African Journal of Biotechnology Vol. 10(55), pp. 11600-11610, 21 September, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1050
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Photosynthetic efficiency and lipid accumulation are affected by the concentration of carbon in microalgae *Micractinium pusillum* Y-002

Xiaodong Deng[1], Bo Gu[1], Xinwen Hu[3], Jianchun Guo[1] and Xiaowen Fei[1,2]*

[1]Key Laboratory of Tropical Crop Biotechnology, Ministry of Agriculture, Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Science, Haikou 571101, China.
[2]Department of Biochemistry, Hainan Medical College, Haikou, 571101, China.
[3]College of Agronomy, Hainan University, Haikou, 571101, China.

Accepted 12 August, 2011

A micro-algae strain was isolated from fresh tropical water in Hainan, China. By phylogenetic analysis with 18S rDNA, we identified it as *Micractinium pusillum* strain Y-002. To study the relationships between nutrient and lipid accumulation of this strain, we cultured the organism either in complete TAP, SE, BG11 or HSM medium, or media deficient of nitrogen and iron, respectively. HSM media deficient in sulfur, phosphorus (P), magnesium (Mg), potassium (K) or calcium (Ca) were also tested. We found that HSM media, N and S starvation led to significant increase in cellular lipid content; and starvation of Ca and K only resulted in moderate increase of lipid content. Under the HSM-N or HSM-S culture condition, the increase in lipid content was accompanied by marked decrease in photosynthesis efficiency, total protein, and total carbohydrate content. Our investigation of the effect of exogenous carbon on lipid accumulation and photosynthetic efficiency revealed that the cell growth rate, photosynthesis efficiency, and lipid content per volume unit peaked in the presence of 15 mM sodium acetate (NaAC) and higher concentrations of NaAC led to decrease in these physiological traits.

Key words: *Micractinium pusillum*, lipid content, photosynthetic efficiency, growth rate, biodiesel.

INTRODUCTION

Due to the foreseeable depletion and the many environmental concerns associated with the use of fossil energy, the entire global society is beginning to realize that renewable fuel sources are the only viable solution to resolve the problem of energy shortage. Among these, production of renewable fuel from biomass consumes CO_2 and lowers its atmospheric concentration. Currently, the majority of biodiesel is produced from vegetable oils derived from oleaginous plants. These vegetable oils are transformed by the trans-esterification process, resulting in the so-called methyl esters or biodiesel. After blended with fossil diesel, biodiesel can be used to power cars and airplanes. However, biodiesel has been strongly criticized because the production of vegetable oils requires extensive cultivation of crops like rapeseed, palm, sunflower and soybean. These agricultural practices compete arable land with food crops (Deng et al., 2009; Li et al., 2008).

For decades, algae have been used in the production of food, feed and nutritional supplements because of its high carbohydrates, proteins and natural oils content in its composition. These organisms have also been employed in wastewater treatment in order to remove nitrogen (N), phosphorus (P) and metals. Further, algae have been considered as a potential source for CO_2 capture and biofuels production (Beer et al., 2009; Gouveia and Oliveira, 2009). Algae have a number of advantages over oleaginous plants:

1) Microalgae have fast growth rates and high lipid content, traits that have been considered as the most important feature for renewable energy. Some scientists believe that algae can potentially completely displace the

*Corresponding author. E-mail: Feixw2000@hotmail.com. Tel: +86-898-66960173. Fax: +86-898-66890978.

fossil fuels in the future (Chisti, 2007; Mata et al., 2009).

2) Microalgae can assimilate carbon dioxide as the carbon source for growth, thus mitigating the atmospheric CO_2 to reduce the environmental cost associated with biodiesel production.

3) Microalgae can be grown on brackish, marine and non-arable land, thus it will not compete for land and water with food crops.

Similar to higher plants, microalgal lipids are composed of neutral and polar lipids. Neutral lipids consist of triacylglycerols (TAG) and ester, mainly in the forms of TAG. Under favorable environmental conditions, microalgae mainly synthesize polar lipids such as glycolipids and phospholipids, which are enriched in chloroplast and cell membrane systems (Guckert and Cooksey, 1990; Harwood, 1998). When the environmental conditions are unfavorable for cell growth, many microalgae tend to accumulate neutral lipids in the form of lipid droplets localized in the cytoplasm as a means to store carbon and energy. Many studies have revealed that nitrogen (N), sulfur (S) or phosphorus (P) starvation induces a significant increase in neutral lipids content in microalgal species (Basova, 2005; Cobelas and Lechado, 1989; Merzlyak et al., 2007; Roessler, 1990; Shifrin and Chisholm, 1981; Spoehr and Milner, 1949; Thompson, 1996 ; Illman et al., 2000; Li et al., 2008; Hu et al., 2008; Khozin-Goldberg and Cohen, 2006; Otsuka, 1961). However, the effects of nutrient starvation on lipid deposition in strain *Micractinium pusillum* are unknown. Moreover, under nutrition starvation conditions, the biomass of the microalgae decreases, thus creating a paradox that requires the establishment of an optimal culture condition to achieve a balance between vegetative growth and lipid production. In this study, we analyzed the factors necessary for an optimal culturing condition for the microalgae strain, *M. pusillum*, for lipid production and biomass accumulation.

MATERIALS AND METHODS

Algal strain and cultivation conditions

The algal strain, *M. pusillum* Y-002, was isolated from a freshwater lake in Haikou, Hainan province, China. In each assay, algae grown and maintained on HSM solid medium were inoculated into 50 mL of TAP, HSM, SE or BG-11 medium or the corresponding nutrient deficiency media (Tables 1 and 2) in a 100 ml Erlenmeyer flasks and the corresponding nutrition restrict media, respectively. All cultures were maintained in an incubator shaker with 220 rpm at 24°C and exposed to a continuous illumination at a light intensity of 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

The biomass and neutral lipid analysis

To analyze the biomass of the cultures, samples were collected at 24 or 48 h intervals in triplicate and the biomass was determined by calculating the cell number as described by Harris (1989). The Nile Red fluorescence method was used to measure neutral lipids (Gao and Xiong, 2008). In these experiments, algal cells were directly stained with 0.1 µg/mL Nile Red (final concentration) for 10 min, and then fluorescence were measured on a GloMax®-Multi Detection System (Promega, USA), with excitation and emission wavelengths of 470 and 570 nm, respectively. The fluorescence was calculated by the equation: FD (470 / 570) = (A2 - A1), where A2 is the fluorescence value of algal cells after staining with Nile Red and A1 is that of the algal cells before staining. The lipid content of the algal cells was calculated using a standard curve based method. Triolein (Sigma, USA) was used as the lipid standard. A linear relationship between the concentrations of triolein and cell density was [0.0004 × FD (470/570) - 0.0038]/cell numbers.

For microscopic assay, images of cells stained with Nile Red (10 µg/ml final concentration) were acquired with a Nikon 80i Fluorescence Microscopes. Nile Red signals were captured using an excitation wavelength of 480 nm, and emission was collected between 560 and 600 nm (Huang et al., 2009; Chen et al., 2009).

Isolation of genomic DNA

M. pusillum cells grown to the mid-log phase were used for genomic DNA extrication according to the method of Deng and Erikson (2007) with minor modifications. Briefly, 1.5 ml of algal cells was pelleted and centrifuged for 30 s at 12,000 rpm. Pelleted cells were resuspended in 150 µl H_2O and 300 µl of SDS-Buffer (2% SDS; 400 mM NaCl; 40 mM Na_2EDTA; 100 mM Tris-HCl, pH 8.0), 5 µl of RNase (20 µg/µl) and 0.1 g of acid-washed glass beads (Sigma, St. Louis, MO) were added. After vortex, the samples were incubated at room temperature for 15 min. The samples were extracted twice with equal volumes of phenol-chloroform before DNA precipitation with two volumes of absolute ethanol. The DNA was washed twice with 70% ethanol, air dried and dissolved in 40 µl of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). Concentration of genomic DNA was determined by spectrophotometer and the DNA integrity was examined by agarose gel electrophoresis.

PCR amplification of 18S rDNA sequence and phylogenetic analysis

18S rDNA gene sequence was amplified using the primers 5'-CAGCMGCCGCGGTAATWC-3' and 5'-ACGGGCGGTGTGTRC-3' (Morgan-Kiss et al., 2008). Polymerase chain reaction (PCR) amplification was performed in a final volume of 25 µl containing 1 × PCR reaction buffer, 2 mM $MgCl_2$, 0.4 µM of each primer, 0.25 mM dNTPs, and 0.5 U Taq DNA polymerase (Promega, USA). Amplification was performed using the following program: 4 min at 95°C; 30 cycles of denaturation for 40 s at 95°C, annealing for 40 s at 68 to 58°C (decreasing 1°C per cycle in the first 10 cycles), and elongation for 20 s at 72°C; 10 min at 72°C. PCR products were purified using EZ Spin Column DNA Gel Extraction Kit (BBI, Canada) and cloned into the pMD18-T vector following the manufacturer's instructions (TaKaRa, Japan). Sequencing was performed by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd (Shanghai, China).

The sequences were aligned and compared with a dataset of algal sequence selected from GenBank using ClustalX 1.83 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Phylogenetic analysis were conducted using MEGA version 4.1 (Tamura et al., 2007), based on the neighbor -joining (NJ) method and 1000 bootstrap replications.

Chlorophyll fluorescence

Cells grown to the exponential phase were collected by centrifuged

Table 1. Media and their nutrition restriction media used in this work.

BG11	Working solution (mg/L)	-N	-Fe
$NaNO_3$	250	172(NaCl)	250
$K_2HPO_4·3H_20$	40	40	40
$MgSO_4·7H_2O$	75	75	75
$CaCl_2·2H_2O$	36	36	36
Citric acid	6	6	6
$FeC_6H_5O_7$	6	6	
EDTA	1	1	1
$NaCO_3$	20	20	20
A_5 + Co solution	1 ml	1 ml	1 ml
SE			
$NaNO_3$	250	172 NaCl)	250
$K_2HPO_4·3H_20$	75	75	75
$MgSO_4·7H_2O$	75	75	75
$CaCl_2·2H_2O$	25	25	25
KH_2PO_4	175	175	175
NaCl	25	25	25
$FeCl_3·6H_20$	5	5	3 (NaCl)
Fe-EDTA	10	10	10 (EDTA/)
A5 solution	1 ml	1 ml	1 ml
TAP			
Tris-Base	2420	2420	2420
Glacial acetic acid	1 ml/L	1 ml/L	1 ml/L
K_2HPO_4	119	119	119
KH_2PO_4	61	61	61
NH_4Cl	400	437 (NaCl)	400
$MgSO_4·7H_2O$	100	100	100
$CaCl_2·2H_2O$	50	50	50

at 3,000 g for 5 min and pellets were resuspended in 80% acetone for several hours until the cell turned gray in the dark. Chlorophyll fluorescence was measured at absorbance OD_{663}, OD_{646} and OD_{750} (Maxwell et al., 2000; Lichtenthaler, 1987) using a 752N UV-visible spectrophotometer (Shanghai Precision & Scientific Instrument CO., Ltd. CHN) and calculated with the formula: Chl a (mg/L) = 12.21*(OD663 - OD750) – 2.81*(OD646 - OD750), Chl b (mg/L) = 20.13*(OD646 - OD750) – 5.03*(OD663 - OD750).

Oxygen evolution and photosynthesis efficiency

Oxygen evolution of intact cells was measured at 25°C with a polarographic (Oxygraph-type) oxygen electrode (Hansatech Instruments Ltd., UK). 4 mM $NaHCO_3$ was added to cells suspended in their original growth medium as a terminal electron receptor. Photosynthesis rates *in situ* were calculated as: Oxygen evolution at 100 µM photons m^{-2} s^{-1} minus oxygen consumption in the dark. For all other oxygen evolution measurements, cells were collected by centrifugation at 3000 × g for 5 min, re-suspended in medium and dark acclimated at 25°C for 10 min. Chlorophyll *a* per sample ranged from 10 to 20 nM/ml. Cells were placed in the cuvette and nitrogen gas was used to purge dissolved oxygen to about 50% saturation. Dark respiration was measured first, followed by measurements of the rate of photosynthesis at sequentially increasing irradiance levels. The linear rate of oxygen evolution was recorded for 2 to 3 min at each irradiation. The maximal photosynthesis rate was estimated from light saturation curves constructed by plotting the total (photosynthesis plus respiration) photosynthesis rate versus light intensities. The photosynthesis efficiency was calculated as the slope of the linear portion of the light saturation curve (Björkman and Demmig, 1987).

Extraction and detection of total protein

Agal proteins were extracted essentially as described by Allen et al. (2007). Cells were collected by centrifugation at 1000 × *g* for 5 min, washed in 10 mM sodium phosphate, pH 7.0, re-suspended in the same buffer to a concentration equivalent of 4 × 10^8 cells/ml. The cells were then broken by sonication (microtip, 30% intensity, two cycles of 30 s). Extracts were separated into soluble and insoluble protein fractions by centrifugation (10,000 × *g* at 10 min). The pellet was washed once and resuspended to the same volume as that of

Table 2. Media and their nutrition restriction media used in this work.

HSM	Working solution (mg/L)	-N	-P	-S
Sodium acetate (hydrate)	2000	2000	2000	2000
NH_4Cl	500	546.7 (NaCl)	500	500
$MgSO_4{\cdot}7H_2O$	20	20	20	16.5 ($MgCl_2.6H_2O$)
$CaCl_2{\cdot}2H_2O$	10	10	10	10
K_2HPO_4	1440	1440	1234 (KCl)	1440
KH_2PO_4	720	720	163 (KCl)	720
Trace	1 ml	1 ml (Trace-N)	1 ml	1 ml (Trace-S)
HSM	**-K**	**-Fe**	**-Mg**	**-Ca**
Sodium acetate (hydrate)	2000	2000	2000	2000
NH_4Cl	500	500	500	500
$MgSO_4{\cdot}7H_2O$	20	20	11.5 (Na_2SO_4)	20
$CaCl_2{\cdot}2H_2O$	10	10	10	4.1(NaCl)
K_2HPO_4	116 (Na_2HPO_4)	1440	1440	1440
KH_2PO_4	63 (NaH_2PO_4)	720	720	720
Trace	1 ml	1 ml (Trace-Fe)	1 ml	1 ml

the soluble fraction. Protein concentration was determined with the Bradford Protein Assay Kit (Beyotime Institute of Biotechnology, CHN).

Detection of total carbohydrate

Anthrone solution was prepared by dissolving 0.2 g of the solute (9, 10-dihydro-9-oxoanthracene) in 100 ml sulfuric acid. The reagent was freshly prepared each day and used within 12 h (Yemm and Willi, 1954). The solution was allowed to stand for 45 min until it was perfectly clear, then 10 ml was pipetted into thick walled Pyrex tubes (150 × 25 mm) against 5 ml of Levoglucosan standard solutions (0.1, 0.2, 0.3, 0.4, 0.5, 1, 1.5, 2, 2.5 and 3 mg/L). The solutions were then transferred to a 100ºC water bath for 6 min to produce the green dye, followed by incubation at 4°C for 5 min. All samples were read in a calorimeter at 620 nm against the deionised water and calibration curve (levoglucosan concentration vs. absorbance) obtained from data for standard glucose solutions (0.1 to 3 mg/L) were drawn. This assay was carried out to determine the concentration of levoglucosan in particulate form 10 (PM) and residue samples using an improved technique in which the order of addition of reagent proved to be essential for data reproducibility. Corrections are made for non carbohydrate materials that may be present in the sample (Fartas et al., 2009; John et al., 1985).

RESULTS

Phylogenetic analysis of the isolated algae

Molecular phylogeny of the isolated algae Y-002 was analyzed using partial sequences of the nuclear 18S rDNA. Based on the nuclear 18S rDNA gene, the algal strain is closely affiliated with organisms of the *M. pusillum*. The closest match to strain Y-002 is *M. pusillum CCAP248/15* (Figure 1). Thus, this strain was placed in this genus.

Nutrition restriction affects the lipid content and the growth rate of *M. pusillum* Y-002

We observed that in the nitrogen-free version of the TAP, HSM, SE or BG11 medium, the growth rate of *M. pusillum* Y-002 was slower than in complete media (Figure 2). Importantly, the lipid content of the cells grown in TAP-N, SE-N or BG11-N moderately increased compared to cells grown the corresponding complete media. On the other hand, cells grown in HSM-N medium contained significantly higher (more than 10 times) lipid than in complete HSM medium (Figure 3 and Table 3). Iron deficiency affected cell growth rates in all tested media, but the effect of iron deficiency on lipid accumulation varied (Figure 2). For examples, lipid content did not change between cells grown in HSM-Fe, SE-Fe or BG11-Fe medium or the complete media, but detectably increased in TAP-Fe (Figure 3). Moreover, some ions such as Mg^{2+}, Ca^{2+}, K^+, or PO_4^{3-} are required for optimal cell growth rates in HSM medium, but only sulfur starvation caused a dramatic lipid accumulation. Lipid content increased more than 6 times in day 4 when this element was subtracted from the HSM medium (Figure 4 and Table 3). Restriction of Ca^{2+} and K^+ led to detectable increases in lipid levels, whereas PO_4^{3-} and Mg^{2+} deficiency did not cause detectable increase in lipids (Figure 4).

The restriction of N or S affects the cell photosynthesis efficiency and the distribution of main components in HSM medium

Since cells grown in HSM-N or HSM-S medium caused a drastic increase of lipid content in *M. pusillum* Y-002, we

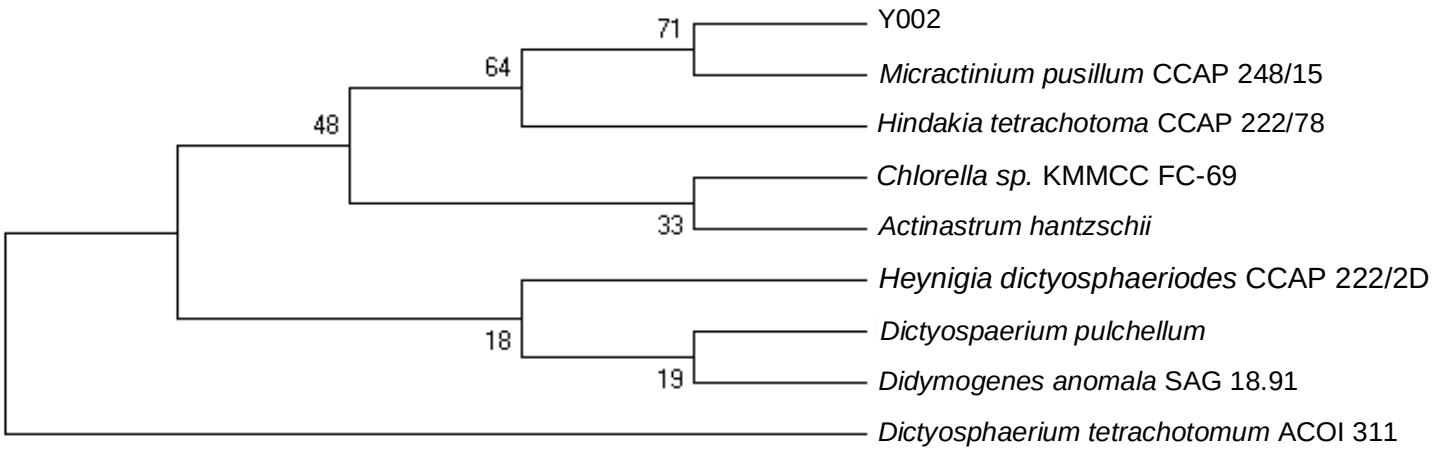

Figure 1. 18S rDNA phylogenetic tree of algae strains Y002. Phylogenetic analysis were done using MEGA version 4.1. Result showed that Y002 was closely related to *M. pusillum* CCAP248/15.

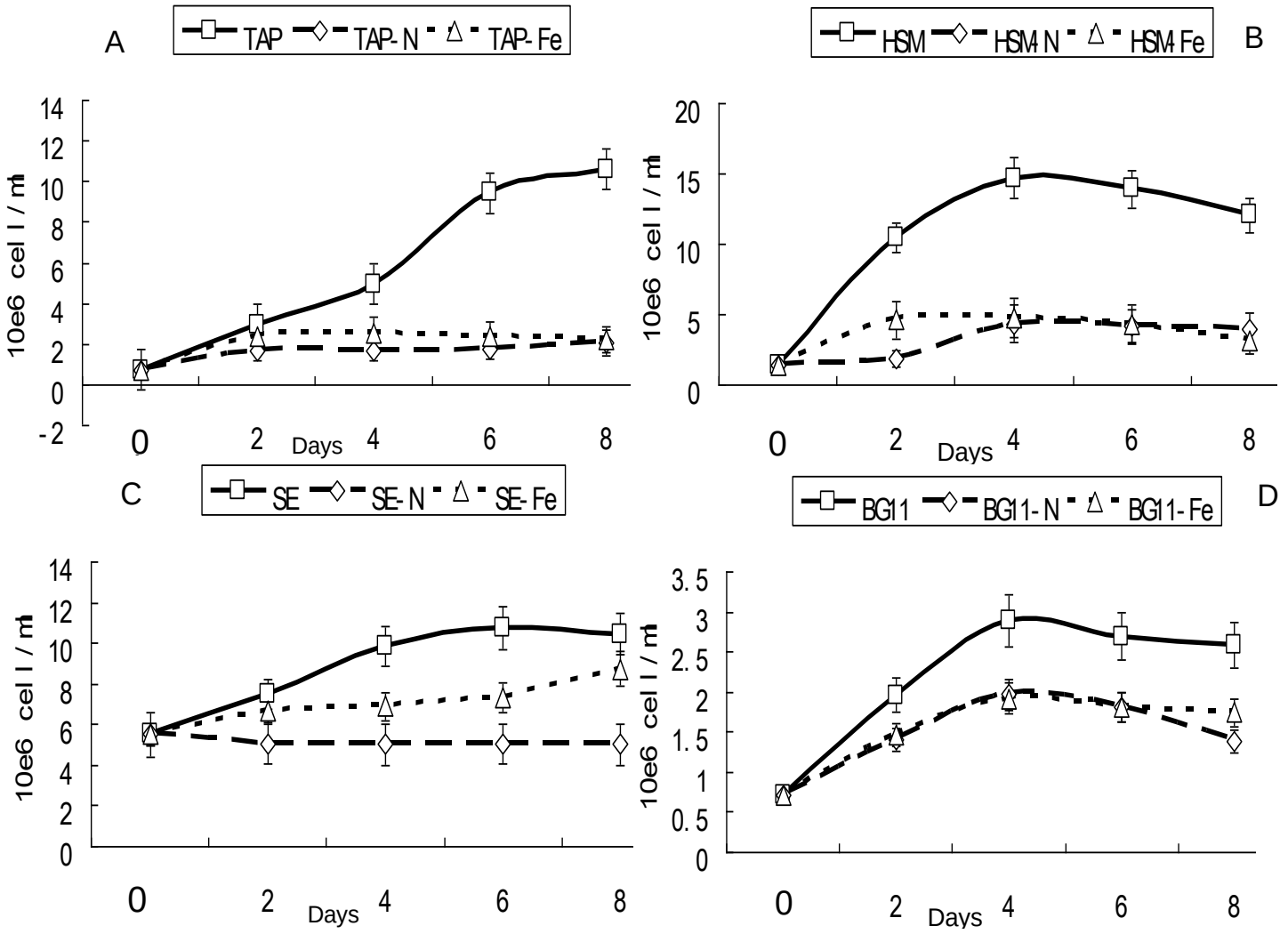

Figure 2. The growth curve of *M. pusillum* Y-002 in TAP, HSM, SE and BG11, and their N free or Fe free medium. A) Growth curve in TAP,TAP-N and TAP-Fe medium; B) Growth curve in HSM, HSM-N and HSM-Fe medium; C) Growth curve in SE, SE-N, and SE-Fe media; D) Growth curve in BG11,BG11-N and BG11-Fe medium.

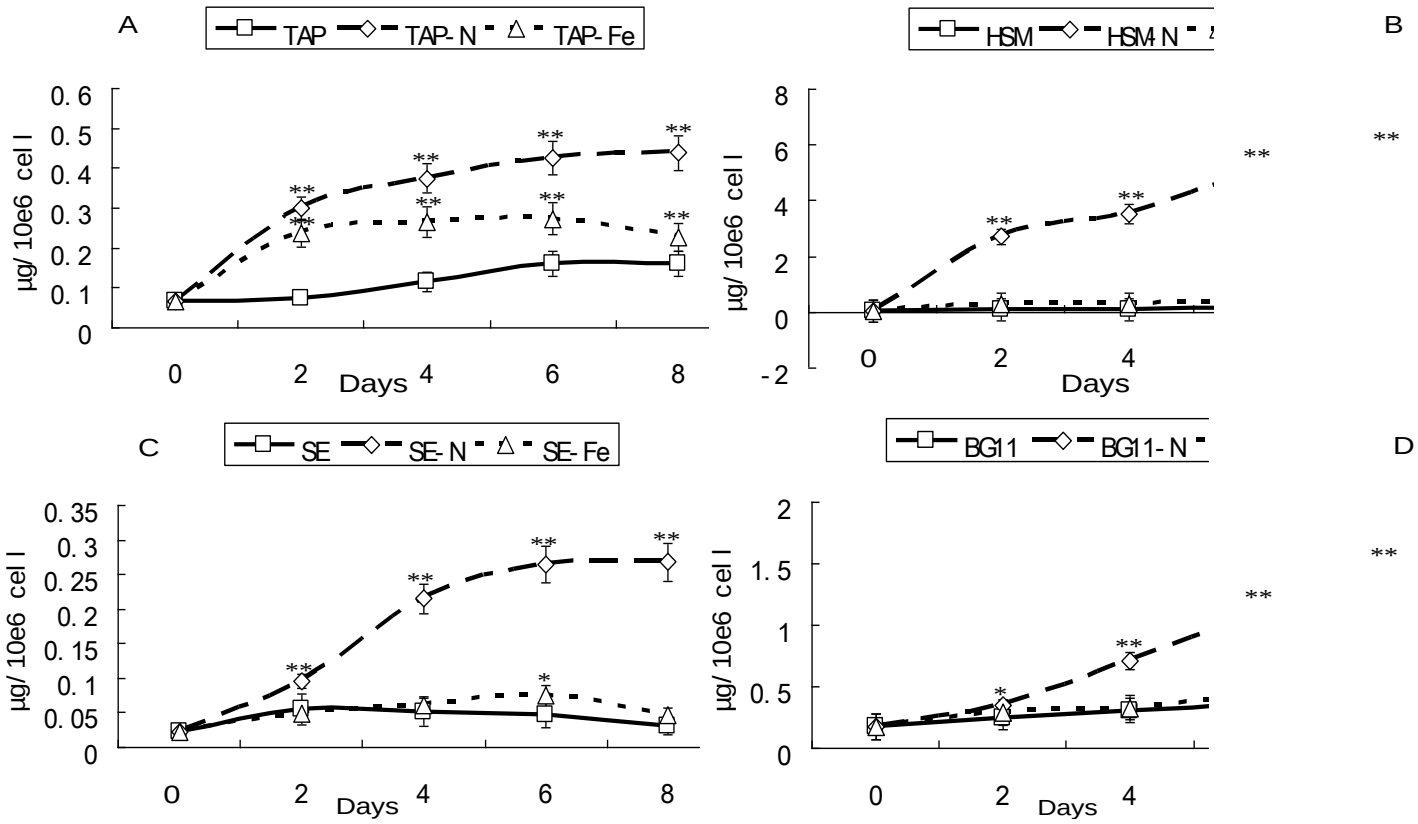

Figure 3. The lipid content of *M. pusillum* Y-002 in TAP, HSM, SE and BG11, and their N free or Fe free medium. A) Lipid content in TAP,TAP-N and TAP-Fe medium; B) Lipid content in HSM, HSM-N and HSM-Fe medium; C) Lipid content in SE, SE-N, and SE-Fe media; D) Lipid content in BG11,BG11-N and BG11-Fe medium. Statistical analysis was performed using SPSS statistical software. Significance is indicated as *$p < 0.05$, ** $p < 0.01$.

thus determine whether there is any change in the physiology of cells grown under these conditions. Thus, we measured the level of dry weight, specific growth rate, photosynthesis efficiency, total protein, sugar and lipids. Our results showed that restriction of nitrogen (N) or sulphur (S) caused a reduction in cell dry weight, specific growth rate and photosynthesis efficiency (Table 3). The major cellular components including sugar, protein and lipid were also reduced in cells grown in HSM-N or HSM-S medium (Figure 5 and Table 3).

The increase in lipid content was accompanied by a decrease in sugar. For examples, compared to complete medium, lipid content increased about 10-fold in HSM-N or HSM-S medium (from 15.14 to 160.14 and 100.32 mg/L, respectively) whereas total sugar decreased from 265.21 to 123.54 (HSM-N) and 113.25 mg/L (HSM-S), respectively. The total protein was also reduced, from 40.45 to 25.29 (HSM-N) and 14.17 mg/L (HSM-S). When cells grew in the P-restricted HSM medium, the cells dry weight decreased but the lipid contents did not display significant change. The results appear to support the carbon flow hypothesis. Under N- or S-restricted conditions, although the photosynthesis efficiency is dramatically decreased, cells can still utilizes sodium acetate as the carbon source via the glyoxylate pathway. The protein synthesis in the cell is inhibited, leading to a decrease in total protein level and an arrest in the tricarboxylic acid cycle, further blocking carbon source flowing into protein synthesis. The N or S restriction also induces the degradation of starch to glucose, which then enter glycolytic pathway to supply carbon source for lipid synthesis.

The relationship between exogenous sodium acetate, cell growth and lipid content in SE-N medium

Our results indicated that nitrogen restriction can significantly enhance lipid content in *M. pusillum* Y-002. One problem associated with this trait is that nitrogen starvation led to significant decrease in biomass accumulation. Such reduction severely compromised the needed biomass for any meaningful biodiesel production (Fabregas et al., 1989; Ratledge et al., 2002). Because sodium acetate (NaAC) is an important carbon source in microalgae cultivation, we investigated cell growth, lipid content and photosynthesis efficiency in cells grown in different concentrations of this compound. Our results

Table 3. The specific growth rate, dry weight, photosynthetic efficiency and the main components of *M. pusillum* Y002 in HSM, HSM-N, HSM-S and HSM-P medium after 4-days cultivation.

Medium	Specific growth rate (μ•d^{-1})	Significance (p < 0.01)	Dry weight (g/L)	Significance (p < 0.01)	Carbohydrate (mg/L)	Significance (p < 0.01)	Protein (mg/L)	Significance (p < 0.01)	Lipid (mg/L)	Significance (p < 0.01)	Others (mg/L)	Photosynthetic efficiency (μmol O2•mg chla-1•h-1) / (μmol photon •m-2•s-1)	Significance (p < 0.01)
HSM	0.86 ± 0.10	A	0.341 ± 0.014	A	265.21 ± 9.13	A	40.45 ± 3.71	A	15.14 ± 2.00	A	20.20 ± 0.84	2.59 ± 0.03	A
HSM-N	0.34 ± 0.02	B	0.321 ± 0.018	A	123.54 ± 4.46	B	25.29 ± 1.09	B	160.2 ± 5.04	B	11.92 ± 1.59	2.36 ± 0.08	AB
HSM-S	0.43 ± 0.03	B	0.239 ± 0.010	B	113.25 ± 4.77	B	14.17 ± 2.84	C	100.32 ± 5.02	C	11.26 ± 2.63	2.24 ± 0.15	BC
HSM-P	0.61 ± 0.05	C	0.218 ± 0.010	B	180.34 ± 8.29	C	12.17 ± 1.16	C	14.95 ± 1.35	A	10.54 ± 0.80	2.04 ± 0.04	C

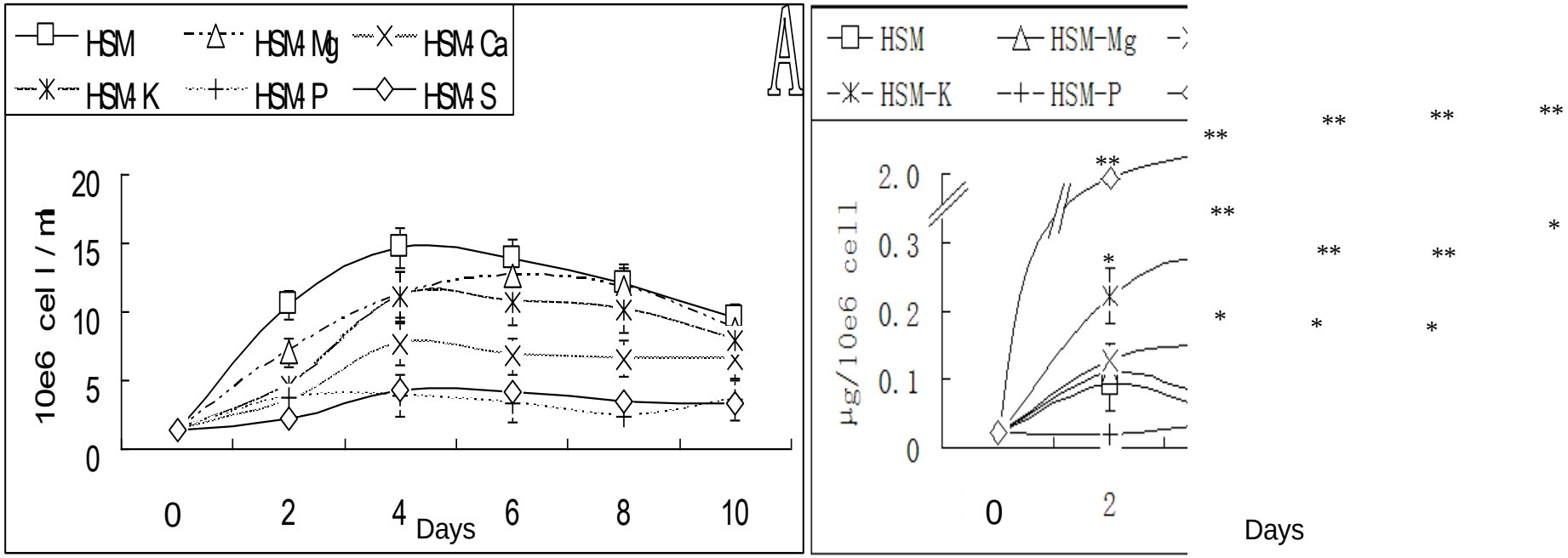

Figure 4. The growth curve (A) and lipid content (B) of *M. pusillum* Y-002 in HSM medium and HSM with P, S, K, Mg and Ca deficiency. Statistical analysis was performed using SPSS statistical software. Significance is indicated as *p < 0.05, ** p < 0.01.

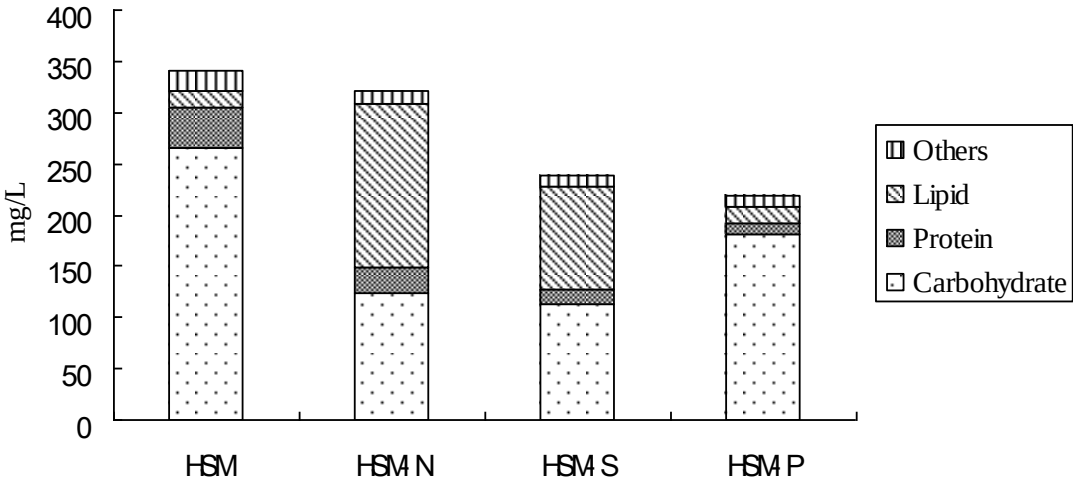

Figure 5. The main components of *M. pusillum* Y002 in HSM, HSM-N, HSM-S and HSM-P medium.

showed that the cell growth rate and lipid content per volume increased when the concentration of NaAC rose from 0 to 15 mM. However, when the concentration of NaAC was more than 15 mM, a reduction in lipid content was observed (Figure 6). A slight increase in photosynthesis efficiency was observed when 15 mM of NaAC was included in the SE-N medium, but a reduction was seen when NaAC was used at 75 mM (Table 4). Similar results were obtained when cellular lipid content was detected by Nile red staining and microscopic analysis. As shown in Figure 7, strong yellow fluorescence, reflecting high level of neutral lipid accumulation was detected in *M. pusillum* Y-002 after 5-day incubation at 15 mM NaAC. On the other hand, only weak fluorescence was detected in cells grown in medium without NaAC.

DISCUSSION

Microalgae as a feedstock for biodiesel production have recently become more attractive due to its many advantages such as easy cultivation and high lipid content. Our study examined changes of cell growth, photosynthesis efficiency, and major cell components including sugar, protein and lipid content in the oleaginous strain, *M. pusillum* Y-002, during growth in various nutrient-limited TAP, HSM, SE or BG11 medium. Our results showed that *M. pusillum* Y-002 accumulates significant neutral lipids by N or S starvation. However, such accumulation was unfortunately associated with low biomass production, which could be one of the major obstacles for potential commercialization of biodiesel production. Therefore, it is important to identify the optimal culture condition, particularly the supplements for carbon sources to achieve the best combination between cell growth and high-level lipid accumulation

Our results showed that supplementation of exogenous carbon sources such as NaAC could result in an increase in biomass yield in SE-N medium. Xing et al. (2008) reported that the cell density of *Chlorella vulgaris* could reach 6.27×10^7 cell/mL and 23.647 g/L cell dry weight. Wijanarko et al. (2008) also reported that a 22.3 g/L cell dry weight was obtained in *C. vulgaris*. Interestingly, exogenous carbon source has little impact on lipid accumulation in N or S deficiency media. Our investigation of the effects of exogenous carbon to lipid accumulation and photosynthesis efficiency revealed that physiological traits such as cell growth rate, photosynthesis efficiency, and lipid content per volume reached the maximal value in the presence of 15 mM NaAC, and began to decrease when higher concentration of this compound was included in the SE-N medium.

It is well established that microalgae usually accumulate more lipids under stress conditions, including nutrient deficiency, high pH value or high temperature. For example, N starvation leads to higher lipid content in many microalgal species (Converti et al., 2009; Hernandez et al., 2009; Tam and Wong, 1996) and S deficiency also enhances lipid content in *Chlorella sp.* (Otsuka, 1960). However, there is little information about the effects of Ca, Mg, Fe and K deficiency on lipid accumulation in microalgae. Our results revealed that N or S starvation led to a dramatic lipid accumulation, while restriction of Ca and K led to detectable increase of lipid levels. The effects of iron deficiency on lipid accumulation varied greatly. For examples, lipid content was not affected in cells grown in HSM-Fe, SE-Fe or BG11-Fe medium, but exhibited detectable increase in TAP-Fe (Figure 2). P and Mg deficiency showed no detectable increase in lipids comparing with cells grown in HSM medium. These results are intriguing because the effects of P starvation on lipid accumulation vary significantly among different species of microalgae. In species *Monodus subterraneus, Phaeodactylum tricornutum, Chaetoceros sp.*, *Isochrysis galbana* and *Pavlova lutheri*, P deficiency induces lipid accumulation (Khozin-Goldberg

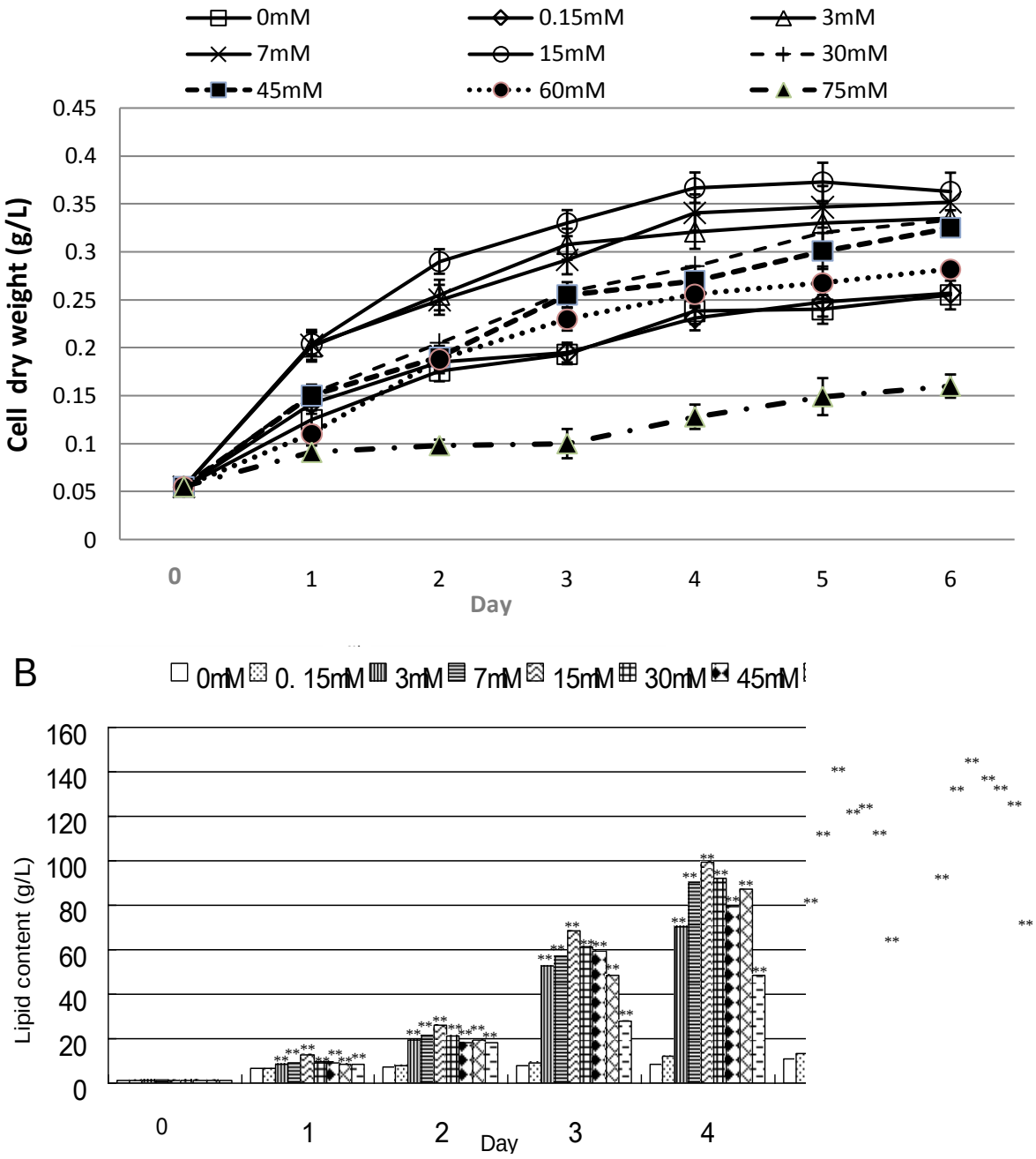

Figure 6. The growth curve (A) and lipid content per volume (B) of *M. pusillum* Y-002 in SE-N medium with addition of 0, 0.15, 3, 7.5, 15, 30, 45, 60 and 75 mM NaAC. Statistical analysis was performed using SPSS statistical software. Significance is indicated as *p < 0.05, ** p < 0.01.

and Cohen, 2006). However, in the cases of *Nannochloris atomus* and *Tetraselmis sp.*, starvation of phosphorus decreased their cellular lipid content (Reitan et al., 1994). Our studies on *Chlamydomonas reinhaditti* and *C. vulgaris* found that cells grown in P deficient medium enhance lipid content. Our analysis of cell photosynthesis efficiency, total protein, and total carbohydrate content revealed the direction of carbon flow under -N or -S condition. Because both N and S are essential elements in protein synthesis, their deficiency is expected to drastically decrease protein synthesis rates (Figure 5), which can cause a feedback inhibition in the citric acid cycle and photosynthesis impairment largely due to insufficient proteins involved in the photosystem reaction center and photosynthesis electron transport. Under such conditions, carbon fixation through photosynthesis is greatly reduced and intercellular carbon will be mainly derived from acetate assimilation via the

Table 4. The chlorophyll content and photosynthetic efficiency of *M. pusillum* Y002 in SE-N and SE-N with different concentrations of sodium acetate after 5-days cultivation.

NaAC (mM)	Chl a (μg/ml)	Significance (p < 0.01)	Chl b (μg/ml)	Significance (p < 0.01)	Photosynthetic efficiency (μmol O2•mg chla-1•h-1)/(μmol photon •m-2•s-1)	Significance (p < 0.01)
0	4.41 ± 0.06	A	2.55 ± 0.03	A	2.07 ± 0.03	AB
0.15	4.56 ± 0.17	A	2.48 ± 0.04	A	2.12 ± 0.02	ABC
3	5.94 ± 0.09	B	3.46 ± 0.05	B	2.19 ± 0.03	BCD
7	6.26 ± 0.16	BC	3.61 ± 0.09	C	2.33 ± 0.06	DE
15	6.30 ± 0.14	C	3.87 ± 0.05	D	2.47 ± 0.09	E
30	5.83 ± 0.18	BD	3.27 ± 0.05	E	2.32 ± 0.11	DE
45	5.68 ± 0.21	BD	3.15 ± 0.10	E	2.27 ± 0.11	CD
60	4.91 ± 0.11	E	2.82 ± 0.06	F	2.21 ± 0.05	BCD
75	2.66 ± 0.05	F	1.50 ± 0.03	G	2.02 ± 0.04	A

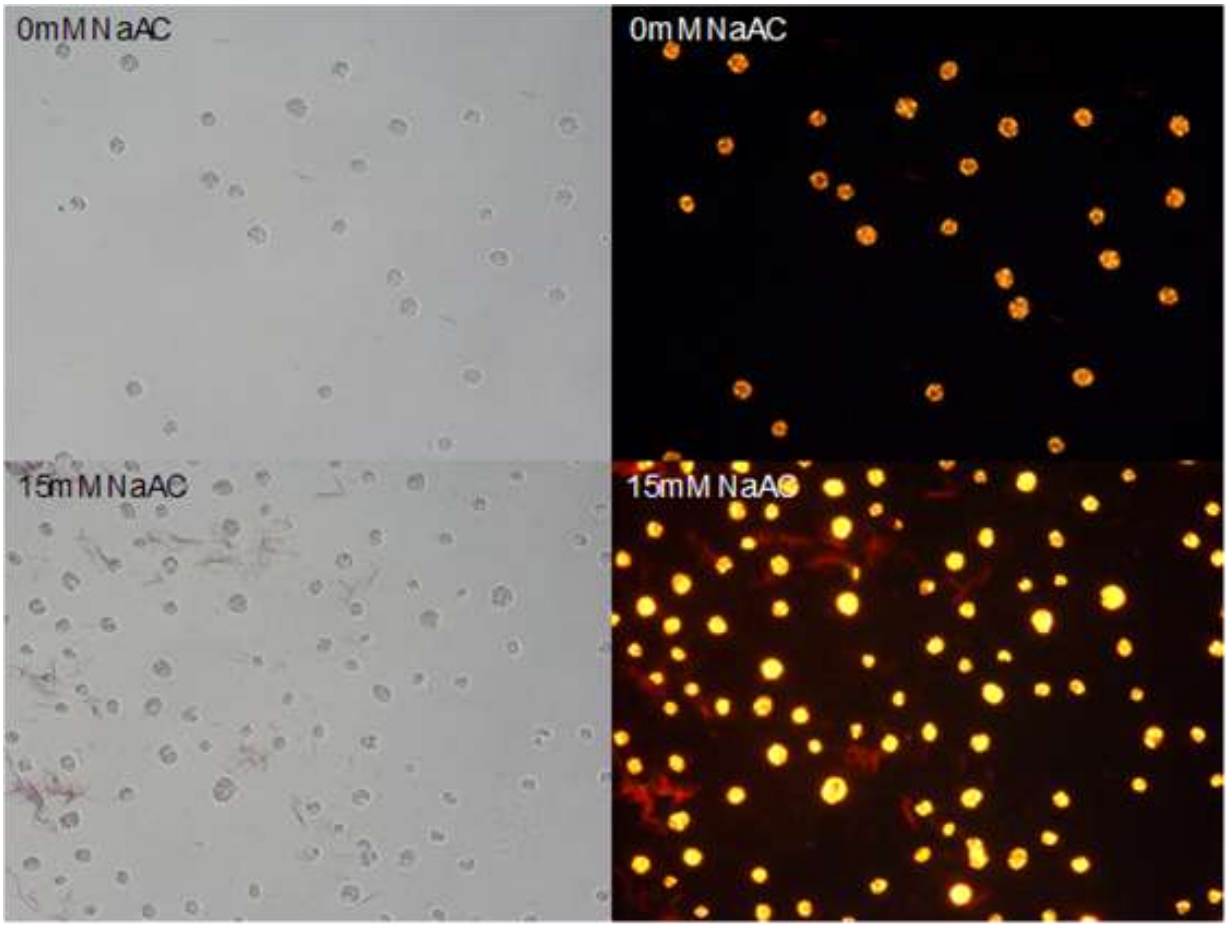

Figure 7. Fluorescence analysis of lipid of *M. pusillum* Y-002 after 5 days cultivation in NaAC concentration of 0 and 15 mM SE-N medium using Nikon 80i Fluorescence Microscopes (400X).

glyoxylate cycle. In the mean time, starch will be degraded to glucose through the glycolytic pathway to produce pyruvate, and thus triacylglycerols (TAGs) via the Kennedy pathway, leading to more lipid accumulation in media deficient for nitrogen and sulfur.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Natural Science Foundation of China (30860028, 30960032), National Nonprofit Institute Research Grants

(CATAS-ITBBZX0841) and National Natural Science Foundation of Hainan Province (061012).

REFERENCES

Allen MD, Del JA, Kropat CJ, Merchant SS (2007). FEA1, FEA2, and FRE1, encoding two homologous secreted proteins and a candidate ferrireductase, are expressed coordinately with *FOX1* and *FTR1* in iron-deficient *Chlamydomonas reinhardtii*. Eukaryot. Cell. 6: 1841-1852.

Basova MM (2005). Fatty acid composition of lipids in microalgae. Int. J. Algae. 7: 33-57.

Beer LL, Boyd ES, Peters JW (2009). Engineering algae for bio-hydrogen and biofuel production. Curr. Opin. Biotechnol. 20: 264-271.

Björkman O, Demmig B (1987). Photon yield of O_2 evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. Planta, 170: 489-504.

Chen W, Zhang C, Song L, Sommerfeld M, Hu Q (2009). A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. J. Microbiol. Methods, 77: 41-47.

Chisti Y (2007). Biodiesel from microalgae. Biotechnol. Adv. 25: 294-306.

Cobelas MA, Lechado JZ (1989). Lipids in microalgae. A review. I. Biochemistry Grasas y Aceites, 40: 118-145.

Converti A, Casazza AA, Ortiz EY (2009). Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. Chemical Engineering and Processing: Process Intensification, 48: 1146-1151.

Deng XD, Eriksson M (2007). Two Iron-Responsive Promoter Elements Control Expression of *FOX1* in *Chlamydomonas reinhardtii*. Eukaryot. Cell, 6: 2163-2167.

Deng XD, Li YJ, Fei XW (2009). Microalgae: a promising feedstock for biodiesel. Afr. J. Microbiol. Res. 3: 1008-1014.

Fabregas J, Abalde J, Herrero C (1989). Biochemical composition and growth of the marine microalga *Dunaliella tertiolecta* (Butcher) with different ammonium nitrogen concentrations as chloride, sulphate, nitrate carbonate. Aquaculture, 83: 289-304.

Fartas FM, Othman MR, Rajeb FA, Latif MT (2009). Determination of levoglucosan in PM10 and biomass close burning residue samples using anthrone-sulfuric acid colorimetric method. J. Appl. Sci. Res. 5: 1663-1667.

Gao CF, Xiong W (2008). Rapid quantitation of lipid in microalgae by time-domain nuclear magnetic resonace, J. Microbilological Methods, 75: 437-440.

Gouveia L, Oliveira AC (2009). Microalgae as a raw material for biofuels production. Ind. Microbiol. Biotechnol. 36: 269-274.

Guckert JB, Cooksey KE (1990). Triacylglyceride accumulation and fatty acid profile changes in Chlorella (Chlorophyta) during high-pH induced cell cycle inhibition. J. Phycol. 26: 72-79.

Harris EH (1989). The Chlamydomonas Sourcebook: A Comprehensive Guide to Biology and Laboratory Use. Academic Press, San Diego, CA.

Harwood JL (1998). Membrane lipids in algae. In Lipids in Photosynthesis: Structure, Function and Genetics (Siegenthaler, P.A. and Murata, N., eds). Dordrecht, The Netherlands: Kluwer Academic Publishers, pp. 53-64.

Hernandez JP, Bashan LE, Rodriguez DJ (2009). Growth promotion of the freshwater microalga *Chlorella vulgaris* by the nitrogen-fixing, plant growth-promoting bacterium *Bacillus pumilus* from arid zone soils. Euro. J. Soil Biol. 45: 88-93.

Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A (2008). Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J. 54: 621-639.

Huang GH, Chen G, Chen F (2009). Rapid screening method for lipid production in alga based on Nile red fluorescence. Biomass bioenergy, 33: 1386-1392.

Illman AM, Scragg AH, Shales SW (2000). Increase in Chlorella strains calorific values when grown in low nitrogen medium. Enzyme. Microb. Technol. 27: 631-635.

Khozin-Goldberg I, Cohen Z (2006). The effect of phosphate starvation on the lipid and fatty acid composition of the fresh water eustigmatophyte Monodus subterraneus. Phytochemistry, 67: 696-701.

Li Y, Horsman M, Wang B, Wu N, Lan CQ (2008). Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. Appl. Microbiol. Biotechnol. 81: 629-636.

Li Y, Horsman M, Wu N, Lan CQ, Dubois-Calero N (2008). Biofuels from microalgae. Biotechnol. Prog. 24: 815-820.

Lichtenthaler HK (1987). Chlorphylls and carotenoids: pigments of photosynthetic biomembranes .Methods Enzymol. 148: 350-382.

Mata TM, Martins AA, Caetano NS (2009). Microalgae for biodiesel production and other applications: A review Renew Sustain Energy Rev. 14: 217-232.

Maxwell K, Johnson GN (2000). Chlorophyll fluorescence: a practical guide. J. Exp. Bot. 51: 659-668.

Merzlyak MN, Chivkunova OB, Gorelova OA, Reshetnikova IV, Solovchenko AE, Khozin-Goldberg I, Cohen Z (2007). Effect of nitrogen starvation on optical properties, pigments, and arachidonic acid content of the unicellular green alga *Parietochloris incisa* (*Trebouxiophyceae, Chlorophyta*). J. Phycol. 43: 833-843.

Morgan-Kiss RM, Ivanov AG, Modla SKC, Hüner NPA, Priscu JC, Lisle JT, Hanson TE (2008). Identity and physiology of a new psychrophilic eukaryotic green alga, Chlorella sp., strain BI, isolated from a transitory pond near Bratina Island, Antarctica. Extremophiles, 12: 701-711.

Otsuka H (1961). Changes of lipid and carbohydrate contents of Chlorella cells during the sulfur starvation, as studied by the technique of synchronous culture. J. Gen. Appl. Microbiol. 7: 72-77.

Ratledge C (2002). The Biochemistry and Molecular Biology of Lipid Accumulation in oleaginous microorganisms. Adv. Appl. Microbiol. 51: 1-51.

Reitan KI, Rainuzzo JR, Olsen Y (1994). Effect of nutrient limitation on fatty acid and lipid content of marine microalgae. J. Phycol. 30: 972-979.

Roessler PG (1990). Environmental control of glycerolipid metabolism in microalgae: commercial implications and future research directions. J. Phycol. 26: 393-399.

Shifrin NS, Chisholm SW (1981). Phytoplankton lipids: interspecific differences and effects of nitrate, silicate and light– dark cycles. J. Phycol. 17: 374-384.

Spoehr HA, Milner HW (1949). The chemical composition of Chlorella; effect of environmental conditions. Plant Physiol. 24: 120-149.

Tam NFY, Wong YS (1996). Effect of ammonia concentrations on growth of *Chlorella vulgaris* and nitrogen removal from media. Bioresour. Technol. 57: 45-50.

Thompson GA (1996). Lipids and membrane function in green algae. Biochim. Biophys. Acta.13: 17-45.

Wijanarko A, Muryanto D, Simanjuntak J, Pembangun P, Wulan DK, Hermansyah H, Gozan M, Soemantojo RW (2008). Biomass Production Chlorella vulgaris Buitenzorg Using Series of Bubble Column Photo Bioreactor with a Periodic Illumination. Makara Technol. 12: 27-30.

Xing X, Zhang XQ, Du ZJ, Zhang LH (2008). Novel Cultivation Method for Chlorella. Sci. Technol. Rev., 26: 56-58.

Yemm EW, Willis AJ (1954). The estimation of carbohydrates in plant extracts by anthrone. Biochem. J. 57: 508-514.