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Full Length Research Paper

The effects of nutritional restriction on neutral lipid accumulation in *Chlamydomonas* and *Chlorella*

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Under nutrient starvation conditions, many microalgae are known to accumulate triacylglycerols (TAG) that can be used for biodiesel production. However, few studies have been performed to analyze the effect of deficiency in nutrient elements such as sulfur, phosphorus, potassium, iron, magnesium and calcium on oil production, particularly in *Chlamydomonas* and *Chlorella*. In this study, we investigated lipid content of *Chlamydomonas reinhaditti* CC124 and *Chlorella vulgaris* Y-019 grown in TAP, HSM, BG11, SE lacking optimal concentrations of these elements. Our results showed that, in high carbon HSM and TAP media, N and S starvation led to significant increase in cellular lipid content in both microalgae species. In addition, *C. reinhaditti* grown in TAP media without P, Fe, K, Ca or Mg or in HSM media without K, Ca or Mg also accumulated detectably higher neutral lipids. In contrast, in *C. vulgaris,* such accumulation was observed only in Mg-free and Fe-free HSM media. In low carbon SE and BG11 media, N starvation resulted in a moderate increase in the lipids content both in *C. reinhaditti* and *C. vulgaris*. On the other hand, P, S, K, Ca or Mg deficiency promoted neutral lipids accumulation in *C. vulgaris*. Finally, we analyzed and discussed the relationships among cell growth rate, lipid accumulation and nitrogen concentrations in *C. reinhaditti*.

Key words: Nutrient limitation, lipid accumulation, nitrogen, sulfur, phosphorus, potassium, iron, magnesium, calcium.

INTRODUCTION

Within a few decades, the world would move to a low carbon or zero carbon economy eras, and renewable energy would become a dominant energy source, displacing the fossil fuels. Microalgae is a large, extremely diverse and widespread group of lower plants that live in freshwater or marine, which has the ability to synthesize lipids that can be used for biodiesel production under conditions of photoautotrophical growth (Deng et al., 2009) . Compared to other biomass materials, microalgae as a feedstock for biodiesel production offers the following advantages: (1) microalgae have fast growth rate and high lipid productivity, which have been considered as the only renewable source that can potentially completely displace the fossil fuels in the future (Chisti, 2007; Mata et al., 2009). (2) Microalgae can assimilate carbon dioxide as the carbon source for growth, which will mitigate atmospheric CO_2 levels and reduce the production costs of biodiesel. (3) Microalgae can grow on brackish, marine and non-arable land, thus avoiding competition for land and water with food crops.

Similar to higher plants, microalgal lipids are composed of neutral lipids 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 phospholipid, which are enriched in chloroplast and cell membrane system (Guckert and Cooksey, 1990; Harwood, 1998) . When the environmental conditions are unfavorable for cell growth, many microalgae tend to accumulate neutral lipids to form

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lipid droplet localized in the cytoplasm as a storage form of carbon and energy. Many reports have described that nitrogen (N) starvation induces a significant increase in neutral lipids content in numerous 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). The accumulation of neutral lipids was also found in *Monodus subterraneus* (Khozin-Goldberg and Cohen, 2006)*, Phaeodactylum tricornutum, Chaetoceros sp., Isochrysis galbana* and *Pavlova lutheri* under phosphate deficiency conditions. On the contrary, Reitan et al. (1994) reported that phosphorus (P) deficiency caused a decrease in neutral lipid levels in *Nannochloris atomus* and *Tetraselmis sp.* There is only one report showed that sulfur (S)-starved *Chlorella sp.* can accumulate neutral lipids in its biomass (Otsuka, 1961). To gain more insight into the effects of nutrient deficiency on cell growth rate and neutral lipids content, our present study investigated the growth and lipids content of *Chlamydomonas reinhaditti* CC124 and *Chlorella vulgari*s Y-019 cells in response to a series of nutrient deficiency including N, P, S, K, Mg, Ca and Fe. Cells grown in normal TAP, HSM, BG11 and SE media were considered as the control, respectively. In addition, an analysis of the effects of different N concentration on lipid accumulation was performed in *C. reinhaditti* CC124 under HSM culture condition. These results would lay the foundation for optimizing the growth conditions for large-scale microalgae cultivation for biofeul production.

MATERIALS AND METHODS

Algal strain and cultivation conditions

Two algal strains, *C. reinhaditti* CC124 purchased from the Duke University and *C. vulgaris Y-019* isolated from a freshwater lake in Haikou, Hainan province, China, were used in this study. In each assay, these strains were grown and maintained on BG11 solid medium, and inoculated into 100 ml Erlenmeyer flasks with 50 ml of TAP, HSM, SE BG-11 and the corresponding nutrition restrict media, respectively (Table 1). All cultures were maintained in an incubator shaker with 230 rpm at 25°C, and exposed to a continuous illumination at a light intensity of 150 $mol \cdot m^{-2} \cdot s^{-1}$. To determine the biomass of the cultures, samples were collected at 24 h intervals with three replications per sample and the biomass was assayed by calculating the cell number as described Harris et al. (1989).

Neutral lipid analysis

A Nile red fluorescence method was applied to the determination of neutral lipids (Gao and Xiong, 2008). The algal cells were directly stained with 0.1 g/ml Nile red (final concentration) for 10 min, and then fluorescence were measured on a GloMax®-Multi Detection System (Promega, USA), which excitation and emission wavelengths were 470 and 570 nm, respectively. The fluorescence was calculated by the equation: FD (470/570) = (A2 to A1), where A2 is the fluorescence value of algal cells after staining with Nile red, A1 is that of the algal cells before staining with Nile red. The lipid content of the algal cell was calculated as using the following formula: Lipid content ($ug/10^6$ cells) = [0.0004*FD (470/570)-0.0038]/cell numbers. For microscopy assay, after staining the cells with Nile red (10 g/ml final concentration), images were acquired using 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).

RESULTS

Nutrition restriction affects the lipid content and the growth rate of *C. vulgaris* Y019

In nitrogen free medium, *C. vulgaris* Y019 always grows slower than in the full N original medium (Figure 1). The lipid content of the cells in high carbon medium TAP-N or HSM-N is significantly higher (more than 10 times) than cells grown in TAP and HSM medium (Figures 2A and B). On the other hand, comparing to SE or BG11 medium, growing in low carbon SE -N and BG11- N medium only led to a moderate increase in lipid (Figure 2C and D). Iron deficiency affected cell growth rates in HSM-Fe, SE-Fe or BG11-Fe, but not in TAP -Fe medium (Figure 1). The effect of iron deficiency on lipid accumulation varies. For examples, lipid content was not affected in cells grown in TAP-Fe or BG11-Fe medium, but exhibiting detectable increase in HSM-Fe and SE-Fe (Figure 2). Interestingly, sulfur starvation in high carbon TAP and HSM medium led to a dramatic lipid accumulation. Lipid content increased more than 20 times when this element was subtracted from the TAP or HSM (Figures 3A and B). In low carbon SE and HSM media, sulphur (S) starvation caused only a moderate increase in lipids and such increases never reach more than 3.5 times (Figures 3C and D). Restriction of other nutrition elements such as phosphorus led to higher lipid levels in TAP, SE or BG11 medium (Figures 3A, C and D). Similarly, deficiency in Ca, K in SE or BG11 medium also detectably increased lipid content in *C. vulgaris* cells. In HSM or BG11 medium, restriction of Mg led to higher cellular lipid level (Figure 3).

The nutrition restriction affects the lipid content and the growth rate of *C. reinhaditti* CC124

C. reinhaditti CC124 exhibits fastest cell growth rates in complete TAP or HSM medium. Whereas this strain grows slower in Fe-deficiency medium it displays the slowest growth rate in a nitrogen restriction (-N) medium (Figures 4A, B, C and D). Interestingly, lipid content varies significantly in different media in response to N restriction. Whereas nitrogen restriction in high carbon TAP or HSM medium led to dramatic increase in cellular lipid content, reaching more than 10 times to cells grown in corresponding complete media, similar restriction in SE or BG11 medium only moderate increases cellular lipid

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

BG11	**Working solution (mg/L)**	**-N**	**-P**	**-S**	**-K**	-Fe	-Mg	-Ca
NaNO3	250	NaCl/172	250	250	250	250	250	250
$K_2HPO_4 \cdot 3H_20$	40	40	KCl/13	40	$Na_2HPO_4 \cdot 12H_20/34$	40	40	40
$MgSO_4 \cdot 7H_2O$	75	75	75	$MgCl_2 \cdot 6H_2O/62$	75	75	Na2SO4/43	75
$CaCl_2 \cdot 2H_2O$	36	36	36	36	36	36	36	NaCl/14.3
Citric acid	6	6	6	6	6	6	6	6
$FeC_6H_5O_7$	6	6	6	6	6	-	6	6
EDTA	1	1	1	1	1	1	1	1
$NaCO_3$	20	20	20	20	20	20	20	20
A_5+Co solution	1 ml	1 ml	1 ml	A5+Co-S	1 ml	1 ml	1 ml	1 ml
SE	Working solution(mg/L)	-N	-P	-S	-K	-Fe	-Mg	-Ca
$NaNO_3$	250	NaCl/172	250	250	250	250	250	250
$K_2HPO_4 \cdot 3H_20$	75	75	KCl/49	75	$Na_2HPO_4 \cdot 12H_20/118$	75	75	75
$MgSO_4 \cdot 7H_2O$	75	75	75	$MgCl_2 \cdot 6H_2O/62$	75	75	Na2SO4/43	75
$CaCl_2 \cdot 2H_2O$	25	25	25	25	25	25	25	NaCl/20
KH_2PO_4	175	175	KCl/96	175	$NaH_2PO_4 \cdot 2H_2O/200$	175	175	175
NaCl	25	25	25	25	25	25	25	25
$FeCl_3 \cdot 6H_20$	5	5	5	5	5	NaCl/3	5	5
Fe-EDTA	10	10	10	10	10	EDTA/10	10	10
A5 solution	1 ml	1 ml	1 ml	A5-S	1 ml	1 ml	1 ml	1 ml
TAP	Working solution(mg/L)	-N	-P	-S	-K	-Fe	-Mg	-Ca
Tris-Base	2420	2420	2420	2420	2420	2420	2420	2420
Glacial acetic acid	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml
K_2HPO_4	119	119	KCl/102	119	Na_2HPO_4 /116	119	119	119
KH_2PO_4	61	61	KCl/33.6	61	NaH_2PO_4/63	61	61	61
NH_4Cl	400	NaCl/437	400	400	400	400	400	400
$MgSO_4 \cdot 7H_2O$	100	100	100	$MgCl_2$/39	100	100	Na_2SO_4/57.7	100
$CaCl_2 \cdot 2H_2O$	50	50	50	50	50	50	50	NaCl/41
Trace	1 ml	Trace-N	1 ml	Trace-S	1 ml	Trace-Fe	1 ml	1 ml
HSM	Working solution (mg/L)	-N	-P	-S	-K	-Fe	-Mg	-Ca
Sodium acetate(hydrate)	2000	2000	2000	2000	2000	2000	2000	2000
NH_4Cl	500	NaCl/546.7	500	500	500	500	500	500
$MgSO_4 \cdot 7H_2O$	20	20	20	$MgCl_2$. $6H_2O/16.5$	20	20	Na_2SO_4/11.5	20
$CaCl_2 \cdot 2H_2O$	10	10	10	10	10	10	10	NaCl/4.1
K_2HPO_4	1440	1440	KCl/KCl/0	1440	Na_2HPO_4/116	1440	1440	1440
KH_2PO_4	720	720	KCl/163	720	NaH_2PO_4/63	720	720	720
Trace	1 ml	Trace-N	1 ml	Trace-S	1 ml	Trace-Fe	1 ml	1 ml

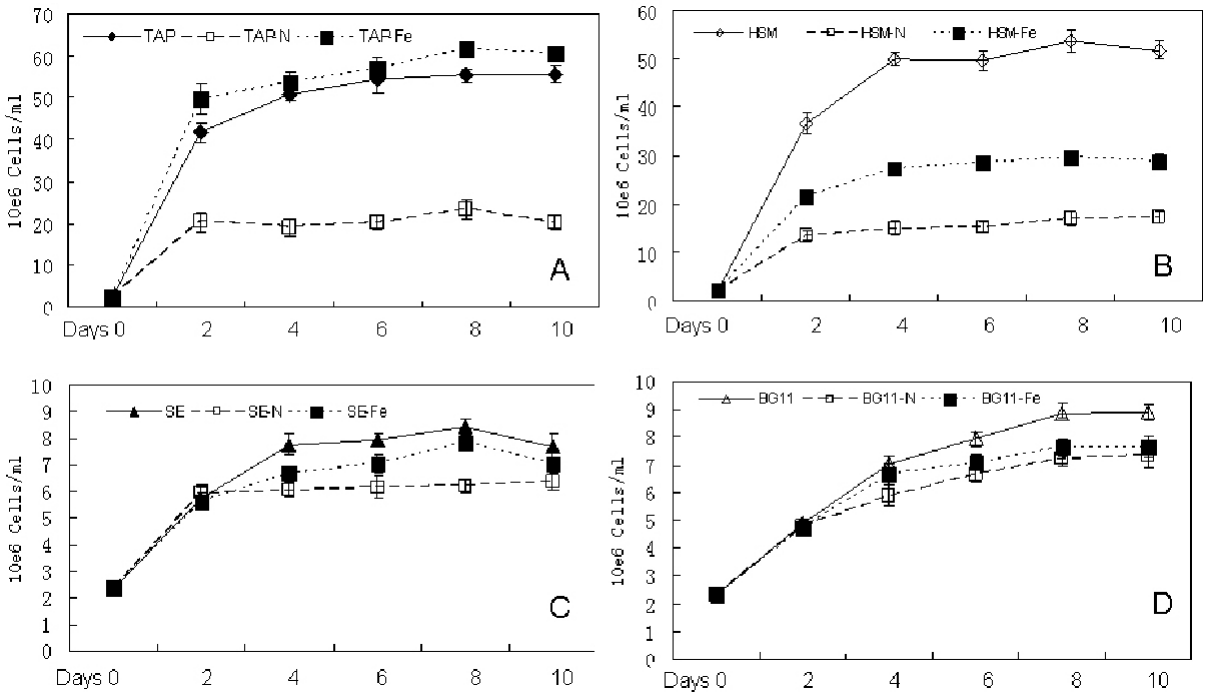


Figure 1. The growth curve of *C. vulgaris* Y019 in high carbon TAP and HSM, and low carbon SE and BG11, and their N free or Fe free media. (A) Growth rate of *C. vulgaris* Y019 in TAP,TAP- N and TAP-Fe media, (B) Growth rate of *C. vulgaris* Y019 in HSM, HSM-N and HSM-Fe media, (C) Growth rate of *C. vulgaris* Y019 in SE, SE-N, and SE-Fe media, (D) Growth rate of *C. vulgaris* Y019 in BG11,BG11-N and BG11-Fe media.

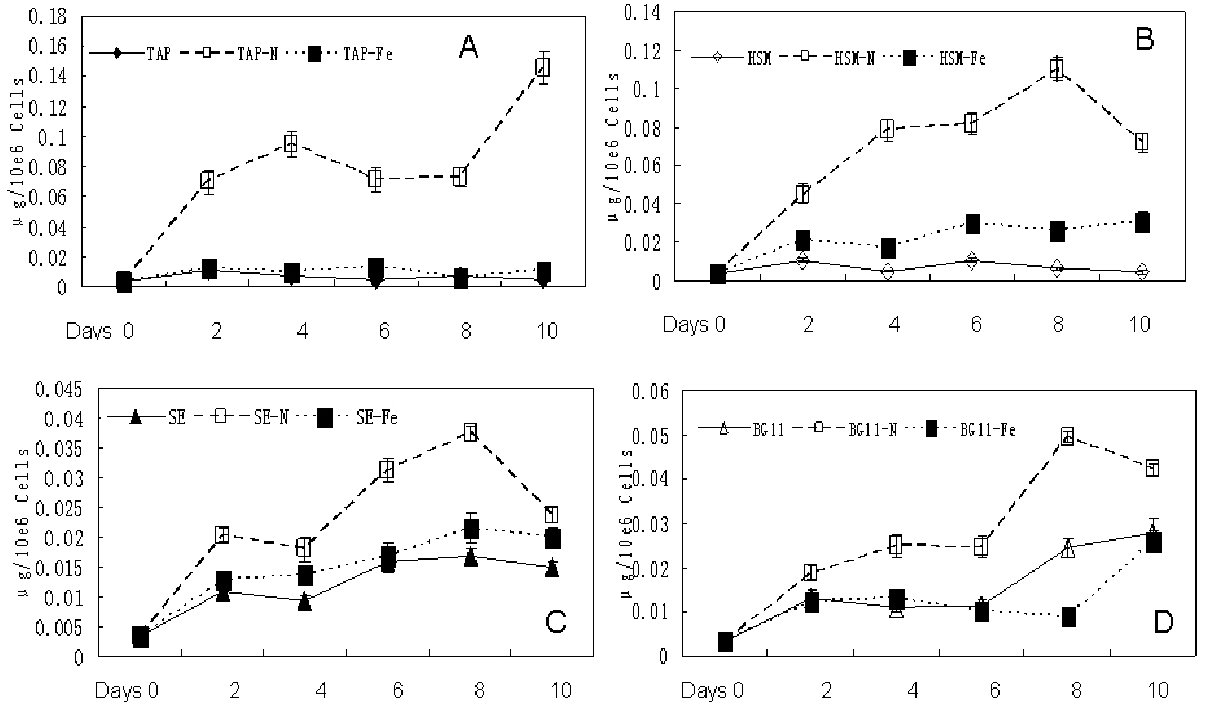


Figure 2. The lipid content of *Chlorella vulgaris* Y019 in high carbon TAP and HSM, and low carbon SE and BG11, and their N free or Fe free media. A) lipid content of *Chlorella vulgaris* Y019 in TAP,TAP-N and TAP-Fe media; B) lipid content of *Chlorella vulgaris* Y019 in HSM, HSM-N and HSM- Fe media; C) lipid content of *Chlorella vulgaris* Y019 in SE, SE-N, and SE-Fe media; D) lipid content of *Chlorella vulgaris* Y019 in BG11,BG11-N and BG11-Fe media.

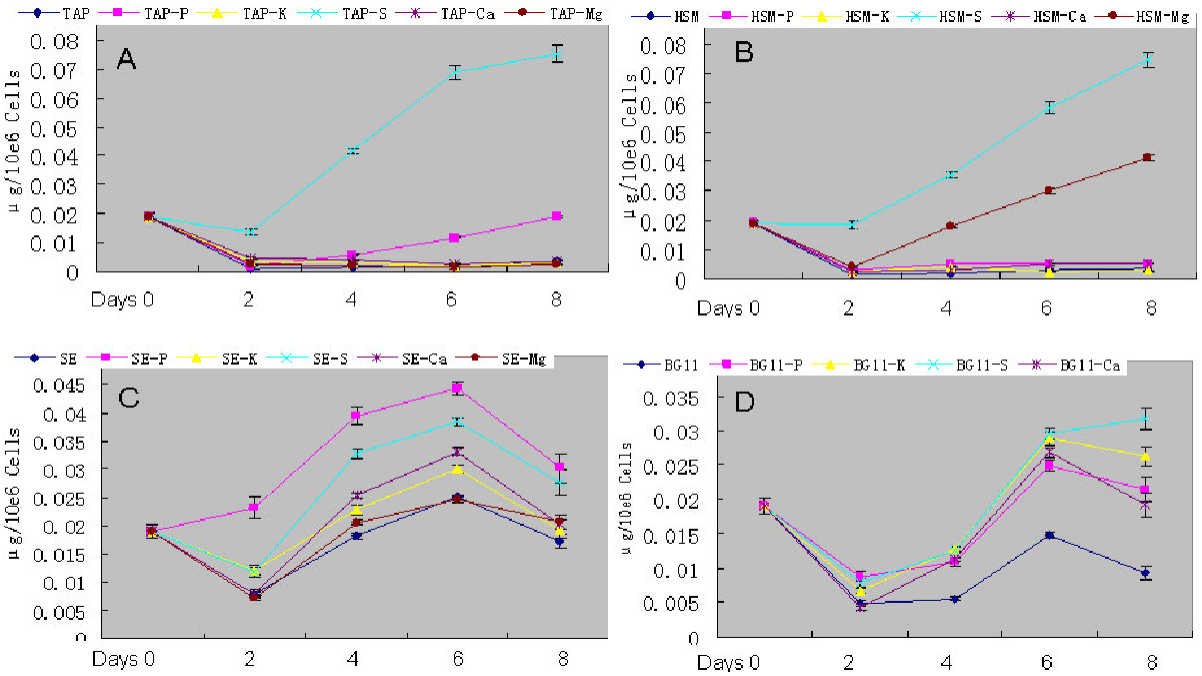
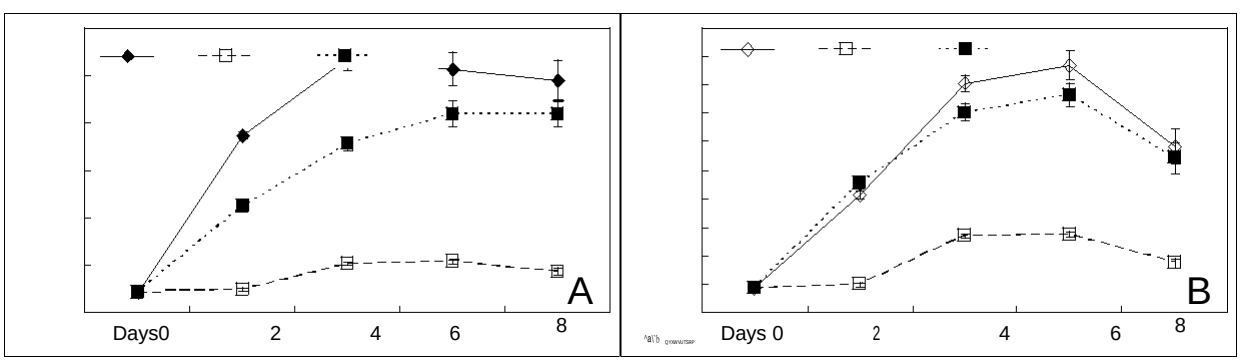


Figure 3. The lipid content of *C. vulgaris* Y019 in high carbon TAP and HSM, and low carbon SE and BG11, and their –S, -P, -K, -Mg and –Ca media. (A) The lipid content of *C. vulgaris* Y019 in TAP,TAP-S, TAP-P, TAP- K,TAP-Mg and TAP-Ca media,(B) The lipid content of *C. vulgaris* Y019 in HSM, HSM-S, HSM-P, HSM-K,HSM-Mg and HSM- Ca media, (C) The lipid content of *C. vulgaris* Y019 in SE, SE-S, SE-P,SE- K,SE-Mg and SE-Ca media, (D) The lipid content of *C. vulgaris* Y019 in BG11,BG11-S,BG11-P,BG11-K,BG11-Mg and BG11-Ca media.



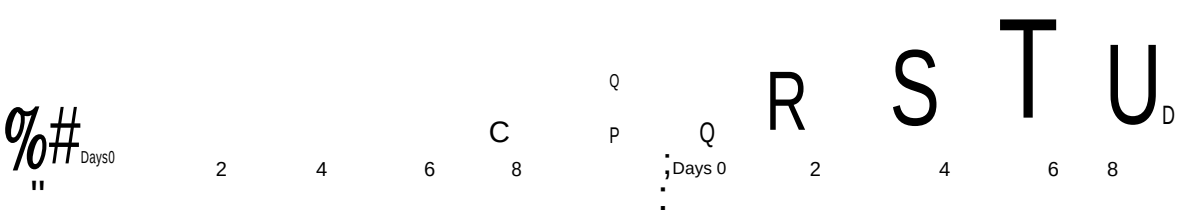


Figure 4. The growth curve of *C. reinhaditti* CC124 in high carbon TAP and HSM, and low carbon SE and BG11, and their N free or Fe free media. A) Growth rate of *C. reinhaditti* CC124 in TAP,TAP-N and TAP-Fe media; B) Growth rate of *C.* of *C. reinhaditti* CC124 in SE, SE-N, and SE-Fe media; D) Growth rate of *C. reinhaditti* CC124 in BG11,BG11-N and BG11-Fe media.

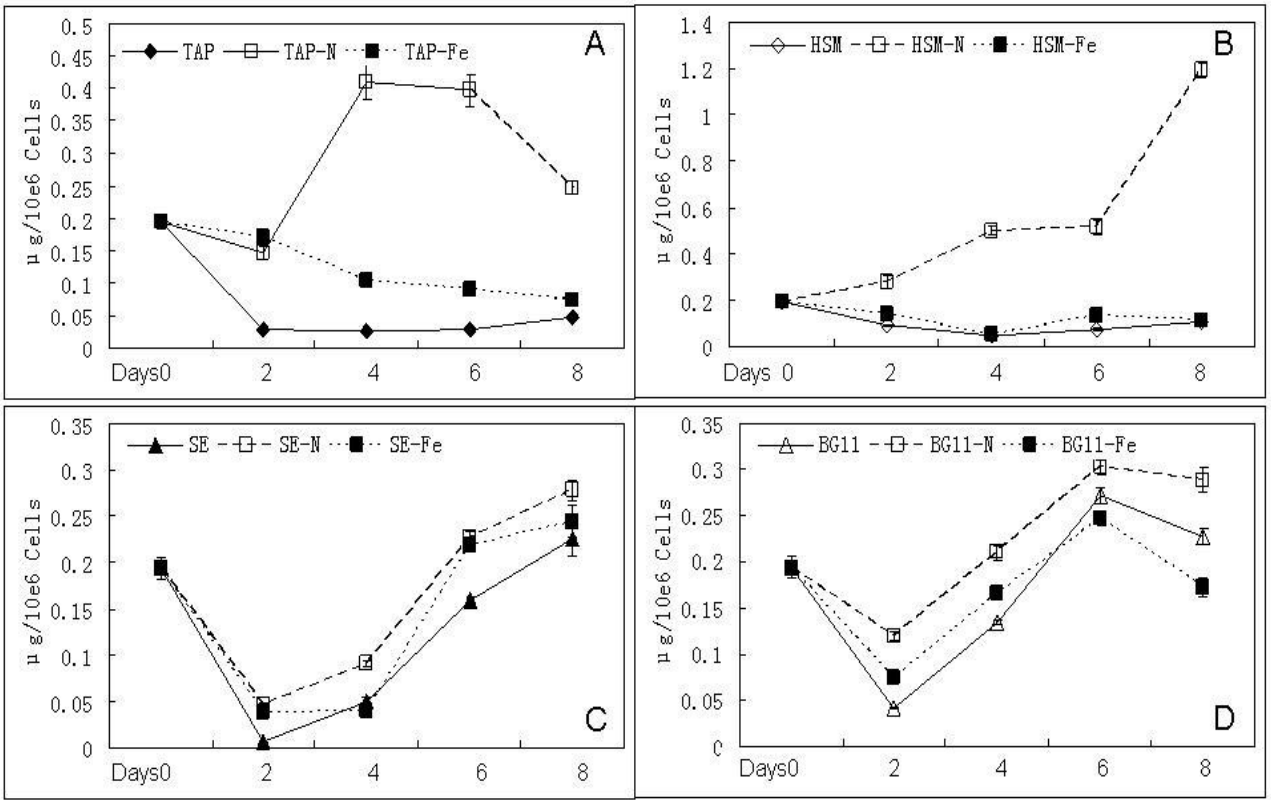


Figure 5. The lipid content of *C. reinhaditti* CC124 in high carbon TAP and HSM, and low carbon SE and BG11, and their N free or Fe free media. A) lipid content of *C. reinhaditti* CC124 in TAP,TAP-N and TAP-Fe media; B) lipid content of *C. reinhaditti* CC124 in HSM, HSM-N and HSM-Fe media; C) lipid content of *C. reinhaditti* CC124 in SE, SE-N, and SE-Fe media; D) lipid content of *C. reinhaditti* CC124 in BG11,BG11-N and BG11-Fe media.

content. Iron deficiency could induce lipid accumulation only in TAP medium (Figure 5). Importantly, sulfur and phosphorus starvation in TAP or HSM medium caused dramatic lipid accumulation. In these media, deficiency in other nutritional elements such as Ca, K or Mg caused a moderate increment in cellular lipid content (Figure 6).

The effects of N concentration on cell growth and lipid accumulation in *C. reinhaditti* CC124

Our results showed that N deficiency induced a dramatic increase of lipid content but such growth condition significantly inhibited cell growth of *C. reinhaditti* CC124 and *C. vulgaris* Y019. To achieve a balance between cell growth and lipid accumulation, we set to determine the effects of different N concentration on cell growth and lipid content. HSM medium with $[NH4]^+$ concentration of 10, 5, 2, 1, 0.1, 0.01 and 0 mM was used to culture *C. reinhaditti* CC124. Cell density and lipid content were detected at 0, 3, 5, 7 and 10 day time points after inoculation. Our results showed that, no significant cell growth inhibition was observed in media with $[NH4]^+$ concentration ranging from 10 to1 mM (Figure 7A). Cell concentration increased from 1.7 x 10^6/ml to 3.8 x 10^6/ml after three days of culture at 1mM concentration. On the other hand, reducing the $[NH4]^+$ concentration to 0.1, 0.01 and 0 mM condition led to complete arrest of cell growth, which consistent with earlier observation concurred with remarkable increase in neutral lipid content (Figure 5B) . After 5 days incubation, lipid content reached up to 2.0 ug/10^6 cells at 0.1, 0.01 and 0 mM concentration, which was approximately 10 times of that obtained in the complete or 5mM $[NH4]^+$ medium. Under our experimental conditions, among the different $[NH4]^+$ concentration between 1 and 0 mM, considerable incubation duration is necessary to reach the peak value of lipid content. For example, at 1 mM $[NH4]^+$ concentration, the highest lipid content (4.38 ug/200 ul) was obtained after 6 days cultivation (Figure 8). Similar results were obtained when cellular lipid content was detected by Nile red staining and subsequent microscopic analysis. As shown in Figure 9, strong yellow fluorescence, reflecting high level of neutral lipid accumulation was detected in *C. reinhaditti* CC124 after 10- day incubation at 1mM or 0 mM $[NH4]^+$ concentration. On the other hand, only red fluorescence, indicating of low-level neutral lipid was captured under 10 or 5 mM condition. The results are in great agreement with data obtained by quantitative analyses.

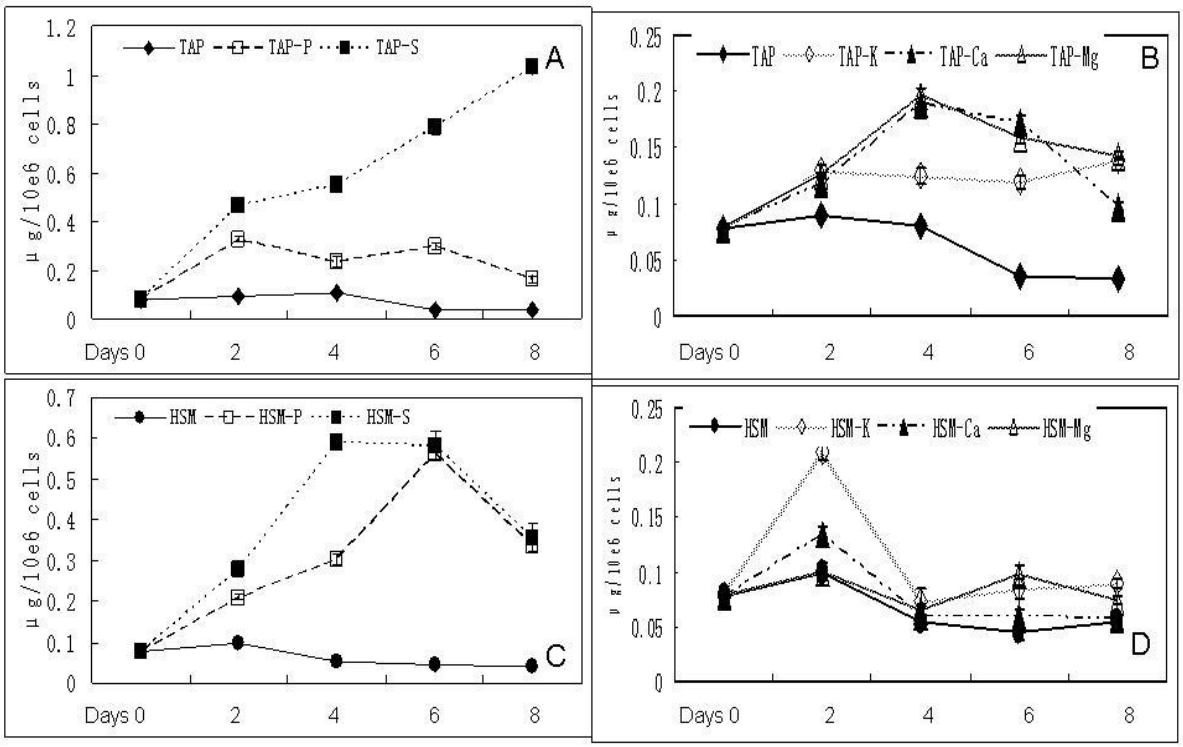


Figure 6. The lipid content of *C. reinhaditti* CC124 in TAP, TAP-P, and TAP-S media (A), in TAP-K, TAP-Ca, and TAP-Mg media (B), in HSM, HSM-P, and HSM-S media (C), in HSM-K, HSM-Ca, and HSM-Mg media (D).

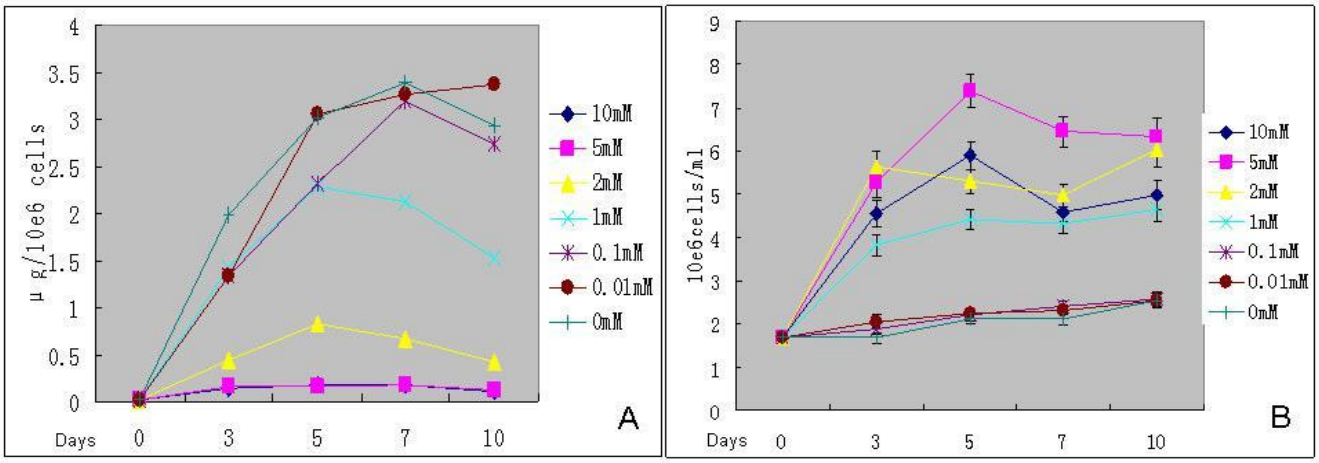


Figure 7. The lipid content(A) and growth curve(B) of *C. reinhaditti* CC124 in $[NH4]^+$ concentration of 10, 5, 2, 1, 0.1 and 0 mM HSM media.

DISCUSSION

Microalgae as a feedstock for biodiesel production have recently become more attractive due to its various advantages such as fast growth rate and high lipid content. Our study examined changes of cell growth and neutral lipid content in the oleaginous strain *C. vulgaris* Y019 and the model organism *C. reinhaditti* CC124 during incubation in various nutrient-limited TAP, HSM, SE or BG11 medium, respectively. The TAP, HSM, SE and BG11 media have been divided into two groups: H0igh carbon (TAP and HSM) and low carbon (SE and BG11), according to the concentration of organic carbon present in medium. Our results indicated that both

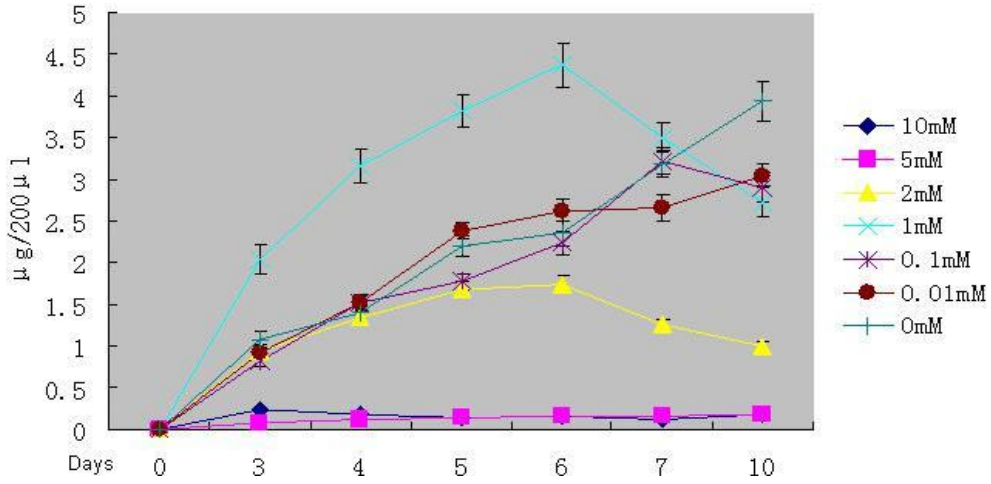


Figure 8. The lipid content of 200 ul of *C. reinhaditti* CC124 in $[NH4]^+$ concentration of 10, 5, ,2, 1, 0.1, 0.01 and 0 mM HSM media.

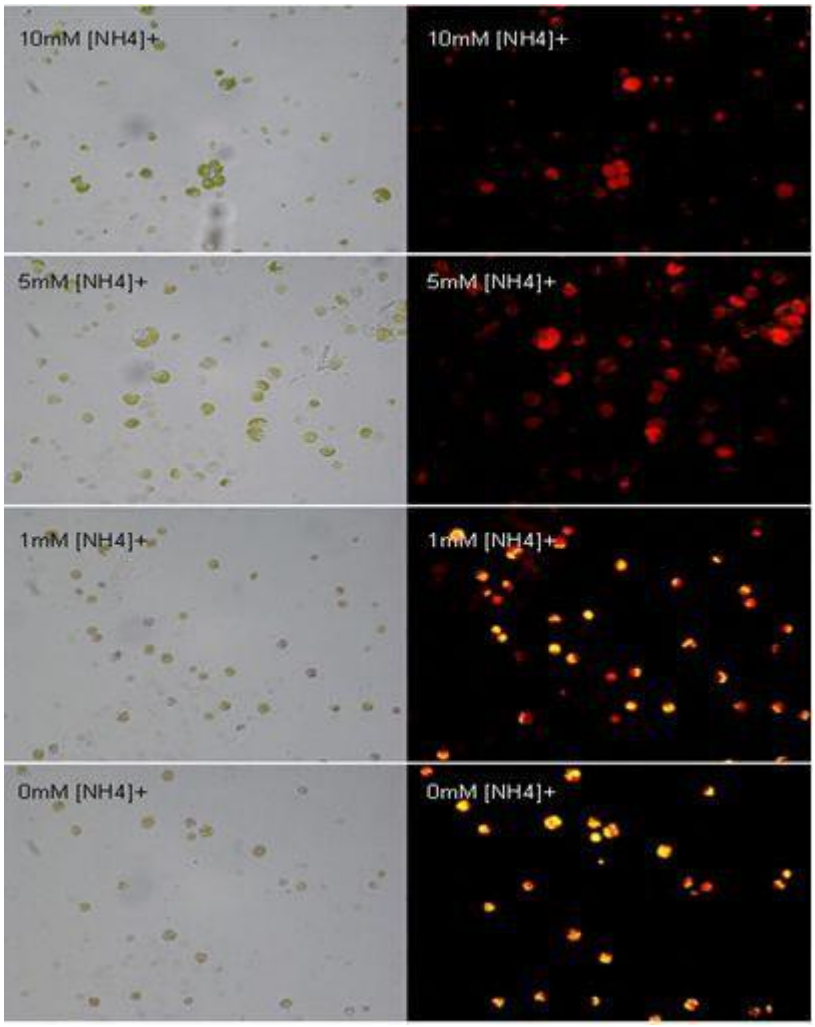


Figure 9. Fluorescence analysis of lipid of *C. reinhaditti* CC124 after 10 days cultivation in $[NH4]^+$ concentration of 10, 5,1 and 0 mM HSM media using Nikon 80i fluorescence microscopes(400X).

organism grew better in high carbon media than in low carbon medium. In general, both strains could accumulate significant neutral lipids by nutrient starvation but such accumulation was unfortunately associated with low biomass production, which is a big obstacle for the commercialization of biodiesel production. Therefore, it is important to identify the optimal nutrition concentration of the medium, especially N source, for the best combination of reasonable cell growth and high lipid accumulation. Alternatively, adding high carbon nutrition such as glucose and sucrose to the culture medium seems to be feasible to enhance the microalgal biomass and lipid production.

The relationship between nutrient deficiency and lipid accumulation in high carbon medium

Our results showed that carbon source could facilitate cell growth. The application of external carbon sources such as glucose and sucrose could result in an increase in biomass yield. Xing et al. (2008) reported that the cell density of *C. vulgaris* could reach 6.27 x 10^7 cell/ml and cell dry weight reach 23.647 g/L. Wijanarko et al. (2008) also reported that a 22.3 g/L cell dry weight was obtained in *C. vulgaris*. However, the external carbon source has little impact on lipid accumulation whereas N or S deficiency in high carbon medium including TAP-N, HSM-N, TAP-S and HSM-S achieved significantly higher lipid accumulation, reaching 10 times of that of similarly grown cells in TAP or HSM. On the other hand, *C. reinhardtii* grown in K, Ca and Mg deficient high carbon media showed a moderate increase in lipid content, whereas *C. vulgaris* could not increase neutral lipids in these media except for in Mg-deficient HSM. Subtraction Fe promoted lipid content mainly in *C. reinhardtii* grown TAP and *C. vulgaris* grown in HSM medium, indicating the complex relationships among the nutrient contents and lipid accumulation in these organisms.

The relationship between nutrient deficiency and lipid accumulation in low carbon medium

In low carbon SE or BG11 medium, N deficiency moderately enhanced the lipid accumulation in *C. reinhardtii* and *C. vulgaris*. Deficiency in other nutritional elements such as P, S, K, Ca or Mg also increased lipid content in *C. vulgaris*. In these media, Fe deficiency had no effect on lipid content in either organism. Understandably, cells grew more considerably slower in nutrient deficiency medium, which constitutes a challenge in the utilization of these growth conditions for biofuel production.

Possible mechanisms of neutral lipid accumulation by nutrient deficiency

It is well established that microalgae usually accumulate more lipids under stress conditions, including nutrient deficiency, high pH value and high temperature. For example, N starvation leads to higher lipid content in many microalgal species (Merzlyak et al., 2007; Illman et al., 2000; Li et al., 2008; Hu et al., 2008). P deficiency induces lipid accumulation in *M. subterraneus* (Khozin-Goldberg and Cohen 2006), *P. tricornutum, Chaetoceros sp.*, *I. galbana* and *P. lutheri*. However, in the cases of *N. atomus* and *Tetraselmis sp.* low phosphorus decreased their cellular lipid content. Otsuka (1960) reported that S deficiency also enhances lipid content in *Chlorella sp.* However, there is little information about the effects of Ca, Mg, K and Fe deficiency on lipid accumulation in microalgae, either much is known about the optimal N concentration for lipid accumulation. Our results revealed that Ca, Mg and K starvation play important roles in the accumulation of neutral lipids in *C. vulgaris* grown in the four media.

Compared to the cells grown in low carbon medium conditions, microalgal cells grown in high carbon media accumulate more lipids after N or S deficiency. Both N and S are essential components of protein synthesis, deficiency in these nutritional elements will drastically decrease protein synthesis rate, resulting in a feed back inhibition in the citric acid cycle and photosynthesis impairment, largely due to insufficient proteins involved in the photosystem reaction center and photosynthetic electron transport. Under such conditions, carbon fixation through photosynthesis is greatly reduced and intercellular carbon will be mainly derived from acetate assimilation via the glyoxylate cycle. A number of carbohydrate intermediate metabolites generated by acetate assimilation are utilized to produce triacylglycerols (TAGs) through the Kennedy pathway (Figure 10B). On the other hand, in high carbon medium a small amount of TAG is biosynthesized, despite under this condition, carbon can be fixed not only by photosynthesis but also from acetate (Figure 10A). The possible reason is that a large portion of carbon source is directed toward the synthesis of starch and protein to support vigorous cell growth. Similarly, comparing to cells grown in N- or S- medium, microalgae cells grown in complete low carbon medium accumulate less neutral lipids (Figure 10C). It is likely that most of the intercellular carbon are used to synthesis TAG because carbon flows toward protein and starch biosynthesis are blocked (Figure 10D). Under nutrient deficiency low carbon condition, carbon flow toward TAG formation may be driven by protein degradation and other intermediate metabolites rather than by utilizing extracellular carbon dioxide and acetate.

Phosphorus is an important component of DNA and RNA; the lack of this element will cause defective cell division, leading to arrest of cell growth. The lack of P also could impair phospholipids synthesis, which will promote the synthesis of TAGs. Our results showed that P deficiency increased the accumulation of neutral lipids both in *C. reinhardtii* and *C. vulgaris* regardless of carbon

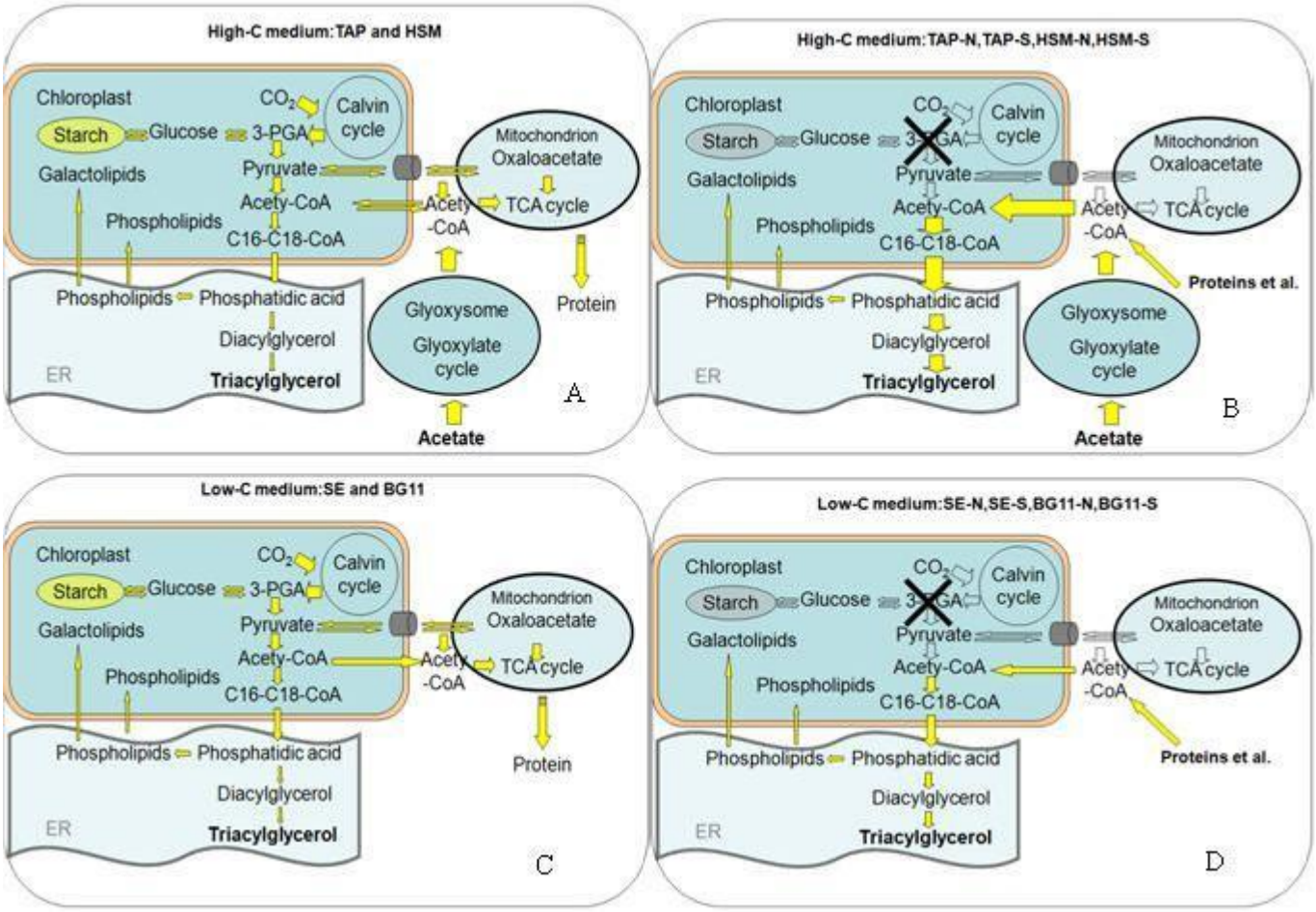


Figure 10. Deduced model for explaining the mechanism of the lipid accumulation in –N and –S conditions.

levels, but high carbon allows the production of significantly higher lipids. The detailed mechanisms underlying the roles of ions deficiency in the induction of lipid accumulation are not fully investigated. Potassium is one of the most abundant positive ions in plant cell and is involved in many important physiological functions such as regulating cellular osmotic pressure, enzymatic activity and protein synthesis (Markova et al., 1995; Tester et al., 1989). Thus, lipid accumulation by potassium deficiency can results multiple intertwined reasons. Interestingly, it has been reported that magnesium starvation induced a dramatic increase of triglyceride and cholesterol in rat blood plasma, accompanying with a decrease of glycogen content and increase of triglycerides in the liver.

Lipid content per cell and lipid content per unit volume

Results from our nitrogen concentration gradient experiments showed that the lower N concentration is in the medium, the higher lipid content per cell is. On the other hand, N concentration is positively correlated with biomass production. For example, cell growth was severely inhibited and little biomass was produced under 0.001 or 0 mM $[NH4]^+$ concentration culture condition. Since fast cell growth and high lipid content are two essential factors for commercialization of microalgae biodiesel, it is important to achieve a balance between cell growth and the cellular lipid content. In our study, 1.4 mg lipid was obtained in *C. reinhardtii* grown in 50 ml culture medium containing 1mM $[NH4]^+$ concentration, whereas 1.0 mg lipid was produced in the same volume of 0 mM $[NH4]^+$ concentration medium (Figure 10). These results highlight the importance of optimal N concentration in large scale microalgae cultivation to obtain high lipid content per unit volume. Our observation that biomass can be harvested 6 days after cultivation suggests that early harvest of the cells for lipid production is necessary in N limited cultivation condition. Clearly, manipulation of the condition that combines nutrient deficiency, growth condition and culture duration is important to achieve algal cells that allow maximal oil production.

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