J. Ocean Univ. China (Oceanic and Coastal Sea Research)
https://doi.org/10.1007/s11802-019-3691-2
ISSN 1672-5182, 2019 18 (2): 474-480
http://www.ouc.edu.cn/xbywb/
E-mail:xbywb@ouc.edu.cn

The Light Regime Effect on Triacylglycerol Accumulation of *Isochrysis zhangjiangensis*

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(Received January 10, 2018; revised April 26, 2018; accepted May 15, 2018)

Abstract Stress state of microalgal cells is caused under unfavorable conditions such as disordered light regime and depleted nitrogen. The stress state can impair photosynthetic efficiency, inhibit cell growth and result in the accumulation of triacylglycerol (TAG) from protective mechanisms. Continuous light or nitrogen starvation was applied on microalgae and performed effectively on inducing TAG production. To evaluate the light regime effect on inducing TAG production, the effect of different light regimes on nitrogen-starved *Isochrysis zhangjiangensis* was investigated in this work. The continuous light and nitrogen starvation elevated TAG content of biomass by 73% and 193%, respectively. Furthermore, the TAG accumulation of *I. zhangjiangensis* cell under nitrogen starvation decreased under aggravated stress from continuous illumination. Our results demonstrated that culturing the cells with 14L: 10D light regime under nitrogen starvation is the optimal mode to achieve maximal accumulation of TAG. A recovery in light regime was necessary for *I. zhangjiangensis* cultivation.

Key words microalgal cultivation; triacylglycerol accumulation; light regime; Nitrogen starvation

1 Introduction

As the next generation source of biofuel, microalgae have great potential in lipids and carbohydrates accumulation. Algal cells are well known for their response to environmental and nutrient stress (Goncalves *et al*., 2016) by shifting the metabolism into energy storage mode as a protective mechanism (Hu *et al*., 2008). Numerous studies have outlined TAG accumulation as a means for carbon and energy storage in microalgae (Thevenieau and Nicaud, 2013). TAG accumulation rates are species-specific since regulation and localization of TAG accumulation are different among microalgae species (Klok *et al*., 2014). The regulation mechanism of TAG synthesis and its responding to environmental stress remain obscure although enzymes or key genes have been proved to be associated with TAG metabolism pathways (Cross and Umen, 2015).

To enhance lipid production, different strategies have been applied on microalgae. Nitrogen starvation is considered as a prevalent approach to induce TAG enrichment (Meng *et al*., 2015). In some cases, light regime has been applied on microalgae to affect the TAG accumulation. Studies proved that continuous light could induce lipid accumulation without direct or indirect nitrogen depletion (Sforza *et al*., 2012; Goold *et al*., 2016).

Energy from light has a direct relationship with productivity in biomass and cell growth of microalgae. Daily illumination length is one of the key factors in microalgae growth (Sforza *et al*., 2012; Singh *et al*., 2015). Furthermore, the light regime has been proved deeply involved in circadian rhythm regulation of plant, such as photosynthesis and lipid metabolism (Nitschke *et al*., 2016; Diamond *et al*., 2015; Hennessey and Field, 1991). Cell cycle and metabolic rate rhythm are also determined by light duration daily (Jacob-Lopes *et al*., 2009). For outdoor cultivations, light energy is available with natural light rhythm in a discontinuous way. The 14L: 10D and 12L: 12D light regimes were often adopted as cultivation natural modes. During the dark, microalgae can recover from photoinhibition effect (Polle *et al*., 1999). Meanwhile, biomass losses were also monitored caused by night respiration, which varied from 1% to 22% (Edmundson *et al*., 2015). Therefore, culturing algae with 24 L: 0D was studied as a strategy to overcome it, improving the yield as well. The 24L: 0D light regime has been successfully explored on many microalgae such as *Chlamydomonas reinhardtii*, *Botryococcus braunii*, *Pycnococcus prova- solii*, *Euglena gracilis* and *Nannochloropsis salina* (Singh *et al*., 2015; Tamburic *et al*., 2012; Yoshimura *et al*., 2013; Sforza *et al*., 2012).

To gain a deeper understanding of the effects of light regime on carbon fixation progress of microalgae, the

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changes of photosynthetic activity were often detected through a chlorophyll fluorometer. The sensitivity of photosystem II (PSII) activity is recognized as an indicator of how microalgal cells respond to stress from environmental change. The data of F_v/F_m from chlorophyll fluorescence measurements represents the maximum quantum efficiency of PSII. Any type of stress that causes inactivation or damage on PS II results in decreasing of F_v/F_m value (Murchie and Lawson, 2013).

Synergistic effect of nitrogen starvation and continuous light stresses was expected to obtain higher TAG production, but the overlay of different stresses sometimes brings in negative results. *Isochrysis zhangjiangensis* is a marine strain with the capacity to accumulate lipids and its regular cultivation has been well documented (Feng *et al*., 2011; Wang *et al*., 2014). In this study, three light regime cultivation modes were used with the same total illumination time of 32 hours to investigate the effect of nitrogen starvation stress and the continuous light regime to find out the combining mode of these two factors for the maximal accumulation of TAG in marine microalga *I. zhangjiangensis*.

2 Materials and Methods

2.1 Strains and Culture Conditions

Isochrysis zhangjiangensis FACHB-1750 was obtained from the Liaoning Institute of Marine Fisheries and maintained by the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences. The microalgal cells were grown at 25 ± 2℃ in modified f/2 medium (Feng *et al*., 2011) with three times NaH_2PO_4 concentration and four times $NaNO_3$ concentration initially. Cultures were inoculated in 3 L Erlenmeyer flasks, filled with 1.2L growth mediums, sealed with sterile cotton gauze and grown under 14L: 10D regime light (60 μmol m^{-2} s^{-1}). The light intensity was measured by a photosynthetically active radiation (PAR) detector (Optometer P9710 with PAR detector 3701, Gigahertz Optik Corporation, Germany).

For the continuous illumination tests, the same strain was adopted in 24L: 0D regime for 10 days to minimize the shock by the direct change of the light regime.

2.2 Light Regimes and N-Deprivation Effect

Experiment started with the re-suspended cell from cultures at the exponential growth phase. N-deprivation (N-) or N- containing (N+) culture was achieved by filling the same volume of N-free or N-containing medium to the re-suspended cell at the initial stage. The N-free medium was prepared as above modified f/2 medium without the addition of $NaNO_3$. The nitrate level in the medium was detected as Chi *et al*., with a UV/VIS spectrophotometer (Jasco V-650, JASCO Corporation, Japan) at 275 and 220 nm (Chi *et al*., 2016). The initial cell density was 2.0×10^6 cells mL^{-1}. The cell density was measured by a UV/VIS spectrophotometer (Jasco V-530, JASCO Corporation, Japan) at 680nm (Chi *et al*., 2016).

To observe the effect of nitrogen starvation and continuous light stress in *I. zhangjiangensis*, a set of batch cultivation was employed. In previous studies, 14L: 10D light regime was used in *I. zhangjiangensis* cultivation to monitor the cell response to nitrogen starvation stress (Wang, *et al*., 2014; Wang, *et al*., 2015). The doubling time for *I. zhangjiangensis* cells was approximately 16 hours, thus both N-free and N-containing cultures were grown under three cultivation modes with total illu-

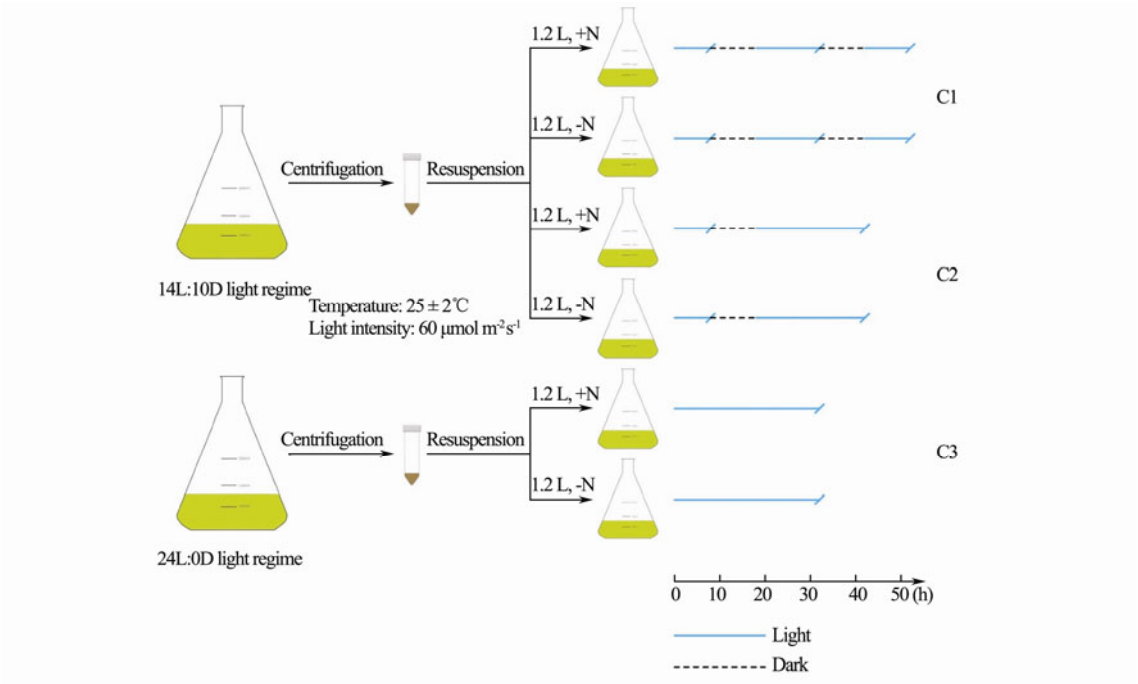

Fig.1 Experimental set-up for the light regimes and N-deprivation effect on *I. zhangjiangensis*. C1 mode: 8 hours' light / 10 hours' dark / 14 hours' light / 10 hours' dark / 10 hours' light; C2 mode: 8 hours' light / 10 hours' dark / 24 hours' light; C3 mode: 32 hours' continuous illumination. This experimental set up was performed in three individual replicates.

mination time of 32 h (Fig.1). Mode 1 (C1): 8 hours' light / 10 hours' dark / 14 hours' light / 10 hours' dark / 10 hours' light; Mode 2 (C2): 8 hours' light / 10 hours' dark / 24 hours' light; Mode 3 (C3): 32 hours' continuous illumination. The above cultures were carried out in independent biological replicates.

2.3 Data Measuring Method

The photosynthetic performance data were measured by a chlorophyll fluorometer (WATER-PAM, Heinz Walz GmbH, Germany). Cells were adapted to dark for 10 min (continuous illumination time less than 16 h) or 20 min (continuous illumination time more than 16 h) before measuring (Wang *et al*., 2014).

For biomass, 50 mL of culture was filtered with pre-weighed 47 mm diameter filter (Whatman GF/C), washed three times with 2 mL 0.5 mol mL^{-1} NH_4HCO_3 solution, and dried to a constant weight at 60℃ (Wang *et al*., 2014).

During fatty acids measurement progress, we used the method of direct transesterification of fresh microalgal cells (Liu *et al*., 2015). Fatty acid methyl esters (FAMEs) analyses from transesterification step were carried out by gas chromatography (7890B GC system, Agilent Technologies, USA) with a DB-23 column (30 m × 0.32 mm × 0.25 μm, Agilent Technologies, USA) and a flame ionization detector (Liu *et al*., 2015).

Total TAG contents were determined by the Nile red method. The culture was diluted to a cell density of 1×10^6 cells mL^{-1} by sea water. Dye Nile red (50 μg mL^{-1}, dissolved in dimethyl sulfoxide) was added to a final concentration of 1 μg mL^{-1}. After staining in the dark for 10 min, the fluorescence intensity was determined by a fluorescence spectrophotometer (Cary Eclipse Fluorescence Spectrophotometer, Agilent Technologies, USA). TAG content was calculated based on fluorescence intensity (Wang *et al*., 2014).

All the measurements of the values used in this study represented the average ±SD of individual replicates during the whole experiment.

3 Results and Discussion

3.1 Effect of Continuous Light Acclimation on *I. zhangjiangensis* seeds

Algal seeds employed for continuous light regime of C3 mode were adopted under 24L: 0D light regime for 10 days. Compared with cells grown under 14L: 10D light regime, seeds adopted under continuous light had higher cellular biomass and TAG content. By Nile red measurement, TAG content in seed cells increased 56% after continuous light acclimation under N+ condition. Notably massive death was observed on *I. zhangjiangensis* seeds without light adaption after directly shifting from 14L: 10D to 24L: 0D under N-condition in the pre-tests. Due to these differences at the initial culture stage, the comparisons among three modes were made by increments of the physiological factors in the later discussion.

3.2 Effect of Light Regime on Cell Growth of *I. zhangjiangensis*

After 32 hours' illumination, the cells grown under N+ condition achieved obviously higher cells density than N-. Under N+ condition, the cell density grew to 4.8×10^6 cells mL^{-1} (OD_{680}=0.46) in C1 and C3 cultivation. At the same time, 4.1×10^6 cells mL^{-1} (OD_{680}=0.40) was obtained in C2 cultivation. These results indicated that the continuous light regime did not inhibit adapted algal seeds under nitrogen repletion, in agreement with the previously study on microalgae *Chaetoceros gracilis* and *Isochrysis galbana* (Toro, 1989). The growth rate of C2 mode was lower than in other two light modes in both N+ and N- (Fig.2a). Comparing the growth of both C1 and C2 modes with acclimated C3, the possible reason may lie in the adaptation of cells to the continuous illumination. Under N-condition, the cell density of C1 and C3 grew to a similar level after 22 hours' illumination, then the C1 cultivation obtained a higher growing rate after

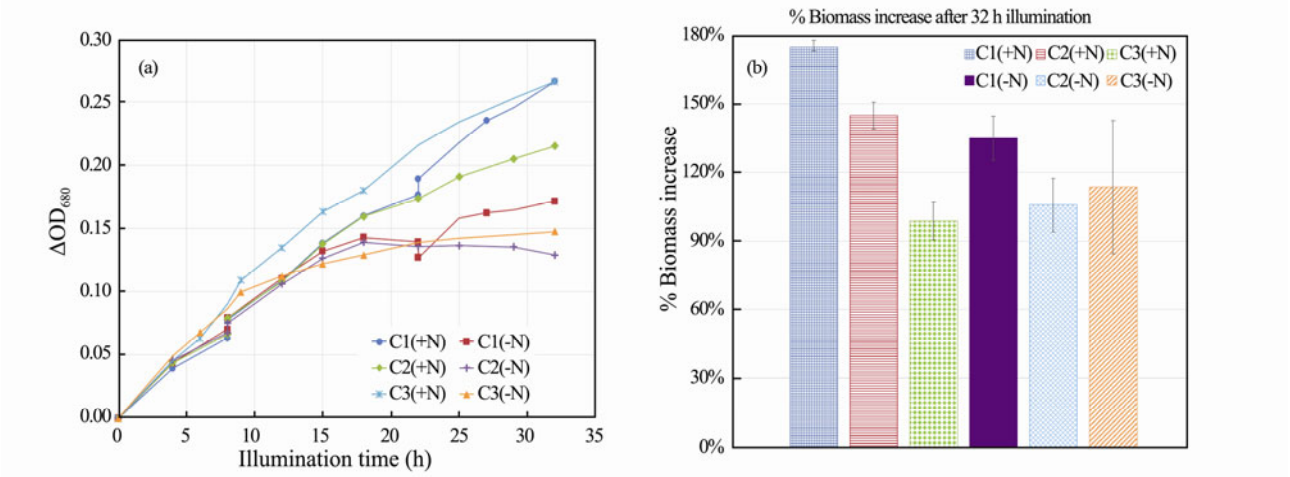

Fig.2 a) The growth of *I. zhangjiangensis* based on real illumination time. Cell density change was presented as ΔOD_{680} = $OD_{680,\ sampling} - OD_{680,\ inoculation}$. Two OD points could be observed on one curve for light switching time. All points represent the mean of three biological replicates with high consistency. b) Biomass changed after 32 hours' illumination. Bars represent the mean and standard deviation of individual replicates, calculated from the equation: biomass increment (%) = $(D_t\text{-}D_0)/D_0 \times 100\%$, where D_t and D_0 are dry weights (g L^{-1}) at 32h and 0h, respectively.

experiencing a 10 hours' dark period. The ΔOD_{680} value of C1 and C3 under N- after 32 hours' illumination was 0.17 and 0.15, respectively. The difference between the two values represented the recovery effect of darkness period under N-condition.

Although the same cell density was obtained under N+ cultivation of both C1 and C3 after 32 hours' illumination, the biomass increase of C3 mode is only 60% of C1 increment (Fig.2b). It indicated that although there should be some biomass loss during the dark, the dark period allowed the algal cells to recover and prepare for the next light regime. Therefore, the overall result for biomass of *I. zhangjiangensis* with regular light regime was better than with continuous light. This was different from some other microalgal species. In the cyanobacterium *Aphanothece microscopica Nägeli*, biomass harvested from continuous light cultivations was 25% more than in photoperiods of 12L: 12D with the same illumination time (Jacob-Lopes *et al*., 2009). The *Botryococcus braunii* KMITL 2 achieved a biomass under 24L: 0D light cycle four times of the biomass under 12L: 12D cycles (Ruangsomboon, 2012). Such difference represented the diversity of microalgal cells under continuous illumination.

3.3 Effect of Light Regime on Photosynthetic Activity of *I. zhangjiangensis*

In the previous study on *I. zhangjiangensis* (Wang *et al*., 2014), F_v/F_m value of cells cultivated under N+ condition remained at 0.70 during 10-days' culture period: in contrast, it decreased to 0.18 under N-condition. As showed in Fig.3, the F_v/F_m value of all N+ cultures was maintained at range 0.65 – 0.69 after 32 hours' illumination, meanwhile, below 0.60 in all three N-cultures after 32 hours' illumination time. Compared with the C3 mode under N+ condition, the F_v/F_m value of C1 and C2 did not decrease after the first dark period until the total illumination time reached 18 hours. The 10 hours' darkness of second dark period allowed cells of C1 to recover again. The cells of C3 mode with long time acclimation showed the adaptability to continuous light under N+ condition. However, this situation of *I. zhangjiangensis* cells was instable with extra stress, which was shown in fast and early drop of F_v/F_m value of C3 under N- condition.

The comparisons among F_v/F_m value of cells cultured under various conditions could evaluate the respective inhibition effect on photosynthesis from two stress factors. In C1 modes, the value of culture under N- condition decreased to 0.60 after 32 hours' illumination, and at the same time, it remained at 0.67 under N+ condition (Fig.3). The difference of two values representing the inhibition effect on photosystem caused by nitrogen starvation about 0.07 varied. Meanwhile, the F_v/F_m value of cells in C3 mode under N+ condition was maintained at 0.69, indicating that the influence on F_v/F_m value was not shown when continuous illumination was applied on nitrogen replete cells. Furthermore, when *I. zhangjiangensis* cells were cultured under nitrogen starvation, the F_v/F_m value started to decline abruptly after 15 hours' illumination in C1 mode as under one stress in contrast, it decreased rapidly after 9 hours' illumination in C3 mode under the synergistic effect of the two stress factors. Based on the stress level under continuous light and N- conditions, it is assumed that algal cells cultured under both factors could accumulate more TAG than under normal light regime and N- condition.

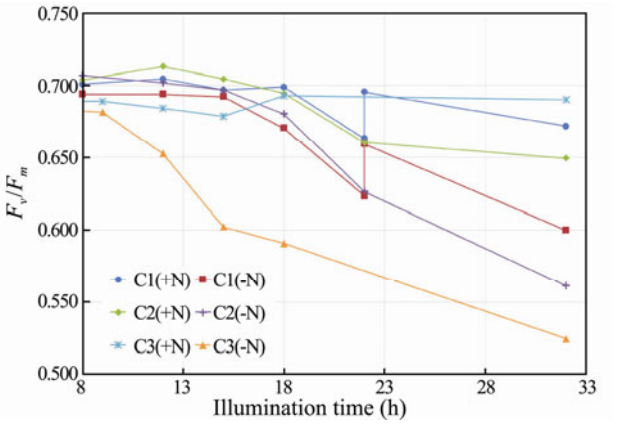

Fig.3 Chlorophyll fluorescence analysis based on reality illumination time. This figure represented the F_v/F_m values after 8 hour's illumination and 10 hours' dark. All points represent the mean of individual replicates.

3.4 Effect of Light Regime on TAG Accumulation of *I. zhangjiangensis*

To validate the above assumption, the change of TAG productivity and FA profile were determined, which could point to differences in the cell response to light regime and nitrogen starvation effect. After 32 hours' illumination, the cellular TAG content of C1 mode increased 60% in N- cultivations. Calculated with the increase of cell density by 92%, the total TAG increased 208% of C1 mode in N- cultivation (Fig.4). In contrast, the TAG increasing percentage was 83%, relatively lower in C3 modes, which was mainly caused by the higher TAG content at the initial stage. Considering the 56% increment of TAG content at the initial stage for continuous light acclimation, the value of total TAG production from seed process was 185% in C3 mode under N- after 32 hours' illumination, which was still 11% lower than the C1 mode in N- cultivation. Considering the results discussed above, cells cultured under the overlay stress of continuous light and nitrogen further inhibited the growth and photosynthesis; however, the TAG productivity was not increased from aggravated stress on nitrogen-starved algal cell caused by continuous illumination.

Among the fatty acid profiles of *I. zhangjiangensis*, the ratio of C14:0 /C16:0, the content of C18:4 and C18:1n9 are important for indicating the corresponding lipids variation (Wang *et al*., 2015). With results of fatty acids composition (Fig.5), the ratio of C14:0 /C16:0 was 1.38 in C3 at the initial stage, lower than the value of C1, which was 1.80. The decrease of the ratio of C14:0 /C16:0 was normally monitored during nitrogen starvation, which was 1.16 of C1 after 32 hours' cultivation.

Therefore, the stress already existed in C3 at the beginning. The content C18:4 was one of the main components of fatty acid, which made up 25% of total lipid in C1 modes at the initial stage and decreased to 18% after 32 hours' illumination under N- condition. In contrast, the C18:4 in C3 mode content began with 21% of total lipid and decreased to 16%, representing the deeper stressed state of C3 mode under N- condition. Significant changes were found on C18:1n9 during the cultivation. The C18:1n9 was also observed as an index to the fatty acid profile under environmental stress, with maximum 32.8% of fatty acid under a particular condition (Wang *et al*., 2015), which was employed as a biomarker of TAG in *I. zhangjiangensis* according to a previous study. The TAG percentage in biomass was calculated based on Eq. (1).

$$\text{TAG (\%) in biomass} = (\text{C18:1n9(\%) in total fatty acids}) \times 0.29 - 1.88 \quad (1)$$

Under 14L: 10D light regime, the TAG content in biomass reached 1.46% and 4.26% after 32 hours' illumination under N+ and N- condition, respectively. Such a 193% increment represented the induction on TAG accumulation applied by nitrogen starvation. Meanwhile, under N+ condition, TAG content in biomass increased from 1.46% with 14L:10D light regime to 2.52% with continuous light. Continuous illumination promoted the TAG production by 73%. The 2.5 times difference between 193% and 73% represented the different stress effect on TAG accumulation between nitrogen starvation and continuous illumination. In C3 mode under N- cultivation, the TAG content in biomass was 4.13% after 32 hours' illumination, calculated with a lower biomass increment. The cells were not able to achieve higher TAG content with overlying of the two stress factors; in contrast, the TAG productivity was lower than singly employed nitrogen starvation as the stress factor.

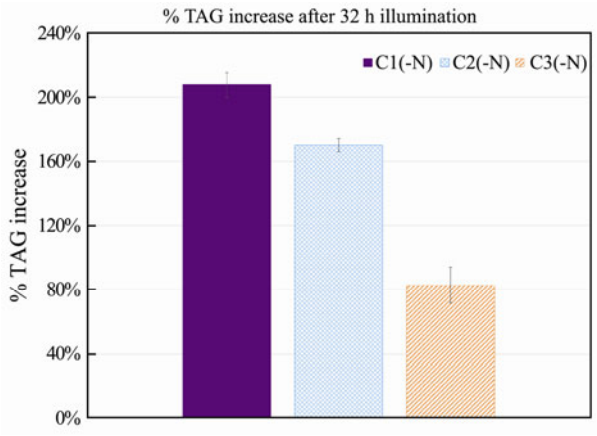

Fig.4 The total TAG increment after 32 hours' illumination measured by Nile red. Bars represent the total TAG increment under nitrogen-starved condition of C1, C2, C3 from left to right, respectively.

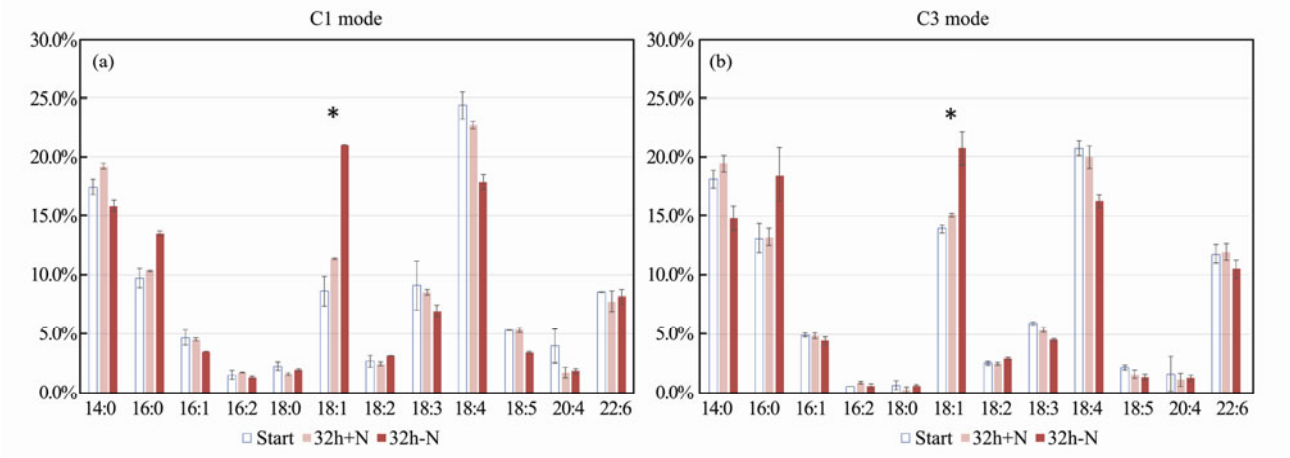

Fig.5 The profiling changes of fatty acid after 32 hours' illumination. The C18:1n9 was employed as the biomarker of TAG in *I. zhangjiangensis*. Left figure represents the fatty acid profiles under C1 mode and right figure represents the fatty acid profiles under C3 mode.

Table 1 The response variables of *I. zhangjiangensis* cells caused by nitrogen starvation and continuous illumination

Factors	C1		C2		C3		*P* value	
	N+	N-	N+	N-	N+	N-	Light	Nitrogen
ΔOD_{680}	0.266	0.172	0.215	0.129	0.266	0.148	0.104	0.009[a]
F_v/F_m	0.672	0.600	0.650	0.562	0.690	0.525	0.684	0.064
%TAG	1.46	4.26	2.04	3.57	2.52	4.18	0.576	0.038[b]

Notes: a, the *P*-value of +N/-N effect on ΔOD_{680} was lower than 0.01; b, the *p*-value of +N/-N effect on TAG content of biomass, $0.01 < P < 0.05$.

To evaluate response variables of *I. zhangjiangensis* cells caused by nitrogen starvation and continuous illumination, we employed the ANCOVA model for the two factors (Quinn and Keough, 2002). From the *p*-values in Table 1, the responses of TAG content and F_v/F_m of algal cells to nitrogen starvation were more prominent than the

response to continuous light. The change of TAG content and ΔOD_{680} caused by nitrogen starvation were considered significant.

3.5 Recovery of *I. zhangjiangensis* Cells from Photodamage During the Dark Time

Compared with C1 mode under nitrogen starvation, lower growth rate and biomass yield were considered as major reasons for lower TAG productivity in C3 mode under nitrogen starvation. Previous studies showed that, in the dark, photodamage on PSII center was slowly removed, even under stress conditions (Nath *et al*., 2013; Polle *et al*., 1999). Thus, the 10 hours' dark time for algal cells in C1 mode allowed the recovery from damage caused by illumination.

From F_v/F_m values before and after the second darkness period of C1 mode in Fig.3, *I. zhangjiangensis* cells cultured under N+ condition had similar recovery range to that under N-, with the value elevated to approximately 0.05 (Fig.3). After the recovery, the F_v/F_m of N- cultivation dropped faster due to the aggravated stress from nitrogen starvation. Under N- cultivation, the F_v/F_m value change of C2 mode showed the same tendency with C1 mode after the second dark period, decreased in parallel with C1 to about 0.05, the same as the value elevated during dark time. In order to ensure the accuracy of recovery value, three extra biological replicates were investigated. Results showed similar recovery levels, indicating that inhibition and damage of PS II system was repaired and recovered during the dark period.

4 Conclusions

From this study, it is concluded that a dark phase in light regime for cell recovery is necessary for *I. zhangjiangensis* cultivation. The continuous light regime (24L: 0D) could aggravate the stress applied by nitrogen starvation on cell, meanwhile, decrease the TAG accumulation rating. The stress from nitrogen starvation was 2.5 times efficient in inducing TAG accumulation than continuous illumination. Culturing the microalgae cells with 14L: 10D light regime under nitrogen depleted state is the optimal strategy for maximal accumulation of TAG. Further research on the regulation mechanism of metabolism pathway in microalgal cells is required for a deeper understanding on the interaction between light regimes and nitrogen starvation stress.

Acknowledgements

This study was supported by the National High Technology Research and Development Program '863' (No. 2012 AA052101) and the Hundred Talent Program of the Chinese Academy of Sciences (No. A1097).

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(Edited by Ji Dechun)