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Simultaneous production of triacylglycerol and high-value carotenoids by the astaxanthin-producing oleaginous green microalga *Chlorella zofingiensis*

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HIGHLIGHTS

- Stress conditions induced TAG and astaxanthin production in *C. zofingiensis*.
- A strong correlation between TAG and astaxanthin accumulation was observed.
- Simultaneous TAG and astaxanthin production in semicontinuous modes was optimized.
- High TAG and astaxanthin productivities (297 and 3.3 mg L^{-1} day^{-1}) were obtained.
- Inhibiting *de novo* fatty acid biosynthesis enhanced astaxanthin accumulation.

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ABSTRACT

The production of lipids and astaxanthin, a high-value carotenoid, by *Chlorella zofingiensis* was investigated under different culture conditions. Comparative analysis revealed a good correlation between triacylglycerol (TAG) and astaxanthin accumulation in *C. zofingiensis*. Stress conditions promoted cell size and weight and induced the accumulation of neutral lipids, especially TAG and astaxanthin, with a concomitant decrease in membrane lipids. The highest contents of TAG and astaxanthin achieved were 387 and 4.89 mg g^{-1} dry weight, respectively. A semi-continuous culture strategy was developed to optimize the TAG and astaxanthin productivities, which reached 297 and 3.3 mg L^{-1} day^{-1}, respectively. Additionally, astaxanthin accumulation was enhanced by inhibiting *de novo* fatty acid biosynthesis. In summary, our study represents a pioneering work of utilizing *Chlorella* for the integrated production of lipids and high-value products and *C. zofingiensis* has great potential to be a promising production strain and serve as an emerging oleaginous model alga.

1. Introduction

Owing to the ever-increasing energy consumption and drastic depletion of fossil fuels, alternative energy forms that are green, renewable and sustainable are highly sought after. Biodiesel, or fatty acid methyl esters (FAMEs) derived from oil transesterification, is renewable, carbon neutral and portable, and has received great interest of the public and research (Knothe, 2009). Currently, biodiesel is produced mainly from plant oils. Nevertheless, plant oil-based biodiesel has inherent limitations and cannot realistically substitute the petroleum fuels (Chisti, 2007). In contrast, microalgae possess significant advantages over plants for biodiesel production and are considered to be the next-generation biodiesel feedstock capable of meeting the existing demand for transportation fuels (Chisti, 2007; Wijffels and Barbosa, 2010).

Although the utilization of microalgae as biodiesel feedstock has achieved substantial progress during the recent decades, significant challenges remain yet to be addressed before the cost-effective production of algal biodiesel can be realized (Richardson et al., 2012). Efforts have been made with an aim to improve the production economics of algal biodiesel, including alga selection, strain trait improvement, exploration of novel cultivation technologies and downstream processes, etc (Radakovits et al., 2010; Breuer et al., 2012; Pribyl et al., 2012; Kim et al., 2013; Liu and Chen, 2014; Wang et al., 2014; Sun et al., 2015). Alga selection,

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the starting point of algal biodiesel production pipeline, is considered fundamentally important. Because of the rapid growth, ease of cultivation, high lipid content and excellent lipid productivity, *Chlorella* has been regarded to be among the most promising producers of biodiesel precursors (Liu and Chen, 2014; Sun et al., 2015). While many studies focus on the optimization of algal culture conditions, the integrated production of lipids with co-products emerges as a new research direction (Liu and Chen, 2014), which is believed to be a promising approach toward offsetting the algal biodiesel production cost. Several oleaginous *Chlorella* species have been proposed to produce other useful components than lipids, including but not restricted to protein, lutein, and astaxanthin (Liu and Chen, 2014; Liu et al., 2014a). Nevertheless, protein and the primary carotenoid lutein inversely correlate with the accumulation of storage lipids (Guccione et al., 2014; Sun et al., 2015), thus impeding the integrated production to a great extent. In contrast, astaxanthin is a secondary carotenoid and tends to accumulate in *Chlorella zofingiensis* under such stress conditions as high light irradiance and nitrogen deprivation (Liu et al., 2010a, 2014b), which are also the typical triggers of storage lipids especially triacylglycerol (TAG), an ideal precursor for making biodiesel (Liu et al., 2012a; Sun et al., 2015). The characteristic that TAG and astaxanthin are induced by the same conditions offers *C. zofingiensis* substantial advantages for the simultaneous production of these two products. While there have been many studies exploiting the production of lipids or astaxanthin by *C. zofingienesis* (Del Campo et al., 2004; Liu et al., 2010a,b, 2011, 2012a, 2012b, 2013, 2014a, 2014b; Sun et al., 2015), to date have been few attempts to concurrently produce both products (Liu et al., 2012b; Mulders et al., 2014). The correlation between TAG and astaxanthin accumulation, and how they respond to the different stress conditions, remains to be elucidated in *C. zofingiensis*.

The main objective of the present study was to investigate the growth and accumulation of TAG and astaxanthin in response to four culture conditions, namely the regular low light (LL) growth condition and three stress conditions of nitrogen deprivation (ND), high light (HL), and the combination of ND and HL (ND + HL). Comparative results revealed that TAG and astaxanthin were induced by stress conditions and accumulated in a well-correlated manner. The production of both TAG and astaxanthin was greatly promoted by the adoption of a semi-continuous cultivation strategy coupled with nitrogen limitation. Our work represents a pioneering effort for comprehensive study of *C. zofingiensis* lipids and carotenoids and provided valuable insights into the utilization of this alga for the integrated production of TAG and astaxanthin.

2. Materials and methods

2.1. Algal strain and culture conditions

C. zofingiensis (UTEX B32) was purchased from the University of Texas Culture Collection of Algae (UTEX, Austin, USA) and maintained at 14 °C on agar plates of the modified BG-11 medium (150 mg L^{-1} of nitrate-N). Briefly, 10 mL of liquid BG-11 was inoculated with cells from agar plates and the alga was grown aerobically in flasks at 25 °C for 6 days with orbital shaking at 150 rpm and illuminated with continuous light of 30 μE m^{-2} s^{-1}. The cells were then inoculated at 10% (v/v) into 100-mL columns provided with constant illumination of 70 μE m^{-2} s^{-1} and aeration of 1.5% CO_2 enriched air, grown to late exponential phase and used as seed cultures for subsequent experiments.

2.2. Batch culture for the induction of oil and astaxanthin

The seed cultures were harvested, washed with N-deficient medium and suspended in N-replete (150 mg L^{-1} of nitrate-N) or N-deficient medium, yielding a final cell density of 0.5 g L^{-1}. Both cultures were grown in 250-mL glass columns aerated with 1.5% CO_2 enriched air, illuminated with 70 μmol photons m^{-2} s^{-1} (LL and ND) or 350 μmol photons m^{-2} s^{-1} (HL and ND + HL).

For the inhibition of fatty acid biosynthesis, cerulenin (Sigma–Aldrich, St. Louis, MO, USA), which specifically inhibits β-ketoacyl-ACP synthase I (KAS I), was added upon N-deprivation at a concentration of 10 μM in the cultures under ND conditions.

2.3. Semi-continuous culture for the production of oil and astaxanthin

The seed cultures were harvested, washed with N-deficient medium and suspended in N-limited (5, 10, or 20 mg L^{-1}) medium, giving a cell density of 0.5 g L^{-1}. The cultures were grown under HL (350 μmol photons m^{-2} s^{-1}) conditions for three days and then subject to semi-continuous cultivation at a daily dilution rate of 0.5 using the corresponding N-limited medium, namely, half of the cultures were taken and replaced by fresh medium manually. Additional semi-continuous cultures (10 mg L^{-1} N) were conducted, with half cultures replaced every 2, 1, and 0.5 days, corresponding to a dilution rate of 0.25, 0.5 and 0.75, respectively.

2.4. Determination of cell number, dry weight, and diameter

Cell numbers were counted using a hemocytometer under a light microscope. For dry weight determination, the algal cells were filtered through a pre-dried Whatman GF/C filter paper (1.2 μm pore size), washed three times with deionized water, dried at 100 °C in a vacuum oven until constant weight was achieved, and were subsequently cooled down to room temperature in a desiccator before weighting. Cell diameter was determined by using a microscope equipped with an internal reticle scale.

2.5. Lipid and pigment extraction

Cell samples were harvested, washed and freeze-dried on a DW3 freeze-drier (Heto Dry Winner, Denmark). The lyophilized cells were disrupted by homogenization with liquid nitrogen and extracted by a solvent mixture of chloroform/methanol/0.75% KCl solution (2:1:0.75, by volume). The lower chloroform phase containing lipids and pigments were collected and dried under nitrogen gas and then dissolved in chloroform or acetone for immediate use or stored at −20 °C.

2.6. Lipid analysis

Neutral lipids were separated on a Silica gel 60 TLC plate (Merck, Darmstadt, Germany) using a mixture of hexane/tert-butylmethyl ether (TBME)/acetic acid (80/20/2, by volume) as the mobile phase, while polar lipids were separated on a TLC plate using a mixture of chloroform/methanol/acetic acid/water (25/4/0.7/0.3, by volume) as the mobile phase. Lipids were detected by spraying the TLC plate with 10% $CuSO_4$ in 8% phosphoric acid, followed by charring at 180 °C for 3 min. For quantification, individual lipids on TLC plate were visualized with iodine vapor, recovered, transesterified and analyzed by Gas Chromatography Mass Spectrometry (GC–MS). The contents of these lipids were expressed as their fatty acids relative to dry weight (%).

Fatty acid methyl esters (FAMEs) were prepared by direct transesterification of lipids with 1% sulfuric acid in methanol. The FAMEs were separated and identified by GC–MS using a PerkinElmer CLarus 680 capillary gas chromatograph equipped with a SQ8 Mass Spectrometry detector and apolar TR-WAX column (Thermo Fisher Scientific; length 30 m, diameter 0.25 μm, film thickness 0.25 μm). Helium was used as the carrier gas. Samples were injected in split mode (5:1 split ratio) at an oven temperature

of 45 °C with an injection volume of 1 μL. The oven temperature program consisted of an initial hold at 45 °C for 1.5 min, ramping to 150 °C at 15 min then to 240 °C at 30 min, and a final hold at 240 °C for 3 min. The injector was kept at 225 °C, the flow rate of carrier gas was 1.45 mL min^{-1}, and the ionization energy was 70 eV. FAMEs were quantified by using a FAME mixture standard (Sigma–Aldrich) with C17:0 as the internal standard.

2.7. Pigment analysis

The extracts in acetone were filtered through a 0.22 μm Millipore organic membrane. Twenty μL of each extract was separated by HPLC on a Waters Spherisorb® 5 μm ODS2 4.6 250 mm analytical column with a Waters HPLC system (Waters, Milford, MA, USA). Pigments were eluted at a flow rate of 1.2 mL min^{-1} with a linear gradient from 100% solvent A (acetonitrile/methanol/0.1 M Tris–HCl (pH 8.0), 84:2:14, by volume) to 100% solvent B (methanol/ethyl acetate, 68:32, by volume) over a 15 min period, followed by 10 min of solvent B. Individual carotenoids were identified by their absorption spectra and their typical retention times compared to standard samples of pure carotenoids.

For thin layer chromatography (TLC) separation of carotenoids, extracts were loaded on a TLC silica gel 60 plate (EMD Millipore, Billerica, MA) using hexane/TBME/acetic acid (80:2:2, by volume) as the development system.

2.8. Statistical analyses

All data were obtained by using at least three biological samples to ensure the reproducibility of the results. Experimental results were expressed as mean value ± SD. Statistical analysis was performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA). Paired-samples *t*-test was used for two group means and one-way ANOVA Tukey's HSD test was used for over two group means. The statistical significance was achieved when $p < 0.05$.

3. Results and discussion

3.1. Cell growth as affected by different culture conditions

We examined the growth and morphology of *C. zofingiensis* in response to LL (70 μE m^{-2} s^{-1}) and HL (350 μE m^{-2} s^{-1}) under both N-replete and N-depleted conditions. Regardless of light intensity, *C. zofingiensis* grew much faster under N-replete conditions than under N-depleted conditions, as evidenced by significantly (*t*-test, $p < 0.05$) higher cell numbers (Fig. 1A). In the presence of N, HL benefited cell growth to a great extent as compared with LL. Generally, *Chlorella* performs photosynthesis proportionally to light intensity and becomes saturated when the light intensity reaches a threshold, above which algal photosynthesis may undergo photoinhibition leading to an impaired growth phenotype (Liu and Chen, 2014). Photoinhibition might not occur under the HL of 350 μE m^{-2} s^{-1} in our experiment. Similar results were observed in our recent report that *Chlorella protothecoides* cells showed no photoinhibition until the light intensity was over 400 μE m^{-2} s^{-1} (Sun et al., 2015). When N was depleted from the medium, HL and LL gave rise to almost no difference in growth. Similar to cell number, dry weight varied considerably under the different growth conditions examined, with HL giving the highest cell dry weight (7.5 g L^{-1} on day 6), followed by LL, ND + HL, and ND (Fig. 1B). Besides, cell weight was monitored and the results showed that the per cell weight declined slightly during the first day and then increased gradually under LL conditions (Fig. 1C). Notably, the stress conditions induced a significantly greater per cell weight than the regular growth conditions (LL), with ND + HL giving the highest followed by ND and HL, which may be

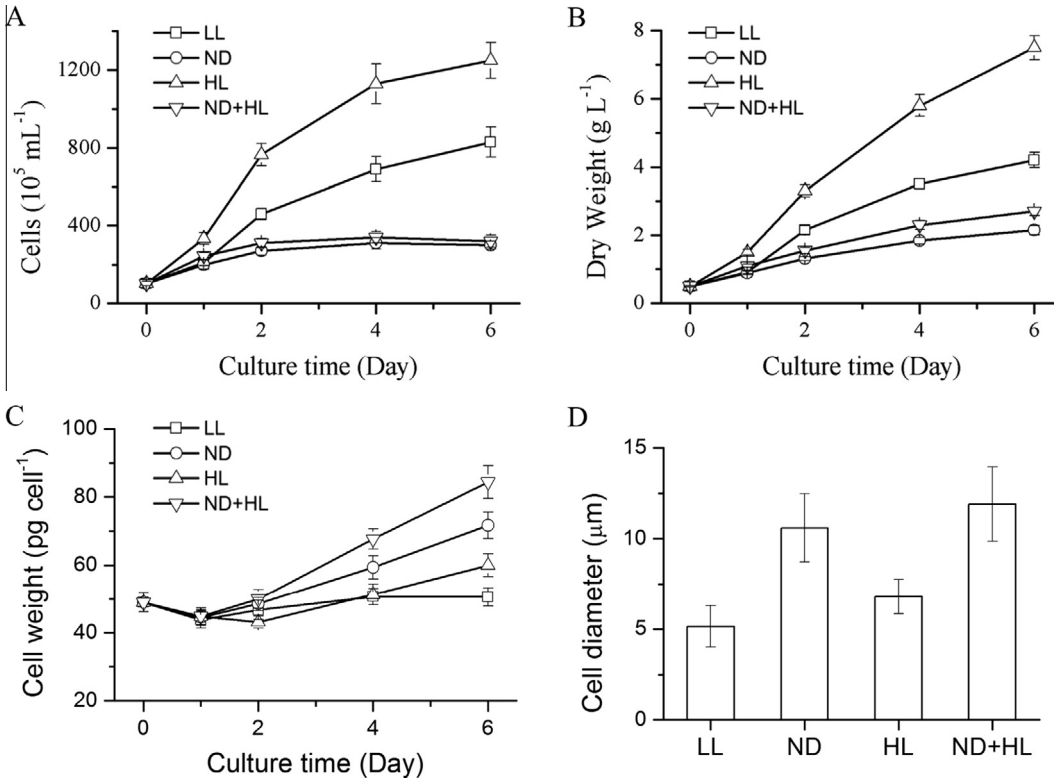

Fig. 1. Growth profiles of *C. zofingiensis* under different stress conditions. A, B, and C, time course of cell number, dry weight and cell weight. D, cell diameter on day 6.

contributed largely by the augmentation in cell size (Fig. 1D) and concurrent accumulation of intracellular compounds such as lipids as well (Fig. 2A).

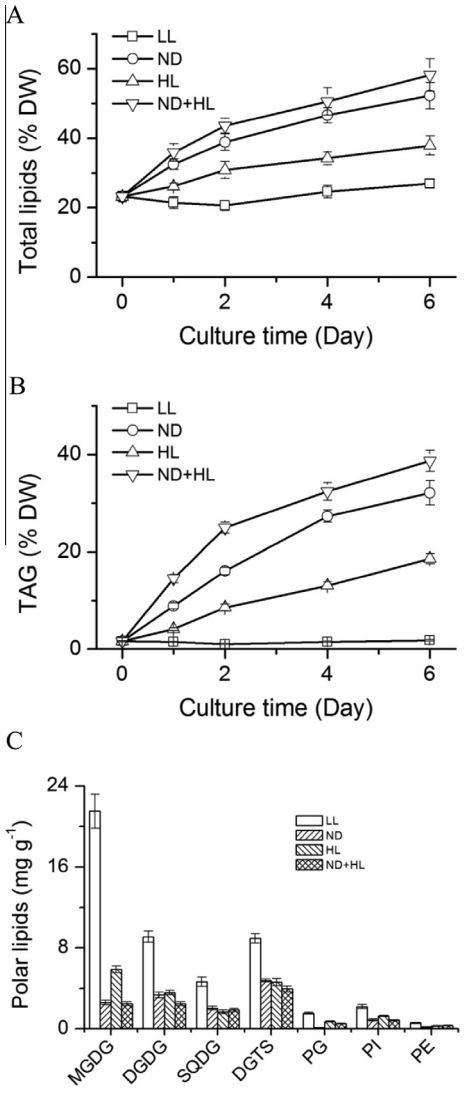

Fig. 2. Lipid profiles of *C. zofingiensis* under different stress conditions. A and B, time course of total lipids (TL) and TAG. C, the contents of polar lipids on day 6.

3.2. Lipid profiles as affected by different culture conditions

The total lipid content in *C. zofingiensis* responded differentially to culture conditions (Fig. 2A). Under LL conditions, *C. zofingiensis* maintained a relatively stable and basal level of lipids during the whole culture period. HL stimulated the accumulation of lipids moderately. In contrast, ND induced a considerable increase in total lipid content, which was 100% and 38% higher than that under LL and HL conditions (day 6), respectively, indicative of ND being more effective than HL for lipid induction. The combination of ND + HL led to the accumulation of more lipids, reaching up to 58% of dry weight at the end of culture period. Triacylglycerol (TAG), the main neutral lipid and ideal precursor for making biodiesel, was also monitored in response to different culture conditions and the results were shown in Fig. 2B. Under LL conditions, *C. zofingiensis* synthesized only a trace amount of TAGs (<2% of dry weight), which was augmented greatly by the stress conditions (HL, ND, and ND + HL) which reached up to 18.6%, 32.1%, and 38.7% of dry weight, respectively. Microalgae tend to accumulate neutral lipids, TAG in particular, with a concomitant decrease in membrane polar lipids (Liu et al., 2016). *C. zofingiensis* contained mainly monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), sulfoquinovosyl diacylglycerol (SQDG), diacylglycerol-N, N,N-trimethylhomoserine (DGTS), phosphatidylglycerol (PG), phosphatidylinositol (PI), and phosphatidylethanolamine (PE) (Fig. 2C). When subjected to stress conditions, ND + HL in particular, a considerable decrease was observed in all polar lipids. Notably, regardless of stress conditions, glycolipids (MGDG, DGDG, and SQDG) especially MGDG that is present exclusively in chloroplast, underwent the most severe decline. The massive augmentation in TAG accompanied by a severe degradation of glycolipids suggested the contribution of recycling of chloroplast lipids to TAG biosynthesis in *C. zofingiensis*. Nevertheless, taking ND + HL as an example, the decrease in glycolipids (up to 30 mg g^{-1}, Fig. 2C) was much less as compared to the TAG augmentation (over 300 mg g^{-1}, Fig. 2B), indicative of a major contribution of the *de novo* synthesized fatty acids to the biosynthesis of TAG under stress conditions.

The fatty acid profile of both total lipids and TAG was monitored in response to different culture conditions (Table 1). *C. zofingiensis* total lipids contained mainly C18:1, C16:0, C18:2, and C18:3n3, which together accounted for 79% of total fatty acids under LL conditions. When exposed to stress conditions, differential changes occurred in the relative abundance of fatty acids: the major C16 fatty acids including C16:0, C16:2, C16:3, and C16:4 declined to different extents; the C18 fatty acids except C18:1 also declined; C18:1, on the other hand, showed a remarkable increase and

Table 1
Fatty acid profiles of *C. zofingiensis* under different growth conditions.

	Total lipids				TAG			
	LL	ND	HL	ND + HL	LL	ND	HL	ND + HL
C16:0	20.5 ± 1.2	15.4 ± 0.3	19.1 ± 0.7	16.8 ± 0.6	26.0 ± 1.1	16.0 ± 0.5	22.5 ± 0.8	18.0 ± 0.5
C16:1	0.7 ± 0.1	0.9 ± 0.0	0.7 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	0.9 ± 0.1	0.6 ± 0.0	1.1 ± 0.1
C16:2	4.7 ± 0.3	1.7 ± 0.1	2.0 ± 0.1	1.7 ± 0.1	1.8 ± 0.1	1.5 ± 0.1	0.9 ± 0.0	1.0 ± 0.0
C16:3	4.6 ± 0.2	3.8 ± 0.2	3.1 ± 0.2	2.8 ± 0.2	1.2 ± 0.0	2.8 ± 0.1	1.0 ± 0.1	1.1 ± 0.1
C16:4	2.0 ± 0.1	0.4 ± 0.0	1.8 ± 0.1	0.6 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.6 ± 0.0	0.1 ± 0.0
C18:0	5.5 ± 0.3	4.7 ± 0.3	3.9 ± 0.2	4.4 ± 0.3	8.1 ± 0.3	4.1 ± 0.2	3.4 ± 0.1	3.8 ± 0.1
C18:1	28.3 ± 1.6	48.5 ± 2.1	40.1 ± 1.7	51.6 ± 2.3	40.1 ± 2.3	52.5 ± 1.8	51.2 ± 2.9	60.4 ± 2.4
C18:2	16.0 ± 0.5	14.3 ± 0.6	14.2 ± 0.9	12.2 ± 0.3	14.7 ± 0.4	14.5 ± 0.6	12.8 ± 0.4	9.5 ± 0.4
C18:3n6	1.3 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	1.1 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
C18:3n3	14.4 ± 0.4	8.3 ± 0.5	12.0 ± 0.4	7.4 ± 0.2	4.5 ± 0.1	6.0 ± 0.2	5.3 ± 0.3	3.5 ± 0.2
C18:4	1.1 ± 0.1	0.6 ± 0.1	0.9 ± 0.1	0.6 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
SFA	26.0 ± 1.1	20.1 ± 1.1	23.0 ± 1.4	21.3 ± 0.8	34.0 ± 2.1	20.1 ± 0.7	25.9 ± 0.9	21.8 ± 1.1
MUFA	29.9 ± 1.5	50.4 ± 2.8	42.4 ± 1.8	53.1 ± 2.9	42.1 ± 2.3	54.2 ± 2.2	53.1 ± 2.5	62.7 ± 2.8
PUFA	44.0 ± 1.8	29.5 ± 1.0	34.6 ± 1.4	25.6 ± 1.1	23.9 ± 0.9	25.7 ± 1.1	21.0 ± 0.4	15.6 ± 0.5

Cells were cultured for 6 days for the fatty acid analysis; fatty acids were expressed as% of total fatty acid;
SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

reached 51.6% of total fatty acids under ND + HL conditions. This is in good agreement with the stearoyl-acyl carrier protein desaturase gene, which is responsible for the conversion of C18:0 to C18:1 and was previously shown to be up-regulated considerably upon stress conditions leading to the greatly enhanced synthesis of C18:1 relative to other fatty acids in *C. zofingiensis* (Liu et al., 2012a). Like total lipids, TAG consisted mainly of C18:1, C16:0, and C18:2, and C18:1 was the only fatty acid enhanced in response to stress conditions (Table 1). Nevertheless, as compared to the total lipids, TAGs were more enriched with C18:1 that reached 60.4% under ND + HL conditions, indicative of TAG being a major reservoir for C18:1 and the stress-triggered TAG accumulation contributed mainly by the *de novo* synthesized fatty acids. It has been suggested that high percentage of C18:1 can provide a compromise solution between oxidative stability and low-temperature properties and is therefore beneficial to biodiesel quality (Knothe, 2009). In this regard, *C. zofingiensis* oils enriched with C18:1 (over 60%) are ideal feedstocks for making biodiesel.

3.3. Carotenoid accumulation as affected by different culture conditions

Under LL conditions, *C. zofingiensis* synthesized only a trace amount of astaxanthin (below 0.08 mg g^{-1} dry weight); conversely, rapid astaxanthin accumulation was observed under stress conditions (Fig. 3A). ND + HL gave rise to the highest level of astaxanthin during the whole culture period, followed by ND and HL, and the highest astaxanthin content reached under these three stress conditions were 4.9, 3.9, and 1.7 mg g^{-1} dry weight, respectively. Similar to astaxanthin, total secondary carotenoids (including astaxanthin, adonixanthin, canthaxanthin) were present in trace amount under LL conditions and were induced to accumulate by stress conditions, with ND + HL leading to the greatest accumulation (Fig. 3B). Although both astaxanthin and total secondary carotenoids were promoted upon stressors, astaxanthin increased to a greater extent, as evidenced by the rise in the ratio of astaxanthin to secondary carotenoids, which reached up to 80% total secondary carotenoids under ND + HL conditions (Fig. 3C). Notably, astaxanthin, which was present in the free form under LL conditions, was predominantly esterified with one (mono-ester) and two (di-ester) fatty acyls under stress conditions (Fig. 3D). The esterified form of astaxanthin is thought to be more stable than the free form in *C. zofingiensis*, and is more amenable for utilization as a value-added co-product, such as fish feed (Sun et al., 2011).

3.4. Simultaneous production of TAG and astaxanthin

To study the relationship between TAG and astaxanthin, their contents under different culture conditions were plotted. Clearly, TAG and astaxanthin had a strong linear relationship especially under stress conditions, with the R^2 being over 0.97 (Fig. 4), which is similar to a previous report in another astaxanthin-producing microalga *Haematococcus pluvialis* (Chen et al., 2015). This indicates that in *C. zofingiensis* the biosynthesis and accumulation of TAG and astaxanthin may be regulated in a somewhat coordinated manner to cope with the abiotic stresses, and also raises the possibility of employing this alga for the simultaneous production of oils and the high-value compound astaxanthin.

As illustrated by Fig. 5, both TAG and astaxanthin yields were marginal with LL, because they were synthesized only in a trace amount under this growth condition (Figs. 2B and 3C). Stressors benefited the accumulation of TAG and astaxanthin thus promoting the production of these two compounds. It is worth noting that although ND and ND + HL conditions gave rise to much higher contents of TAG and astaxanthin than HL, their yields were

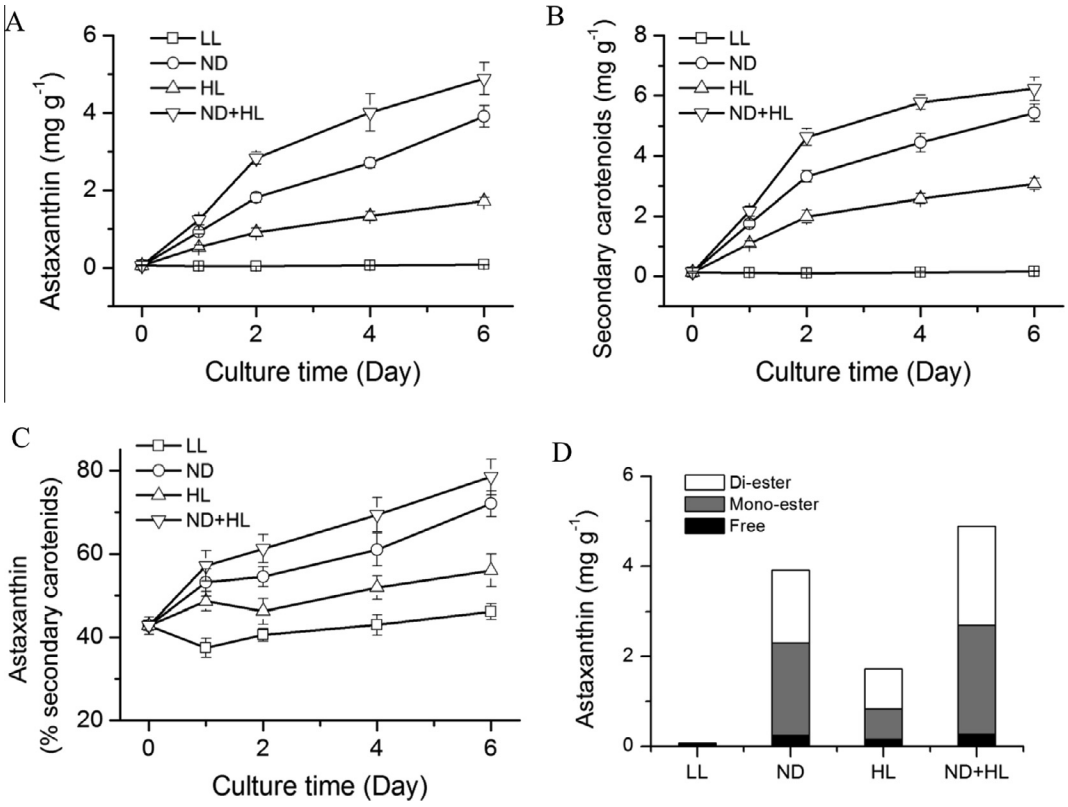

Fig. 3. Carotenoid profiles of *C. zofingiensis* under different stress conditions. A, B, and C, time course of astaxanthin, secondary carotenoids and the relative abundance of astaxanthin per secondary carotenoids. D, the distribution of astaxanthin in the forms of free, mono-ester, and di-ester.

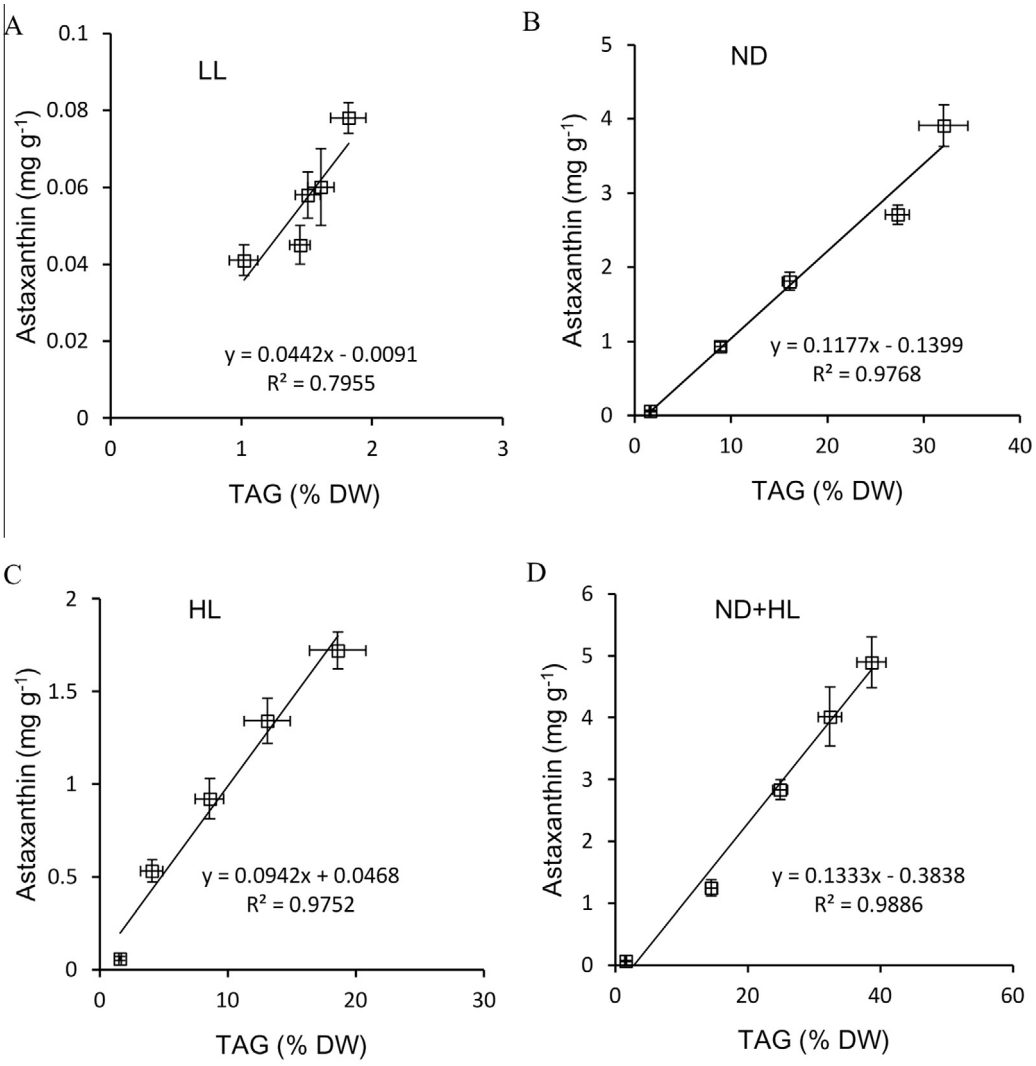

Fig. 4. Linear relationship between astaxanthin and TAG under different culture conditions of LL (A), ND (B), HL (C), and ND + HL (D).

compromised by the severely impaired biomass production (Fig. 5 and Table 2). Consequently, the highest productivities of TAG and astaxanthin were achieved under HL conditions, which were 174 mg L^{-1} day^{-1} and 2 mg L^{-1} day^{-1}, respectively.

3.5. Enhanced production of TAG and astaxanthin by semi-continuous culture with nitrogen limitation

Nitrogen deprivation was able to enhance the intracellular contents of TAG and astaxanthin but at the expense of biomass production. This conflict may be partially addressed by the employment of a nitrogen limitation strategy couple with a semi-continuous culture system. Nitrogen limitation provides a compromise solution to cell growth and accumulation of storage compounds (Griffiths et al., 2014), while semi-continuous culture allows the relatively stable production (Liu et al., 2012b).

Three nitrogen concentrations of 5, 10, and 20 mg L^{-1} were first tested in a semi-continuous culture with a dilution rate of 0.5 day^{-1}. Clearly, the higher the nitrogen concentration, the higher the biomass productivity but the lower the TAG and astaxanthin contents; the nitrogen concentration of 10 mg L^{-1} gave rise to the highest productivities of TAG (297 mg L^{-1} day^{-1}) and astaxanthin (3.3 mg L^{-1} day^{-1}) (Table 3). With this nitrogen concentration, the intracellular contents of TAG and astaxanthin were also maintained at a relatively high level, which were 286 and 3.2 mg g^{-1}, respectively. Next, the effect of different dilution rates on productivities were studied, with the nitrogen concentration fixed at 10 mg L^{-1}. Three dilutions rates, namely, 0.25, 0.5 and 0.75 day^{-1}, were evaluated. The contents of TAG and astaxanthin decreased as the dilution rate increased, accompanied by a rise in biomass productivity. While for the productivities of TAG and astaxanthin, they showed no difference between the dilution rate of 0.5 and 0.75 day^{-1}, which were higher than that with the dilution rate of 0.25 day^{-1}. In view of the fact that higher dilution rate leads to more usage of medium input, the dilution rate of 0.5 day^{-1} is superior to 0.75 day^{-1} for the production of TAG and astaxanthin by *C. zofingiensis*.

3.6. Inhibition of de novo fatty acid biosynthesis by cerulenin promotes astaxanthin accumulation

The fatty acid biosynthesis inhibitor cerulenin, which specifically targets β-ketoacyl-ACP synthase I (KAS I) and blocks the *de novo* fatty acid synthesis (Liu et al., 2016), was employed to study the astaxanthin production by *C. zofingiensis* under ND conditions. The presence of 10 μM cerulenin led to a drastic decrease in TAG

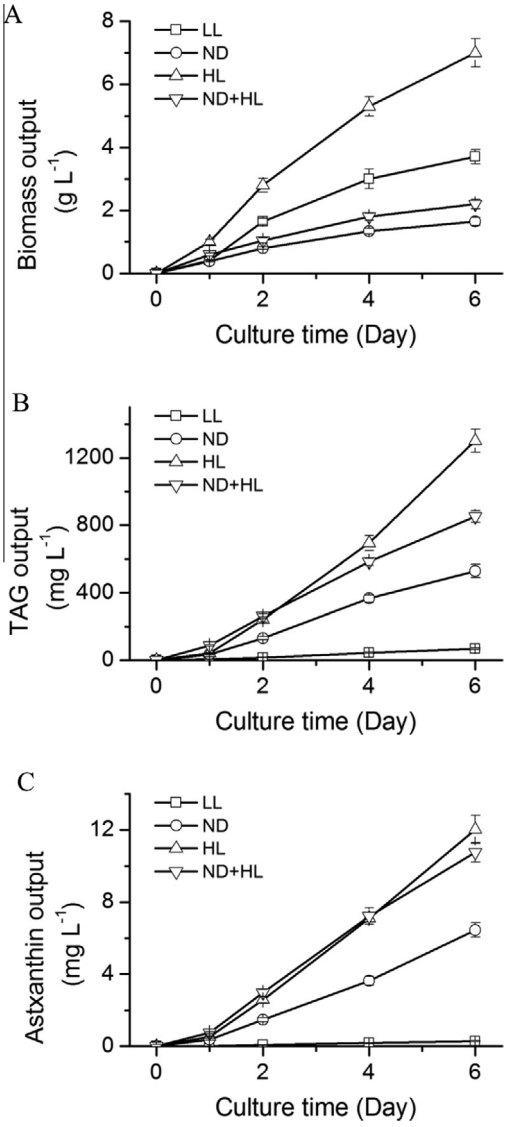

Fig. 5. Output of biomass (A), TAG (B), and astaxanthin (C) under different culture conditions.

Table 2
The production of TAG and astaxanthin by *C. zofingiensis* under different conditions.

	Maximum productivities		
	Biomass ($g\ L^{-1}\ day^{-1}$)	TAG ($mg\ L^{-1}\ day^{-1}$)	Astaxanthin ($mg\ L^{-1}\ day^{-1}$)
LL	0.83 ± 0.05^{a}	11.3 ± 0.7^{a}	0.05 ± 0.01^{a}
ND	0.41 ± 0.02^{b}	91.5 ± 5.5^{b}	1.08 ± 0.06^{b}
HL	1.40 ± 0.09^{c}	173.6 ± 11.2^{c}	2.01 ± 0.14^{c}
ND + HL	0.53 ± 0.03^{d}	145.8 ± 9.7^{d}	1.79 ± 0.17^{c}

Values in each column followed by different letters are significantly different (*t*-test, $P < 0.05$).

content, which persisted during the whole culture period (Fig. S1A). Intriguingly, a considerable increase in astaxanthin content was observed (Fig. S1B), reaching by up to 40% quantitatively. In contrast to the increased astaxanthin, other carotenoids declined leading to a considerable increase in the ratio of astaxanthin to carotenoids or astaxanthin 'purity' (data not shown). These results provided alternative approaches to enhanced astaxanthin production and purity in *C. zofingiensis* by inhibiting its competing pathways. TAG and astaxanthin biosyntheses share the common precursors acetyl-CoAs. Cerulenin blocked the fatty acid biosynthesis leading to the buildup of acetyl-CoAs, which could be reallocated to carotenoid biosynthetic pathway for enhanced astaxanthin accumulation. Besides, severe fatty acid reduction may cause the rise of NADPH relative to $NADP^+$, as NADPH is the main reducing power for fatty acid synthesis. Thus, molecular oxygen serves as the electron acceptor to offset insufficient $NADP^+$ supply resulting in the generation of excess reactive oxygen species (ROS) (Liu et al., 2012a). Consistent with our hypothesis, cerulenin treatment promoted ROS level in *C. zofingiensis* cells (Fig. S1C). The overproduction of ROS, in turn, may trigger the synthesis of more astaxanthin. The underlying mechanism, however, remains to be elucidated. Interestingly, it was reported that cerulenin treatment abolished astaxanthin accumulation in *H. pluvialis* (Zhekisheva et al., 2005; Chen et al., 2015). This may be explained by the fact that these two algae may possess different mechanism in response to cerulenin, which yet needs experimentally support.

3.7. Comparison of lipid and astaxanthin production by *C. zofingiensis* with other algae

The lipid productivity depends on not only algal growth rate but also intracellular lipid content. There have been many reports of using microalgae, *Chlorella* in particular, for lipid production (Table 4). In the present study, high volumetric biomass and lipid productivities of 1.04 and 0.47 $g\ L^{-1}\ day^{-1}$ were achieved in *C. zofingiensis*, respectively, which are higher or comparable to the previous reports. Furthermore, *C. zofingiensis* produced predominantly C16 and C18 fatty acids with C18:1 being the major fatty acid and accounting for up to 52% of total fatty acids (Table 1), thus representing a good biodiesel feedstock as C18:1 is believed to help improve the biodiesel quality (Knothe, 2009).

Although microalgae have long been cited as a promising alternative to oil plants for biodiesel, the commercial production has yet to be achieved owing largely to the relatively high production cost. Thus, the future direction for algal biofuels may lie in the exploration of integrated production of value-added products. *C. zofingiensis* is capable of accumulating astaxanthin, a potent antioxidant component with great global market. The astaxanthin productivity obtained in our study reached 3.3 $g\ L^{-1}\ day^{-1}$, comparable to the previous reports in either *C. zofingiensis* or *H. pluvialis*, though its content was relatively low (Table 5). Metabolic engineering for enhanced astaxanthin biosynthesis may represent one of the future research directions for *C. zofingiensis* and be worth of further investigation. In a word, the promising integrated production of astaxanthin will offer a great advantage to the use of *C. zofingiensis* for biofuel production and *C. zofingiensis* may serve as an emerging production strain and model oleaginous alga.

4. Conclusions

C. zofingiensis was capable of accumulating TAG and astaxanthin in a well-correlated manner under stress conditions of ND, HL, and ND + HL. ND + HL gave the highest contents of TAG and astaxanthin, though their productivities were compromised by the impaired growth as compared to HL. The TAG and astaxanthin productivities were promoted by a semi-continuous culture strategy coupled with nitrogen limitation, reaching 297 and 3.3 $mg\ L^{-1}\ day^{-1}$, respectively. Taken together, our study represents a pioneering work of utilizing microalgae for the integrated production of lipids and high-value products and underscores *C. zofingiensis* great potential to serve as a promising production strain.

Table 3
The production of TAG and astaxanthin by *C. zofingiensis* by semi-continuous culture with nitrogen limitation.

	Content (mg g^{-1})		Productivity (mg L^{-1} day^{-1})		
	TAG	Astaxanthin	Biomass	TAG	Astaxanthin
Nitrogen (mg L^{-1})[1]					
5	314 ± 14[a]	3.4 ± 0.2[a]	790 ± 40[a]	248 ± 13[a]	2.7 ± 0.2[a]
10	286 ± 16[a]	3.2 ± 0.1[a]	1040 ± 60[b]	297 ± 10[b]	3.3 ± 0.2[b]
20	224 ± 18[b]	2.5 ± 0.1[b]	1200 ± 50[c]	269 ± 15[ab]	3.0 ± 0.1[b]
D (day^{-1})[2]					
0.25	308 ± 16[a]	3.3 ± 0.2[a]	690 ± 40[a]	213 ± 10[a]	2.3 ± 0.1[a]
0.5	279 ± 21[ab]	3.2 ± 0.1[a]	980 ± 40[b]	273 ± 13[b]	3.2 ± 0.2[b]
0.75	261 ± 18[b]	2.8 ± 0.1[b]	1070 ± 60[c]	279 ± 18[b]	3.0 ± 0.2[b]

Values in each column within the same experiment followed by different letters are significantly different (*t*-test, $P < 0.05$).
[1] Half of the cultures were replaced once a day, corresponding to a dilution rate (*D*) of 0.5.
[2] The nitrogen content was 10 mg L^{-1}; half of the cultures were replaced every 2, 1, and 0.5 days, corresponding to a dilution rate (*D*) of 0.25, 0.5 and 0.75, respectively.

Table 4
Photoautotrophic lipid production of *C. zofingienesis* in comparison with previously reported *Chlorella* strains.

Algal strain	Culture conditions	Biomass productivity (g L^{-1} day^{-1})	Lipid content (%DW)	Lipid productivity (mg L^{-1} day^{-1})	References
C. zofingiensis	I, 100-mL columns	1.04	45.5	473	This study
C. protothecoides	I, 100-mL columns	0.57	48.3	280	Sun et al. (2015)
	O, 50-L panel PBRs	1.25	50.4	590	
C. protothecoides	I, 200-mL glass tubes	0.41	16.8	69	Sirisansaneeyakul et al. (2011)
C. sp	I, 300-mL glass tubes	0.5	50.8	250	Guccione et al. (2014)
	O, 10-L panel PBRs	0.6	26.6	160	
C. vulgaris	I, 250-mL flasks	0.49	35.4	170	Breuer et al. (2012)
C. vulgaris	I, 50-mL glass tubes	1.05	57.3	604	Pribyl et al. (2012)
	O, 150-L thin layer PBRs	1.25	30.6	330	
C. vulgaris	O, 30-L panel PBRs	0.67	44.6	390	Munkel et al. (2013)
C. zofingiensis	I, 250-mL flasks	0.67	44.8	301	Breuer et al. (2012)

I, indoor; O, outdoor

Table 5
Astaxanthin production of *C. zofingienesis* in comparison with previously reported algal strains.

Strain	Culture conditions	Biomass productivity (g L^{-1} day^{-1})	Astaxanthin content (mg g^{-1})	Astaxanthin productivity (mg L^{-1} d^{-1})	References
C. zofingiensis					
UTEX32	P, semi-continuous	1.04	3.2	3.3	This study
CCAP211/14	P, batch	0.74	3.7	2.8	Del Campo et al. (2004)
ATCC30412	H, batch	1.6	1.18	1.7	Liu et al., 2012b
ATCC30412	H, batch	2.1	1.31	2.3	Liu et al., 2013
	H, fed-batch	4.7	1.19	5.6	
H. pluvialis					
CCAP34/7	P, batch	0.02	27	0.44	Harker et al. (1996)
UTEX16	M, batch	0.13	20.1	2.6	Zhang et al. (2009)
	M, fed-batch	0.14	23.5	3.2	
CCAP34/8	P, batch	0.41	11.0	4.4	López et al. (2006)
CCAP34/8	P, continuos	0.6	8	5.6	Del Rio et al. (2005)
NIES-144	P, fed-batch	0.2	36	7.2	Ranjbar et al. (2008)
CCAP34/8	P, continuous	0.7	10	7	García-Malea et al. (2009)

P, photoautotrophic; H, heterotrophic; M, mixotrophic.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2016.04.112.

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