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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 μE 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 μE 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 (μ) was calculated as the slope of the following equation:

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

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

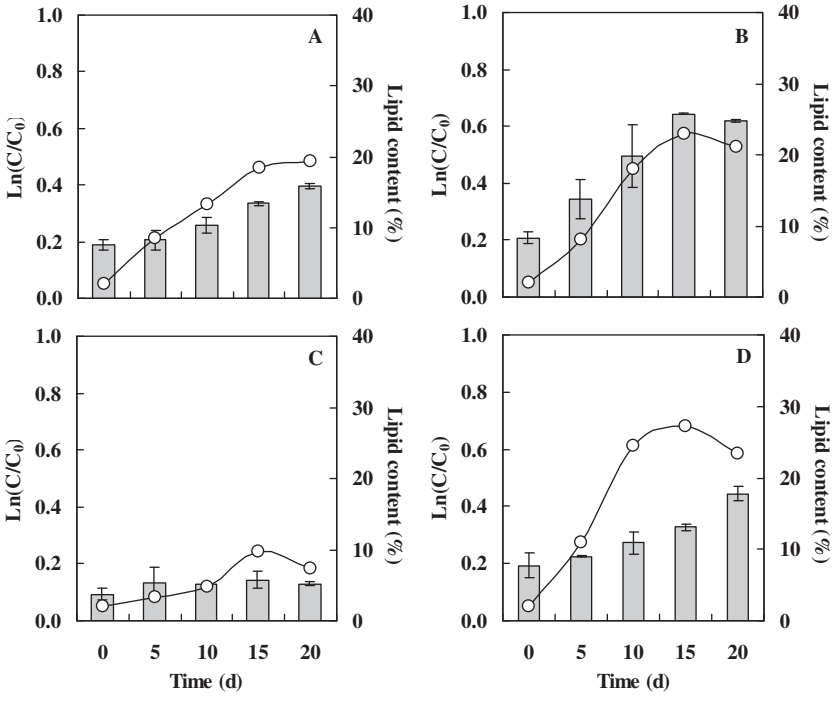

Fig. 1. Growth (line) and lipid content (bar) of four strains. A: SK, B: TRG, C: PSU and D: KB. The algal growth was represented as Ln (C/C_0) versus cultivation time.

(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} 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)

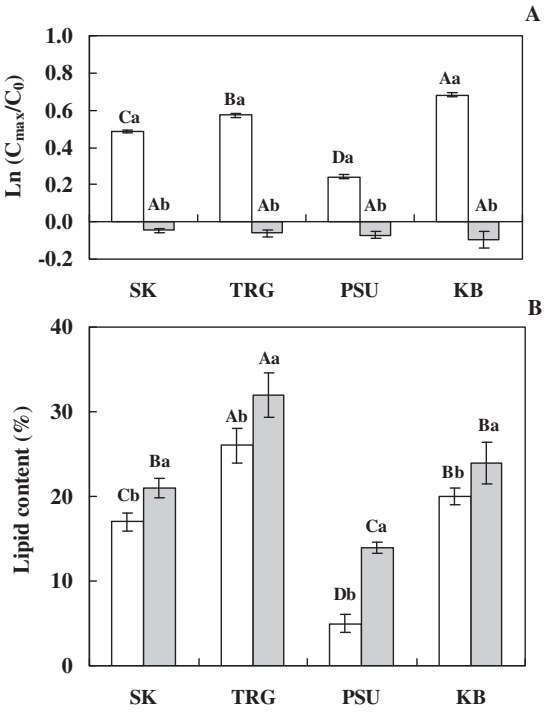

Fig. 2. Comparison of growth (A) and lipid content (B) of four strains under nitrogen-rich (white bar) and nitrogen-deficient (grey bar) conditions. The maximum algal growth was represented as Ln (C_{max}/C_0). Different capital letters on the bars indicate significant difference between strains in the 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 μE m^{-2} s^{-1}, but decreased when the light intensity was increased up to 82.5 μE 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 μE 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

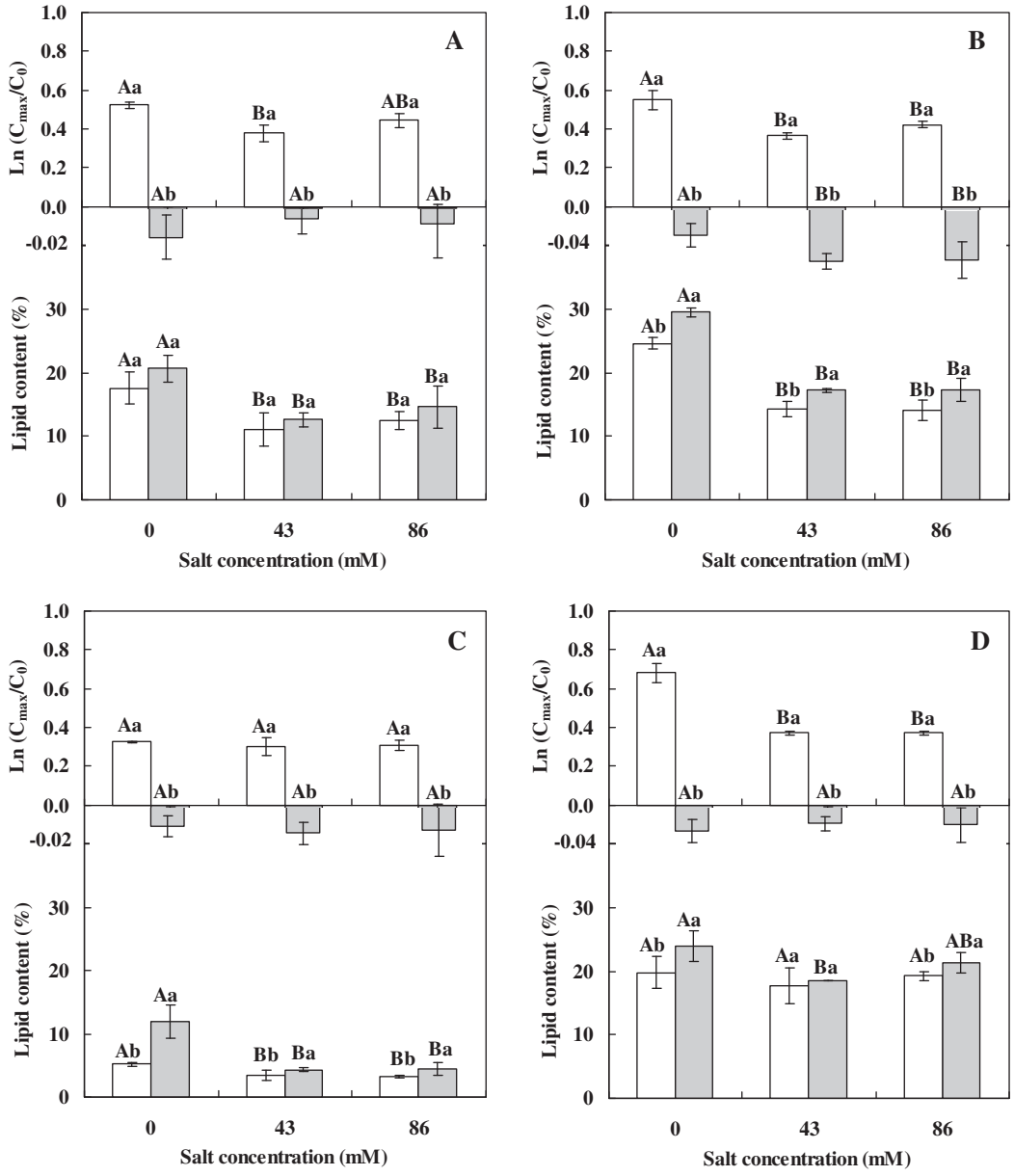

Fig. 3. Effect of salinity on growth and lipid content of four *Botryococcus* strains under nitrogen-rich (white bar) and nitrogen-deficient (grey bar) conditions. A: SK, B: TRG, C: PSU and D: KB strains. The maximum algal growth was represented as Ln (C_{max}/C_0). Different capital letters on the bars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

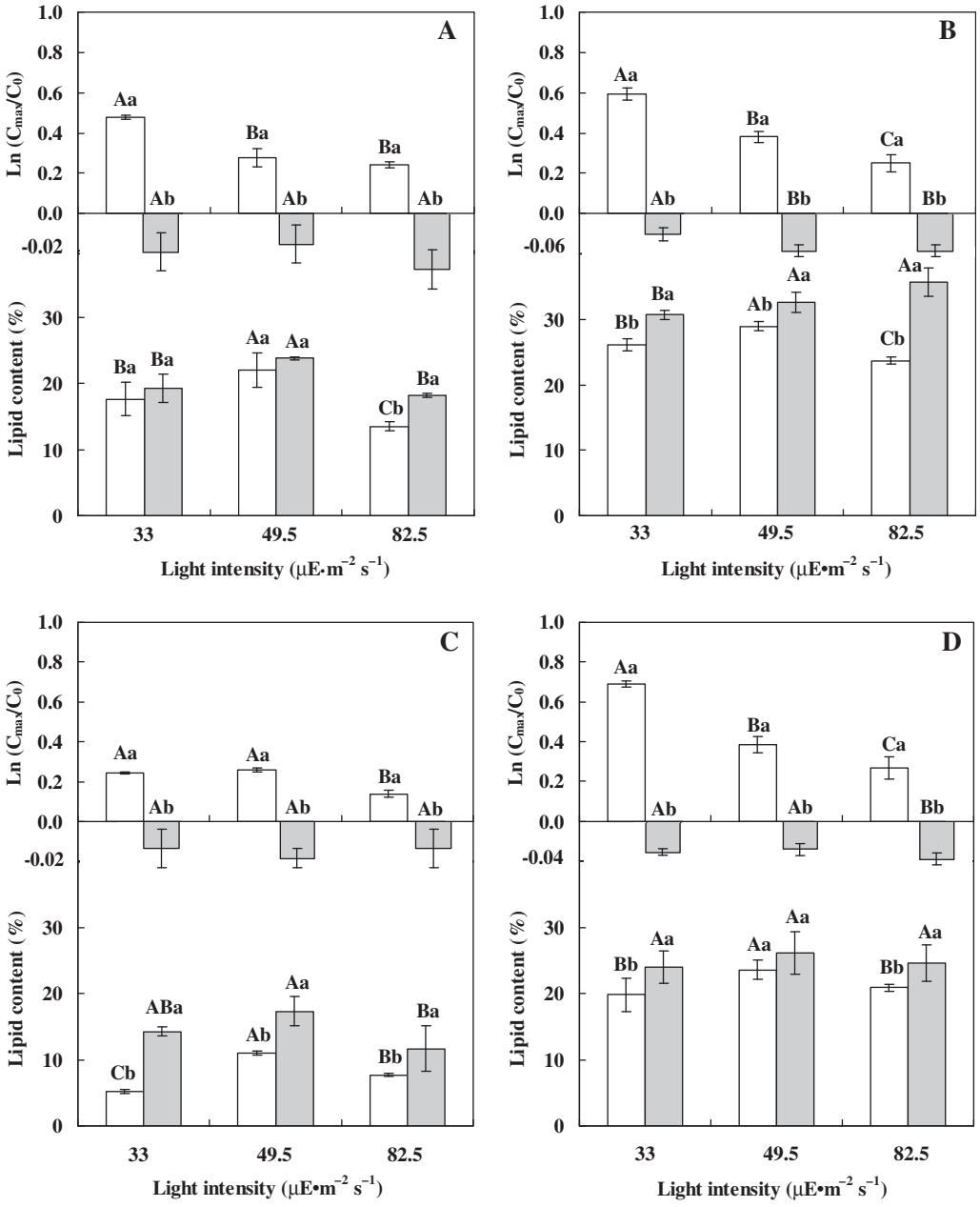

Fig. 4. Effect of light intensity on growth and lipid content of four *Botryococcus* strains under nitrogen-rich (white bar) and nitrogen-deficient (grey bar) conditions. A: SK, B: TRG, C: PSU and D: KB strains. The maximum algal growth was represented as Ln (C_{max}/C_0). Different capital letters on 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

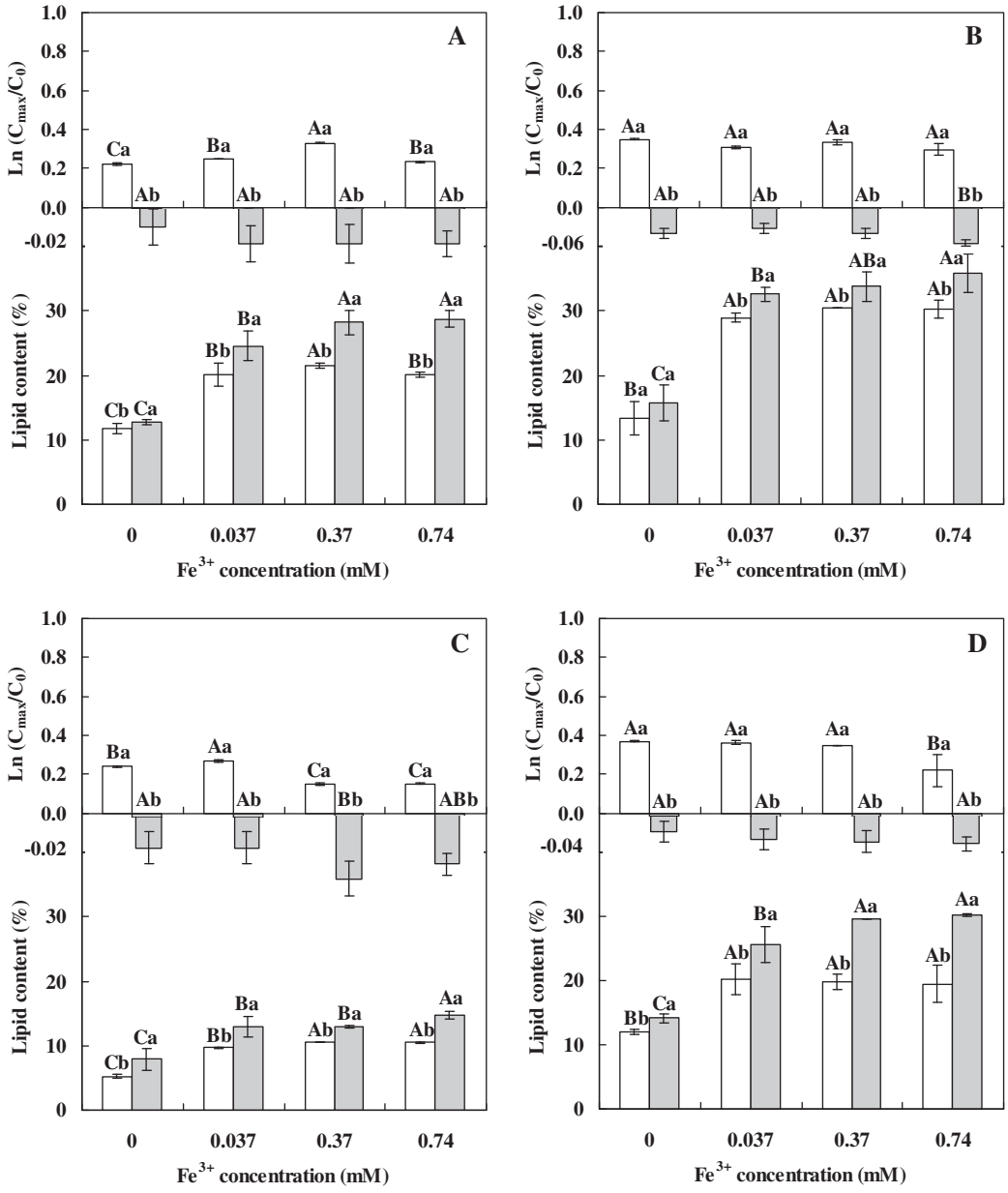

Fig. 5. Effect of Fe^{3+} concentration on growth and lipid content of four *Botryococcus* strains under nitrogen-rich (white bar) and nitrogen-deficient (grey bar) conditions. A: SK, B: TRG, C: PSU and D: KB strains. The maximum algal growth was represented as Ln (C_{max}/C_0). Different capital letters 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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