documented in microalgae. Liu *et al*. (2008) showed that high iron concentration could also induce considerable lipid accumulation in marine strain *C. vulgaris*. The results also suggested that some metabolic pathways related to the lipid accumulation in *C. vulgaris* were probably modified by high level of iron concentration in the initial medium.

**4. Lipid production and wastewater treatment**

Microalgal culture systems show a great deal of versatility that allows their use in different processes, such as wastewater treatment, production of animal food, production of fertilizers, and production of common and fine chemicals (Gonzalez *et al*., 1997). It was reported that the ability of microalgae to assimilate inorganic nitrogen into their biomass may be an effective method for nitrogen compound detoxification (An *et al*., 2003).

Many stock farms in the world and livestock wastewater is not treated adequately, taking into account its high contents of nitrogen and phosphorus, low C/N ratio, fluctuation of loading rate, toxicity of free ammonia, *etc*. Gustafsson (1997) reported that piggery wastewater contains very high concentrations of inorganic and organic nitrogen compounds, and other nutrients. Therefore, the use of algae to treat such a wastewater has been investigated. Since, microalgae can reduce excess nitrogen and phosphorus compound levels, they have been used in livestock wastewater treatment processes (Pizarro *et al*., 2006)

Unicellular microalgae *Chlorella vulgaris* and *Scenedesmus dimorphus* were capable of removing up to 55% of the phosphates from dairy industry and pig farming wastewaters (Gonzalez *et al*., 1997). Another strain of *Scenedesmus* sp. grown in artificial wastewater, also removed more than 50% of the phosphates. Production of starch yielded wastewater with a unique C:N:P ratio of 24:0.14:1. This effluent supported good growth of *Spirulina platensis*. Reductions in phosphate levels of the digested effluent reached over 99% (Phang *et al*., 2000). Ogbonna *et al*. (2000) also reported that *S. platensis* could efficiently remove nitrates, ammonia, and phosphates from synthetic wastewater.

Potentially, domestic sewage that has been pretreated by activated sludge treatment can be used as a medium for hydrocarbon production by *B. braunii*. Using sewage can reduce the cost of producing the hydrocarbons. Secondary stage-treated sewage (STS) has been characterized, and found to contain a large amount of nitrogen (as nitrate) and phosphorus (as $PO_4^{3-}$). When *B. braunii* was cultured on STS aerated with 1% $CO_2$ at 25°C and without controlling the pH, the nitrate content could be reduced from 7.67 g/m$^3$ to a level below detection (<0.01 g/m$^3$ ). The alga was able to consume $PO_4^{3-}$ present at quite low levels (0.02 g/m$^3$). $NO_2^-$ appeared to be consumed after $NO_3^-$, but $NH_4^+$ was not utilized. The growth rate was 0.35 g/m$^3$ per week and the hydrocarbon content was 53% compared with 58% in modified Chu-13 medium under the same conditions (Sawayama *et al*., 1992).

The use of *B. braunii* for tertiary treatment of municipal sewage and the conversion of the biomass to energy can reduce global warming and eutrophication. *Botryococcus* cannot grow on industrial wastewater containing a high concentration of inorganic ions. Attempts have been made to develop continuous biomass production processes using secondary-stage treated wastewater as the culture medium. A 2-l continuous flow bioreactor operated at a dilution rate of 0.57 d$^{-1}$ attained a biomass productivity of 196 g/m$^3$ per week. The bioreactor was aerated with 1% $CO_2$-enriched air (500 ml/min) and illuminated continuously at 68 μEm$^{-2}$s$^{-1}$. Under these conditions, nitrates and phosphates decreased from 5.5 g/m$^3$ and 0.08 g/m$^3$ to 4.0 g/m$^3$ and 0.03 g/m$^3$, respectively (Sawayama *et al*., 1994). Potentially, the productivity of continuous algal culture can be improved by optimizing the dilution rate. The optimal dilution rate is expected to depend on the strength of wastewater and the intensity of illumination. Productivity can be enhanced further by culturing the cells at an optimal light–dark cycling frequency (Molina *et al*., 1999, 2000, 2001).

*B. braunii* grew well and was the dominant species in mixed cultures of other bacteria and algae (specially *Oscillatoria*) when cultivated on swine wastewater in an outdoor photobioreactor. At 25°C, the removal rates of chemical oxygen demand

(COD), the total dissolved carbon (TDC), total nitrogen and phosphorus were 0.83, 0.61, 0.69, and 0.16 g/$m^3$ per day, respectively. The lipid content of the alga grown in swine wastewater and the modified Chu-13 medium were 32.8 ± 3.2% and 33.2 ± 2.6%, respectively (Lee *et al*., 1999).

An *et al*. (2003) studied the conduct on the removal of nitrogen and phosphorus from piggery wastewater during growth of *B. braunii* UTEX 572, together with measurements of hydrocarbon formation by this alga. The influence of the initial nitrogen and phosphorus concentrations on the optimum concentration range for alga culture in secondarily treated piggery wastewater was tested. A high cell density (> 7 $g.l^{-1}.d^{-1}$) was obtained with 510 mg/l $NO_3$-N. Growth increased with nitrogen concentration at the basal phosphorus concentration (14 mg P/l). A dry cell weight of 8.5 mg/l and hydrocarbon level of 0.95 g/l were obtained, and nitrate was removed at a rate of 620 mg N/l. These results indicated that pretreated piggery wastewater provides a good culture medium for the growth and hydrocarbon production by *B. braunii*.

Dumrattana and Tansakul (2006) cultivated *B. braunii* in effluent (nitrate 78 mg/l) from seafood processing plant and adjusting effluent to a three-fold reduction in nitrate (nitrate 26 mg/l) compared with synthetic medium (Modified Chu 13) under continuous illumination. The highest algal growth was obtained in effluent with a dry weight of 13.61 g/l. *B. braunii* could reduce nitrate, phosphorus and ammonium-nitrogen concentrations in the effluent by 73%, 74% and 79%, respectively. In addition, the hydrocarbon synthesis by the alga *B. braunii* was 34% of its dry weight.

## 5. Applications of hydrocarbon from *B. braunii*

### 5.1 Botryococcenes

*B. braunii* is noteworthy for synthesizing and accumulating high amounts of hydrocarbons and ether lipids. The wide biodiversity of *B. braunii* results in the production of three types of hydrocarbons, associated with three chemical races: A (alkadienes, trienes), B (triterpenes) and L (tetraterpene). In addition, hydrocarbon levels and distributions vary with algal origin. Botryococcenes and methylated squalenes, hydrocarbons of race B, have received much attention due to an original terpenoid structure, a close biosynthetic relationship with squalene and an important role in geochemistry. Indeed, the fully hydrogenated derivatives, botryococcanes and tetrametylsqualane, are specific markers of *B. braunii* which are found in petroleum and oil shales, sometimes in high amounts (Summons *et al*., 2002; Metzger and Largeau, 2005). Botryococcenes, like methylated squalenes, originate from the non-mevalonate terpenoid pathway, likely via presqualene diphosphate as a close precursor. Successive methylations of $C_{30}$ botryococcene and squalene give rise to their respective higher homologues.

In the literature, the oil from *B. braunii* is often associated almost exclusively with hydrocarbons. However, numerous other compounds, such as ether lipids, are present in the extracts. These high-molecular-weight ether lipids derive from lower metabolites, notably hydrocarbons, via the coupling of epoxide derivatives. Strains rich in these unusual compounds could be the source of novel enzymes responsible for the formation of ether bonds (Metzger and Largeau, 2005).

In addition to hydrocarbons, *B. braunii* also synthesizes classic lipids such as fatty acids, triacylglycerols and sterols (Metzger and Largeau, 1999). A second feature of this alga is the production of numerous ether lipids of a new type which are not glycerol derivatives, like those occurring in all other living organisms. In each race, ether lipids are closely related to hydrocarbons; and in some strains their production can be largely dominant. Lastly, non-polysaccharide biopolymers of very high molecular weight ($10^4$ Da to $4\times10^6$ Da), polyaldehydes and polyacetals, have been isolated from lipid extracts of *B. braunii*. Their occurrence and possible functions as precursors of the insoluble polymeric material building up the outer walls of the algae were recently reviewed by Metzger and Largeau (2002). Furthermore, oil production via $CO_2$ fixation could also mitigate the emission of greenhouse gases (Pedroni *et al*., 2001).

Botryococcenes can be chemically converted into fuels. Transesterification can not be used to make biodiesel from botryococcenes. This is because botryococcene is not a regular 'vegetable oil' (which is a fatty acid triglyceride) but is instead a triterpene, and lacks the free oxygen for transesterification. It can be used as feedstock for hydrocracking in an oil refinery to produce octane (gasoline, a.k.a. petrol), kerosene, and diesel. Up to 86% of its dry weight can be long chain hydrocarbons. Hydrocarbon oils extracted from *B. braunii* produced a distillate comprising of 67% gasoline fraction, 15% aviation turbine fuel, 15% diesel fuel fraction and 3% residual oil when hydrocracked (Gelpi *et al*., 1970 by Banerjee *et al*., 2002)

**5.2 Biodiesel from algae**

While a number of bio-feedstock are currently being experimented for biodiesel (and ethanol) production, algae have emerged as one of the most promising sources especially for biodiesel production, for main reasons, the yields of oil from algae are orders of magnitude higher than those for traditional oilseeds, and algae can grow in places away from the farmlands and forests, thus minimising the damages caused to the eco- and food chain systems. Algae can be grown in sewages and next to power-plant smokestacks where they digest the pollutants and give us oil. Though research into algae oil as a source for biodiesel is not new, the current oil crises and fast depleting fossil oil reserves have made it more imperative for organizations and countries to invest more time and efforts into research on suitable renewable feedstock such as algae.

Just by way of history, petroleum is widely believed to have had its origins in kerogen, which is easily converted to an oily substance under conditions of high pressure and temperature. Kerogen (Wikipedia, 2008d) is formed from algae, biodegraded organic compounds, plankton, bacteria, plant material, etc., by biochemical and/or chemical reactions such as digenesis and catagenesis. Several studies have been conducted to simulate petroleum formation by pyrolysis. On the basis of these findings, it can be inferred that algae grown in $CO_2$-enriched air can yield oil that can be converted into biodiesel. Such an approach can contribute to solving two major problems: air pollution resulting from $CO_2$ evolution, and future crises due to a shortage of energy sources.

The biodiesel from algae in itself is not significantly different from biodiesel produced from vegetable or plant oils. All biodiesel essentially are produced using triglycerides (commonly called fats) from the plant oils. Some differences could exist, though: Algae produce a lot of polyunsaturated, which may present a stability problem since higher levels of polyunsaturated fatty acids tend to decrease the stability of biodiesel. But polyunsaturated also have much lower melting points than monounsaturated or saturates, thus algal biodiesel should have much better cold weather properties than many other bio-feedstock. Since one of the disadvantages of biodiesel is their relatively poor performance in cold temperatures, it appears that algal biodiesel might score well on this point. The most significant different is however in the yield of algal oil, and hence biodiesel. According to some estimates, the yield of oil from algae is over 200 times the yield from the best-performing plant oils (Oilgae.com, 2008).

Miao and Wu (2006) study integrated method for the production of biodiesel from heterotrophic microalgal oil. They suggested that heterotrophic growth of *Chlorella protothecoides* resulted in the accumulation of a large amount of lipid in cells. Most of determine parameters of microalgal oil comply with the limits established by ASTM related to biodiesel quality (Antolin *et al*., 2002). The physical and fuel properties of biodiesel from microalgal oil in general were comparable to those of diesel fuel. The biodiesel from microalgal oil showed much lower cold filter plugging point of -11 °C in comparison with the diesel fuel.

**6. Photobioreactors**

Algae can be cultured in raceway pond and lakes. Because these systems are open to the elements, sometimes called "open-pond" systems, they are much more vulnerable to contamination by other microorganisms, such as invasive algal species or bacteria. Because of these factors, the number of species successfully cultivated in an "open-pond" system for a specific purpose (such as for food, for the production of oil, or for pigments) are relatively limited. In open systems one does not have control over water temperature and lighting conditions. The growing season is largely dependent on location and, aside from tropical areas, is limited to the warmer months. A major benefit to this type of system is that it is one of the cheaper ones to construct, in the very least only a trench or pond needs to be dug. It can also have some of the largest production capacities relative to other systems of comparable size and cost. This type of culture can be viable when the particular algae in question requires (or is able to survive) some sort of extreme condition that other algae can not survive. For instance, *Spirulina* sp. can grow in water with a high concentration of sodium bicarbonate and *Dunaliela salina* will grow in extremely salty water.

Algae can also be grown in a photobioreactor. A photobioreactor is a bioreactor which incorporates some type of light source. Virtually any translucent container could be called a photobioreactor, however the term is more commonly used to define a closed system, as opposed to an open tank or pond. Because these systems are closed, all essential nutrients must be introduced into the system to allow algae to grow and be cultivated. Essential nutrients include ($CO_2$ sequestration/carbon dioxide), water, minerals and light. A pond covered with a greenhouse could be considered a photobioreactor. A photobioreactor can be operated in batch mode but it is also possible to introduce a continuous stream of sterilized water containing nutrients, air, and carbon dioxide. As the algae grows, excess culture overflows and is harvested. If sufficient care is not taken, continuous bioreactors often collapse very quickly, however once they are successfully started, they can continue operating for long periods. An advantage of this type of algae culture is that algae in the log phase are produced which is generally of higher nutrient content than old algae. It can be shown that the maximum productivity for a bioreactor occurs when the "exchange rate" (time to exchange one volume of liquid) is equal to the "doubling time" (in mass or volume) of the algae.

Different types of photobioreactors include:

- tanks provided with a light source
- (polyethylene) sleeves or bags
- glass or plastic tubes.

Unlike open raceways, photobioreactors permit essentially single-species culture of microalgae for prolonged durations. Photobioreactors have been successfully used for producing large quantities of microalgal biomass (Carvalho *et al.*, 2006).

A tubular photobioreactor consists of an array of straight transparent tubes that are usually made of plastic or glass. This tubular array, or the solar collector, is where the sunlight is captured. The solar collector tubes are generally 0.1 m or less in diameter. Tube diameter is limited because light does not penetrate too deeply in the dense culture broth that is necessary for ensuring a high biomass productivity of the photobioreactor. Microalgal broth is circulated from a reservoir to the solar collector and back to the reservoir (Figure 4).

Instead of being laid horizontally on the ground, the tubes may be made of flexible plastic and coiled around a supporting frame to form a helical coil tubular photobioreactors (Figure 5). These types of photobioreactors are potentially useful for growing a small volume of microalgal broth, for example, for inoculating the larger tubular photobioreactors that are needed for producing biodiesel. Other variants of tubular photobioreactors exist (Carvalho *et al*., 2006), but are not widely used. Artificial illumination of tubular photobioreactors is technically feasible, but expensive compared with natural illumination. Nonetheless, artificial illumination has been used in large-scale biomass production particularly for high-value products (Carvalho *et al*., 2006; Chisti, 2007).

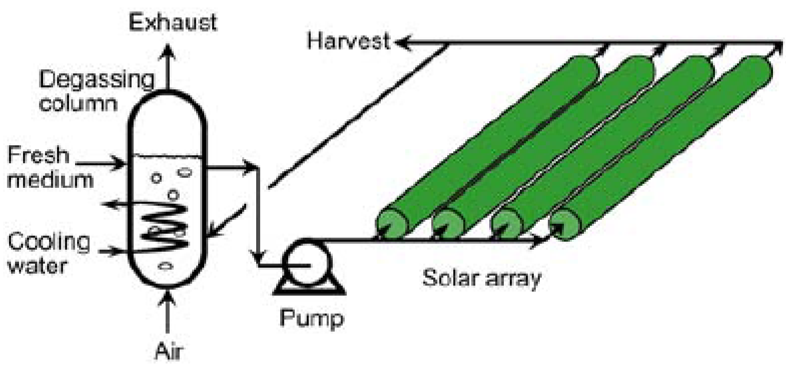


Figure 4 : A tubular photobioreactor with parallel run horizontal tubes.
Source: Chisti (2007)

Figure 5 : A 1000 L helical tubular photobioreactor at Murdoch, University, Australia. Source: Chisti (2007)

**7. Reduction of $CO_2$ by microalgae culture in photobioreactor**

Carbon dioxide ($CO_2$) is the principal greenhouse gas and its concentrations have increased rapidly since the onset of industrialization. In 1997, 7.4 billion tons of $CO_2$ were released into the atmosphere from anthropogenic sources; by the year 2100, this number will increase to 26 billion tons. During the last two decades, many attempts have been made to reduce atmospheric $CO_2$, for example by the use of renewable energy sources or by terrestrial sequestration of carbon (IPCC, 2001). One of the most understudied methods of $CO_2$ reduction is the use of microalgae that convert $CO_2$ from a point source into biomass. Microalgae use $CO_2$ efficiently because they can grow rapidly and can be readily incorporated into engineered systems, such as photobioreactors (Carvalho *et al*., 2006; Lee and Lee, 2003; Suh and Lee, 2003). The $CO_2$ fixation rate is related directly to light utilization efficiency and to cell density of microalgae. Microalgal $CO_2$ fixation involves photoautotrophic growth in which anthropogenically derived $CO_2$ may be used as a carbon source. Therefore, biomass measurements or growth rate evaluations are critical in assessing the potential of a microalgal culture system for directly removing $CO_2$. Microalgal photobioreactor can be used for $CO_2$ mitigation from waste gas with high concentration of $CO_2$ efficiently, if the effects of the $CO_2$ concentration in airstreams on microalgal cell growth could be well controlled (Chiu *et al*., 2008).

The New York State Energy Research and Development Authority (NYSERDA) was funding the project, which will test Green Fuel's $CO_2$ recycling technology (as shown in Figure 6). They explained around 40 % of U.S. carbon dioxide emissions come from fossil-fueled power plants. Green Fuel will use a mini-bioreactor system to assess the technical and economic viability of its technology, which would use algae to consume $CO_2$ emitted by the power plant. The algae could then be converted into biofuel. In a press release, the partners described the process this way: In the presence of light, the single-celled algae take up $CO_2$ to produce the energy that fuels plant life with a general rule of thumb being that two tons of algae remove one ton of $CO_2$. Once the algae are harvested, they can be converted to generate commercially viable by products such as ethanol or biodiesel (NYSERDA, 2008).

Figure 6 : Algae stored in tubes are busy capturing carbon dioxide in this reactor
Source: NYSERDA (2008)

Chiu *et al*. (2008) cultured microalga *Chlorella* sp. in a photobioreactor to assess biomass, lipid productivity and $CO_2$ reduction. They also determined the effects of cell density and $CO_2$ concentration on the growth of *Chlorella* sp. by using microalgal photobioreactor as $CO_2$ mitigation system is a practical approach for elimination of waste gas from the $CO_2$ emission (Figure 7). The results found that during an 8-day interval cultures in the semicontinuous cultivation, the specific growth rate and biomass of *Chlorella* sp. cultures in the conditions aerated 2–15% $CO_2$ were 0.58–0.66 $d^{-1}$ and 0.76–0.87 g/L, respectively. At $CO_2$ concentrations of 2%, 5%, 10% and 15%, the rate of $CO_2$ reduction was 0.261, 0.316, 0.466 and 0.573 g/h, and efficiency of $CO_2$ removal was 58%, 27%, 20% and 16%, respectively. The efficiency of $CO_2$ removal was similar in the single photobioreactor and in the six-parallel photobioreactor. However, $CO_2$ reduction, production of biomass, and production of lipid were six times greater in the six-parallel photobioreactor than those in the single photobioreactor.

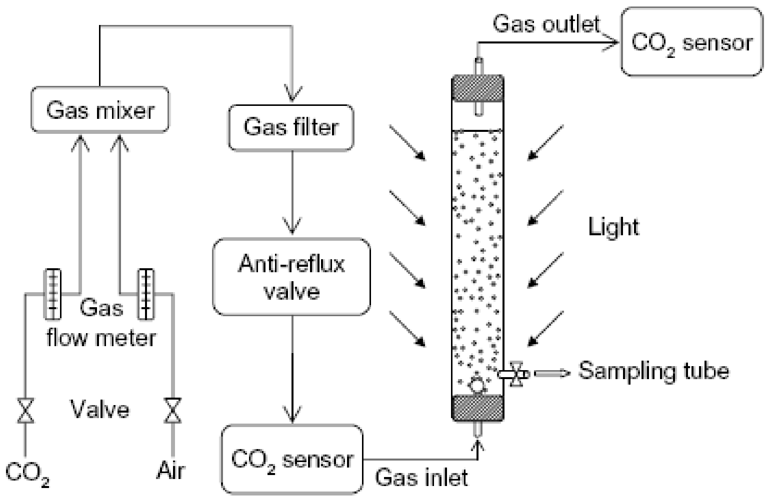


Figure 7 : Schematic diagram of the photobioreactor for the experiments on $CO_2$ reduction for batch and semicontinuous microalgal cultures.

Source : Chiu *et al*. (2008)

# CHAPTER 2

# MATERAILS AND METHODS

## Materials

### 1. Microalga Samples

The microalgal strains were isolated from lake and reservoir pond in the southern region of Thailand by plankton net. For *Botryococcus* PSU strain was obtained from Prince of Songkla University's reservoir.

### 2. Medium preparation

Samples were grown on modified Chu 13 medium which 1 L contains 0.2 g $KNO_3$, 0.04 g $K_2HPO_4$, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.054 g $CaCl_2 \cdot 2H_2O$, 0.01 g Fe citrate, 0.1g citric acid, 0.036 g $NaHCO_3$ and 1 mL of micro element, pH 6.7. The micro element 1 L consists of 2.85 g $H_3BO_3$, 1.8 g $MnCl_2 \cdot 4H_2O$, 0.02 g $ZnSO_4 \cdot 7H_2O$, 0.08 g $CuSO_4 \cdot 5H_2O$, 0.08 g $CoCl_2 \cdot 6H_2O$ and 0.05 g $Na_2MoO_4 \cdot 2H_2O$ (Largeau *et al*., 1980).

### 3. Effluent from seafood processing plant

The effluent used in this study was the secondarily pretreated (activated sludge pond) effluent from seafood processing plant CP factory (Songkhla, Thailand). The characteristics of the effluent were as follows: COD 740 mg/L, $NO_3^-$ 24.1 mg/L, $NH_4^+$-N 4.6 mg/L and $NO_2^-$-N 0.04 mg/L.

### 4. Chemicals

All chemicals and solvents used was reagent grade, and purchased from various suppliers.

**Methods**

**Analytical methods**

**1. Characteristics of effluent from seafood processing plant**

The effluent from seafood processing plant was characterized based on pH, Chemical Oxygen Demand (COD), ammonia (Phenate method), total nitrogen (Kjeldahl method) and nitrate (Cadmium reduction method) according to the standard method (APHA, AWWA and WPCF, 1995). Total sugar and reducing sugar in the broth were determined by Antrone method and DNS method, respectively. Effluent was filtered through mesh for discharged suspension solid and will be sterilized with autoclave (121◦C, 15 min) before used.

**2. Growth determination**

The cells were harvested by centrifugation at 1,585 x g for 15 minutes. Then the cell pellets were freeze-dried by freeze-drier (Type Labvista, FTS Duradry Freeze Dryer System, Triad Scientific Ltd., USA). The dry weight of the algal biomass was determined gravimetrically and growth was expressed in terms of dry weight. The specific growth rate ($\mu$) was calculated as the slope of the following equation:

$$\mathrm{Ln}\frac{C}{C_0} = \mu dt \qquad (1)$$

where $C_0$ is the initial biomass concentration (g/L) and $C$ is the biomass concentration (g/L) at any time $t$(Ceron Garcia *et al*., 2005).

**3. Lipid extraction**

Freeze-dried algal 100 mg were extracted with 50 mL of n-hexane and sonicated at 70 Hz intensity with sonicator (Model Transsonic 460/H, εlma Ltd, Germany) at room temperature (Mexwell *et al*., 1968). The extraction process was repeated twice. The suspension was filtered through Whatman No. 40 filter paper and the filtrate was taken into pre-weighed glass vial. The hexane solution was evaporated to dryness at 30°C under vacuum. Hydrocarbon content was measured gravimetrically and expressed as dry weight percentage (Dayananda *et al*., 2006).

**4. Fatty acid composition analysis**

The method for the production of biodiesel (fatty acid methyl esters; FAME) from extracted hydrocarbon involved the hydrolysis of the lipids followed by esterification (Jham *et al*., 1982). The fatty acid composition in the FAME were analyzed using a HP6850 Gas Chromatography equipped with a cross-linked capillary FFAP column (FFAP, a reaction product of polyethylene glycol 20000 and 2-nitrophthalic acid, Crompton, 2000) length 30 m, 0.32 mm I.D, 0.25 μm film thickness and flame ionization detector. Operating condition were as follows: inlet temperature 290 °C, oven temperature initial 210 °C hold for 12 min then ramp to 250 °C at 20°C/min, hold 8 min and detector temperature was 300°C. Fatty acids were identified by comparing their retention times with those of standard ones, and quantified based on their respective peak areas and normalized.

**5. Statistical analysis**

The data was calculated with mean values, and standard deviations (mean±SD) was determined from at least duplicate trials. Statistical significance of the results was evaluated by one way ANOVA (analytical of variance) and Duncan's multiple range tests ($P < 0.05$) using SPSS 10 software.

**Experimental methods**

**Experiment 1: Isolation and purification of lipid producing microalgae**

The samples (10-12 μm x 7-9 μm in size) were collected from lakes and freshwater ponds in southern Thailand (Songkhla, Trang, and Krabi provinces) with a plankton net (22μm). The *Botryococcus*-like green colonies were separated using a sterile micropipette washing method (Stein, 1973) and cultured in modified Chu 13 medium (Largeau *et al*., 1980). The algae were subjected to purification by serial dilution followed by plating. The individual colonies were isolated and inoculated into liquid medium (modified Chu 13 medium) and incubated at 25 ± 1ºC under 33 μmol.photon.$m^{-2}s^{-1}$ light intensity with 16:8 hrs light photoperiod. The purity of the culture was ensured by repeated plating and by regular observation under microscope. The experiment was carried out in Erlenmeyer flasks of 50 ml capacity, containing 20 ml modified Chu 13 medium for a period of 20 days. The culture flasks were inoculated (10% v/v) and incubated with 125 rpm agitation at 25 ± 1ºC under 33 μmol.photon.$m^{-2}s^{-1}$ light intensity with 16:8 hrs light and dark cycles. Cultures were harvested and dry biomass and lipid content were estimated at 5 days of intervals. All the experiments were carried out at least in duplicate.

**Experiment 2: Effect of physio-chemical environments**

**2.1 Effect of nitrogen limitation**

Isolated microalgae were inoculated in 50 mL Erlenmeyer flasks containing 20 mL modified Chu 13 medium and incubated with 125 rpm agitation at 25±1ºC under 33 μmol.photon.$m^{-2}s^{-1}$ light intensity with 16:8 h light photoperiod for 7 days. The nitrogen limitation trail was performed followed the method of Dayananda *et al*. (2005). The culture was then centrifuged at 1,585 x g for 15 minutes. Cell pellets washed twice with sterilized distilled water and re-suspended in modified Chu 13 medium without any nitrogen source (a nitrogen-deficiency condition) (Siron *et al*., 1989) and with a nitrogen source (a nitrogen-rich condition) as a control. The culture was incubated for 20 days. The dry biomass and lipid content were measured. All the experiments were carried out at least in duplicate.

**2.2 Effect of salt concentration**

Three levels of 0, 43 and 86 mM NaCl were added to the culture medium of four microalgae. Culture medium without NaCl addition was used as control. The algae were cultured at 25 ◦C under 33 μmol.photon.$m^{-2}s^{-1}$ light density with 16:8 hours light photoperiod and 125 rpm agitation. The experimental duration and other protocols were the same as those in the nitrogen limitation trial. The responses of isolated microalgae in terms of dry biomass and lipid content under nitrogen-rich and nitrogen-limited conditions were monitored. The concentration of NaCl which gives the highest cell growth and lipid content was chosen for the next study.

**2.3 Effect of light intensity**

Three irradiances of 33, 49.5 and 82.5 μmol.photon.$m^{-2}s^{-1}$ with 16:8 hours light photoperiod were used to test the response of the four microalgae in terms of growth and lipid content. The algae were culture at 25 ◦C with 125 rpm agitation. The experimental duration and other protocols were the same as those in the nitrogen limitation. The light intensity which gives the highest cell growth and lipid content was chosen for the next study.

**2.4 Effect of iron ($Fe^{3+}$) concentration**

Since ferric citrate was an iron compound used in modified Chu 13, the effect of its concentration was investigated. Five levels of 0, 0.037, 0.37 and 0.74 mM of ferric citrate were added to the culture medium of four microalgae. Culture medium supplements 0.037 mM of ferric citrate was used as control as it is the concentration of ferric citrate in modified Chu medium. The algae were cultured at 25 ◦C under 49.5 µmol.photon.$m^{-2}s^{-1}$ light intensity with 16:8 hours light photoperiod and 125 rpm agitation. The experimental duration and other protocols were the same as those in the nitrogen limitation trial.

**Experiment 3: Fatty acid composition of biodiesel from isolated microalgal lipid**

The best of each condition from Experiment 2 was chosen to study the profile of growth and lipid production of four isolated strains. Then, the fatty acid composition of each strain under optimal condition was determined.

**Experiment 4: Growth and lipid production by selected microalga in effluent from seafood processing plant**

**Starter preparation**

Isolated microalga that gives highest lipid production from the previous section was selected. It was inoculated into 100 ml of modified Chu 13 medium, adjusted pH to 6.7 with 1M KOH in 250-ml flask and incubated with shaking at 125 rpm at 25°C under 33 µmol.photon.$m^{-2}s^{-1}$ light intensity with 16:8 hours light photoperiod for 7 day. The culture broth was filtered through Whatman No. 40 filter paper and diluted with the medium to obtain proper dilution with $OD_{435}$ = 2 and used as starter culture. 10% inoculums of starter were used.

**4.1 Dilution of effluent from seafood processing plant**

Selected microalga was cultured in the effluent that was diluted one, two, and four times compared with non-diluted effluent. The effluent was sterilized with autoclave before used and pH was adjusted to 6.7 with 1M KOH. The microalga was incubated at 25°C, 125 rpm agitation under 49.5 µmol.photon.$m^{-2}s^{-1}$ light intensity with 16:8 hours light photoperiod. The samples were taken intervals 5 day. Cell growth

($OD_{435}$) and dry cell weight was used to calculate specific growth rate, doubling time and lipid content was measured. Total nitrate was measured to investigate the removal efficiencies of nitrate by cultivation of this microalga.

In batch kinetic, the initial substrate removal rate, $R_i$, was used to determine the coefficients of nitrate removal. The initial substrate removal rate was calculated as given in Eq. (2)

$$R_i = \frac{S_1 - S_2}{t_2 - t_1} \tag{2}$$

Where $S_1$ is the initial $NO_3$-N concentrations, $S_2$ is the corresponding substrate concentration at $t_t$ which is the time at which concentration of the substance did change (Aslan and Kapdan, 2006). The effluent medium which gives the highest cell growth and lipid production was chosen for the next study.

### 4.2 Effect of concentration of carbon dioxide

A two-tier culture vessel consisting of two 250 ml small neck Erlenmeyer flask was use for photoautotrophic growth experiments (Figure 8). The lower compartment of the flask contained 100 ml of a 3 M buffer mixture ($KHCO_3/K_2CO_3$) at specific ratios which generated specific $CO_2$ partial pressure in the two-tier flask (as shown in Table 4). The concentration of buffer mixture was determined to get a $CO_2$ partial pressure of 0.5, 1.0 and 2.0% (v/v), respectively, as given by Tripathi *et al*., (2000). The culture of isolated microalga was performed in 250 ml of the optimal effluent medium**,** pH 6.7. The culture was grown at best condition of light intensity from previous section. The percentage of $CO_2$ which gives the highest cell growth and lipid production was chosen for the next study.

Figure 8. A modified two-tier vessel for phototrophic growth containing effluent medium in upper flask and $KHCO_3/K_2CO_3$ buffer mixture in the lower compartment.

Table 4: The establish of $CO_2$ partial pressure by $KHCO_3/K_2CO_3$

| Stock solution $KHCO_3/K_2CO_3$ (mole/l) | Buffer mixture $KHCO_3/K_2CO_3$ (ml) | $CO_2$ partial pressure (%v/v) |
|---|---|---|
| 3.0 | 50/50 | 0.5 ± 0.1 |
| 3.0 | 62/38 | 1.0 ± 0.1 |
| 3.0 | 73/27 | 2.0 ± 0.1 |

**Experiment 5: Growth and lipid production of selected microalga under photoautotrophic, heterotrophic and mixotrophic conditions**

**5.1 Effect of culture condition**

The starter of selected strain was inoculated in modified Chu 13 medium and incubated with 125 rpm agitation at 25±1ºC under 33 μmol.photon.$m^{-2}s^{-1}$ with 16:8 hours light and dark cycles for 7 days. The culture was inoculated in 20 mL modified Chu 13 medium and incubated with 125 rpm agitation at 25 ºC under the optimum intense illumination 49.5 μmol.photon.$m^{-2}s^{-1}$ with 16:8 hours light and dark cycle as photoautotrophic condition. For mixotrophic and heterotrophic condition, glucose was used as a carbon source at a concentration of 5 g/L with and without light, respectively. Dry weight and lipid content were estimated at 5 days of interval. All the experiments were carried out at least in duplicate.

**5.2 Effect of glucose and molasses concentrations under mixotrophic condition**

Cultures were performed in 50 ml conical flasks containing 20 ml modified Chu 13 medium broth with supplement varied glucose and molasses concentration (5-20 g/L) as carbon source. The algae were cultured at 25 ◦C under 49.5 μmol.photon.$m^{-2}s^{-1}$ light intensity with 16:8 hours light photoperiod and 125 rpm agitation. The experimental duration and other protocols were the same as those in the photoautotrophic condition.

**Experiment 6: Batch and semi-continuous cultures**

Batch culture was carried out in a 2 L stirred tank photobioreactor with 1.5 L working volume. The selected microalga was grown in undiluted effluent. The cultures were carried out with 125 rpm agitation at 25 ºC under the optimum intense illumination 49.5 µmol.photon.m$^{-2}$s$^{-1}$ with 16:8 hours light and dark cycle. A pH of the cultural was controlled at 6.7 by injection of carbon dioxide. Carbon dioxide was fed in response to signals from pH sensor.  Dry cell weight and lipid content were estimated daily. In semi-continuous culture, 500 mL of medium was withdraw and replaced with fresh effluent medium in the same volume at 4 days of intervals. The effluent was sterilized with autoclave before used.

Figure 9. The set apparatus for *Botryococcus* TRG strain cultivation under batch and semi-continuous regime

**Experiment 7: Outdoor cultivation in a horizontal tubular photobioreactor**

The reactors consisted of four-tubes of polyacrylic tubes (length: 1 m, diameter: 2.2 cm, wall thickness: 0.4 cm, volume: 4 L) which acts as solar receiver, linked by PVC tubes to 20 L reservoir tank and air pump to feed and circulate the culture through the tubes. The overall starting volume was 14 L. The experiment was conducted in undiluted effluent medium with semi-continuous regime feeding strategy. A pH of the cultural was controlled at 6.7 by injection of carbon dioxide. Carbon dioxide was fed in response to signals from pH sensor. Temperature was maintained approximately at 25 ºC with circulating cooling water. Aliquots of the culture were manually sampled once a day. The biomass and lipid content were measured. The system was operated in semi-continuous regime, supporting a pre-established cell density by removal of part of the cell suspension (5 L) and replacement with fresh medium. The effluent was sterilized with autoclave before used. The photobioreactor was sterilized by 2% (v/v) chlorine solution.

Figure 10. A tubular photobioreactor for Botryococcus TRG strain outdoor cultivation

# CHAPTER 3

# RESULTS AND DISCUSSION

## 1. Isolation and purification of lipid producing microalgae

The samples of *Botryococcus*-like green microalgae were collected from lakes and reservoir ponds in southern region of Thailand by 22 μm nylon plankton net. The sample site was selected based on its good water quality. The characteristics of water from each site are shown in Table 5. The pH of these sites was neutral (6.7-7.2), most of the sites have clear water with a slight blue tinge as shown the optical density at 435 nm in the range of 0.074-0.126. Those of lakes and reservoir ponds have low COD value (328-1138 mg/l) that is green algae tend to dominate in lakes with a low COD. Banerjee *et al*. (2002) reported that microalgal *B. braunii* can grow better in acidic to neutral than alkaline. It was also reported that the colonial microalga, *B. braunii* is widespread in fresh and brackish waters of all continents. The cosmopolitan nature of this alga is confirmed by the strains originating in the USA, Portugal, Bolivia, France, Ivory Coast, Morocco, Philippines, Thailand, and the West Indies (Banerjee *et al*., 2002). It has been reported that, *B. braunii* exists in the form of blooms in freshwater bodies like ponds, lakes and reservoirs (Metzger and Largeau, 2005).

Four isolated strains were isolated from lakes and reservoir ponds in southern Thailand (Songkhla, Trang and Krabi provinces). They were named as SK (isolated from Kukud lake, Songkhla province), TRG (isolated from Thalae Song Hong, Trang province), PSU (isolated from PSU reservoir, Songkhla province) and KB (isolated from Liknite reservoir, Krabi province). The morphological examination under a microscope found that SK showed bright green color, regularly pyramid shaped colloidal cell and held together by a lipid biofilm matrix. While TRG, PSU and KB strains showed dark green color, irregular colonies consisting of hundreds of elliptical cells interconnected by strands of tough mucilage (as shown in Figure 11). The morphology of isolated *Botryococcus* spp. in this study was similar to *B. braunii* in the previous reports. Benerjee *et al*. (2002) reported that the cells of *B. braunii* are embedded in a communal extracellular matrix (or “cup”), which is impregnated with oils and cellular exudates. Cells are attached to each other by a refringent material that sometimes links two or more distinct clumps of cell. Metzger and Largeau (2005) reported that within each chemical race and for the same strain the morphology of the alga could vary in relation to age and culture conditions.

The growth and lipid content profiles of four strains are shown in Figure 12. The specific growth rate of each strain was calculated as described in Materials and Methods. Among four strains, KB strain grew fastest and showed highest specific growth rate of 0.223 $d^{-1}$ followed by TRG strain (0.182 $d^{-1}$) and SK strain (0.135 $d^{-1}$). While PSU strain grew slowly and showed the lowest specific growth rate (0.061 $d^{-1}$). Under growth condition with rich nitrogen source, TRG strain gave the highest amount of lipid content of 25.8% based on its dry biomass weight and showed the highest lipid productivity of 46.9 mg $L^{-1}d^{-1}$ (Table 6). Although KB strain gave the highest specific growth rate, the lower lipid productivity of 39.7 mg $L^{-1}d^{-1}$ was obtained due to its lower lipid content (17.8%) compared with TRG strain. The lipid contents in SK and PSU strains were 15.8% and 5.7%, respectively.

Some *B. braunii* strains produced quite a small amount of hydrocarbon because they accumulate a large amount of polysaccharides instead of hydrocarbons (Fernandes *et al*., 1989). Comparison of lipid contents of newly isolated four *Botryococcus* strains in the present study with other reports are shown in Table 7. Okada *et al*. (1995) isolated four new strains of *B. braunii* (Yamanaka, Yayoi, Kawaguchi-1 and Kawaguchi-2) from Japanese waters. The Yayoi strain gave highest hydrocarbon content of 37.9% by dry weight and that of another strains ranged from 9.7-18.8%. Tansakul *et al*. (2005) isolated *B. braunii* from Prince of Songkla University's reservoir which gave hydrocarbon content 17.89% of its dry weight. In addition, Dayananda *et al*. (2007) collected microalga *B. braunii* from Bear Shola Falls at Kodaikanal, Tamil Nadu, India. Hydrocarbon content of the isolated organism was in the range of 13-18% of its dry biomass. Although the lipid content of a newly isolated TRG strain in the present study was lower than Yayoi strain isolated by Okada *et al*. (1995), it was much higher than the other reports (Okada *et al*., 1995, Tansakul *et al*., 2005, Dayananda *et al*., 2007). In addition, it would be useful to conduct further study on the local isolated strains to investigate the feasibility of the mass production of biodiesel using microalgae.

Table 5 : Characteristics of sample collected sites

| Site | Characteristic | | |
|---|---|---|---|
| | pH | $OD_{435}$ | COD ($mg.L^{-1}$) |
| PSU reservoir pond, Songkhla province : (PSU) | 6.47 | 0.074 | 328 |
| Kukud lake, Songkhla province : (SK) | 7.23 | 0.214 | 557 |
| Thalae Song Hong lake, Trang Province : (TRG) | 6.72 | 0.088 | 1138 |
| Liknite reservoir pond, Krabi Province : (KB) | 6.86 | 0.126 | 342 |

Table 6 : Growth and lipid content and productivity of four isolated *Botryococcus* strains

| Strain | Specific growth rate ($d^{-1}$) | Lipid content (%) | Lipid productivity ($mg\ L^{-1}d^{-1}$) |
|---|---|---|---|
| SK | 0.135 | 15.8 | 21.3 |
| TRG | 0.182 | 25.8 | 46.9 |
| PSU | 0.061 | 5.7 | 3.5 |
| KB | 0.223 | 17.8 | 39.7 |

Notes: The lipid productivity was calculated by the maximum hydrocarbon content multiplied by the specific growth rate.

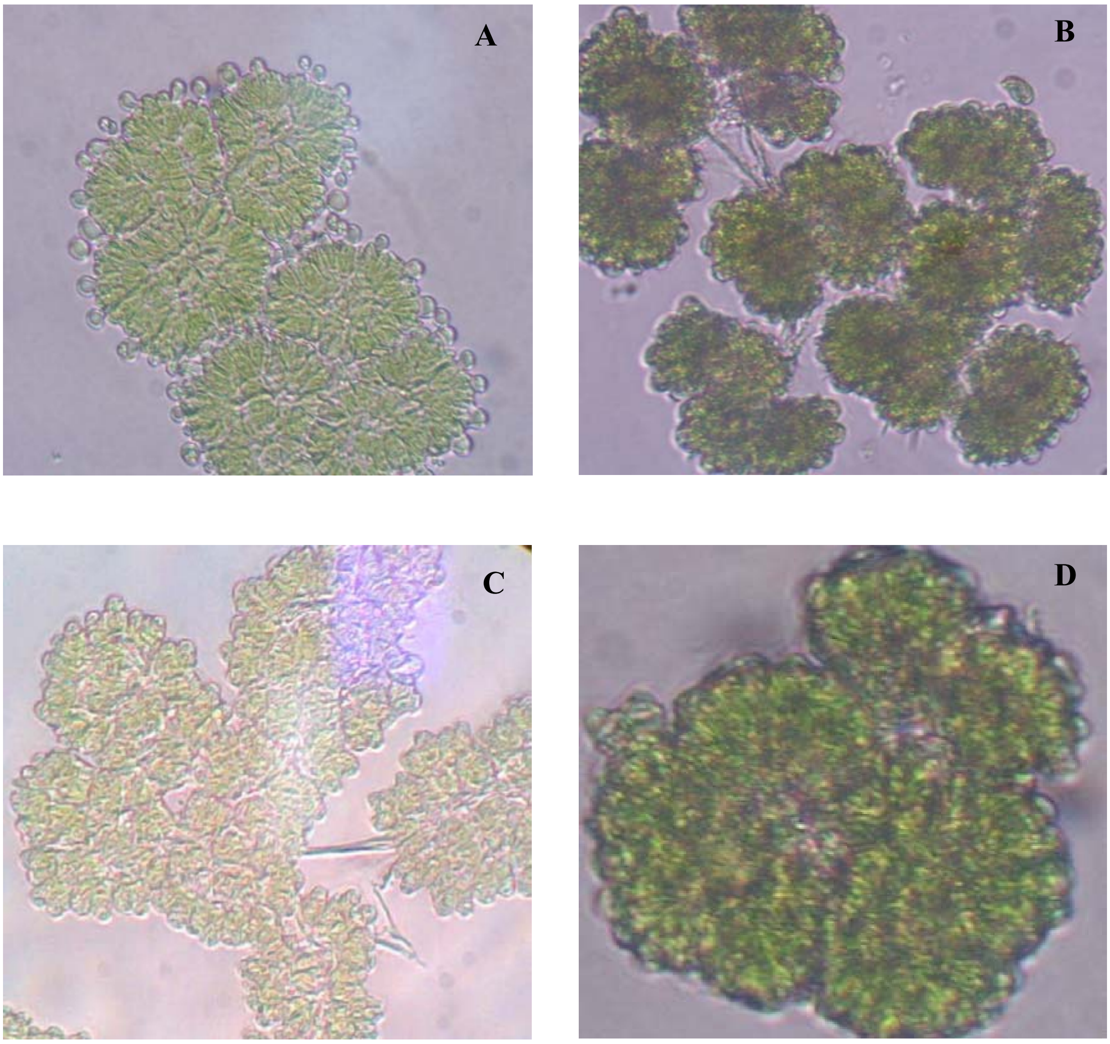


Figure 11. The algal microstructure of *Botryococcus* spp. isolated from lakes and fresh water ponds in southern region of Thailand under light microscopic (40x). A: SK strain, B: TRG strain, C: PSU strain, and D: KB strain.

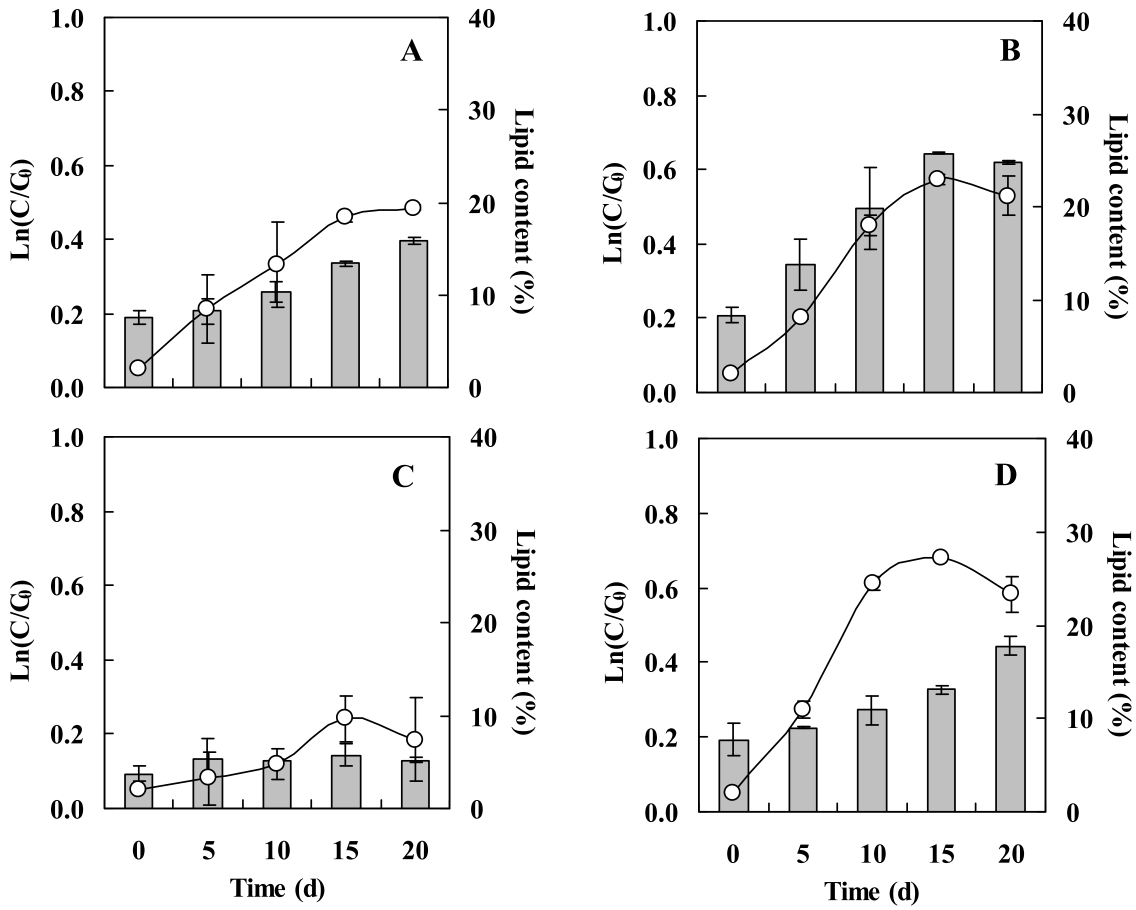


Figure 12 : Growths (line) and lipid contents (bar) of four *Botryococcus* strains. A: SK, B: TRG, C: PSU and D: KB strains. The algal growth was represented as Ln ($C/C_0$) versus cultivation time.

Table 7: Lipid content of newly isolated *Botryococcus* strains in the present study and the previous reports

| Strain | Lipid content (%) | Reference |
|---|---|---|
| SK | 15.8 | In present study |
| TRG | 25.8 | In present study |
| PSU | 5.7 | In present study |
| KB | 17.8 | In present study |
| Yayoi | 33.0 | Okada *et al*., 1995 |
| Yamanaka | 16.1 | Okada *et al*., 1995 |
| Kawaguchi-1 | 18.8 | Okada *et al.,* 1995 |
| Kawaguchi-2 | 9.7 | Okada *et al*., 1995 |
| *B. braunii* N-836 | 13-18 | Dayananda *et al*., 2007 |
| *B. braunii* CFTRI- Bb1 | 13-18 | Dayananda *et al*., 2007 |

## 2. Effect of physio-chemical environments

### 2.1 Effect of nitrogen limitation

Under nitrogen starvation, the content of photosynthetic pigments in the cell decrease, and the rate of photosynthesis are reduced. Microalgae appear to respond to nitrogen starvation condition by degrading nitrogen containing macromolecules and accumulating carbon reserve compounds, such as polysaccharides and fats (Banerjee *et al*., 2002; Dayananda *et al*., 2005). In this study, the lipid production of four isolated *Botryococcus* strains under nitrogen-limited condition was evaluated.

The impact of nitrogen limitation on algal growth and lipid production are showed in Figure 13. The maximum algal growth was represented as Ln (Cmax/C0). The maximum algal biomass for TRG and PSU was obtained at 15 days while, the maximum algal biomass for SK and KB was obtained at 10 days. An increase in algal biomass (the positive value of Ln (Cmax/C0)) was found under nitrogen-rich condition for all strains. In the absence of a nitrogen source there was no growth observed and the cell was bleached. Although some loss in algal biomass (the negative value of Ln (Cmax/C0)) was found when the nitrogen source was limited, the lipid contents of four strains were increased. This result was in agreement with that of Singh and Kumar (1992). They reported that nitrogen starvation gave a 1.6-fold increase in lipid content compared with normal medium. The highest value of lipid content (32.3%) was found in TRG strain under nitrogen limitation. The lipid content in SK, PSU and KB strains under nitrogen limitation were also increased up to 20.7%, 14.3% and 23.9%, respectively. Nitrogen deficiency apparently decreases the rate of synthesis of δ-aminolevulenic acid, the first committed precursor of chlorophyll, which in turn lowers the extent of chlorophyll synthesis and cells were shifted to produce the reserve energy in the cell (Singh and Kumar, 1992).

It was also reported that *Botryococcus* spp. are capable of synthesizing exopolysaccharides (Casadevall *et al*., 1985). Cells of *Botryococcus* strain posses an internal fibrillar layer made of mucilaginous polysaccharides. The polysaccharide production occurs both during growth and stationary phases, but higher production rate are seen as the growth rate declines. This enhanced production is associated with the development of nitrogen limitation in the culture medium (Banerjee *et al*., 2002). However, in our experiment since the culture broth did not become viscous during 20 days of the cultivation, it was thought that the isolated strains might not produce exopolysaccharides.

As it has been previously reported as nitrogen (N)-limited algae, nitrogen-limited condition greatly reduces the synthesis of chloroplastic proteins, and among the pigments, chl a decreases, whereas carotinoids increase. During nitrogen-limited condition, algal cells have a surplus of carbon (C) metabolites that often accumulate as lipids (Ahlgren and Hyenstrand, 2003). For example, analyses of 18 freshwater and 11 marine algal species showed in most cases an increased lipid content with nitrogen-limited condition, often two to three times higher than cultures with repletion nitrogen (Shifrin and Chisholm 1981). Correlation of lipid productivity with the biomass yield reveals that the lipid production is growth associated. In the absence of nitrogen source the growth was poor and the cell was bleached. It was also reported that, the reduction of nitrogen source in the medium increased the lipid content in another species of microalgae including *Chlorella* strains (Illman *et al*., 2000) and *Dunaliella* spp. (Kacka and Donmez, 2008).

Ahlgren and Hyenstrand (2003) reported that during the nitrogen-limited condition, algal cells often accumulate a surplus of carbon (C) metabolites as lipids. From these results it can be confirmed that the *B. braunii* is indeed unique among the alga in being able to produce and accumulate large amounts of lipid comprising mainly hydrocarbon under specific condition (Singh and Kumar, 1992; Zhila *et al*., 2005).

Both under nitrogen-limited condition and in the control condition, the amount of lipid in *Botryococcus* TRG strain were represent by 32.29% and 26.08%, respectively and these contents were higher than other strains (Figure 13). It was clear that the response of the algal strains to nitrogen-limited was varied in term of dry cell weight. The growth of four strains was lower with nitrogen depleted medium but the final lipid yield was higher than nitrogen rich medium (Figure 13).

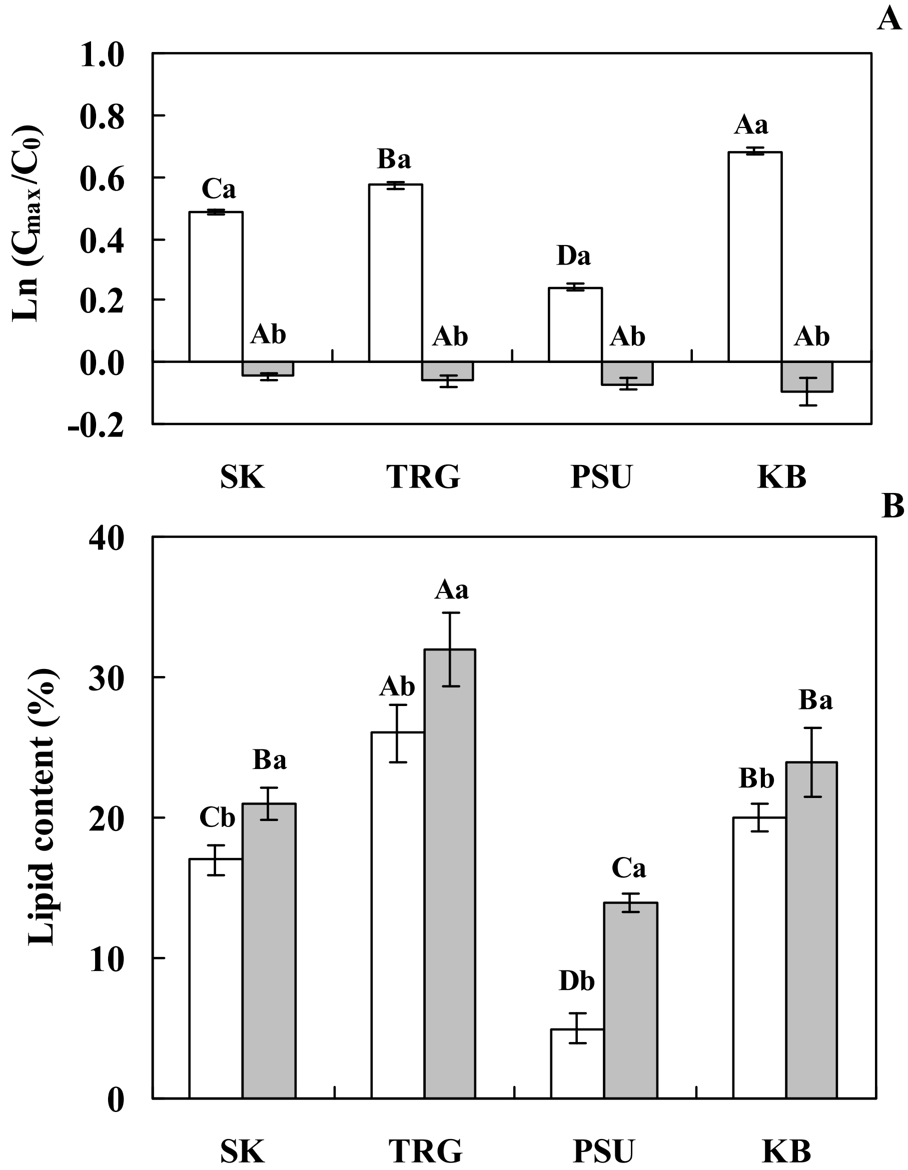


Figure13: Comparison of growths (A) and lipid contents (B) of four *Botryococcus* strains under nitrogen-rich (white bar) and nitrogen-limited (grey bar) conditions. The maximum algal growth was represented as Ln ($C_{max}/C_0$). Different capital letters on the bars indicate significant difference between strains ($P< 0.05$). Different small letters on the bars indicate significant difference between nitrogen-rich and nitrogen-limited conditions ($P< 0.05$).

## 2.2 Effect of salt concentration

The present study focused on the influence of salinity on four strains by varying the range of salt concentration and investigating their effect on growth and lipid production (Figure 14). Under nitrogen-rich condition, all strains could survive at high salinity but the biomass of SK, TRG and KB strains were decreased when the salt concentration was attempted. While, there was no significant effect of salinity on the biomass of PSU strain. In addition, the salt concentration did not significantly affect the loss of biomass in all strains under nitrogen-limited condition. The lipid content in SK, TRG and PSU strains were drastically decreased when the salt concentration increased. The negative effect of salt concentration on the lipid content of KB strain was less than on the other strains. In the absence of salt concentration, TRG strain gave the highest lipid content under both nitrogen-rich and nitrogen-limited conditions. However, the lipid content of KB strain at 43 mM and 86 mM salt concentrations was higher than that of TRG strain. In the case of *B. braunii* race A, it was reported that the biomass yield and the cellular composition were affected by salt concentration (Banerjee *et al*., 2002). With increasing salt concentration a slight increase in the lipid content was also observed in the study of Vazquez-Duhalt and Arredondo-Vega (1991) and Hart *et al*. (1991). Sarada *et al*. (2002) reported that high level of salinity (>170 mM) were lethal for freshwater green alga, *Heamatococcus* strain.

It is well known that microalgae modify their biochemical composition in response to environmental factors including nutrient availability, light, temperature and salinity. Algae differ in their adaptability to salinity and based on their tolerance extent they are grouped as halophilic (salt requiring for optimum growth) and halotolerant (having response mechanism that permits their existence in saline medium). In either case, the algae produce some metabolites to protect from salt injury and also to balance as per the surroundings osmotic (Richmond, 1986). The halotolerance property of KB strain was suitable for cultivation in conditions of high salinity. It was reported that the sampling source is an important role in salt sensitivity in microalgae (Ferroni *et al*., 2007). In this study, the salinity in each source of sampling was determined by Brix refractometer, then converted and expressed as mM of NaCl (Reep, 2009). It was shown that, the sampling source of each strain had various salt concentrations; KB source had highest salinity of 54.6 mM follow by SK source 38.8 mM, TRG source 21.6 mM and PSU source 17.2 mM NaCl. There are some differences in the degree of tolerance to salinity stress among freshwater microalgae (Imai *et al*., 1997). Since KB strain was isolated from a lake near by the sea (close to blackish water), this could explain why it was halotolerant. In the view point of the high lipid production, TRG strain was considered as a suitable strain for cultivating under a condition without salinity stress. However, since the lipid content of KB strain was less affected by salinity stress, it shows a high potential to be used in an open pond which is saline.

It was reported that *B. braunii* could not only survive in freshwater, but could also adapt to large salinity variations (Qin, 2005). Our results were contrast with the results of Rafael and Bertha (1991) who reported that the highest salinity that *B. braunii* could survive was 3 M salt concentration. A decreased yield of biomass and product at high salinity was reported by Vazquez- Duhalt and Arredondo-Vega (1991). This was probably due to the non adaptability of the organism to higher salinity (0.1–0.75 M). Li and Qin (2005) compared the effect of four salinities (0, 0.15, 0.25, and 0.5 M salt concentrations) on the growth of three *B. braunii* strains from China (CHN), United Kingdom (UK) and Japan (JAP). They found that the specific growth rate of microalga significantly declined when the salinity increase. In this present study, although the isolated strains could adapt to the high concentration of salt, the salt concentration did not improve the lipid content in all strains. On the contrast, Ben-Amotz *et al.* (1985) found that the lipid content of *B. braunii* grown in 0.50 M salt concentration was higher than that with no salt addition. This can be attributed to the different characteristics of microalgae. Since high concentration of salt did not increase the lipid content of four strains, the medium without adding NaCl was chosen for next study.

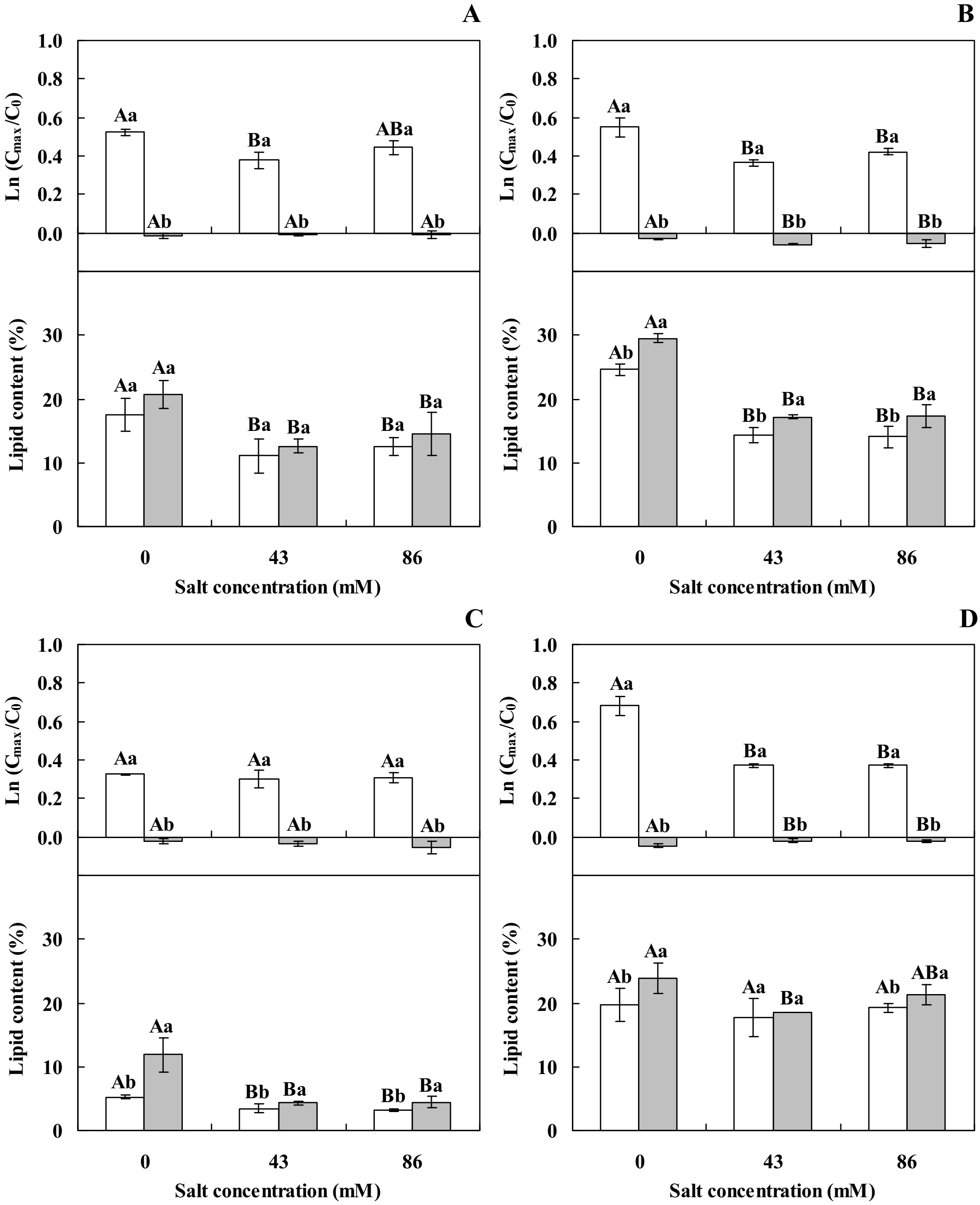


Figure 14: Effect of salt concentration on growths and lipid contents of four strains under nitrogen-rich (white bar) and nitrogen-limited (grey bar) conditions. A: SK, B: TRG, C: PSU and D: KB strains. The maximum algal growth was represented as Ln ($C_{max}$/$C_0$). Different capital letters on the bars indicate significant difference between salinities ($P$< 0.05). Different small letters on the bars indicate significant difference between nitrogen-rich and nitrogen-limited conditions ($P$< 0.05).

**2.3 Effect of light intensity**

The effect of light intensity on lipid production profile of four strains are shown in Figure 15. In the light trail, the TRG strain produced the higher amounts of lipid at all light intensity about 23-28% of its dry weight in nitrogen-riched and 30-35% of its dry weight in nitrogen-limited condition, whereas the lipid content of PSU strain was the lowest amounts about 5-10% of its dry weight in nitrogen-riched and 11-17% of its dry weight in nitrogen-limited condition at all light intensity. The lipid contents in all strains increased with increasing light intensity from 33 to 49.5 μmol.photon.$m^{-2}s^{-1}$ then decreased when the light intensity was increased up to 82.5 μmol.photon.$m^{-2}s^{-1}$. From Figure 15, it was observed that at 49.5 μmol.photon.$m^{-2}s^{-1}$ light intensity gave highest lipid content in all strains. It is interesting to note here that the synergic effect of nitrogen-limited and light intensity was found in TRG strain. The lipid content in TRG strain under nitrogen-limited condition at 49.5 μmol.photon.$m^{-2}s^{-1}$ was 37.1% higher than that under nitrogen-riched and 35.67% higher at 82.5 μmol.photon.$m^{-2}s^{-1}$ where as at 33 μmol.photon.$m^{-2}s^{-1}$ only 30.70% higher. The photoinhibition at 82.5 μmol.photon.$m^{-2}s^{-1}$ in all strains was obvious under nitrogen-riched condition. However, under nitrogen-limited condition the lipid in TRG and KB strains was found unchanged at the light intensity higher than 49.5 μmol.photon.$m^{-2}s^{-1}$. This result is contrast to that of the previous reports which had shown that a high light irradiance was favored for more lipid and hydrocarbon contents rather than more biomass (Metzger and Largeau, 1999; Tansakul et al., 2005).

The previous studies have attempted to identify the optimal irradiance levels for supporting growth and lipid production. A high intensity of light increases the carotenoid-to-chlorophyll ratio, and this affects the color of algal colonies (Wolf *et al*., 1985a by Benerjee *et al*., 2002). Carbohydrate concentration cellular nitrogen, and phosphorus content of *B. braunii* UTEX 572 are decreased by extended exposure to a light intensity of 25 to 72 μE·$m^{-2}$ $s^{-1}$ (Oh *et al*., 1997). Under intense illumination (10 klx, or ~140 μE·$m^{-2}$ $s^{-1}$) algal cells that had been adapted to a high irradiance during preculture could attain a higher biomass concentration (7 kg·$m^{-3}$) and hydrocarbon content (50% of dry weight) compared with cells that had been adapted to low level irradiance (3 klx, or ~42 μE·$m^{-2}$ $s^{-1}$) (Kojima and Zhang, 1999). The lower productivity of dark-adapted culture was because it was highly susceptible to photoinhibition.

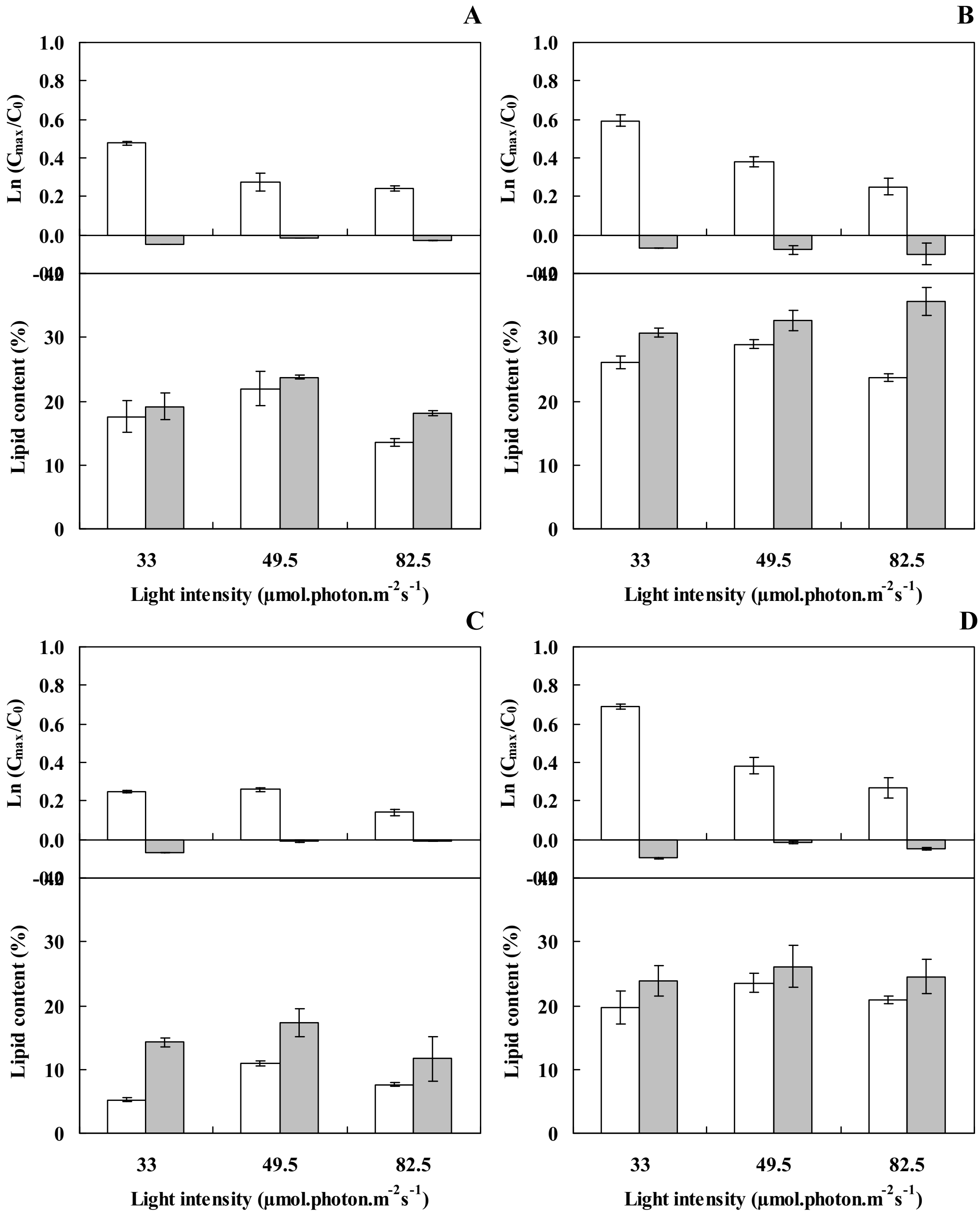


Figure 15: Effect of light intensities on growths and lipid contents of four strains under nitrogen-rich (white bar) and nitrogen-limited (grey bar) conditions. A: SK, B: TRG, C: PSU and D: KB strains. The maximum algal growth was represented as Ln ($C_{max}/C_0$). Different capital letters on the bars indicate significant difference between light intensities ($P< 0.05$). Different small letters on the bars indicate significant difference between nitrogen-rich and nitrogen-limited conditions ($P< 0.05$).

Casadevall *et al*. (1985) reported that the optimal light range for *B. braunii* strain Droop 807/1 was between 30 and 60 $Wm^{-2}$. Similarly, Li and Qin (2005) found the specific growth rate of the CHN strain was reduced when irradiance exceeded 60 $Wm^{-2}$ while, the growth rates of the UK and JAP strains were independent of irradiance from 60 to 300 $Wm^{-2}$. Cepak and Lukavsky (1994) suggested that *B. braunii* could survive a wide range of irradiance from 15 to 180 $Wm^{-2}$. However, Metzger and Largeau (2005) argued that hydrocarbon synthesis should be favored by irradiances between 40 and 90 $Wm^{-2}$. *B. braunii* is identified as an untapped resource for production of hydrocarbons. Successful use of this organism as an alternate source of energy depends on its growth rate, hydrocarbon productivity and their fuel efficiency. Similarly, Qin (2005) had examined the algal growth and lipid content of *B. braunii* (China strain 1) under various light and photoperiod in an attempt to obtain the optimal of light condition for the maximum biomass and hydrocarbon production. In the experiment of low light range, the 30 and 60 $W/m^2$ treatments were more suitable to algal culture than high light intensities. Total lipid content at 30 and 60 $W/m^2$ was significantly higher than that at other light intensity. Lipid content decreased sharply when light intensity increased from 60 to 150 $W/m^2$. However, lipid content did not differ when the light intensity was above 100 $W/m^2$ ($P>0.05$).

However, each *B. braunii* strain has a unique requirement in nutrients and condition. Incidentally, lipid content of each strain was not affected by condition than native characterization of itself. The present microalga strain might also have a narrow range of light intensity and could not be tolerant to high light intensity. Therefore, 49.5 $\mu mol.photon.m^{-2}s^{-1}$ light intensity was chosen for next study.

### 2.4 Effect of iron ($Fe^{3+}$) concentration

On the basis of fluorescence measurements taken over 12 years, Behrenfeld *et al.* (2006) demonstrated that iron had a key function in regulating phytoplankton biomass in both HNLC (high nitrate low-chlorophyll) and oligotrophic waters. However, whether the ‘‘bioavailable’’ iron deficiency is one of the main factors limiting algal biomass productivity in batch culture conditions is, to the best of our knowledge, unknown. And some biochemical components such as hydrocarbon in response to iron have not been well documented in microalgae. Previous studies had displayed that lipid content in some microalgae could be modified by various growth conditions such as nitrogen deprivation (Illman *et al*., 2000), silicon deficiency (Lynn *et al*., 2000), phosphate limitation (Reitan *et al*., 1994), high salinity (Rao *et al*., 2007), some heavy metals stress such as cadmium (Guschina and Harwood, 2006) or co-immobilized in alginate beads with the bacterium *Azospirillum brasilense* (Lebsky *et al*., 2001; de-Bashan *et al*., 2002). Significant increase in lipid content occurred in many microalgae after being subjected to such conditions. Oil levels of 20–50% by weight of dry biomass are quite common and some can exceed 80%.

As mentioned above, iron has been demonstrated to be a key factor in enhancing phytoplankton biomass in oceanic waters. Moreover, certain culture conditions could result in higher quantities of lipids. Therefore, the effects of iron ($Fe^{3+}$) on the growth and the lipid content in four strains were investigated to find if iron could promote the biomass productivity or lipid accumulation under laboratory conditions. Since ferric citrate was an iron compound used in modified Chu 13, it was used as $Fe^{3+}$ source in this study.

The present study showed that high $Fe^{3+}$ concentration could also induce considerable lipid accumulation in all strain of isolated microalgae (Figure 16). The lipid content was increased with increasing $Fe^{3+}$ concentration. From Figure 16, it was observed that at 0.74 mM of $Fe^{3+}$ concentration gave highest lipid content in both of condition (nitrogen riched and nitrogen limited). It is interesting to note here that the synergic effect of nitrogen-limited and $Fe^{3+}$ concentration was found. The lipid content in four strain under nitrogen-limited condition was higher than that under nitrogen-riched in all treatments (Figure 16). Among four strains, the TRG strain produced the highest amount of lipid content, while the PSU strain produced the least amount of lipid at all treatments. Similarly, the lipid accumulation of the SK and KB strain in all $Fe^{3+}$ concentrations. The highest amount of lipid was found at 0.74 mM $Fe^{3+}$ as 35.9% of its dry weight in TRG strain. This suggested that some metabolic pathways related to the lipid accumulation in TRG strain were probably modified by high level of iron concentration in the initial medium. Likewise, Liu *et al*. (2008) reported the effect of iron on growth and lipid accumulating in *Chlorella vulgaris* They were found that, total lipid content in cultures supplemented with $1.2\times10^{-5}$ mol $L^{-1}$ $FeCl_3$ was up to 56.6% biomass by dry weight and was 3–7 fold that in other media supplemented with lower iron concentration.

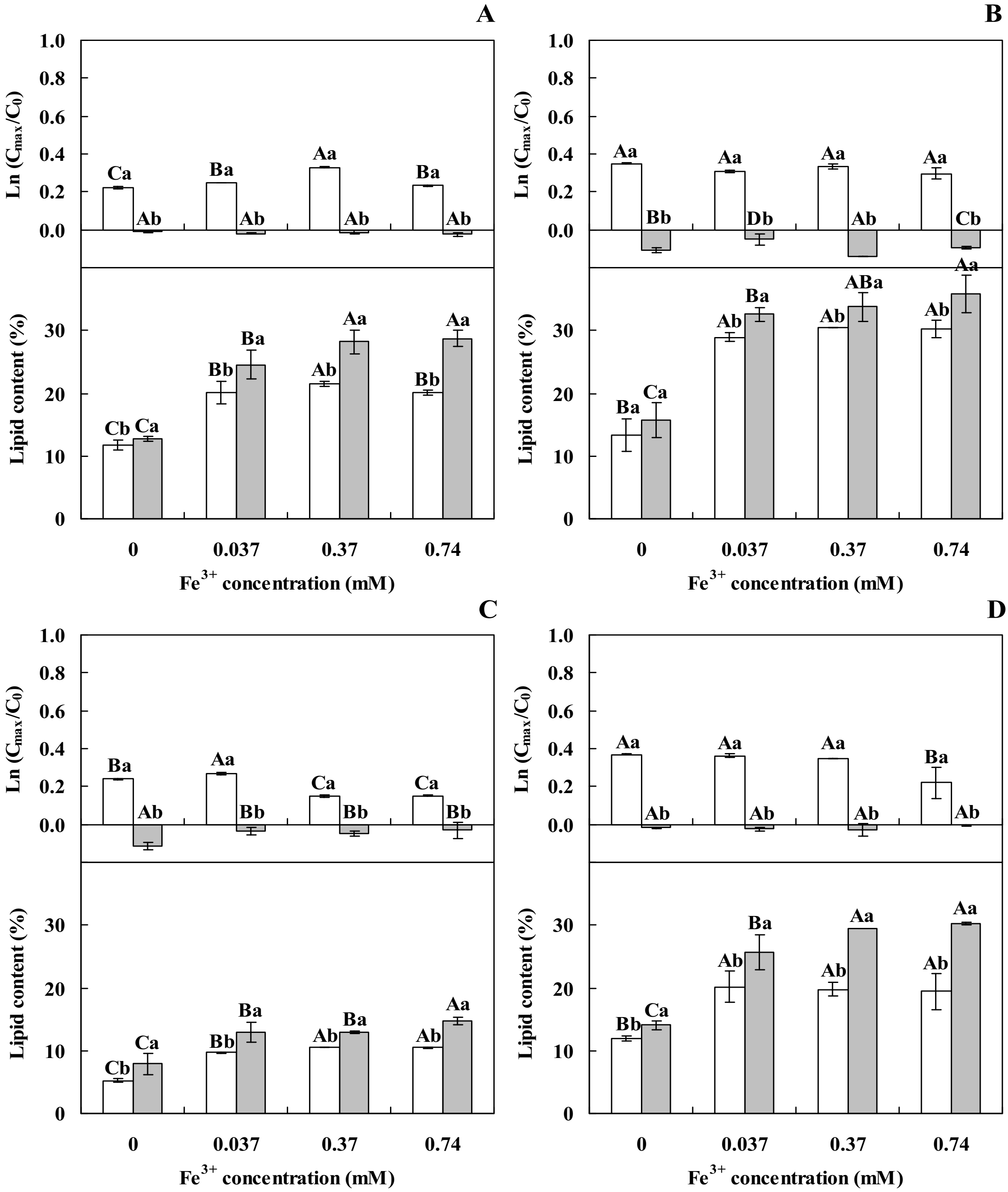


Figure 16: Effect of $Fe^{3+}$ concentrations on growths and lipid contents of four strains under nitrogen-rich (white bar) and nitrogen-limited (grey bar) conditions. A: SK, B: TRG, C: PSU and D: KB strains. The maximum algal growth was represented as Ln ($C_{max}/C_0$). Different capital letters on the bars indicate significant difference between $Fe^{3+}$ concentrations ($P$< 0.05). Different small letters on the bars indicate significant difference between nitrogen-rich and nitrogen-limited conditions ($P$< 0.05).

**3. Fatty acid composition of biodiesel from microalgal lipid**

Four isolated strains were cultured under the optimal condition from the previous section. The medium contained 0.74 mM of ferric citrate was used without adding nitrogen source (nitrogen-limited) and salt. The culture was incubated with 125 rpm agitation at 25±1ºC under 49.5 µmol.photon.$m^{-2}s^{-1}$ with 16:8 hours light and dark cycles for 20 days. The fatty acid of isolated SK, TRG, KB and PSU strains were extracted and the biodiesel (fatty acid methyl esters) was produced. The fatty acid compositions of the biodiesel obtained from each strain are shown in Table 8. Four strains accumulated fatty acid profiles of C12:0 to C22:0. Palmitic and oleic acids were the major fatty acids found in all strains. The palmitic acid was the highest fatty acid found in TRG (49.5%), PSU (46.7%) and KB (41.1%), while the highest fatty acid found in SK strain was oleic acid (37.68%). Similar observations were made by Fang *et al*. (2004) and Dayananda *et al*. (2006). They found that palmitic acid and oleic acid were the major components in the *B. braunii* hydrocarbon. Banerjee *et al*. (2002) demonstrated that *B. braunii* race A produces nonisoprenoid hydrocarbons alkadienes and trienes. The structural similarity (locations of double bonds), and stereochemistry of alkadienes resemble that of oleic acid (18:1, cis-ω9). These features and other evidence suggest that oleic acid is the direct precursor of the n-alkadienes. Moreover, some fatty acid like as lauric acid (C12:0) and palmitoleic acid (C16:1) was found only in PSU strain while tridecanoic acid (C13:0) and behenic acid (C22:0) was found only in KB and SK strain, respectively. Most of fatty acids found in isolated microalgae were similar to those found in plant oil. It was remarkable that, arachidic acid (C20:0) which is a saturated fatty acid found in fish oil and peanut oil (1.1-1.7%), also found in SK, KB and PSU (0.22-0.63%).

Palmiltic, linoleic and α-linolenic acids were described as the dominant fatty acid in *B. braunii* (Ben- Amotz *et al*., 1985). Two different strains of *B. braunii* (Austin, Berkeley) were found to have high contents of oleic acid (>50% of the sum of fatty acid), about 12% of C20:1 and a very low content of polyenoic acids represented only by linoleic and eicosapentaenoic acids (Yamaguchi *et al*., 1987 by Kalacheva *et al*., 2002). According to other authors these strains synthesize lipids with a broad spectrum of polyenoic, C16–C18 dienoic and trienoic and trace amounts of eicosapentaenoic acids, but the main fatty acid were palmitic and oleic (70–80% of the sum fatty acid) (Vazquez- Duhalt & Arrendondo-Vega, 1991). In the three races, the even acids were in the range C14-C32, the main being acids were palmitic, oleic and octacosenoic acids (Metzger *et al*., 1989). Oleic acid predominates in the *B. braunii* race A (more 80% of the total fatty acids) (Metzger et al., 1999). The fatty lipid of lipids of the field sample from lake and freshwater pond in southern Thailand were represented mostly by saturated and monoenoic acids with a chain length ranging from C12 to C22. The dominant fatty acids are palmitic and oleic acids. Identification results of fatty acid composition of the organism isolated from lake and freshwater pond in southern Thailand are in good agreement with the literature data related to the known strain of *Botryococcus* sp. race A.

The fatty acids of biodiesel derived from SK strain were represented mostly by unsaturated acids (>50%) while those in KB, PSU and TRG strains were 41.1%, 34.5% and 30.6%, respectively. The high content of unsaturated fatty acids was evidence of lower oxidative stability, but excellent fuel properties at low temperatures, which is an advantage for operating in winter. However, compared with the commonly used soybean oil and rapeseed oil as feedstock for biodiesel production in the US and the EU, the biodiesel derived from microalgae in this study were more saturated. Soybean oil contains mostly linoleic and oleic acids at 53.7% and 23.3% respectively, while rapeseed oil also contains similar fatty acids at 23.3% and 64.4% respectively (O'Brien, 1988). Therefore, the biodiesel derived from *B. braunii* strains in this study tends to give favorable properties compared with the biodiesel derived from soybean oil and rapeseed oil. These include an increased cetane number (CN), decreased NOx emissions, a shorter ignition delay time, and oxidative stability.

Table 8: Fatty acid composition of biodiesel from microalgal lipid

| Fatty acid (%) | SK strain | TRG strain | KB strain | PSU strain |
|---|---|---|---|---|
| C12:0 | - | - | - | 0.27 |
| C13:0 | - | - | 0.18 | - |
| C14:0 | 3.95 | 3.33 | 3.35 | 2.36 |
| C15:0 | 1.56 | 6.43 | 3.55 | 2.54 |
| C16:0 | 34.04 | 49.54 | 41.13 | 46.65 |
| C16:1 | 0.94 | - | - | 0.41 |
| C17:0 | 1.54 | - | 0.55 | 0.49 |
| C18:0 | 12.02 | 10.11 | 10.73 | 18.93 |
| C18:1 | 37.68 | 28.51 | 35.21 | 30.63 |
| C18:2 | 5.01 | 1.34 | 2.73 | 2.00 |
| C18:3 | 7.35 | 0.74 | 2.73 | 1.04 |
| C20:0 | 0.63 | - | 0.35 | 0.22 |
| C20:1 | - | - | 0.39 | 0.44 |
| C22:0 | 0.28 | - | - | - |

Note: Four isolated strains were cultured under the optimal condition from the previous section. The medium contained 0.74 mM of ferric citrate was used without adding nitrogen source (nitrogen-limited) and salt. The culture was incubated with 125 rpm agitation at 25±1ºC under 49.5 µmol.photon.$m^{-2}s^{-1}$ with 16:8 hours light and dark cycles for 20 days.

Comparison among four strains, TRG fulfills the major requirements for lipid production, including high lipid content, yield and productivity in the growth medium which contained nitrogen source and 0.74 mM of ferric citrate. After growing in the growth medium, the lipid content of TRG could be further increased from 25.8 % to 35.9 % by exposing the algal cells to the medium without nitrogen source but contained 0.74 mM of ferric citrate with 49.5 μmol.photon.$m^{-2}s^{-1}$ light intensity. With further understanding on the cultivation of TRG in photobioreactors, much greater productivity of algal lipid would be obtained. Moreover, the use of primary-treated wastewater and outdoor cultivation could also make algal lipid production more economical by eliminating the need to supply nutrients and light, respectively.

**4. Growth and lipid production by TRG strain in effluent from seafood processing plant**

**4.1 Dilution of effluent from seafood processing plant**

Based on the results from the previous experiment, isolated TRG strain which showed the highest growth and lipid production was chosen to evaluate its cultivation in the effluent from seafood processing plant. It was reported that *Botryococcus* cannot grow on industrial wastewater containing a high concentration of inorganic ions. Therefore, the secondary-stage treated wastewater was used to cultivate *Botryococcus* (Banerjaa *et al*., 2002). In this study, the effluent used was secondarily pretreated wastewater from a seafood processing plant in Songkhla province, Thailand. The characteristic of the effluent was shown in Table 9. Both of $CO_2$ in the air (normally contain 0.04%) and complex organic carbon in the effluent was used as carbon source in the culture. As shown in Table 9, since the amount of $NO_2^-$-N, and $NH_3$-N in the secondarily pretreated effluent was very low, nitrate was used as a main nitrogen source.

The effluent was diluted one, two, and four times compared with non-diluted effluent (Figure 17). The non-diluted effluent with an initial nitrate concentration of 21.12 mg.$L^{-1}NO_3^-$ gave highest dry cell weight and lipid content. The growth and lipid content of TRG strain was affected by dilution times. Both of value was decreased when the effluent was diluted due to the lower nitrate concentration. The one and two time diluted effluent gave half of specific growth rate (0.20 $d^{-1}$ and 0.25 $d^{-1}$, respectively) than non-diluted effluent (0.47 $d^{-1}$). While the four times diluted effluent, cell growth was severely limited and the specific growth rate was only 0.10 $d^{-1}$.

Table 9. Characteristics of secondarily pretreated seafood effluent

| Virable | Value ($mg.L^{-1}$) |
|---|---|
| COD | 740 ± 5.31 |
| $NO_3^-$ | 24.1 ± 2.13 |
| TKN | 58.8 ± 1.47 |
| $NH_4^+$-N | 4.6 ± 0.12 |
| $NO_2^-$-N | 0.04 ± 0.01 |
| pH | 8.66 ± 0.05 |

Moreover, in the two and four times diluted effluent, the color of alga cell changed from green to brown (Figure 18). This could be due to a decrease in chlorophyll content. The lipid accumulation seemed to be proportional to algal growth. The undiluted and one time diluted effluent gave similarly lipid content (16.9% and 17.4%, respectively). In the two and four times diluted effluent, the lipid accumulation (Figure 15) was only 8.22% and 5.83%, respectively, on day 4 of cultivation and it was continuously decreased to 3.5% at the end of cultivation. This could be due to the utilization of lipid as reserve energy by this microalga under nitrogen starvation condition. Thus, supply of a sufficient level of nitrate is required for high cell density culture and increased lipid content.

After 8 days of cultivation, the amount of nitrate was decreased to a half of initial concentration (Figure 15A and 15B), from 24.09 to 12.49 $mg.L^{-1}NO_3^-$ in the undiluted effluent culture and from 16.97 to 9.25 $mg.L^{-1}NO_3^-$ in one time diluted effluent culture. While, in two times and four times diluted effluent (Figure 15C and 15D), only 4 days of cultivation the initial nitrate was decreased to half (from 11.25 to 4.53 $mg.L^{-1}NO_3^-$ and from 6.51 to 3.68 $mg.L^{-1}NO_3^-$, respectively). Prolonged incubation (more than 12 days) did not result in additional removal of nitrate in the effluent. At the end of cultivation, the nitrate concentration in the effluent with and without dilution was in the range of 1.41- 4.21 $mg.L^{-1}NO_3^-$. The results indicated that more than 80% of the initial nitrate was removed by cultivation of TRG strain. Similarly, An *et al*. (2003) demonstrated the hydrocarbon production from secondarily treated piggery wastewater by the green alga *Botryococcus braunii* UTEX 572. They found that after 12 days of cultivation, a biomass of 8.5 g dry cell weight $L^{-1}$ and hydrocarbon level of 0.95 g hydrocarbon $L^{-1}$ were obtained. The initial nitrate concentration was 620 mg N $L^{-1}$ and the final nitrate concentration was 168 mg N $L^{-1}$, indicating that 80% of the initial nitrate was removed by microalgal cultivation. Tansakul *et al*. (2005) reported that *B. braunii* grew well in adjusted effluent from seafood processing plant with 1 L/min aeration rate and 1% (v/v) $CO_2$. The alga reduced nitrate and phosphate concentration in the effluent by 90% and 56%, respectively. With this medium the hydrocarbon content of alga were 16.98%.

Even, the lipid content in undiluted and one time diluted was similarly. By comparison, the nitrate removal efficiency of undiluted effluent was higher than one time diluted. Undiluted effluent was chosen to used as medium culture in the next study.

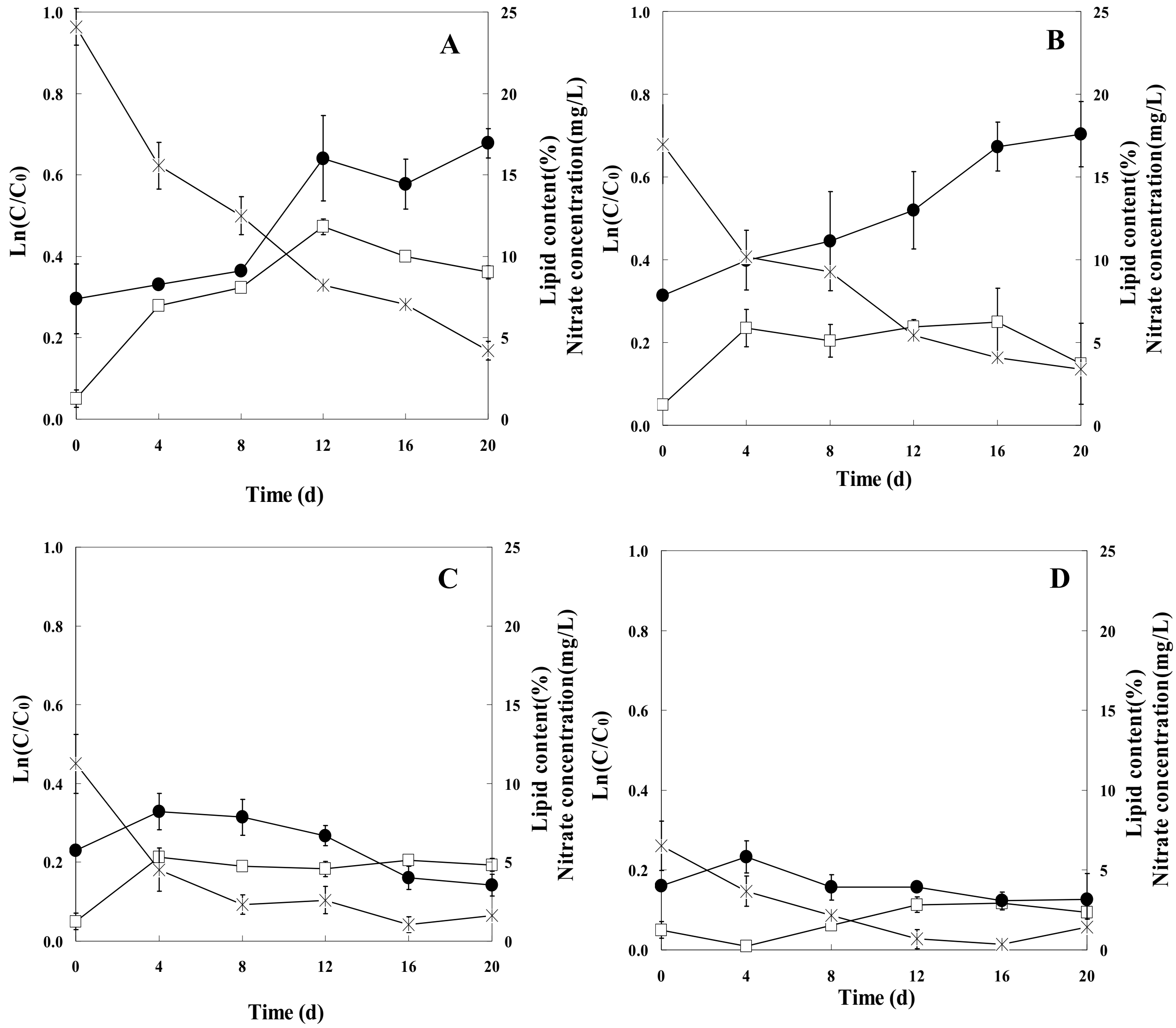


Figure 17: Cultivation of TRG strain in undiluted (A), one time diluted (B), two times diluted (C) and four times diluted (D) effluent medium. $LnC/C_0$ (open square), lipid content (close circle) and nitrate concentration (star).

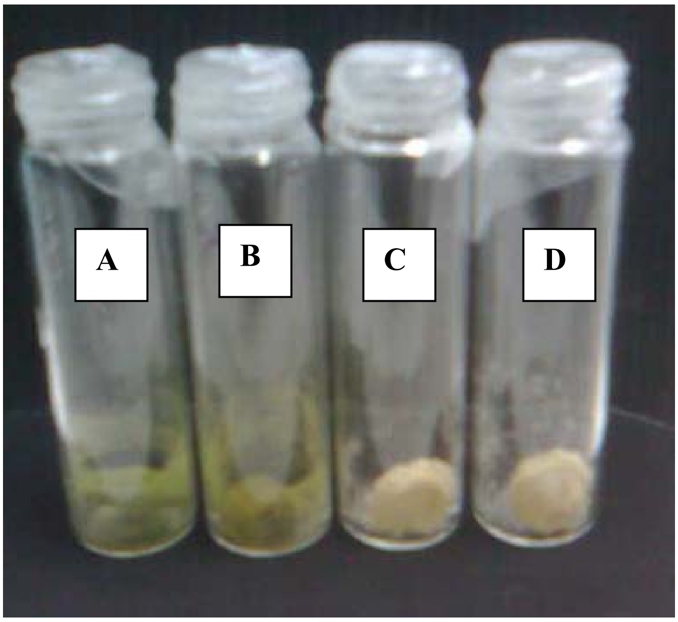


Figure 18: Cell pallet of TRG strain in undiluted (A), one time diluted (B), two times diluted (C) and four times diluted (D) effluent medium.

## 4.2 Effect of carbon dioxide concentrations

The undiluted effluent was used in this experiment. The effect of carbon dioxide concentrations on growth and lipid content of TRG strain were investigated in a two-tier flask provided with different levels of $CO_2$ (Figure 19). TRG strain grew better in flask supplemented with $CO_2$. At 2% $CO_2$, TRG strain grew most rapidly with the specific growth rate of 0.77 $d^{-1}$. At 1% $CO_2$, TRG strain also grew well with the specific growth rate of 0.72 $d^{-1}$. The specific growth rate markedly fell to 0.48 and 0.31 $d^{-1}$ with 0.5% $CO_2$ and non-supplemented with $CO_2$, respectively. It is worth to emphasize that the biomass at 2% $CO_2$ was 255 mg/L. The values were significantly increased as compared with non-supplemented with $CO_2$, 0.5% and 1% $CO_2$ (116 mg/L, 167 mg/L and 172 mg/L, respectively) (data not shown). However, the growth of TRG strain ceased after 10 days of incubation under the conditions of 1% and 2% $CO_2$. The enhancement by supplemented with $CO_2$ may due to the enrichment of available $CO_2$ as a carbon source. This result is in agreement with the previous report that the cultivation of microalgae with supplemented with $CO_2$ resulted in high cell density (Lee *et al*., 2002; Chiu *et al*., 2007).

The lipid content of TRG strain increased with increasing $CO_2$ concentration from 0.5% to 2.0%. The maximum lipid content of 30.33% was obtained at 2% $CO_2$ followed by 1% $CO_2$ (23.80%) and 0.5% $CO_2$ (20.73%). While non-supplemented with $CO_2$ gave the lowest lipid content only 16.94%. As shown in Figure 19, after 10 days of cultivation the lipid content of the culture with non-supplemented with $CO_2$ was almost constant about 16%, while that in the culture with supplemented with $CO_2$ continuously increased until day 20. It was reported that photosynthetic cultures of *Botryococcus* require $CO_2$ and this microalga utilizes exogenous carbon sources for improved growth and lipid production (Banerjee *et al*., 2002). However, our result was not consistent with the previous studies. Chiu *et al*. (2007) revealed the lipid content of *Chlorella* sp. was not responding with higher $CO_2$ concentration. Such divergent results for lipid content of microalgae cultured under $CO_2$ aeration may be due to differences in microalgal species, content of culture medium, and culture condition.

The amount of nitrate concentration in effluent medium was reduced sharply with high level of $CO_2$ concentration. The amount of nitrate concentration at the end of cultivation was 4.21 mg/L, 3.38 mg/L, 2.64 mg/L and 2.21 mg/L for the cultures without supplemented with $CO_2$, supplemented with $CO_2$ at 0.5%, 1% and 2%, respectively (from initial nitrate concentration 24.09 mg/L). Thus, the overall efficiency of nitrate reduction in the cultures was higher than 85%.

In this study, 2.0% $CO_2$ was favored for rapid growth and lipid accumulation. The lipid content varied in the range of 14 to 28% at different $CO_2$ levels. Rao *et al*. (2007) studied the effect of $CO_2$ on biomass and products accumulation using a two-tier flask. The products included hydrocarbon, carbohydrate, fatty acid and carotenoid in various species of *B. braunii*. They reported that the level of $CO_2$ at 2.0% (v/v) enhanced growth and carotenoid contents of all strains. Similarly Dayananda *et al*. (2007) reported that an increase of 1.1 to 1.3 fold in biomass yield was observed in *B. braunii* culture supplemented with $CO_2$ over the control. In this study, lipid accumulation also increased with the supplementation of $CO_2$. Increase in chlorophyll contents was also observed with increasing $CO_2$ supplementation up to 2% (v/v) as shown in Figure 20. By comparing the control culture (without supplemented with $CO_2$) with the cultures supplemented with $CO_2$ at 0.5%, 1% and 2%, 2% $CO_2$ showed dark green colony of cells. In contrast, without supplemented with $CO_2$ cell culture was bleach.

This shows its effective utilization of $CO_2$ through photosynthesis. Tripathi *et al*. (2001) reported photoautotrophic growth of different microalgae for higher growth and carotenoid production. It was evident that different algae require different levels of $CO_2$ for their photoautotrophic adaptability.

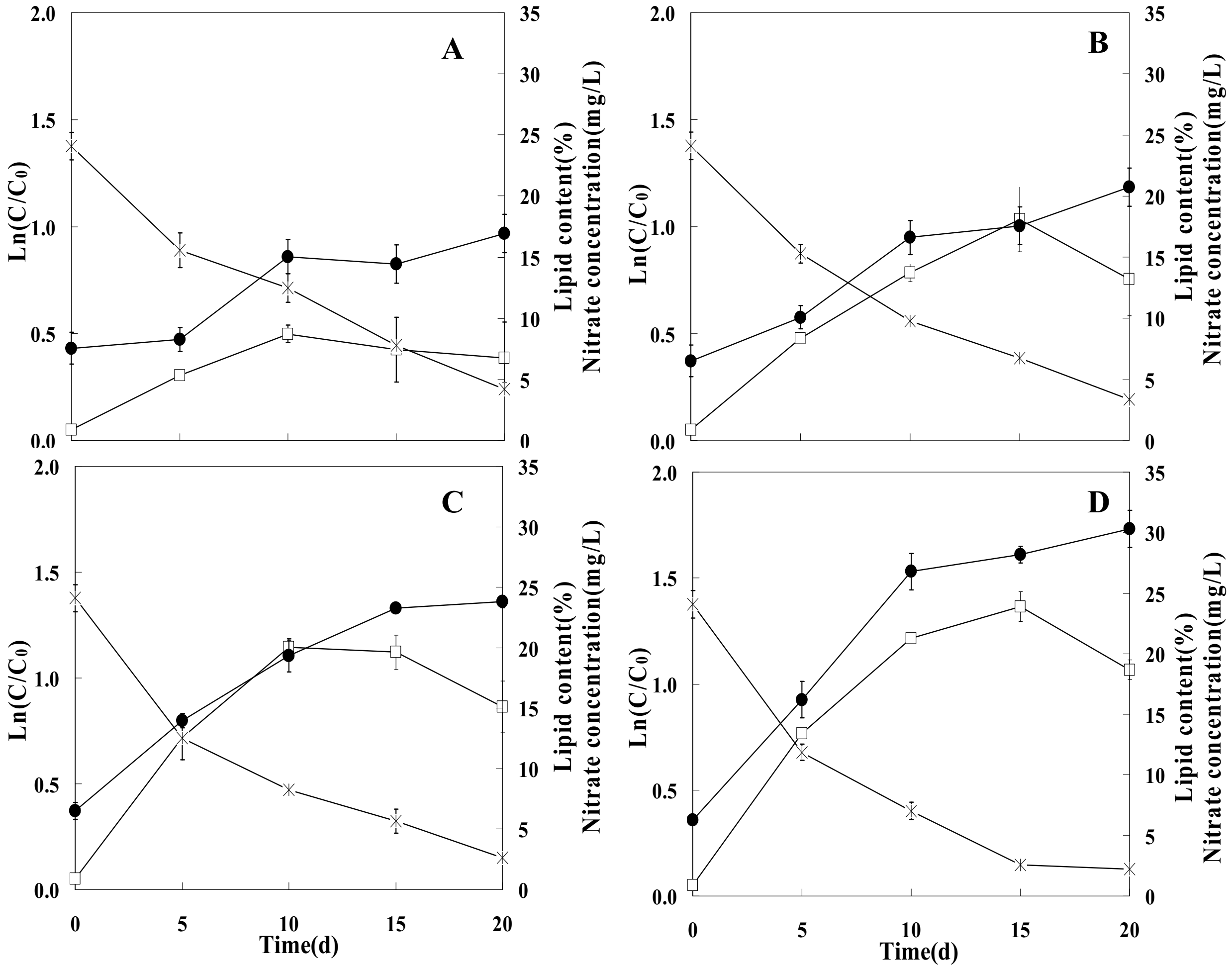


Figure 19: Effect of $CO_2$ concentration on growth, lipid production and nitrate removal of TRG strain in effluent medium. Control (0.04% $CO_2$) (A), 0.5% $CO_2$ (B), 1.0% $CO_2$ (C) and 2.0% $CO_2$ (D). $LnC/C_0$ (open square), lipid content (close circle) and nitrate concentration (star).

Many reports have suggested using flue gas as carbon origin for microalgal cultivation, which could combine biofuel production with current $CO_2$ mitigation strategies (Vunjak-Novakovic *et al*., 2005; Wang *et al*., 2008; Yoo *et al*., 2010). Ge *et al*. (2011) evaluated the potential of cultivating *B. braunii* with flue gas (normally containing high $CO_2$) for biofuel production, growth characteristics of *B. braunii* 765 with 2–20% $CO_2$ aeration. The hydrocarbon content of *B. braunii* 765 increased with increase of $CO_2$ concentration, being 16.43%, 18.25%, 21.03% and 24.45% (w/w) with 2%, 5%, 10% and 20% $CO_2$ aeration, respectively. Likewise, Morais and Costa (2007) tested the effect of $CO_2$ limitation and high $CO_2$ concentration on the growth of *Scenedesmus obliquus* and *Spirulina* sp. They found that after 5 days of cultivation under conditions of carbon limitation both organisms showed cell death. For *Spirulina* sp., the maximum specific growth rate (0.44 $d^{-1}$) and maximum productivity (0.22 g/L $d^{-1}$) was obtained with 6% (v/v) $CO_2$, while the maximum cellular concentration (3.50 g/L) was obtained with 12% (v/v) $CO_2$. However, according to several authors (Berenguel *et al*., 2004 and Chae *et al*., 2006), the excess supply of $CO_2$ could favour the availability of the carbon source to the cells, so as not to limit metabolic activity. Nevertheless, the excess addition of this compound can be used in the culture volume more than 1 L and $CO_2$ could provokes losses that are not used by the cultures, resulting in unnecessary environmental pollution.

The increase in biomass yields and lipid content in TRG strain in response to a $CO_2$-enriched atmosphere when compared with control culture (normally containing 0.04% $CO_2$) indicated the improved growth and metabolite production of the organism to the tested levels of $CO_2$. The pH of the cultures were about 6.7–6.43, 6.4, 6.28 and 6.05 for the cultures supplemented with 0.04%, 0.5%, 1.0%, and 2.0% $CO_2$, respectively (data not shown). Obviously, the culture pH just slightly reduced with the increase of $CO_2$ concentration from 0.04% to 2.0%. Similar results were also found in the culture of *Chlorella* sp. supplemented with 2–15% $CO_2$ (Chiu *et al*., 2008). Similarly, Dayananda *et al*. (2007) reported that the pH of the culture had no significant effects on the biomass yield and hydrocarbons production of *B. braunii* when it ranged from 6.0 to 8.5. *Dunaliella tertiolecta* was also found to be able to grow sufficiently without controlling pH, even under flushing with 24% $CO_2$-enriched air (Suzuki *et al*., 1995). This ability of algae might be related to osmoregulation which was achieved biochemically by synthesis or dissimilation of intracellular glycerol (Suzuki *et al*., 1995). Goyal and Gimmler (1989) had reported that *D. tertiolecta* could maintain a constant intracellular pH over a wide range of external pH values (6.5–8.5). The present algal strain might also have such adjustment mechanisms which would be of significance when the strain was cultivated with aerated flue gas containing 10–20% $CO_2$ (Suzuki *et al*., 1995).

It was also reported that bubbling of $CO_2$ at higher levels (>2%) or continuous bubbling at 2% level resulted in significant decrease in the pH of the culture medium. This may be possibly due to a poor buffering capacity of the medium as the salt strength was low (Rao *et al*., 2007)

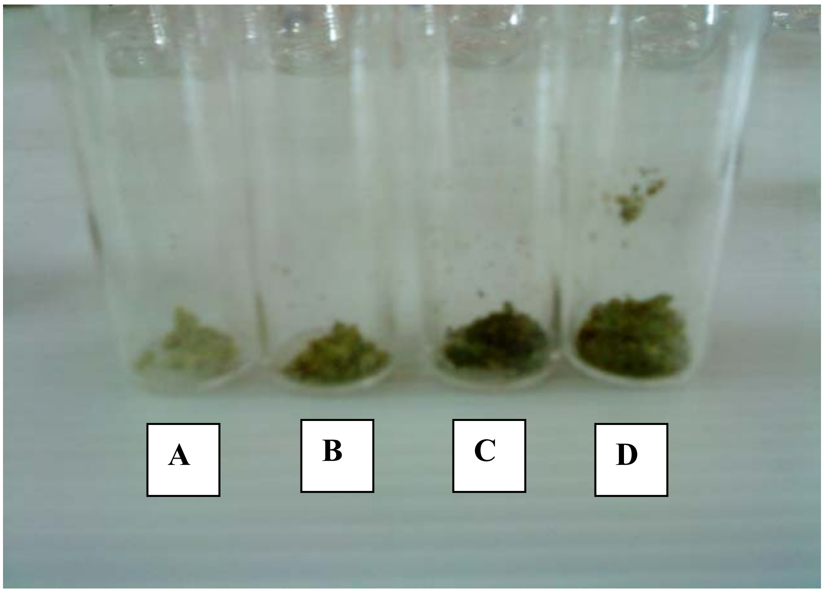


Figure 20: Cell pallet of TRG strain in undiluted effluent medium with control (0.04% $CO_2$) (A), 0.5% $CO_2$ (B), 1.0% $CO_2$ (C) and 2.0% $CO_2$ (D).

**5. Growth and lipid production of TRG strain under photoautotrophic, heterotrophic and mixotrophic conditions**

**5.1 Effect of culture conditions**

In order to improve the performance of microalgal cultures, a good understanding of the carbon and energy metabolisms in microalgal cells is needed. Microalgae can perform oxygenic photosynthesis and fix carbon dioxide through Calvin cycle like plant cells under photoautotrophic condition. That is, microalgal cells can trap light energy as the carbon source. The energy source and assimilate $CO_2$. Moreover, organic substrates can also be utilized as the carbon and energy sources by many microalgae under heterotrophic (without light) and mixotrophic (with light) conditions (Yang *et al*., 2000).

The growth and lipid production of TRG strain was studied under photoautotrophic, heterotrophic and mixotrophic conditions. In this study, 5 g/L of glucose was used as a carbon source for heterotrophic and mixotrophic conditions. The results are shown in Figure 21. The maximum cell concentration under mixotrophic condition was obtained at day 10 (214 $mg.L^{-1}$). The maximum cell concentration under photoautotrophic and heterotrophic conditions was obtained at day 15 which were 114 $mg.L^{-1}$and 174 $mg.L^{-1}$, respectively. Among three cultural conditions, the mixotrophic condition gave fastest growth and highest specific growth rate of 0.77 $d^{-1}$ followed by heterotrophic condition (0.65 $d^{-1}$). TRG strain under photoautotrophic cultural condition grew slowly and showed the lowest specific growth rate (0.49 $d^{-1}$). It was reported that *Botryococcus* strain was able to utilize exogenous carbon source for improved growth and lipid production. Various carbon source, including $C_1$-$C_6$ compounds and disaccharide (lactose, sucrose), have been screened in attempts to decrease the mass doubling time of this alga (Weetal, 1985; Banerjee *et al*., 2002). The mixotrophic condition gave highest lipid content of 32.69%. The results are in agreement with those of Martínez *et al*. (1997) and Marques *et al*. (1995) who also found that the growth of *Chlorella* under mixotrophic condition supplemented with glucose was much better than that under photoautotrophic growth condition.

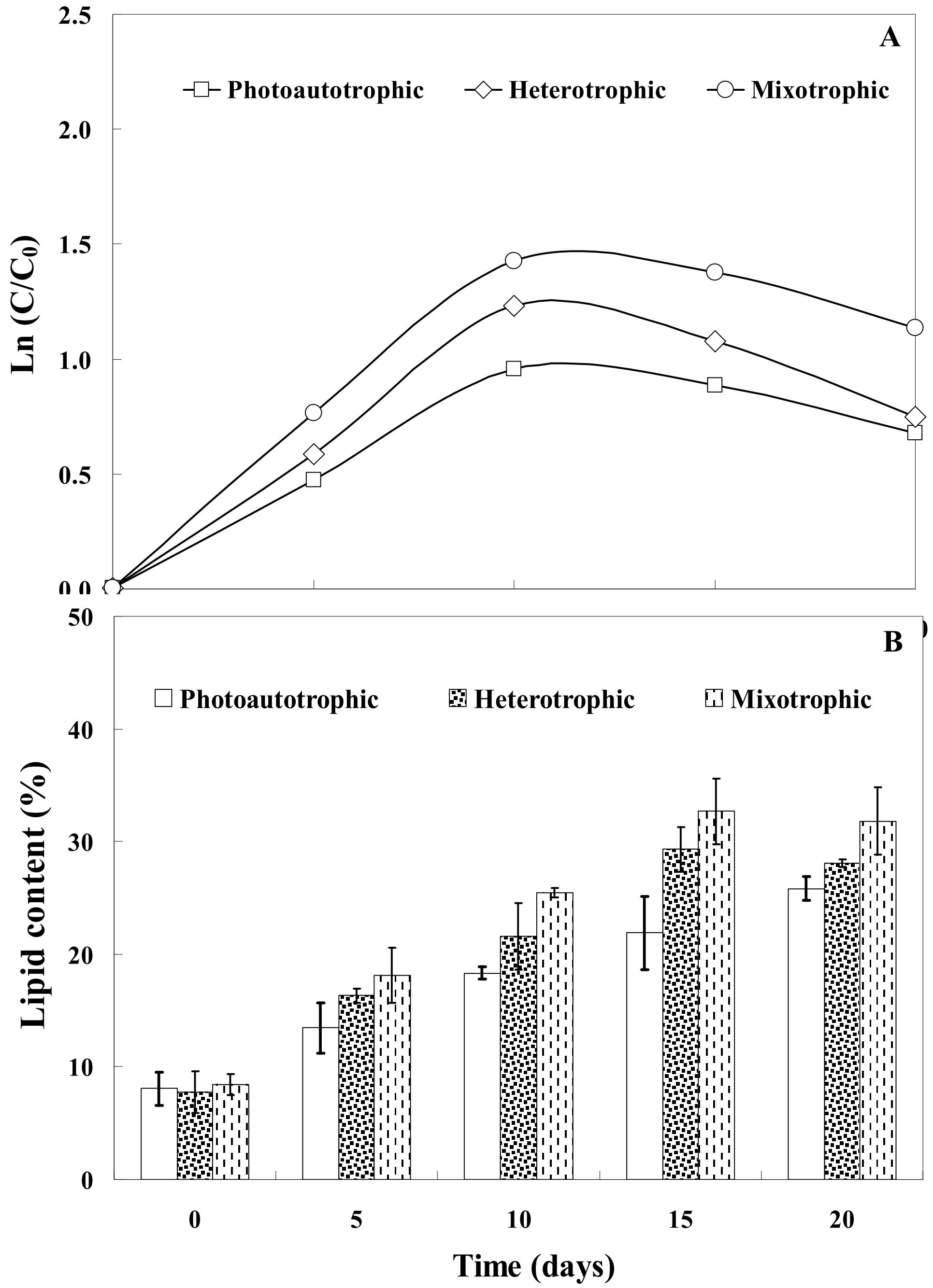


Figure 21: Growths (A) and lipid contents (B) of TRG strain cultivated under photoautotrophic, heterotrophic and mixotrophic condition. The algal growth was represented as Ln $(C/C_0)$ versus cultivation time

In the absence of light under heterotrophic condition, the use of suitable carbon source is crucial for attaining high biomass yield in algal culture. Many types of organic and inorganic carbon sources such as glucose and acetate are of the commonly used for heterotrophic cultivation of green microalgae such as *Chlorella zofingiensis* (Ip *et al*., 2004), *Chlamydonmonas reinhardtii* (Chen and Johns, 1994) and *Haematococcus pluvialis* (Jeon *et al*., 2006). However, severe growth inhibition was also observed in these algae even when carbon source was applied at low concentrations. In the case of *C. reinhardtii*, the alga started to be inhibited at an acetate concentration above 0.4 $g.L^{-1}$ (Chen and Johns, 1995).

Heterotrophic growth can be performed in conventional microbial bioreactors, which can improve the yield of biomass and reduce the cost of microalgal biomass production (Chen, 1996; Apt and Behrens, 1999; Borowitzka, 1999). It was reported that the heterotrophic process of *C. protothecoides* resulted in a yield 3.4 times higher than that from an autotrophic process (Shi *et al*., 2000). Chojnacka and Noworyta (2004) evaluated the growth of *Spirulina* sp. under photoautotrophic, heterotrophic and mixotrophic culture condition. They found that, *Spirulina* sp. was able to grow under photoautotrophic (in the light), heterotrophic (on glucose) and mixotrophic (simultaneously in the light and on glucose). The highest specific growth rate was reached under mixotrophic culture condition (0.055 $h^{-1}$, above light intensity 30 $W.m^{-2}$, glucose concentration 5 $g.L^{-1}$).

Although heterotrophic growth and photosynthesis has been reported to occur simultaneously and independently in mixotrophic *Spirulina* cultures (Marquez *et al*., 1993), the presence of organic carbon can alter both the photosynthetic and heterotrophic metabolism of *Chlorella* (Villarejo *et al*., 1995) and decreases production of photosynthetic pigments as compared with the amounts present in the absence of organic carbon source (Ogbonna and Tanaka, 1998). In this study, the lipid production under mixotrophic condition was higher than that obtained in photoautotrophic and heterotrophic conditions indicating that the organic carbon source did not negatively affected the product of TRG strain. Provided light and organic carbon simultaneously as energy sources, the mixotrophic culture reached the maximum final cell concentration, but formed a much less content of chlorophyll resulting in yellowish cell in comparison to the autotrophic culture (Figure 22). It was obvious that the heterotrophic and mixotrophic growth of TRG strain resulted in not only the disappearance of chlorophyll in cells but also accumulation of high lipid content in cells. Similarly Xu *et al*. (2006) revealed that the lipid soluble compounds from autotrophic cells appeared in a blackish green with chlorophyll and carotenoid as the major components, whereas the lipid-soluble compounds from the heterotrophic cells appeared in a state of light yellow grease, which are mainly lipid compounds (referred as oil).

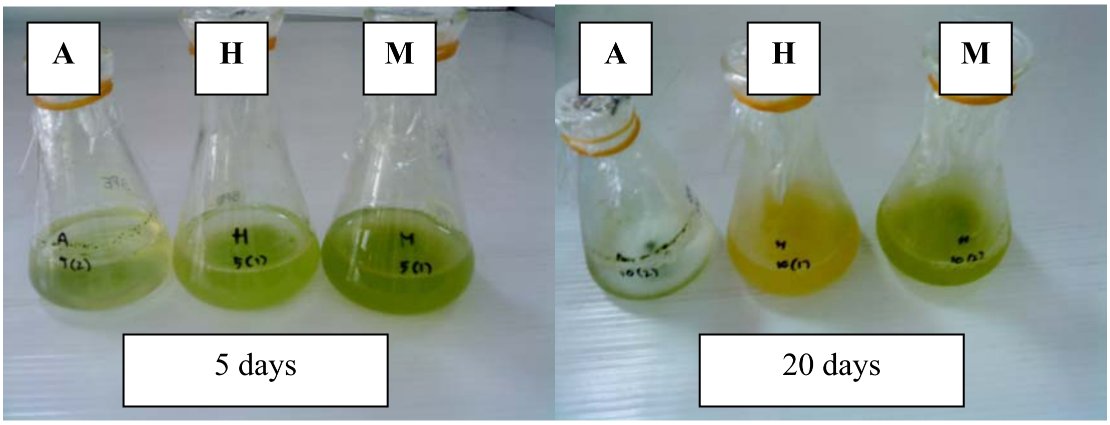


Figure 22 **:** Cell pellet of TRG strain cultivated under photoautotrophic (A), heterotrophic (H) and mixotrophic (M) condition.

### 5.2 Effect of glucose and molasses concentrations under mixotrophic condition

From the previous experiment, the results showed that TRG strain can grow well under mixotrophic condition with glucose as a carbon source. This experiment was conducted to evaluate the effect of glucose concentration on growth and lipid production of TRG strain under mixotrophic condition. The results are shown in Figure 23. The growth of TRG strain increased when the concentration of glucose was increased from 5 $g.L^{-1}$ to 15 $g.L^{-1}$. A further increase in glucose concentration did not encourage the growth of this strain. The highest level of dry cell weight (275 $mg.L^{-1}$) was obtained at 15 $g.L^{-1}$ glucose concentration. Although the glucose concentration of 15 $g.L^{-1}$ gave fastest growth and highest specific growth rate of 1.10 $d^{-1}$ followed by 10 $g.L^{-1}$ (0.97 $d^{-1}$), the highest lipid content of 37.5% was obtained at 10 $g.L^{-1}$ glucose concentration. The lipid content increased when glucose concentration was increased from 5-10 $g.L^{-1}$, and then decreased when increased glucose concentration up to 15-20 $g.L^{-1}$.

At low glucose concentration of 5 $g.L^{-1}$, glucose was depleted at day 5. At glucose concentration of 10 and 15 $g.L^{-1}$, glucose was gradually decreased and almost completely depleted at the end of cultivation. At high glucose concentration of 20 $g.L^{-1}$, glucose was remained about 5 $g.L^{-1}$. At this glucose concentration, the dry cell weight and lipid content were reduced to 156 $g.L^{-1}$ and 27%, respectively. There are two possible reasons for this phenomenon. One is that a high concentration of glucose could result in a high osmotic pressure, and the other may be substrate inhibition on cell growth and had affected to lipid production. This result was similar to the study of Oh *et al*. (2009) who also found that the highest lipid accumulation level of *Porphyridium cruentum*, 19.3% (w/w), was achieved under dark condition (heterotrophic) with 10 $g.L^{-1}$ of glucose concentration. While Zhu *et al*. (2008) explained the similar evident of higher lipid content in the heterotrophic than photoautotrophic condition may be because the excessive glucose consumption led to a sharp decrease in pH. It was reported that under mixotrophic condition the presence of an organic substrate mean that the cell growth is not strictly dependent on photosynthesis and hence light stops being an indispensable growth factor. In addition, it was found that the growth of *Spirulina* sp. was stimulated during the light phase in media supplemented with glucose (Marques *et al*., 1993; Chojnacka and Noworyta, 2004) and there was less biomass loss in the dark phase (Torzillo *et al*., 1993).

In order to reduce the cost of lipid production, molasses was used as a carbon source in the cultivation of TRG strain. The effect of molasses concentrations on the mixotrophic growth was evaluated. Figure 24 illustrates the influence of the molasses concentration on the growth and lipid production of TRG strain. Similar to the culture using glucose, the highest dry cell weight of 304 mg.L$^{-1}$ and lipid content of 36.9% were obtained at 15 g.L$^{-1}$ of molasses concentration. Both values increased markedly with increasing molasses concentration from 5 g.L$^{-1}$ to 15 g.L$^{-1}$ and tended to level off at molasses concentration higher than 15 g.L$^{-1}$. This might be due to substrate inhibition likewise in experiment of glucose. On the other hand, at highest concentration of molasses (20 g.L$^{-1}$) showed dark brown color. It might be obstacle of light exposure. As shown in Figure 25, both of specific growth rate and specific lipid production rate when using molasses as carbon source was higher than glucose. The highest specific growth rate (1.13 d$^{-1}$) and specific lipid production rate (0.453 g.L$^{-1}$d$^{-1}$) were obtained at 15 g.L$^{-1}$ of molasses concentration. From this result, it can be assumed that molasses was favour for growth and especially lipid accumulation of TRG strain than glucose. It might be due to the composition of molasses. It had been reported that molasses contains trace amounts of vitamins and significant amounts of several minerals. Blackstrap molasses is a source of calcium, magnesium, potassium, and iron; one tablespoon provides up to 20% of the daily value of each of those nutrients (Ensminger *et al*., 1983). In term of trace element, molasses contain with high iron concentration 2.39 mg. g$^{-1}$ dry weights (Chen and Chou, 1993). It was clarified that the effective of component in molasses leading yield enhancement.

Even, glucose is the final product of the photosynthesis, thus allowing the assumption that any photosynthetic microorganism must be able to incorporate it to its metabolism but the complex composition of molasses might be resulting in enhance of growth and lipid accumulation of TRG strain. From these results, it was suggested that both of glucose and molasses plays a vital role in promoting cell growth of TRG strain in mixotrophic culture. Supplementation of glucose or molasses not only led to a significant improvement in growth, but also lipid content. However, a high glucose (>15 g.L$^{-1}$) or molasses (>15 g.L$^{-1}$) level caused reduction in growth and lipid yield.

Similar results were found in another green microalga, *Chlorella protothecoides*, in which better growth was observed with increasing glucose concentration from 10 to 80 g.L$^{-1}$, but a further increasing glucose concentration (up to 100 g.L$^{-1}$) resulted in decreases in both the specific growth rate and cell growth yield (Shi *et al*., 1999), which might probably due to substrate inhibition (Chen and Johns, 1994: Chen and Johns, 1996).

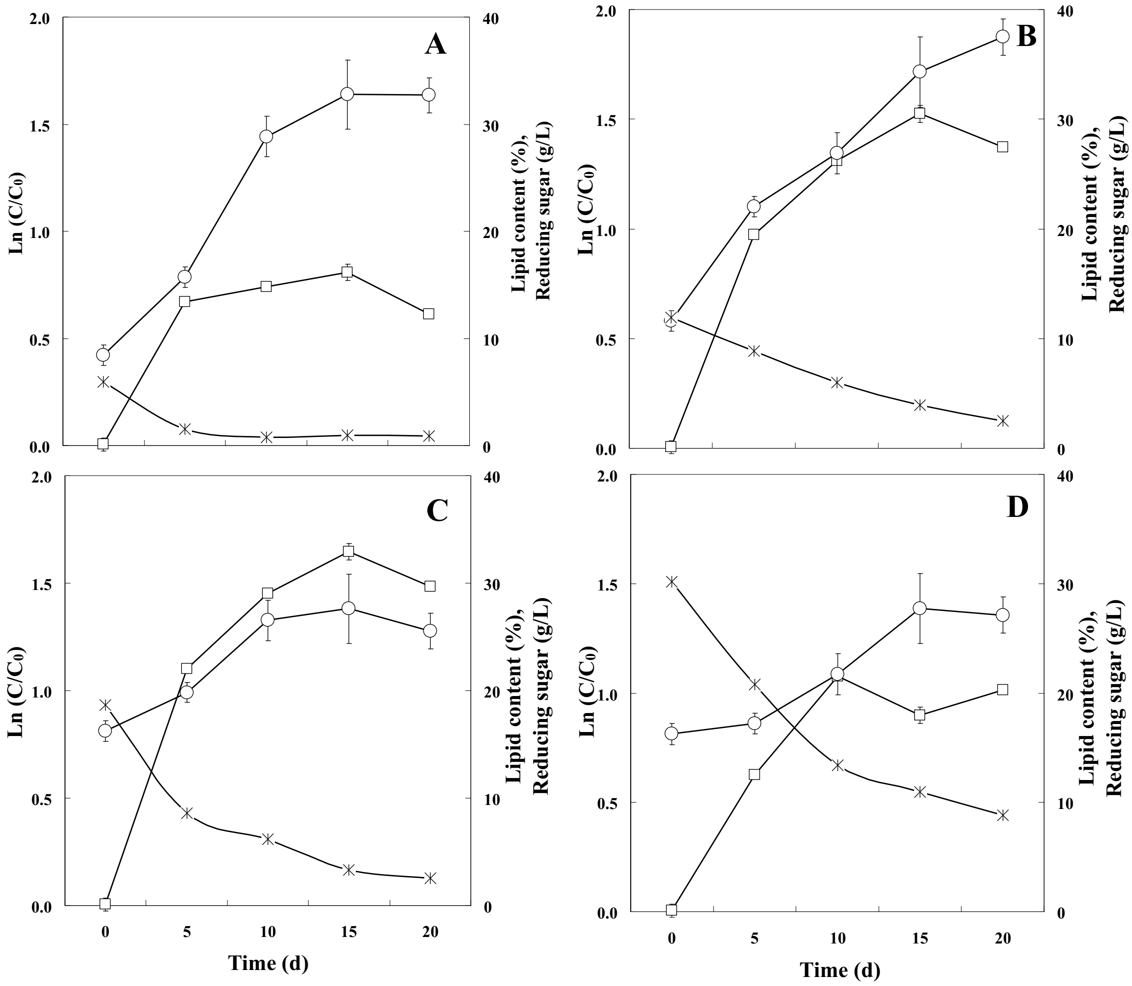


Figure 23 : Effect of glucose concentrations on growths (open square), lipid contents (open circle), and glucose consumption (star) of TRG strain. A: 5 g/L; B: 10g/L; C: 15g/L and D: 20g/L. The algal growth was represented as Ln ($C/C_0$) versus cultivation time

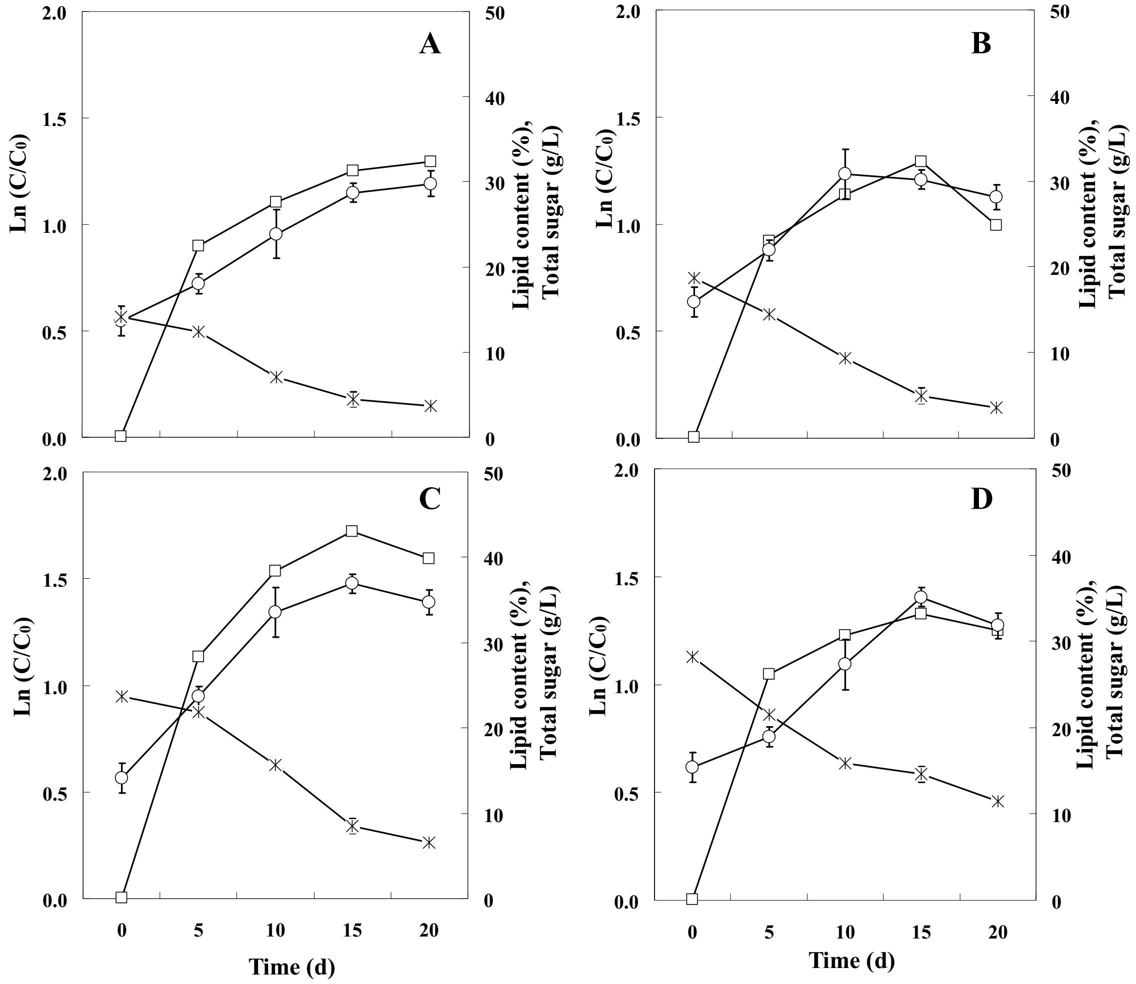


Figure 24: Effect of molasses concentrations on growths (open square), lipid contents (open circle), and total sugar (star) of TRG strain. A: 5 g/L; B: 10g/L; C: 15g/L and D: 20g/L. The algal growth was represented as Ln ($C/C_0$) versus cultivation time

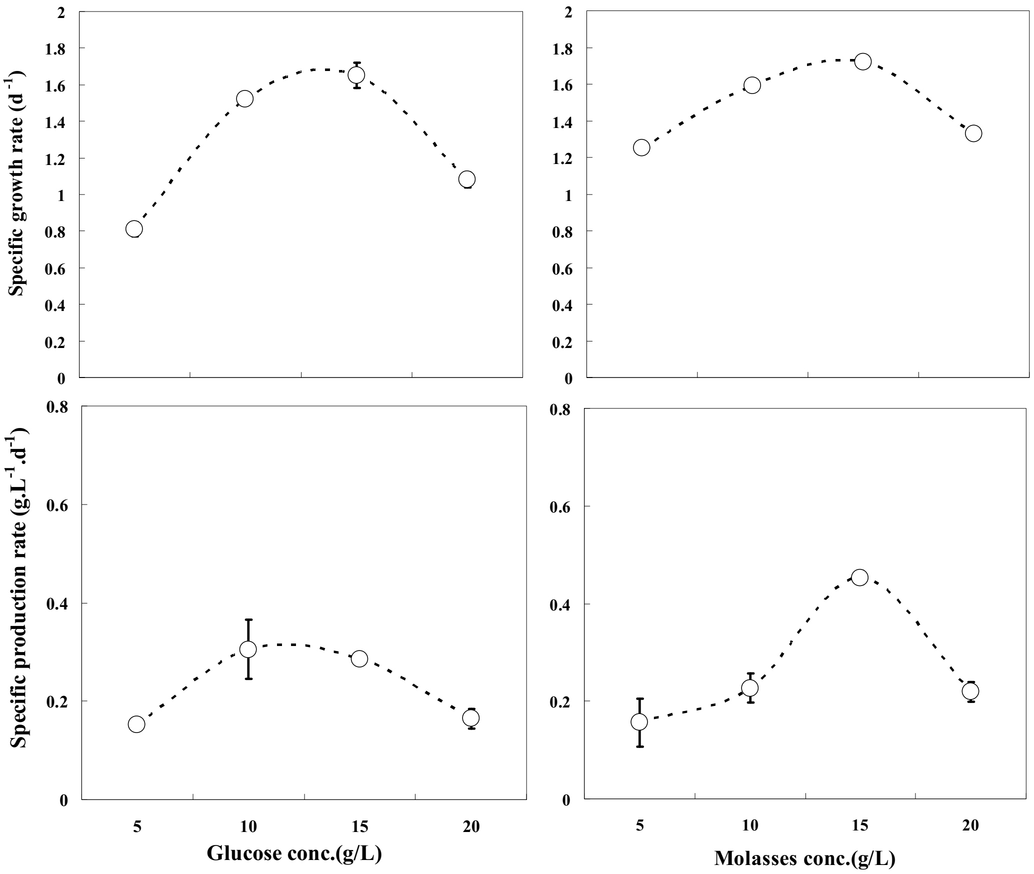


Figure 25: Effect of glucose (left side) and molasses concentrations (right side) on specific growth rate and specific lipid production rate of TRG strain.

**6. Batch and semi-continuous cultivation in a stirred tank photobioreactor**

TRG strain was cultured using the effluent in a stirred tank photobioreactor working volume 2 L under batch and semi-continuous mode. The growth and lipid content were investigated (Figure 26). The cell growth reached the highest value at day 11 (243 mg.L$^{-1}$) with highest specific growth rate of 1.70 d$^{-1}$. On day 10 of cultivation, the microalga grew into stationary phase. This could be due to the depletion of nitrogen source. The nitrate concentration in the effluent sharply decreased and closely depleted at the end of cultivation (Figure 24B). The lipid content showed increasing trend in parallel with biomass and reached the maximum of 30.95% at day 12. Since it was reported that nitrogen deficiency enhanced lipid accumulation in microalgal cell (Illman *et al*., 2000), the nitrogen depletion in effluent medium may be the reason for the increased lipid content in this study.

Nitrate removal was nearly coincident to that the alga consumed nutrient for cell growth. More than 80% of the initial nitrate was removed by TRG strain batch cultivation. The rate of nitrate removal decreased with prolonging the cultivation time. The maximum removal rate of nitrate was obtained at the beginning of cultivation (first day) as 3.5 mg.L$^{-1}$d$^{-1}$. The nitrate removal efficiencies achieved in this study was similar to the removal rate by *Chlorella pyrenoidosa* (3.4 mgN.L$^{-1}$d$^{-1}$) (Tam and Wong, 2000).

To achieve high cell density of the TRG strain, semi-continuous cultivation was attempted. As shown in Figure 27, 500 mL medium was removed every 5 days and the same volume of the fresh medium was added. The growth rate of TRG strain continually increased when the fresh effluent medium was added. The highest dry cell weight was achieved at day 21 (508 mg.L$^{-1}$) with highest specific growth rate of 2.65 d$^{-1}$. During the 20 days of the cultivation, the lipid accumulation continually increased along with the cell growth of TRG strain. The highest lipid content of TRG strain in semi-continuous cultivation was 32.2% of its dry weight and remained constant until the end of cultivation. Noteworthy, the biomass of TRG strain in semi-continuous culture was 2 times higher than that of the batch culture. TRG strain removed nitrate from effluent more than 92%. The pattern of decline of nitrate in effluent medium with batch mode decreased as a consequence over time. While in semi-continuous mode, the nitrate removal rate showed the dominant peak at the day as interval replacing the fresh effluent medium into the culture and gave the constant rate in another day. This evidence was resulted the intermittent curve. However, the maximum rate of nitrate removal in semi-continuous mode was occurred with a concomitant increase nitrate in the culture. The maximum rate of nitrate removal in batch mode (3.4 $mg.L^{-1}d^{-1}$) was 2 times lower than that in semi-continuous mode (7.24 $mg.L^{-1}d^{-1}$).

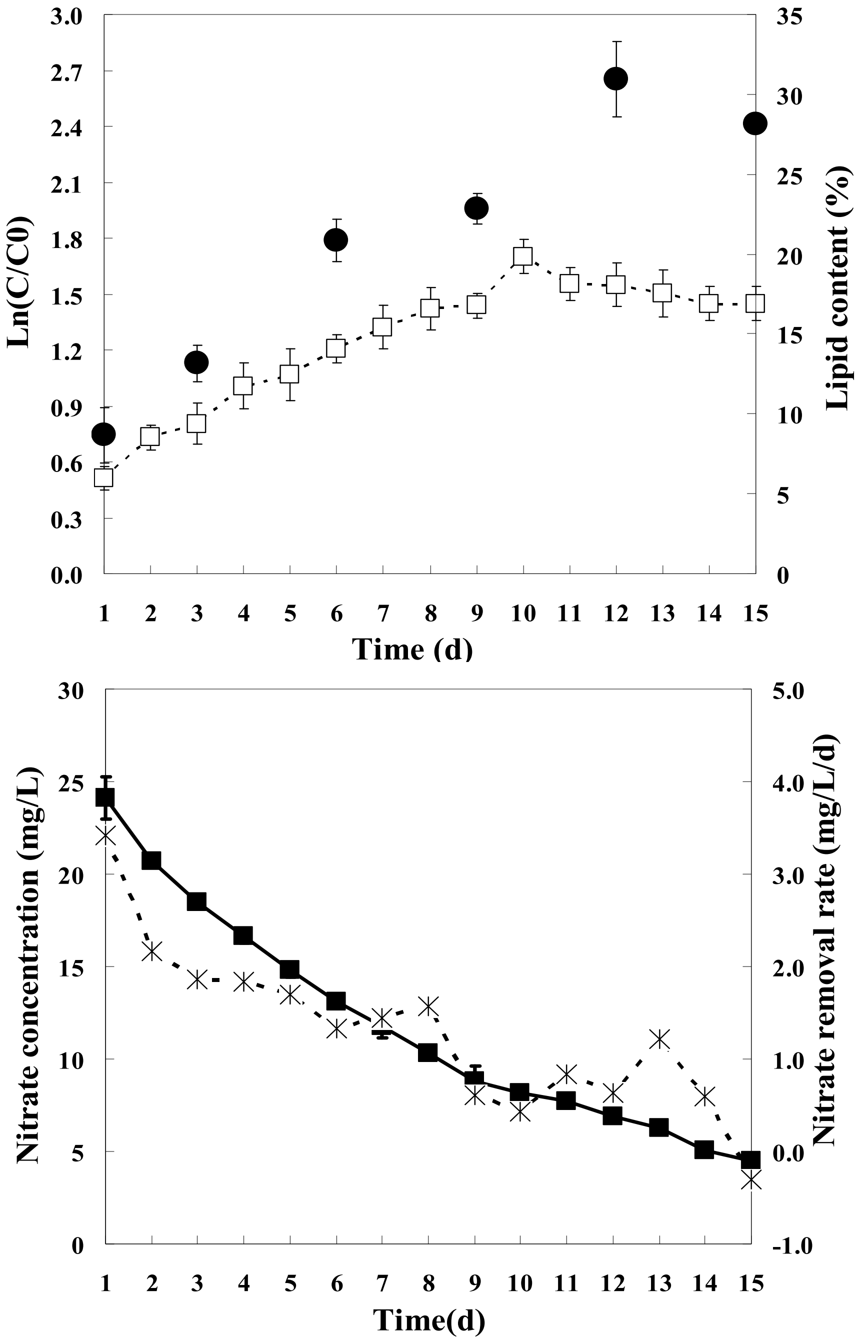


Figure 26: Batch cultivation of TRG strain in the undiluted effluent. The cultures were carried out with 125 rpm agitation at 25 ºC at 49.5 µmol.photon.$m^{-2}.s^{-1}$ light intensity with 16:8 hours light and dark cycle. The pH was controlled at pH 6.7 by automatically injecting carbon dioxide, as activated by the signal from pH sensor. $LnC/C_0$ (open square), lipid content (close circle), nitrate concentration (close square) and nitrate removal rate (star).

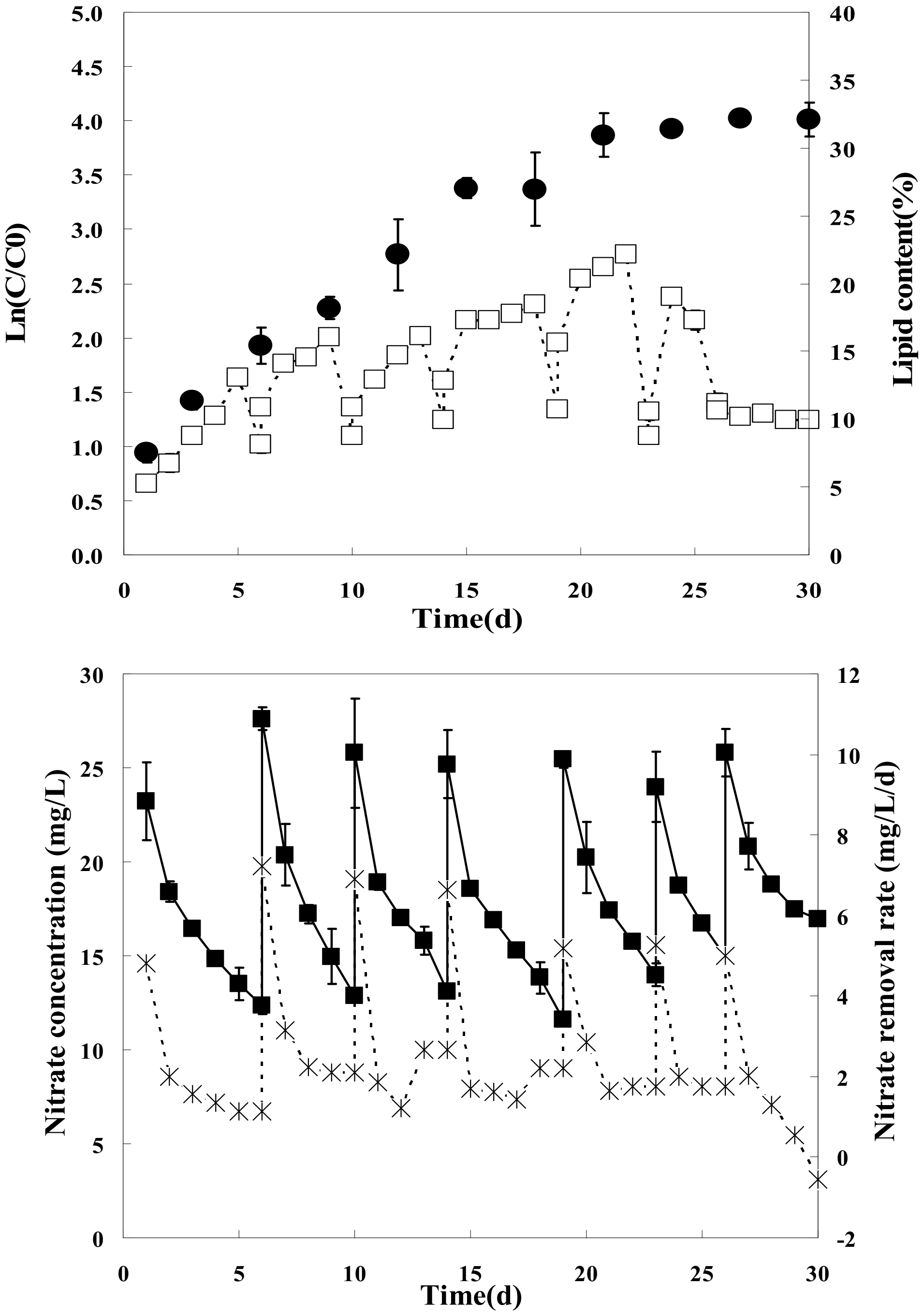


Figure 27: Semi-continuous cultivation of TRG strain in the undiluted effluent. The cultures were carried out with 125 rpm agitation at 25 ºC at 49.5 μmol.photon.m$^{-2}$.s$^{-1}$ light intensity with 16:8 hours light and dark cycle. The pH was controlled at pH 6.7 by automatically injecting carbon dioxide, as activated by the signal from pH sensor. 500 mL of medium was withdraw and replaced with fresh effluent medium in the same volume at 4 days of intervals. LnC/$C_0$ (open square), lipid content (close circle), nitrate concentration (close square) and specific nitrate removal rate (star).

**7. Outdoor cultivation in tubular photobioreactor**

The outdoor cultivation of TRG strain was attempted in a 20 L closed tubular photobioreactor with the working volume of 14 L using undiluted effluent from seafood processing plant under the climatic conditions of southern Thailand. The experiment was operated in semi-continuous system. The culture was replaced 5 L of broth with fresh medium daily. The reactor was provided with intermittent injection of pure $CO_2$ for controlled pH at 6.7. The tank was circulated by 25 ºC cooling water. The light intensity was in the range of 49.5-160.05 µmol.photon.$m^{-2}s^{-1}$ with the natural light–dark cycle. The operation under semi-continuous regime of tubular photobioreactor at outdoor resulted in a low dry cell weight of 145-157 mg/L as shown in Table 10. TRG strain accumulated a low lipid content of 26.7%. Changing of cell color from dark green to yellowish was observed at 10 days and subsequent until at the end of cultivation (as shown in Figure 28). This might be due either to the higher solar irradiance to the cultures or to the excessively high temperatures during the day (the range of mean temperatures of 30–33°C were recorded in the cultures during the midday). Although, the control unit including a thermostat provided temperature regulation of the outdoor units by automatically activating cooling water onto inside of reactors, when the culture temperature exceeded the preset value of 25°C, unfortunately, this cooling system was inadequate to maintain the culture temperature at the optimum value during the hours of strongest irradiation, particularly during the midday. It was reported that the cultivation of microalgae in outdoor photobioreactors could be hampered by some problems, including overheating and accumulation of oxygen to toxic levels (Tredici and Materassi, 1992). Prolonged exposure to supra optimal diurnal temperatures, together with high irradiances at midday, were considered the main cause of the low performance of the cultures. However, this finding in this study differs from suggestion of Garciıa-Gonzalez *et al*. (2005) who found that the outdoor cultivation of *Dunaliella salina* in a closed tubular photobioreactor could enhance biomass enriched in the valuable 9-cis-isomer of β-carotene and lutein.

Zittelli et al. (1999) demonstrated the feasibility of outdoor cultivation of *Nannochloropsis* sp. in tubular reactors and the potential of this eustigmatophyte as an alternative source of EPA. The transfer of the cultures from laboratory to outdoor conditions, the exposure to natural light–dark cycles, factors that caused lasting modifications in the fatty acid content and composition of *Nannochloropsis* sp., did not significantly affect the EPA content of the biomass. Chiu *et al*. (2008) cultured microalga *Chlorella* sp. in a photobioreactor to assess biomass, lipid productivity and $CO_2$ reduction. They found that during 8-day interval cultures in the semi-continuous cultivation aerated with 2–15%, the specific growth rate and biomass of *Chlorella* sp. cultures in the conditions $CO_2$ were 0.58–0.66 $d^{-1}$ and 0.76–0.87 $g.L^{-1}$, respectively. While, the lipid content in the cells cultured under different $CO_2$ aeration were very similar (approximately 32–34% of dry weight).

Table 10: Growth, lipid content and nitrate removal rate of outdoor cultivation TRG strain in tubular photobioreactor.

| Days | Specific growth rate ($d^{-1}$) | Lipid content (%) | Nitrate removal rate ($mg.L^{-1}d^{-1}$) |
|---|---|---|---|
| 0-3 | 0.116-0.470 | 9.87 | 2.48 |
| 3-6 | 0.294-0.571 | 16.92 | 1.62 |
| 6-9 | 0.179-0.544 | 23.07 | 1.21 |
| 9-12 | 0.189-0.560 | 26.71 | 0.63 |
| 12-15 | 0.144-0.463 | 25.93 | 0.50 |

The development of tubular photobioreactor is expected a potential technology for application of outdoor cultivation in tropical countries with elevated solar light availability. In addition, the tubular photobioreactors, considered to be more promising for the large scale production of bio-products obtained from the cultivation of microalgae, with the simultaneous removal of $CO_2$. In the future work, the use of combustible $CO_2$ from the factory to culture this alga should be attempted. Moreover, since it was reported that *Botryococcus* strain undergoes a color change because of an accumulation of secondary carotenoids (carotenoids produced in large quality under stress conditions such as nitrogen deficiency and high light intensity) (Banerjee *et al*., 2002), the development of tubular photobioreactor for valuable biochemical production should also be considered.

**1d 2d 3d 4d 5d 6d 7d 8d 9d 10d 11d 12d 13d 14d 15d**

Figure 28 **:** Broth color of outdoor tubular photobioreactor cultivation of TRG strain in the undiluted effluent. The cultures were carried out with 125 rpm agitation at 25 ºC at 49.5 µmol.photon.m-2.s-1 light intensity with 16:8 hours light and dark cycle. The pH was controlled at pH 6.7 by automatically injecting carbon dioxide, as activated by the signal from pH sensor. 1 L of medium was withdraw and replaced with fresh effluent medium in the same volume daily.

**8. Benefit and potential of isolated strain**

**8.1 Algal growth and lipid production**

Comparison among four strains, TRG fulfils the major requirements for lipid production, including high lipid content, yield and productivity in the growth medium which contained nitrogen source and a suitable level of $Fe^{3+}$. After growing in the growth medium, the lipid content of TRG could be further increased from 25.8% to 35.9% by exposing the algal cells to the medium without nitrogen source but contained higher level of $Fe^{3+}$ and higher light intensity. The results have proved that the modification of the culture condition can tailor to the specific demands of highly productive microalgae to attain a consistently good yield of lipid.

Moreover, under the optimal culture condition using effluent from seafood processing plant and molasses, the lipid content was also highest at 36.9%. Although comparing with the previous reports TRG strain has shown a relatively slow growth rate, its higher lipid content has attracted more attention for the possibility of being exploited as a renewable source.

**8.2 Co-process**

The commercial production of lipid and hydrocarbon from *B. braunii* cultivation is not viable because of its slow growth rate and high production costs (Sawayama *et al*., 1992; Banerjee *et al*., 2002). Although *B. braunii* forms spectacular blooms in nature, today its culture in open ponds is far from under control, essentially due to competition with fast-growing microalgae (Banerjee *et al*., 2002). In point of view, a co-process of wastewater treatment and lipid production in this study showed the strong realistically satisfy this demand. Since TRG strain was able to grow in undiluted effluent from seafood processing plant supplemented with 2.0% $CO_2$, this suggests the possibility of using industrial flue gas to supply cultures. Although the flue gas was not used in this study, according to Yoo *et al*. (2010) they found that *B. braunii* could grow with 10% $CO_2$ and flue gas (containing 5.5% $CO_2$) during 14-day cultivation. In those tested $CO_2$ concentrations, 2.0% was regarded as the best for *B. braunii* growth. Likewise, Ge *et al*. (2011) confirmed the tolerance of *B. braunii* to grow in high $CO_2$ concentration (20% $CO_2$).

The co-process of by-product, molasses, utilization and lipid production was also one of the methods to reduce the production cost of lipid. The highest lipid content of 36.9% was obtained at 15 $g.L^{-1}$ of molasses concentration. This was higher than the lipid content reported by Tansakul *et al*. (2005). In their study, *B. braunii* cultured in effluent from seafood processing plant accumulated only 16.98% of lipid content. Successive operation of this co-process gave rise to higher amount of lipid production and the concentration of nitrate in the effluent was significantly decreased.

# CHAPTER 4

# CONCLUSIONS AND SUGGESTIONS

## Conclusions

### Strain selection

1. Four microalga *Botryococcus* sp. (SK, TRG, PSU and KB) were isolated from lake and reservoir pond.
2. The combined trials of nitrogen limitation, moderate light intensity were preferred for lipid accumulation in all strains. High iron concentrations also improved considerable lipid accumulation.
3. The highest amount of lipid content 35.9% found in TRG strain and 30.2%, 28.4% and 14.7% of total algal dry weight in KB, SK and PSU strains, respectively
4. Palmitic and oleic acid were dominant fatty acid in lipid of isolated microalgae.

### Economical lipid production

5. Undiluted effluent with 2.0% $CO_2$ was optimum for growth and lipid production of TRG strains. Besides the lipid accumulating ability, TRG strain also removed 80% of nitrate in the effluent.
6. TRG strain could grow under photoautotrophic, heterotrophic and mixotrophic culture conditions with glucose and molasses as carbon source. The highest dry cell weight of 245 $mg.L^{-1}$ and lipid content of 37.4% were achieved under mixotrophic condition using glucose concentration of 10 $g.L^{-1}$. TRG strain also grew well when 15 $g.L^{-1}$ molasses was used instead of glucose.

### Scale Up in Photobioreactors

7. The batch culture in a stirred tank photobioreactor gave the highest dry cell weight of 267 $mg.L^{-1}$ and lipid content of 30.9%.

8. In semi-continuous culture, the higher dry cell weight of 508 $mg.L^{-1}$ and lipid content of 32.2% were achieved at day 21 of cultivation.
9. Outdoor cultivation of TRG strain was performed in a 20 L closed tubular photobioreactor. TRG strain gave a lower dry cell weight of 157 $mg.L^{-1}$ and lower lipid content of 26%.

**Suggestion**

1. With further understanding on the cultivation of another strain (KB, PSU and SK) in photobioreactors, much greater productivity of algal lipid would be obtained.
2. Based on the results that highlight the potential use of molasses as an organic substrate for the mixotrophic cultivation of TRG strain, the further study should be the use of another low cost agricultural by-product for the growth of this strain.
3. Since the results from the present study showed that in outdoor cultivation the biomass and lipid content changed with the incident irradiation, a sunshade could be an inexpensive alternative method for protecting growth performance against photoinhibition.
4. The use of combustible $CO_2$ from the factory to culture this alga should be attempted.

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# APPENDIX

## A. Modified Chu 13 medium

### 1. Definition

CHU 13 medium is a culture medium used in microbiology for the growth of certain algal species, first published by S.P. Chu in 1942. It is used as growth medium for the biofuel candidate alga *Botryococcus braunii*. CHU 13 includes essential minerals and trace elements that are required by algae for growth, but does not include a carbon source and so is only appropriate for growth of phototrophs. It can be prepared as either a liquid medium or as an agar medium.

### 2. Compound

Modified CHU13 Medium, one liter

| **Compound** | **mg/L** |
|---|---|
| $KNO_3$ | 400 |
| $K_2HPO_4$ | 80 |
| $CaCl_2$ dihydrate | 107 |
| $MgSO_4$ heptahydrate | 200 |
| Ferric Citrate | 20 |
| Citric acid | 100 |
| $CoCl_2$ | 0.02 |
| $H_3BO_3$ | 5.72 |
| $MnCl_2$ tetrahydrate | 3.62 |
| $ZnSO_4$ heptahydrate | 0.44 |
| $CuSO_4$ pentahydrate | 0.16 |
| $Na_2MoO_4$ | 0.084 |
| 0.072 N $H_2SO_4$ | 1 drop |

### 3. Preparing

The remaining volume is pure, de-ionized water. Because it is difficult to weigh out some of the trace minerals, it is advisable to create a mixture of all components at a large concentration, such as a thousand times these measures, and then mix with the appropriate amount of (pure, de-ionized) water. Correct pH to 7.5, and then autoclave.

**B. Nitrate assay: Ultraviolet Spectrophotometric Screening Method**
(APHA, AWA, WPCF., 1995)

**1. Apparatus**

a. Spectrophotometer for use at 220 nm and 275 nm with matched silica cells of 1-cm of longer light path.

**2. Reagents**

a. Nitrate-free water: Use redistilled or diluted, deionized water of highest purify to prepare all solutions and dilutions.

b. Stock nitrate solution: Dry potassium nitrate ($KNO_3$) in an oven at 105°C for 24 h. Dissolve 0.7218 g in water and dilute to 1000 mL; 1.00 mL = 100 μg.$NO_3^-$-N. Preserve with 2 mL $CHCl_3.L^{-1}$. This solution is stable for at least 6 months.

c. Intermediate nitrate solution: Dilute 100 mL stock nitrate solution to 1000 L with water; 1.00 mL = 10.0 μg.$NO_3^-$-N. preserve with 2 mL $CHCl_3.L^{-1}$. This solution is stable for 6 months.

**3. Procedure**

a. Treatment of sample: To 50 mL clear sample, filtered if necessary, add 1 mL HCl solution and mix thoroughly.

b. Preparation of standard curve: Prepare $NO_3^-$ calibration standard in the range 0-7 mg. $NO_3^-$ -N.$L^{-1}$ by dilute to 50 mL the following volumes of intermediate nitrate solution: 0, 1.00, 2.00, 4.00, 7.00,…35.0 mL. Treat $NO_3^-$ standard in same manner as samples.

c. Spectrophotometric measurement: Read absorbance or 100% transmittance. Use wavelength of 220nm to obtain $NO_3^-$ reading and a wavelength of 275 nm to determined interferce due to dissolve organic matter.

**4. Calculation**

For samples and standard, substract two times the absorbance reading at 275 nm from the reading at 220 nm to obtain absorbance due to $NO_3^-$. Construct a standard curve by plotting absorbance due to $NO_3^-$ against $NO_3^-$—N concentration of standard. Using corrected sample absorbances, obtain sample concentration directly from standard curve.

**Note**: If correction value is more than 10% of the reading at 220 nm, do not used this method.

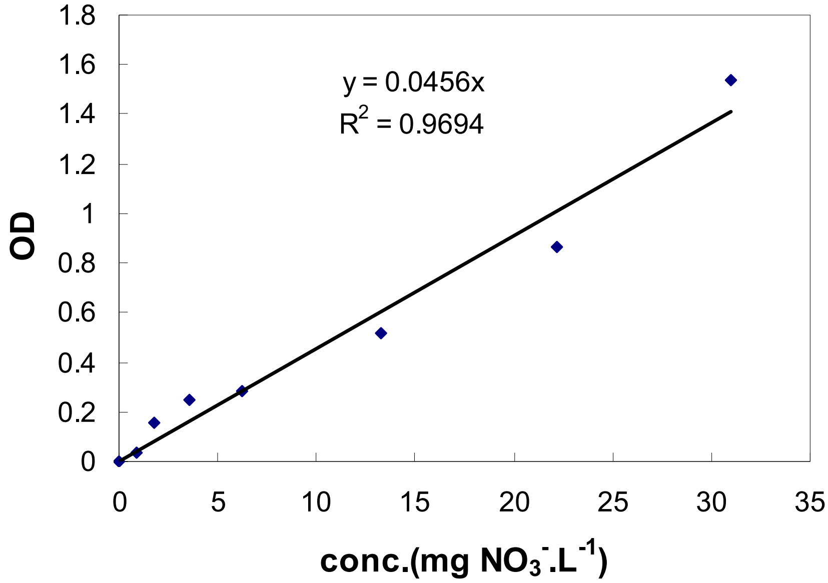


Figure 29: Standard curve of nitrate concentration

# Outputs

## Publication

**Yeesang, C.** and Cheirsilp, B. 2011. Effect of nitrogen, salt, and iron content in the growth medium and light intensity on ipid production by microlagae isolated from freshwater sources in Thailand. Bioresour. Technol. 102(3): 3034-3040

**Yeesang, C.** and Cheirsilp, B. 2010. Cost-effective production of lipid by green microalga cultivated in effluent from seafood processing plant. (Preparing).

**Yeesang, C.** and Cheirsilp, B. 2010. Enhanced growth and lipid production of green microalga under mixotrophic culture condition. (Preparing).

## Conference

### Poster presentation

**Yeesang, C.** and Cheirsilp, B. 2008. Isolation and cultivation of green microalga, *Botryococcus* sp. rom lake and freshwater pond in southern region of Thailand. The 20$^{th}$ Annual Meeting and International Conference of the Thai Society for Biotechnology. Taksila Hotel, Mahasarakarm, Thailand. 14-17 October 2008. pp.78.

### Oral presentation

**Yeesang, C.** and Cheirsilp, B. 2010. Hydrocarbon production by *Botryococcus* braunii TRG strain cultivated in industrial wastewater under mixotrophic condition. TSB 2010 International Conference on Biotechnology for Healthy living. Prince of Songkla University, trang campus, Trang, Thailand. 20-22 October 2010.


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# Effect of nitrogen, salt, and iron content in the growth medium and light intensity on lipid production by microalgae isolated from freshwater sources in Thailand

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ABSTRACT

Four green microalgae (TRG, KB, SK, and PSU) identified as *Botryococcus* spp. by morphological criteria were isolated from lakes and freshwater ponds in southern Thailand. In nitrogen-rich medium the strains achieved a lipid content of 25.8%, 17.8%, 15.8% and 5.7%, respectively. A combination of nitrogen deficiency, moderately high light intensity (82.5 $\mu E\ m^{-2}\ s^{-1}$) and high level of iron (0.74 mM) improved lipid accumulation in TRG, KB, SK, and PSU strains up to 35.9%, 30.2%, 28.4% and 14.7%, respectively. The lipid contents and plant oil-like fatty acid composition of the microalgae suggested their potential as biodiesel feedstock.



## 1. Introduction

*Botryococcus* species are colonial green alga that synthesize and accumulate an unusually high level of lipids in a range of 25–75% of their dry weights (Kalacheva et al., 2002; Metzger and Largeau, 2005). These types of alga would be useful for the production of biofuels, chemicals or chemical precursors. For commercial production of these compounds, locally adapted algae strains and optimized cultivation conditions are required.

A number of factors are known to influence the lipid content of microalgae, such as nitrogen (Illman et al., 2000) and silicon (Lynn et al., 2000) deficiency, phosphate limitation (Reitan et al., 1994), high salinity (Rao et al., 2007), stress from cadmium (Guschina and Harwood, 2006) or co-immobilization in alginate beads with the bacterium *Azospirillum brasilense* (Lebsky et al., 2001; de-Bashan et al., 2002). Light intensity (Kojima and Zhang, 1999) and iron content of the medium also affect algal growth (Liu et al., 2008).

The present study focused on the isolation of indigenous algae from lakes and freshwater ponds in southern Thailand and determined the effect of the nitrogen deficiency, high salinity, light intensity and iron concentration on growth and lipid production by the isolates. The fatty acid composition of the algal lipid was analyzed and the methyl esters were produced.

## 2. Methods

### 2.1. Isolation and purification

Microalgae were collected from lakes and freshwater ponds in southern Thailand (Songkla, Trang, and Krabi provinces) with a plankton net (10–12 μm × 7–9 μm in size). The pH values of these sites were neutral pH (6.7–7.2). Most sites had clear water with a slight blue tinge with an optical density at 435 nm in the range of 0.074–0.126 and a COD value of 328–1138 mg/L. *Botryococcus*-like green colonies of microalgae were separated using a sterile micropipette washing method (Stein, 1973) and cultured in modified Chu 13 medium which contained (g/L) $KNO_3$, 0.2; $K_2HPO_4$, 0.04; $MgSO_4{\cdot}7H_2O$, 0.1; $CaCl_2{\cdot}2H_2O$, 0.054; Fe citrate, 0.01; citric acid, 0.1; $NaHCO_3$, 0.036; and one mL of a microelement solution consisting of (g/L) $H_3BO_3$, 2.85; $MnCl_2{\cdot}4H_2O$, 1.8; $ZnSO_4{\cdot}7H_2O$, 0.02; $CuSO_4{\cdot}5H_2O$, 0.08; $CoCl_2{\cdot}6H_2O$, 0.08; and $Na_2MoO_4{\cdot}2H_2O$, 0.05 (Tansakul et al., 2005). The pH was 6.7. The algae were subjected to purification by serial dilution followed by plating. The individual colonies were isolated and inoculated into liquid modified Chu 13 medium and incubated at 25 ± 1 °C under 33 $\mu E\ m^{-2}\ s^{-1}$ light intensity with 16:8 h light and dark cycles. The purity of the culture was ensured by repeated plating and by regular observation under microscope. The isolated microalgae were tentatively identified as belonging to the genus *Botryococcus* according to morphological properties (Banerjee et al., 2002).

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*2.2. Algal cultures*

The isolates were grown in 50-mL Erlenmeyer flasks containing 20 mL of modified Chu 13 medium with agitation at 125 rpm at 25 ± 1 °C under 33 μE $m^{-2}$ $s^{-1}$ light intensity with 16:8 h light and dark cycles for 20 days. The dry biomass and lipid content were measured at 5-day intervals. All the experiments were carried out at least in duplicate. The specific growth rate ($\mu$) was calculated as the slope of the following equation:

$$\mathrm{Ln}\frac{C}{C_0} = \mu dt \qquad (1)$$

where $C_0$ is the initial biomass concentration (g/L) and $C$ is the biomass concentration (g/L) at any time $t$ (Ceron Garcia et al., 2005).

For the nitrogen deficiency trial, the isolates were inoculated in 50-mL Erlenmeyer flasks containing 20 mL modified Chu 13 medium and incubated with 125 rpm agitation at 25 ± 1 °C under 33 μE $m^{-2}$ $s^{-1}$ light intensity with 16:8 h light and dark cycles for 7 days. The culture was then centrifuged at 1585*g* for 15 min. Cell pellets were re-suspended in modified Chu 13 medium (a nitrogen-rich condition) and modified Chu 13 medium without addition of $KNO_3$ (a nitrogen-deficient condition). The culture was incubated for 14 days. The dry biomass and lipid content were measured. All the experiments were carried out at least in duplicate.

The effects of 0, 43 and 86 mM NaCl in the medium and light intensities of 33, 49.5 and 82.5 μE $m^{-2}$ $s^{-1}$ with 16:8 h light and dark cycles (Rao et al., 2007) were tested under nitrogen-rich and nitrogen-deficient conditions. Similarly, the effect of the addition of 0, 0.037, 0.37 and 0.74 mM $Fe^{3+}$ (Liu et al., 2008) to the culture medium were tested.

*2.3. Analytical method*

Cells were harvested by centrifugation at 1585*g* for 15 min, and pellets were freeze-dried in a freeze-drier. The dry weight of the algal biomass was determined gravimetrically and growth was expressed in terms of dry weight. Freeze-dried algal were extracted with 50 mL of *n*-hexane and sonicated at 70 Hz intensity with a sonicator (Transsonic model 460/H, Elma, Singen, Germany) at room temperature (Mexwell et al., 1968). The extraction process was repeated twice. The suspension was filtered through Whatman 1 No. 40 filter paper and the filtrate was transferred into pre-weighed glass vial. The hexane solution was evaporated to dryness at 30 °C under vacuum. The lipid content was measured gravimetrically and expressed as dry weight percentage (Dayananda et al., 2006).

Fatty acid methyl esters (FAME) from extracted lipid involved the hydrolysis of the lipids followed by esterification (Jham et al., 1982). The fatty acid composition in the FAME were analyzed using a HP6850 Gas Chromatography equipped with a cross-linked capillary FFAP column (Aligent Technologies, Palo Alto, CA) (length 30 m, 0.32 mm I.D, 0.25 μm film thickness) and flame ionization detector. Operating condition were as follows: inlet temperature 290 °C, oven temperature initial 210 °C hold for 12 min then ramp to 250 °C at 20 °C/min, hold 8 min and detector temperature was 300 °C. Fatty acids were qualified by comparing their retention times with those of standard ones.

## 3. Results and discussion

*3.1. Morphology, growth and lipid content of isolates*

Under the microscope, isolate SK showed a bright green color, a regularly pyramid shaped colloidal cell and was held together by a lipid biofilm matrix. The TRG, PSU and KB strains showed a dark green color, with irregular colonies consisting of hundreds of elliptical cells interconnected by strands of tough mucilage. The morphology of the isolates resembles those of *Botryococcus* spp. Banerjee et al. (2002) reported that the cells of *Botryococcus braunii* are embedded in a communal extracellular matrix (or "cup"), which is impregnated with oils and cellular exudates. Cells are attached to each other by a refringent material that sometimes links two or more distinct clumps of cells, but Metzger and Largeau

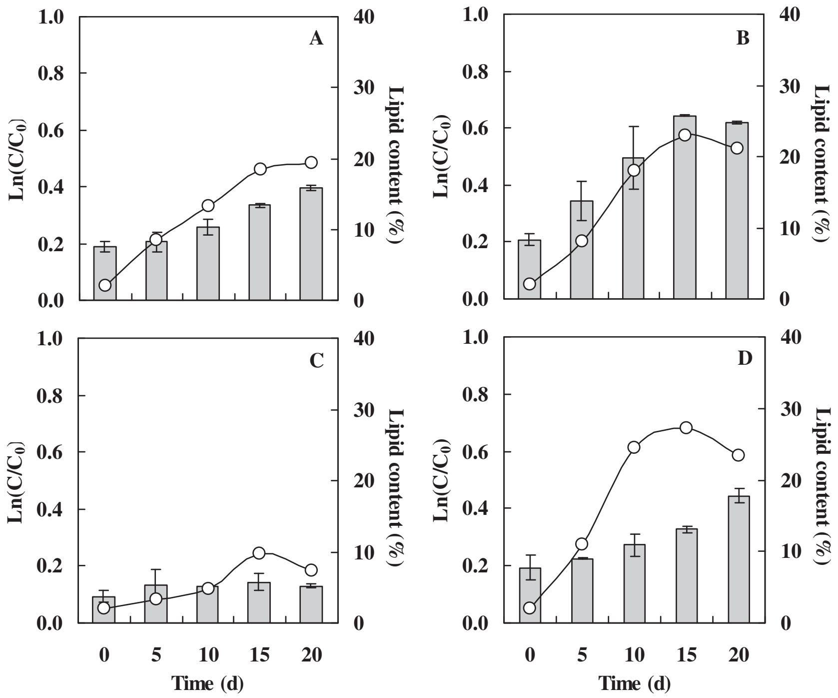


**Fig. 1.** Growth (line) and lipid content (bar) of four strains. A: SK, B: TRG, C: PSU and D: KB. The algal growth was represented as Ln ($C/C_0$) versus cultivation time.

(2005) reported that the morphology of the alga for the same strain could vary in relation to age and culture conditions.

Growth and lipid content profiles of the four isolates are shown in Fig. 1. Among the four strains, KB grew fastest and showed the highest specific growth rate of 0.223 $d^{-1}$ followed by TRG (0.182 $d^{-1}$) and SK (0.135 $d^{-1}$). PSU strain grew slowly and showed the lowest specific growth rate (0.061 $d^{-1}$). In nitrogen-rich medium TRG showed the highest lipid content of 25.8% based on its dry biomass weight and showed the highest lipid productivity of 46.9 mg $L^{-1}$ $d^{-1}$ (Table 1). Although KB gave the highest specific growth rate, a lower lipid productivity of 39.7 mg $L^{-1}$ $\mathbf{d^{-1}}$ was obtained due to its lower lipid content (17.8%) compared with TRG. The lipid contents in SK and PSU were 15.8% and 5.7%, respectively.

A comparison of the lipid contents of the newly isolated strains with those reported for other algae is shown in Table 2. The lipid content of TRG was lower than that of strain Yayoi isolated by Okada et al. (1995), but was higher than those reported for other strains (Okada et al., 1995; Tansakul et al., 2005; Dayananda et al., 2007).

### 3.2. Lipid production under nitrogen-deficient condition

The impact of nitrogen deficiency on algal growth and lipid production are shown in Fig. 2. An increase in algal biomass (the positive value of Ln ($C_{max}/C_0$)) was found under nitrogen-rich condition for all strains. In the absence of a nitrogen source, no growth was observed and the cells appeared bleached. Although some loss in algal biomass (the negative value of Ln ($C_{max}/C_0$)) was found, the lipid contents of four strains increased. The highest lipid content (32.3%) was found in TRG under nitrogen-deficient condition. The lipid content in SK, PSU and KB also increased up to 20.7%, 14.3% and 23.9%, respectively. Ahlgren and Hyenstrand (2003) reported that under nitrogen-deficient conditions, algal cells often accumulate a surplus of carbon metabolites as lipids. It was also reported that microalgae respond to the nitrogen starvation condition by degrading nitrogen containing macromolecules and accumulating carbon reserve compounds, such as polysaccharides and fats (Banerjee et al., 2002; Dayananda et al., 2005).

**Table 1**
Growth and lipid content and productivity of four isolated strains.

| Strain | Specific growth rate ($d^{-1}$) | Lipid content (%) | Lipid productivity (mg $L^{-1}$ $d^{-1}$) |
|---|---|---|---|
| SK | 0.135 | 15.8 | 21.3 |
| TRG | 0.182 | 25.8 | 46.9 |
| PSU | 0.061 | 5.7 | 3.5 |
| KB | 0.223 | 17.8 | 39.7 |

Notes: The lipid productivity was calculated as the maximum lipid content multiplied by the specific growth rate.

**Table 2**
Lipid content of strains isolated in the present study and of other strains reported in the literature.

| Strain | Lipid content (%) | Reference |
|---|---|---|
| SK | 15.8 | In present study |
| TRG | 25.8 | In present study |
| PSU | 5.7 | In present study |
| KB | 17.8 | In present study |
| Yayoi | 33.0 | Okada et al. (1995) |
| Yamanaka | 16.1 | Okada et al. (1995) |
| Kawaguchi-1 | 18.8 | Okada et al. (1995) |
| Kawaguchi-2 | 9.7 | Okada et al. (1995) |
| *B. braunii* N-836 | 13–18 | Dayananda et al. (2007) |
| *B. braunii* CFTRI-Bb1 | 13–18 | Dayananda et al. (2007) |

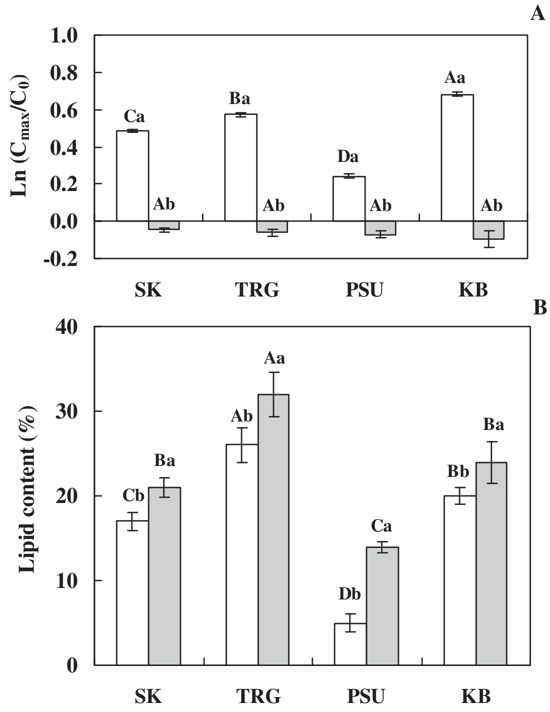


**Fig. 2.** Comparison of growth (A) and lipid content (B) of four strains under nitrogen-rich (white bar) and nitrogen-deficient (grey bar) conditions. The maximum algal growth was represented as Ln ($C_{max}/C_0$). Different capital letters on the bars indicate significant difference between strains in the same condition ($P < 0.05$). Different small letters on the bars indicate significant difference between nitrogen-rich and nitrogen-deficient conditions for the same strain ($P < 0.05$).

### 3.3. Effect of salinity

Since the natural habitat of each strain had various salt concentrations (54.6, 38.8, 21.6 and 17.2 mM for KB, SK, TRG and PSU strains, respectively), growth and lipid accumulation by these microalgae could be affected by salinity. Under nitrogen-rich condition, all strains survived at high salinity but growth of SK, TRG and KB strains decreased (Fig. 3). There was no significant effect of salinity on the biomass growth of PSU. The lipid contents of SK, TRG and PSU strains decreased when the salinity increased. This finding differs from that of Ben-Amotz et al. (1985) who found that the lipid content of *B. braunii* grown in 0.5 M salt concentration was higher than that with no salt.

### 3.4. Effect of light intensity

The effect of light intensity on growth and lipid production of the four strains is shown in Fig. 4. Photoinhibition on growth was observed in all strains under nitrogen-rich condition. The loss of biomass under nitrogen-deficient condition also increased with increasing the light intensity for all strains. The lipid contents in all strains increased with increasing light intensity from 33 to 49.5 $\mu E\ m^{-2}\ s^{-1}$, but decreased when the light intensity was increased up to 82.5 $\mu E\ m^{-2}\ s^{-1}$. TRG produced the highest amount of lipid compared with the other strains at all light intensities. A synergic effect of nitrogen deficiency and high light intensity was found in TRG. When the light intensity was increased to 82.5 $\mu E\ m^{-2}\ s^{-1}$, the lipid content in TRG strain under nitrogen-deficient condition was much higher than that under nitrogen-rich condition.

### 3.5. Effect of $Fe^{3+}$ concentration

The optimum levels of $Fe^{3+}$ concentration for the growth of SK and PSU were at 0.37 and 0.037 mM, respectively (Fig. 5). A

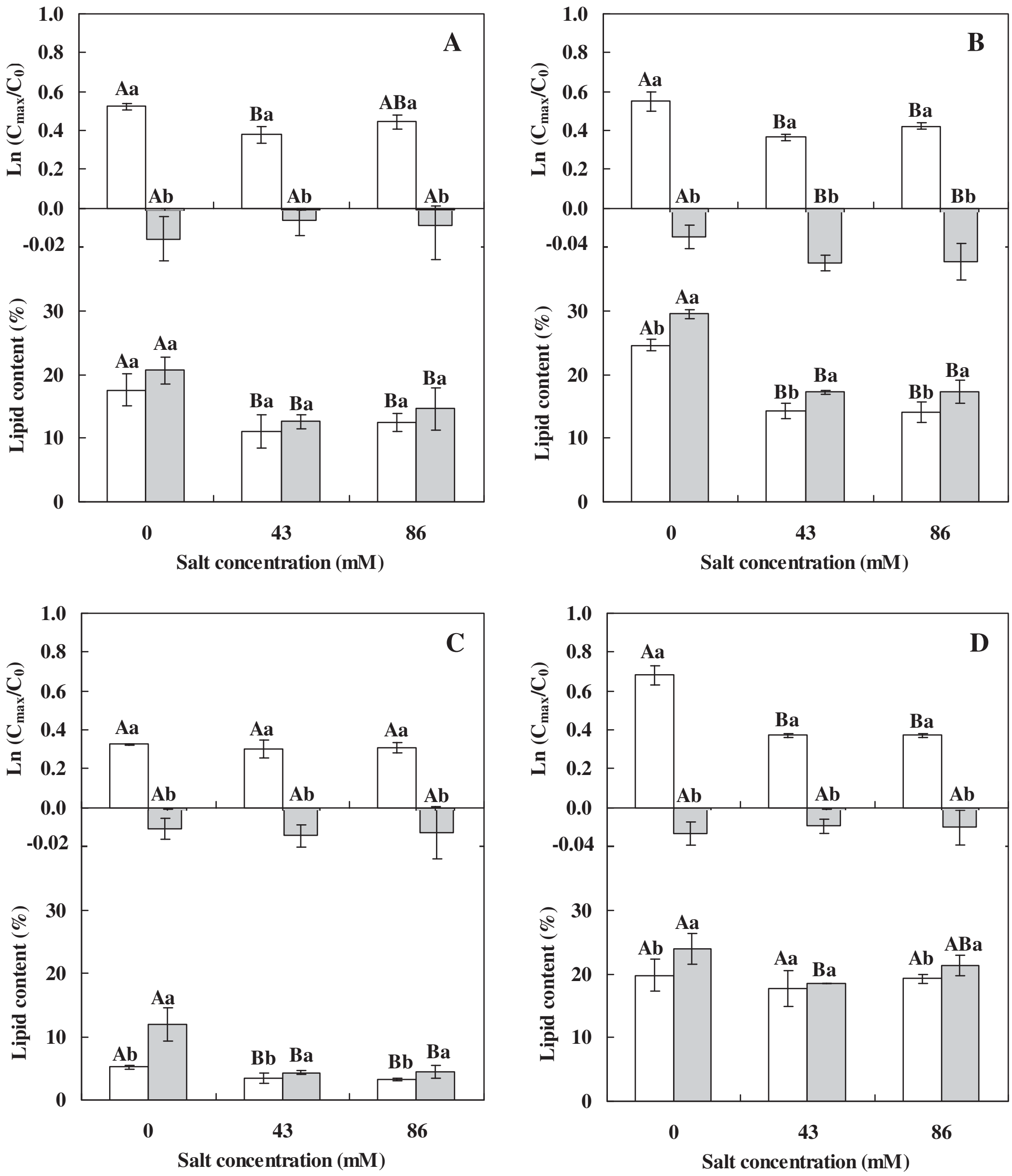


**Fig. 3.** Effect of salinity on growth and lipid content of four *Botryococcus* strains under nitrogen-rich (white bar) and nitrogen-deficient (grey bar) conditions. A: SK, B: TRG, C: PSU and D: KB strains. The maximum algal growth was represented as Ln ($C_{max}/C_0$). Different capital letters on the bars indicate significant difference between salinities in the same nitrogen condition ($P < 0.05$). Different small letters on the bars indicate significant difference between nitrogen-rich and nitrogen-deficient conditions in the same salinity ($P < 0.05$).

negative effect of $Fe^{3+}$ on growth of KB was observed at 0.74 mM. There was no significant effect of $Fe^{3+}$ addition on growth of TRG in the examined range. Under nitrogen-deficient condition, the loss of biomass increased slightly with increasing $Fe^{3+}$ concentrations. The lipid content in all strains under nitrogen-deficient condition increased with increasing $Fe^{3+}$ up to 0.037 mM. A further increase in $Fe^{3+}$ concentration did not improve the lipid content. Liu et al. (2008) also found that the total lipid content of *Chlorella vulgaris* in cultures supplemented with 0.012 mM $Fe^{3+}$ was increased 3–7-fold compared with those supplemented with lower iron concentrations. Under nitrogen-deficient condition, a greater improvement in the lipid content was observed when the $Fe^{3+}$ was increased up to 0.74 mM. Among the four strains, the combination of nitrogen deficiency, moderate high light intensity and high $Fe^{3+}$ concentration enhanced the lipid content in TRG up to the highest level of 35.9%. The lipid yield (the multiple value of $C_{max}$ and lipid content) of TRG was also highest compared with other isolates.

### 3.6. Fatty acid composition of biodiesel from microalgal lipid

The fatty acid composition of SK, TRG, KB and PSU is shown in Table 3. The isolated strains accumulated fatty acid in the range of C12:0 to C22:0. Palmitic acid was the predominant fatty acid in TRG (49.5%), PSU (46.7%) and KB (41.1%), while oleic acid predominated in SK (37.68%). These two fatty acids were also the major fatty acids in *B. braunii* reported by Fang et al. (2004) and Dayananda et al. (2006), whereas palmitic, linoleic and α-linolenic acids were described as the dominant fatty acids in *B. braunii* reported by Ben-Amotz et al. (1985). Lauric acid (C12:0) and palmitoleic acid, (C16:1) were found only in PSU, and tridecanoic acid

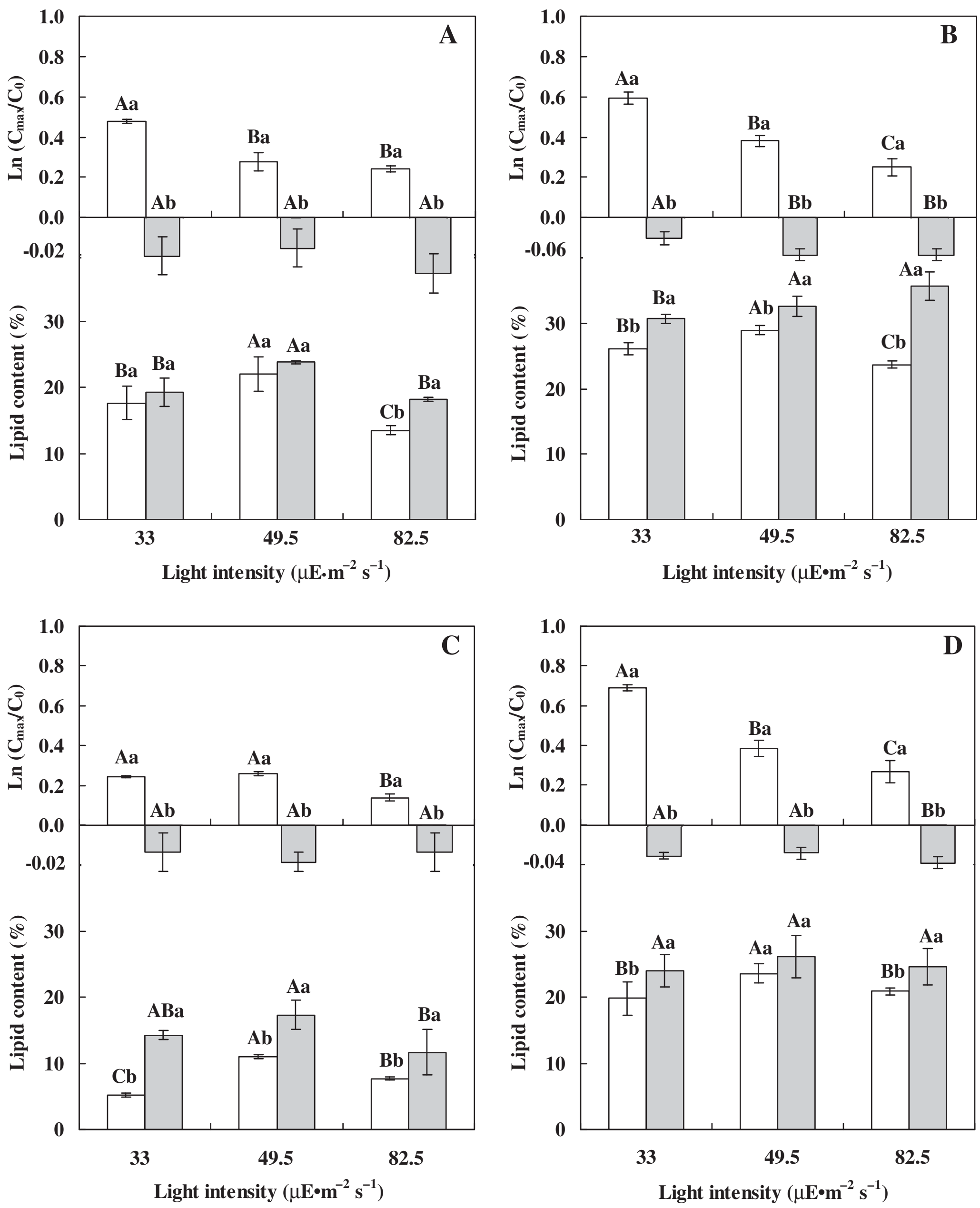


**Fig. 4.** Effect of light intensity on growth and lipid content of four *Botryococcus* strains under nitrogen-rich (white bar) and nitrogen-deficient (grey bar) conditions. A: SK, B: TRG, C: PSU and D: KB strains. The maximum algal growth was represented as Ln ($C_{max}/C_0$). Different capital letters on the bars indicate significant difference between light intensities in the same nitrogen condition ($P < 0.05$). Different small letters on the bars indicate significant difference between nitrogen-rich and nitrogen-deficient conditions in the same light intensity ($P < 0.05$).

(C13:0) and behenic acid (C22:0) were found only in KB and SK, respectively. Most of fatty acids found in isolated microalgae were similar to those found in plant oil (Fang et al., 2004). In addition, arachidic acid (C20:0), which is a saturated fatty acid found in fish oil and peanut oil (1.1–1.7%), was also found in SK, KB and PSU (0.22–0.63%).

Over 50% of the fatty acids in SK were unsaturated acids, while in KB, PSU and TRG, 41.1%, 34.5% and 30.6%, respectively, belonged to this group. Compared with the commonly used soybean oil as feedstock for biodiesel production in the US and the EU (linoleic and oleic acids contents of 53.7% and 23.3%, respectively), the algal fatty acids were more saturated. It was reported that the more saturated oil could provide biodiesel with higher cetane number (CN), decreased NOx emissions, a shorter ignition delay time, and oxidative stability (Antolin et al., 2002).

## 4. Conclusions

Comparison among four strains, TRG fulfils the major requirements for lipid production, including high lipid content, yield and productivity in the growth medium which contained nitrogen source and a suitable level of $Fe^{3+}$. After growing in the growth medium, the lipid content of TRG could be further increased from 25.8% to 35.9% by exposing the algal cells to the medium without nitrogen source but contained higher level of $Fe^{3+}$ and higher light intensity. The results have proved that the modification of the culture condition can tailor to the specific demands of highly productive microalgae to attain a consistently good yield of lipid. With further understanding on the cultivation of TRG in photobioreactors, much greater productivity of algal lipid would be obtained. Moreover, the use of primary-treated wastewater and outdoor

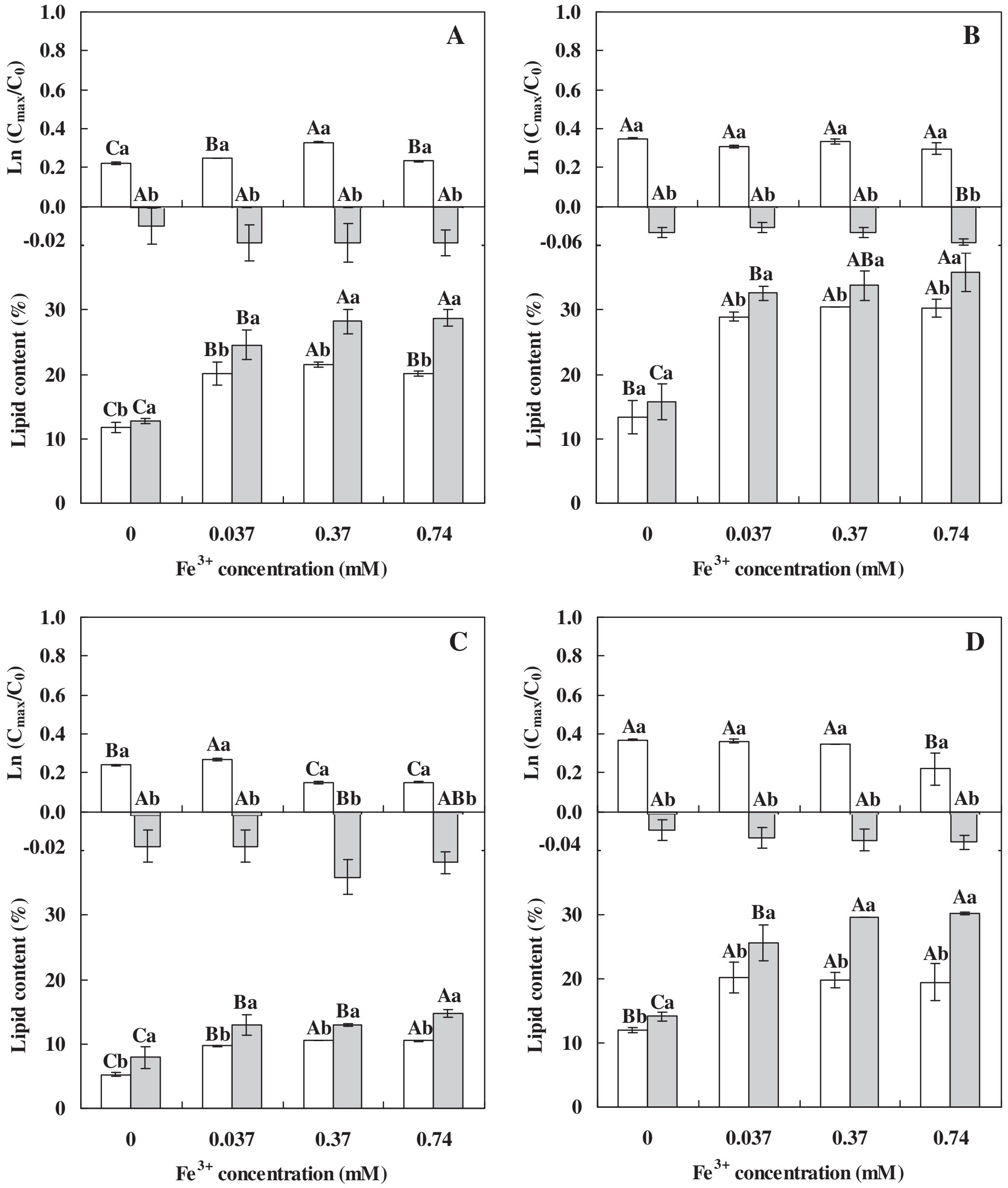


**Fig. 5.** Effect of $Fe^{3+}$ concentration on growth and lipid content of four *Botryococcus* strains under nitrogen-rich (white bar) and nitrogen-deficient (grey bar) conditions. A: SK, B: TRG, C: PSU and D: KB strains. The maximum algal growth was represented as Ln ($C_{max}/C_0$). Different capital letters on the bars indicate significant difference between $Fe^{3+}$ concentrations in the same nitrogen condition ($P < 0.05$). Different small letters on the bars indicate significant difference between nitrogen-rich and nitrogen-deficient conditions in the same $Fe^{3+}$ concentration ($P < 0.05$).

**Table 3**
Fatty acid composition of biodiesel derived from microalgal lipid.

| Fatty acid (%) | SK strain | TRG strain | KB strain | PSU strain |
|---|---|---|---|---|
| C12:0 | – | – | – | 0.27 |
| C13:0 | – | – | 0.18 | – |
| C14:0 | 3.95 | 3.33 | 3.35 | 2.36 |
| C15:0 | 1.56 | 6.43 | 3.55 | 2.54 |
| C16:0 | 34.04 | 49.54 | 41.13 | 46.65 |
| C16:1 | 0.94 | – | – | 0.41 |
| C17:0 | 1.54 | – | 0.55 | 0.49 |
| C18:0 | 12.02 | 10.11 | 10.73 | 18.93 |
| C18:1 | 37.68 | 28.51 | 35.21 | 30.63 |
| C18:2 | 5.01 | 1.34 | 2.73 | 2.00 |
| C18:3 | 7.35 | 0.74 | 2.73 | 1.04 |
| C20:0 | 0.63 | – | 0.35 | 0.22 |
| C20:1 | – | – | 0.39 | 0.44 |
| C22:0 | 0.28 | – | – | – |

cultivation could also make algal lipid production more economical by eliminating the need to supply nutrients and light, respectively.

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