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Screening and characterization of oleaginous *Chlorella* strains and exploration of photoautotrophic *Chlorella protothecoides* for oil production

Zheng Sun [a], Zhi-gang Zhou [a], Henri Gerken [c], Feng Chen [b], Jin Liu [b,d,*]

[a] College of Fisheries and Life Science, Shanghai Ocean University, Shanghai 201306, PR China
[b] Institute for Food and Bioresource Engineering, College of Engineering, Peking University, Beijing 100871, PR China
[c] Department of Applied Sciences and Mathematics, Arizona State University Polytechnic Campus, Mesa, AZ 85212, USA
[d] Institute of Marine and Environmental Technology, University of Maryland Center for Environmental Science, Baltimore, MD 21202, USA

HIGHLIGHTS

- The growth and oil production of nine *Chlorella* strains were compared.
- Culture conditions were optimized for oil production by *C. protothecoides* CS-41.
- Outdoor oil production by *C. protothecoides* CS-41 was assessed in a panel PBR.
- A two-stage culture strategy was proposed for oil production in PBRs.
- High oil content (55% of dry weight) and productivity (0.59 g L^{-1} d^{-1}) were obtained.

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ABSTRACT

The growth and oil production of nine *Chlorella* strains were comparatively assessed and *Chlorella protothecoides* CS-41 demonstrated the greatest lipid production potential. The effects of different nitrogen forms and concentrations, phosphorus concentrations and light intensities on growth and oil production were studied in laboratory columns. *C. protothecoides* CS-41 accumulated lipids up to 55% of dry weight, with triacylglycerol and oleic acid being 71% of total lipids and 59% of total fatty acids, respectively. High biomass and lipid productivities were achieved in outdoor panel PBRs, up to 1.25 and 0.59 g L^{-1} day^{-1}, or 44. 1 and 16.1 g m^{-2} day^{-1}, respectively. A two-stage cultivation strategy was proposed to enhance the algal biomass and lipid production. This is the first comprehensive investigation of both indoor and outdoor photoautotrophic *C. protothecoides* cultures for oil production, and *C. protothecoides* CS-41 represents a promising biofuel feedstock worthy of further exploration.

1. Introduction

Fossil fuels are the primary global energy sources and are drastically depleting because of ever-increasing energy consumption. Thus, alternative forms of energy that are green, renewable and sustainable are highly sought after. Biodiesel, or fatty acid methyl esters (FAMEs) produced by transesterification of oils, attracts much attention because it is renewable, carbon neutral and portable (Knothe, 2009). Currently, plant oils serve as the main feedstocks for biodiesel production, but biodiesel produced from plant oils cannot realistically replace the petroleum-derived transport fuels in the foreseeable future (Chisti, 2007). Conversely, microalgae, which potentially possess several significant advantages over plants for biodiesel production, are considered the next-generation biodiesel feedstock and have the potential to meet the existing demand for transportation fuels (Chisti, 2007; Wijffels and Barbosa, 2010).

Exploration of microalgae as the feedstock for biodiesel has attracted ever-increasing attention and substantial progress has been achieved during the recent decades (Suali and Sarbatly, 2012). Nevertheless, there are significant challenges yet to be addressed, making the current production of algal biodiesel not economically viable (Richardson et al., 2012). Attempts have been made to lower the production cost of algal biodiesel

* Corresponding author at: Institute of Marine and Environmental Technology, University of Maryland Center for Environmental Science, Baltimore, MD 21202, USA. Tel.: +1 410 234 8864; fax: +1 410 234 8896.
E-mail address: jliu@umces.edu (J. Liu).

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through strain selection, strain improvements, exploration of next-generation culture systems, and development of state-of-the-art downstream processes (Radakovits et al., 2010; Breuer et al., 2012; Singh and Sharma, 2012; Suali and Sarbatly, 2012; Kim et al., 2013). The first step of algal biodiesel production pipeline, strain selection, is of fundamental importance. It is expected that an ideal algal strain for biodiesel production should grow fast with high cell density, accumulate a large quantity of oils and perform well in downstream processes (Wijffels and Barbosa, 2010). Rapid growth brings high biomass productivity and, at the same time, has the potential to reduce the risk of contamination by outcompeting slowly-growing organisms when cultured outdoors. High oil content helps increase the process yield coefficient and lower the cost of downstream extraction and purification steps.

Among the oleaginous microalgae, *Chlorella* is thought to be a promising candidate biodiesel feedstock in that it is able to grow robustly to high cell density and produce a high level of triacylglycerol (TAG), an ideal precursor for making biodiesel (Liu et al., 2010; Yang et al., 2011; Breuer et al., 2012; Pribyl et al., 2012). *Chlorella* has long been cited for food use and is the first genus of microalgae achieving commercial success at large scale (Liu and Hu, 2013). There have been many studies using *Chlorella* strains for biodiesel exploration, under either photoautotrophic, mixotrophic, or heterotrophic conditions (Liu et al., 2011, 2012a; Yang et al., 2011; Breuer et al., 2012; Wang et al., 2013). Heterotrophic growth of *Chlorella* requires organic carbon sources, commonly sugars. But the conversion of organic carbon to biomass is usually below 0.5, and the untransformed carbon is released into the atmosphere in the form of carbon dioxide, leading to a relatively high production cost of heterotrophic algae (Liu et al., 2010; Yang et al., 2011). As such, *Chlorella* fermentation is regarded as less favorable for the low-cost commodity products such as biofuels. Previous studies indicate that the growth and oil content may vary considerably across *Chlorella* strains and culture conditions (Liu et al., 2008, 2011; Yang et al., 2011; Breuer et al., 2012; Pribyl et al., 2012; Wang et al., 2013). Therefore, it is imperative to employ a high performance *Chlorella* strain along with an optimal operational protocol for oil production. In the past decades, many *Chlorella* strains have been tested, but mainly in flasks or small columns under laboratory conditions. Only a limited number of attempts were made outdoors with *Chlorella* for oil production (Feng et al., 2011; Zhou et al., 2013; Pribyl et al., 2012; Munkel et al., 2013; Guccione et al., 2014).

The main objective of the present study is to select a high-performance *Chlorella* strain and optimize several key biological and engineering parameters for enhanced oil production. Nine *Chlorella* strains from three most outstanding oleaginous species of *Chlorella vulgaris*, *Chlorella zofingiensis*, and *Chlorella protothecoides* were employed and comparatively analyzed with respect to their photoautotrophic growth, lipid content, lipid productivity and fatty acid profile. The best strain *C. protothecoides* CS-41, which showed the greatest lipid productivity and highest level of oleic acid, was investigated comprehensively in laboratory glass columns to assess its biomass and lipid production by manipulating several nutritional and environmental factors. The biomass and oil production potential of *C. protothecoides* CS-41 were further evaluated in an outdoor panel photobioreactor (PBR) by optimizing the initial cell density and length of optical path. A two-stage cultivation strategy was proposed to enhance the algal biomass and lipid production. This is the first comprehensive investigation of photoautotrophic *C. protothecoides* for lipid production. The biomass and lipid productivities are higher or comparable to previous reports of *Chlorella* strains, warranting the further exploration of *C. protothecoides* CS-41 for oil production in pilot-scale PBRs or open ponds.

2. Methods

2.1. Chlorella strains and culture conditions

The *Chlorella* strains used in this study include: #1 *C. zofingiensis* (ATCC 30412), #2 *C. zofingiensis* (UTEX B32), #3 *Chlorella pyrenoidosa* (Carolina 15-2070), #4 *C. pyrenoidosa* (Carolina 15-2071), #5 *C. pyrenoidosa* (HKU 03), #6 *C. vulgaris* (HKU 04), #7 *C. vulgaris* (Carolina 15-2075), #8 *C. vulgaris* (CS-42), and #9 *C. protothecoides* (CS-41). #1 is purchased from the American Type Culture Collection (ATCC, Rockville, USA), #2 is from the University of Texas Culture Collection of Algae (UTEX, Austin, USA), #3, 4, and 7 are from Carolina Biological Supply Co. (Burlington, USA), #5 and 6 are from the University of Hong Kong (HKU, Hong Kong, China), and #8 and 9 are from the Commonwealth Scientific and Industrial Research Organization (CSIRO, Hobart, Australia). These algae were maintained at 4 °C on agar slants of the modified BG-11 medium (50 mg L^{-1} of nitrate-N). Briefly, 10 ml of liquid BG-11 was inoculated with cells from slants and the alga was grown aerobically in flasks at 25 °C for 4 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 illumination of 100 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. Strain selection in 100-mL bubble columns

Chlorella cultures were grown at 22 °C in 100-mL modified BG-11 medium in columns (3 cm of inner diameter) aerated with 1.5% CO_2 enriched air and illuminated with continuous light of 100 μE m^{-2} s^{-1}. The cultures were harvested at late exponential growth phase for the screening of growth characteristics and oil production.

C. protothecoides CS-41 showed the highest oil productivity and was therefore selected for further study. The growth and oil production of *C. protothecoides* were investigated in response to different nitrogen sources, nitrate concentrations, phosphorus concentrations, and light intensities. The culture conditions were the same as the above mentioned (N, 50 mg L^{-1}; light intensity, 100 μE m^{-2} s^{-1}) except where otherwise indicated. For the nitrogen source experiment, nitrate, ammonia and urea were tested at a concentration of 50 mg L^{-1} N. For the nitrate concentration experiment, nitrate concentrations were 6.25, 12.5, 25, 50, 100 and 200 mg L^{-1}. For the phosphorus concentration experiment, phosphorus concentrations were 0.89, 1.78, 3.56, 7.13, 14.26 mg L^{-1}. For the light intensity experiment, light intensities were 25, 50, 100, 200, 400, and 800 μE m^{-2} s^{-1}.

The experiment of nitrogen deficiency was also conducted, where the seed cultures were pelleted by centrifugation and resuspended with nitrogen-deplete or -replete medium at a concentration of 50 mg L^{-1}.

2.3. Outdoor cultures in panel photobioreactors

C. protothecoides CS-41 was grown in outdoor panel photobioreactors (PBRs) in Shanghai, China (latitude 31° 14′ N, longitude 121° 29′ E). The reactors are 140-cm high and 120-cm long with an internal width of 3.5 cm. Compressed air was bubbled at the bottom of the reactors through a perforated plastic tube to produce a turbulent flow in the culture suspension. A stainless iron tube with circulation of cooling water was placed in the cultures to prevent the culture temperature from exceeding 30 °C. During the night, the cooling system was turned off and the culture temperature was allowed to equilibrate to ambient. The seed cultures were

maintained in 20-L indoor panel PBRs with continuous illumination of 100 μE m^{-2} s^{-1} to late exponential growth phase and inoculated into outdoor panel PBRs at 6:00 PM (day 0). Cell samples were collected every day at 6:00 PM for analyses. Light intensity was monitored by a quantum sensor (Li-Cor LI-190), which was attached to the surface center of both PBR sides and connected to a Li-Cor LI-1400 data logger.

2.4. Analytical methods

Cell samples were centrifuged at 3800×*g* for 5 min. The pellet was re-suspended in distilled water and filtered through a pre-dried Whatman GF/C filter paper (1.2 μm pore size). The algal cells on the filter paper disks were dried at 100 °C in a vacuum oven until constant weight and were cooled to room temperature in a desiccator before weighting. The supernatant was collected to measure the residual nitrate-N by using a Quickchem 8500 (Lachat, Loveland, Colorado, USA) according to the instructions. The specific growth rate (μ) at the exponential phase was calculated according to the equation $\mu = (\ln X_2 - \ln X_1)/(t_2 - t_1)$, where X_2 and X_1 are the dry cell weight concentration (g L^{-1}) at time t_2 and t_1, respectively.

For biochemical analysis, cell samples were centrifuged as mentioned above, washed 3 times with distilled water, and lyophilized on a DW3 freeze-drier (Heto Dry Winner, Denmark). The residual nitrate-N and phosphate-P in the supernatant were determined as stated in Liu et al. (2013). Protein was determined as described by Meijer and Wijffels (1998). Carbohydrate was determined by the colorimetric method after hydrolysis with 4 M H_2SO_4 (Renaud et al., 1999). Total lipids were analyzed gravimetrically after extraction with chloroform–methanol (2:1) as previously described (Liu et al., 2010).

Total lipid extracts were fractionated into neutral lipids (NLs), glycolipids (GLs), and phospholipids (PLs) on silica cartridges (Waters, Milford, MA, USA) by sequential elution with chloroform, acetone, and methanol, as previously described (Liu et al., 2010). Neutral lipids were further resolved to subclasses by TLC (silica gel 60, 20 × 20 cm plates, 0.25 mm thickness; Merck, Whitehouse Station, NJ, USA) using a solvent system of petroleum ether: diethyl ether: acetic acid (70:30:1, by vol.). Lipids were visualized by brief exposure to 2,7-dichlorofluorescein (Sigma, St. Louis, MO, USA) vapors and were identified by comparison with the standards (Sigma).

Fatty acid methyl esters (FAMEs) were prepared by direct transmethylation of samples with sulfuric acid in methanol (Liu et al., 2010). The FAMEs were analyzed by using a HP 6890 capillary gas chromatograph (Hewlett–Packard, Palo Alto, CA) equipped with a flame ionization detector (FID) and a HP-INNOwax capillary column (30 m × 0.32 mm) (Agilent Technologies, Inc., Wilmington, DE). Nitrogen was used as carrier gas. Initial column temperature was set at 170 °C, which was subsequently raised to 230 °C at 1 °C/min. The injector was kept as 250 °C with an injection volume of 2 μL under splitless mode. The FID temperature was set at 270 °C. FAMEs were identified by chromatographic comparison with authentic standards (Sigma). The quantities of individual FAMEs were estimated from the peak areas on the chromatogram using heptadecanoic acid as the internal standard.

The biodiesel properties including kinematic viscosity, specific gravity, cloud point, cetane number, iodine value, and higher heating value were predicated based on the FAME composition using the equations described by Hoekman et al. (2012).

2.5. RNA isolation and RT-PCR assay

RNA was isolated from aliquots of about 10^8 cells using the TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. The concentration of total RNA was determined spectrophotometrically at 260 nm. Total RNA (1 μg) extracted from different samples was reverse transcribed to cDNA by using a SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) for reverse transcription PCR (RT-PCR) primed with oligo(dT) according to the manufacturer's instructions. PCR amplification was carried out according to Liu et al., 2012b using the degenerate primers of *BC* (forward, 5′-GGTGGTGGCGGCMGNGGTATG-3′, and reverse, 5′-GTGTT CATYTCCATGAARTARAA-3′) and *SAD* (forward, 5′-GGTGATAT GATHACTGARGARGC-3′, and reverse, 5′-GCATGCCKTGCTGTRTT NCCRTG-3′). *Chlorella* actin (*ACT*) primers (forward, 5′-TGCC GAGCGTGAAATTGTGA-3′, and reverse, 5′-CGTGAATGCCAG CAGCCTCCA-3′) were used to demonstrate equal amounts of templates and loading. Amplification of the cDNA was done by conventional PCR [94 °C for 2 min followed by 22 cycles (for *ACT* gene) or 26 cycles (for *BC* and *SAD* genes) of 94 °C for 15 s, 58 °C for 20 s, 72 °C for 30 s]. PCR products were separated on a 2% agarose gel and stained with ethidium bromide for photography (Bio-Rad, Hercules, CA, USA).

2.6. Statistical analyses

All experiments were determined in biological triplicate to ensure the reproducibility. Experimental results were obtained as the mean value ± SD. Statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA). Paired-samples *T*-test was applied. The statistical significances were achieved when $p < 0.05$.

3. Results and discussion

3.1. Comparative analyses of growth, lipids, and fatty acids of nine Chlorella strains

Nine *Chlorella* strains within three species, namely, *C. vulgaris*, *C. zofingiensis*, and *C. protothecoides*, were used for the screening of high-performance oleaginous organisms. A comparative analysis with respect to biomass productivity, lipid content and lipid productivity was conducted and the results are shown in Fig. 1. Under the tested conditions, biomass productivity ranged from 0.41 to 0.58 g L^{-1} day^{-1}, indicating that the growth potential of *Chlorella* is species/strain dependent. *C. protothecoides* CS-41 (#9) and *C. pyrenoidosa* (#3) were the fastest growing strains, with the biomass productivity being 0.55 and 0.58 g L^{-1} day^{-1}, respectively (Fig. 1A). The lipid content of *Chlorella* is also species/strain dependent with the range of 36–49% of dry weight, and *C. protothecoides* CS-41 accumulated the highest level of lipids (Fig. 1B). *C. protothecoides* CS-41 also had the highest lipid productivity (Fig. 1C; 0.28 g L^{-1} day^{-1}).

The fatty acid profiles of *Chlorella* strains were investigated and compared, since the composition and structure of fatty acid esters determines important properties of biodiesel, such as cetane number, viscosity, cold flow and oxidative stability (Knothe, 2009). Fatty acids are either in saturated or unsaturated form, and unsaturated fatty acids can be classified as mono-unsaturated (MUFAs) and poly-unsaturated (PUFAs). As shown in Table 1, the fatty acid composition among *Chlorella* strains was very similar, consisting mainly of C16:0, C16:1, C16:2, C16:3, C18:0, C18:1, C18:2 and C18:3, but the level of individual fatty acids varied greatly, e.g., C16:0 ranging from 14.9% to 36.6% of total fatty acids and C18:1 from 17.7% to 51.3% of total fatty acids. In general, saturated fatty esters have high cetane number and thus improve the oxidative stability of the biodiesel, whereas unsaturated fatty esters have better low-temperature properties. It has been suggested that C18:1 ester may act as a balance between oxidative stability and

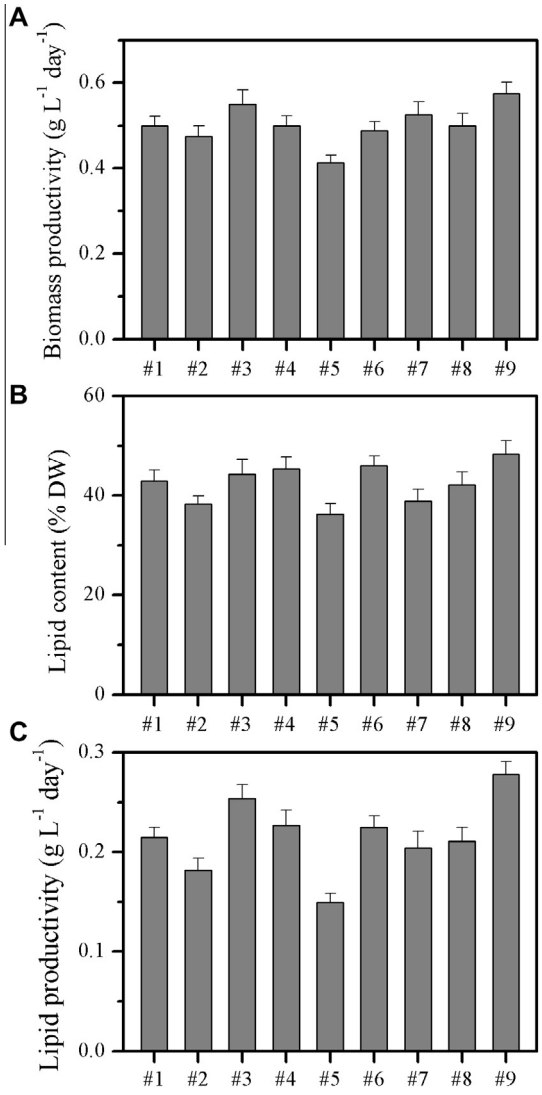

Fig. 1. Biomass productivity (A), lipid content (B), and lipid productivity (C) of the tested 9 *Chlorella* strains. Cells harvested in the late stationary growth phase were used for analysis. See Section 2 for the detailed description of *Chlorella* strains.

low-temperature properties, and the proportion of C18:1 is considered an important index to assess the biodiesel quality of microalgal oils (Knothe, 2009). In this regard, *C. prototheocoides* CS-41 and *C. pyrenoidosa* (#5) contained the highest level of C18:1 (51.3% and 50.1% of total fatty acids, respectively), and are therefore superior to other *Chlorella* strains. The predicted biodiesel properties of *Chlorella* oils including kinematic viscosity, specific gravity, cloud point, cetane number, iodine value, and higher heating value were shown in Table 2. In general, the oils from all nine *Chlorella* strains meet the specification established by both US (ASTM D6751) and Europe (EN 14214) standards. Taken together, *C. prototheocoides* CS-41 demonstrated the greatest potential for biodiesel production and was thus selected for further investigation.

3.2. Effect of nitrogen sources and concentrations on growth and lipid production of *C. prototheocoides* CS-41

Chlorella species are capable of utilizing a broad range of nitrogen sources and nitrate, urea, and ammonia are three common forms used for algal cultivation. As indicated by Fig. 2A, when fed with nitrate and urea, *C. prototheocoides* CS-41 showed almost identical growth pattern and substantial biomass was produced. On the other hand, ammonia could only sustain the cells for 4 days, less than half of the whole cultivation period. The maximum biomass obtained was only 0.8 g L^{-1} which is much lower than what was obtained with nitrate or urea (4.5 g L^{-1}), indicating that ammonia was not suitable for the growth of *C. prototheocoides* CS-41 under our culture conditions. This may be explained by the acidification of the culture medium resulting from the consumption of ammonia (Fig. 2B). Similar observations were reported in several previous studies, in which ammonia consumption led to a drastic decrease in the pH of unbuffered culture medium and thus greatly impaired biomass production (Liu and Hu, 2013; Liu et al., 2013). In addition to growth, the biochemical composition (lipids, carbohydrates, and proteins) of algal cells fed with nitrate or urea was comparatively analyzed. Algal samples were collected in logarithmic phase, early stationary phase, and late stationary phase. No significant difference in composition was observed in any of the three growth phases for cells grown on nitrate compared to urea (Fig. 2C).

Nitrogen is an essential nutrient for algal growth and can greatly influence oil production. In general, low concentrations of nitrogen favor the accumulation of lipids and total fatty acids, but at the same time limit the algal growth. Therefore, the nitrogen concentration needs to be optimized to induce lipid accumulation while maintaining algal growth for maximum production of lipids. Six concentrations of nitrate-N, 6.25, 12.5, 25, 50, 100 and 200 mg L^{-1} N, were tested. As shown in Fig. 2D, *C. prototheocoides* CS-41 had almost the same cell density under all tested N

Table 1
Fatty acid profiles of 9 *Chlorella* strains in stationary growth phase.

Fatty acids [a]	#1	#2	#3	#4	#5	#6	#7	#8	#9
C14:0	–	–	2.8 ± 0.1	2.9 ± 0.2	–	3.0 ± 0.1	2.9 ± 0.2	2.6 ± 0.2	–
C16:0	21.6 ± 1.2	23.4 ± 1.1	35.7 ± 1.6	36.6 ± 1.6	15.8 ± 0.6	36.2 ± 1.6	31.3 ± 1.5	35.3 ± 1.9	14.9 ± 0.7
C16:1	2.1 ± 0.1	1.9 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	3.1 ± 0.2	1.7 ± 0.1	1.3 ± 0.1	1.8 ± 0.2	2.3 ± 0.1
C16:2	7.4 ± 0.4	6.5 ± 0.2	0.6 ± 0.0	0.6 ± 0.0	6.4 ± 0.3	1.1 ± 0.0	0.8 ± 0.0	1.0 ± 0.1	5.1 ± 0.3
C16:3	1.9 ± 0.2	2.0 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	2.5 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.3 ± 0.1	4.3 ± 0.2
C18:0	1.6 ± 0.1	2.0 ± 0.2	5.0 ± 0.3	3.9 ± 0.3	2.0 ± 0.1	3.7 ± 0.2	8.5 ± 0.3	4.2 ± 0.2	3.6 ± 0.2
C18:1	38.2 ± 2.0	35.7 ± 1.9	21.2 ± 1.2	19.9 ± 1.2	50.1 ± 2.8	19.0 ± 1.1	17.7 ± 0.9	18.3 ± 1.2	51.3 ± 2.4
C18:2	19.7 ± 1.1	21.4 ± 1.0	14.9 ± 0.6	13.5 ± 0.5	14.8 ± 0.8	14.7 ± 0.8	16.7 ± 0.6	15.9 ± 0.8	10.2 ± 0.4
C18:3	7.6 ± 0.3	7.1 ± 0.4	17.2 ± 0.9	19.7 ± 1.1	5.2 ± 0.2	19.5 ± 0.9	19.8 ± 1.1	19.6 ± 0.7	8.1 ± 0.6
MUFA[b]	40.3 ± 1.8	37.5 ± 1.2	22.9 ± 1.2	21.6 ± 0.9	53.3 ± 2.4	20.7 ± 1.2	19.0 ± 1.0	20.1 ± 1.3	53.6 ± 2.4
PUFA[c]	36.5 ± 1.7	37.0 ± 1.7	33.7 ± 1.5	35.1 ± 1.5	28.9 ± 1.6	36.4 ± 1.7	38.2 ± 1.5	37.8 ± 2.2	27.8 ± 1.4
UFA[d]	76.8 ± 3.6	74.5 ± 3.0	56.5 ± 2.8	56.7 ± 2.6	82.2 ± 3.9	57.1 ± 2.8	57.2 ± 2.7	57.9 ± 3.4	81.4 ± 3.8
DUS[e]	1.20 ± 0.05	1.21 ± 0.04	1.08 ± 0.06	1.13 ± 0.04	1.19 ± 0.07	1.14 ± 0.05	1.16 ± 0.03	1.17 ± 0.06	1.22 ± 0.05

[a] Data were expressed as percentage of total fatty acids (%); –, under detectable level.
[b] MUFA, monounsaturated fatty acids.
[c] PUFA, polyunsaturated fatty acids.
[d] UFA, unsaturated fatty acid.
[e] DUS (▽/mol), the degree of fatty acid unsaturation = [1.0 (% monoenes) + 2.0 (% dienes) + 3.0 (% trienes) + 4.0 (% tetraenes)]/100.

Table 2
The predicted properties of biodiesel from the oils of nine *Chlorella* strains.

Properties	#1	#2	#3	#4	#5	#6	#7	#8	#9	Biodiesel	ASTM D6751	EN14214
Kinematic viscosity 40 °C ($mm^2\ s^{-1}$)	4.43	4.44	4.52	4.50	4.46	4.49	4.47	4.47	4.44	4–5	1.9–6.0	3.5–5.0
Specific gravity ($kg\ L^{-1}$)	0.879	0.879	0.879	0.879	0.879	0.879	0.879	0.879	0.879	0.87–0.89	0.85–0.90	
Cloud point (°C)	3.60	3.87	5.52	4.95	4.13	4.76	4.48	4.42	3.74			
Cetane number	54.69	54.83	55.65	55.36	54.95	55.27	55.13	55.10	54.76	44–55	Min 47	Min 51
Iodine value ($g\ I_2/100\ g$)	103.97	102.48	93.33	96.49	101.07	97.51	99.10	99.43	103.24			Max 120
Higher heating value (MJ/kg)	40.69	40.66	40.44	40.52	40.63	40.54	40.58	40.59	40.68	38–41		

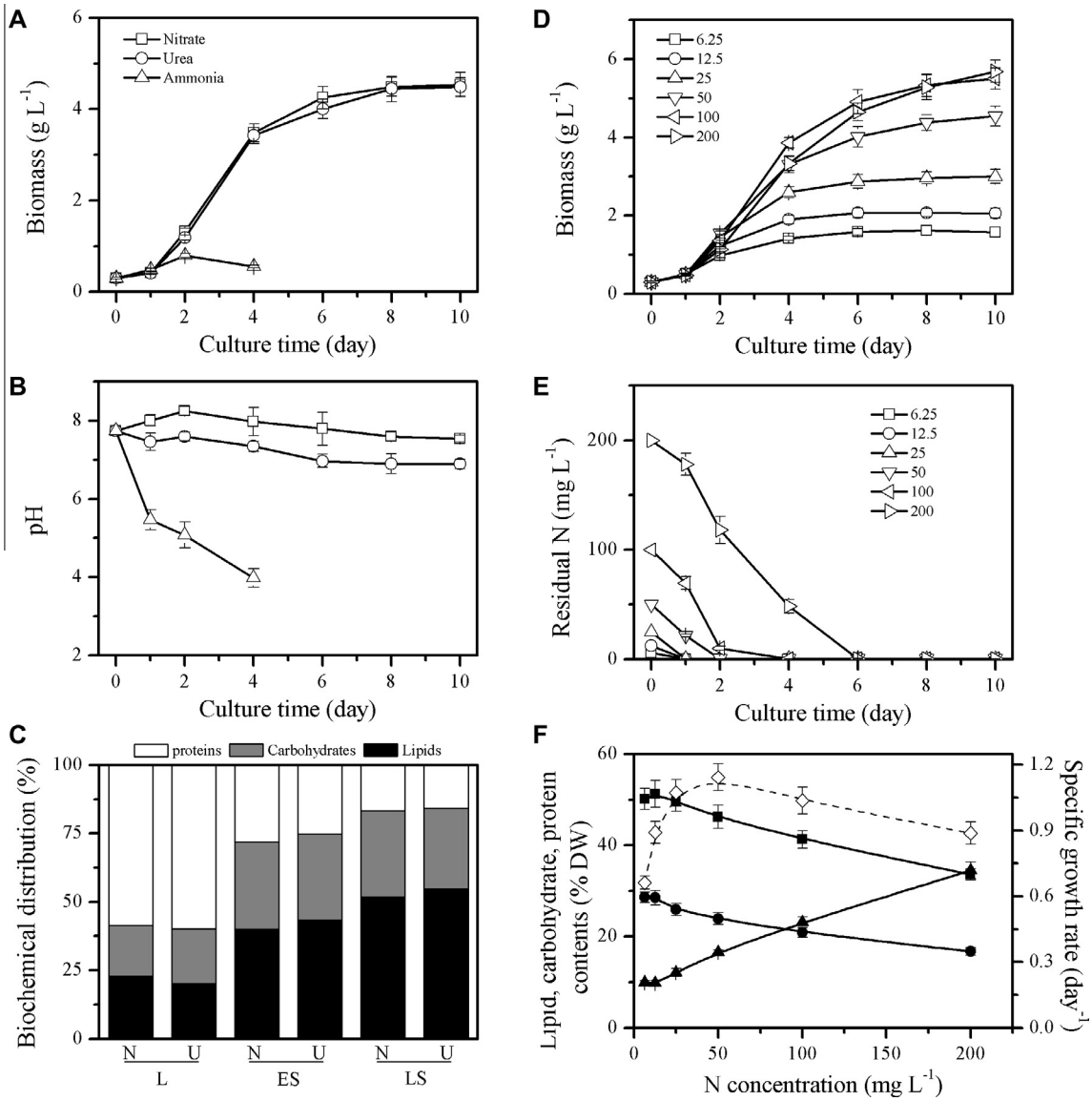

Fig. 2. Effect of nitrogen sources and concentrations on growth and oil production of *C. prototheocoides* CS-41. Biomass (A), pH (B), and biochemical composition (C) of *C. prototheocoides* as affected by nitrogen sources. N, nitrate; U, urea; L, logarithmic phase; ES, early stationary phase; LS, late stationary phase. Biochemical composition was normalized to the sum of proteins, lipids and carbohydrates. Biomass (D), nitrogen consumption (E), and biochemical composition (F) of *C. prototheocoides* CS-41 as affected by various nitrogen concentrations of 6.25, 12.5, 25, 50, 100 and 200 mg L^{-1}. (■), Lipid; (●), carbohydrate; (▲), protein; (◇), specific growth rate. The lipid, carbohydrate, and protein were determined using 10-day cells.

concentrations in the first two days. Thereafter, the production of biomass was positively related to the initial N concentrations, and the highest yield reached up to 5.7 g L^{-1}. On the other hand, the maximum N consumption rate appeared almost identical under all the tested N concentrations (Fig. 2E). The algal biochemical composition was also examined and the data were shown in Fig. 2F. Clearly, low N concentration benefited the accumulation of storage compounds such as lipids and carbohydrates, while high N concentration favored the protein biosynthesis. From a lipid productivity point of view, the N concentration between 50 and 100 mg L^{-1} provided a compromise between algal growth and lipid accumulation and gave rise to the highest lipid productivity.

3.3. Nitrogen deficiency enhanced accumulation of TAG and oleic acid

Triacylglycerol (TAG), the most energy dense lipid, is believed to be superior to other lipids for biodiesel production. TAG accumulation in algae is usually induced by stress conditions, of which N deficiency is the best characterized. Upon N deficiency, *C. prototh-ecoides* CS-41 produced substantial amounts of lipids, which reached up to 55% of dry weight and was 77% higher than that under N replete conditions (Fig. 3A). Under N deficient conditions, TAG was the dominant lipid species and accounted for up to 71% of total lipids or 39% of dry weight; in contrast, the TAG content was only 21% of total lipids or 6% of dry weight under N replete conditions (Fig. 3B). Similar to TAG, oleic acid (C18:1) was the major fatty acid produced under N deficient conditions, which represented 59% of total fatty acids after 6-day cultivation, much higher than that under N replete conditions (28%) (Fig. 3C).

In microalgae, TAG appears to be stored in lipid bodies located in cytoplasm. It may be synthesized either from acyl-CoA dependent Kennedy pathway or acyl-CoA independent pathway that utilizes membrane polar lipids as the acyl donor (Liu and Benning, 2013). We examined the lipid profiles of *C. prototheecoides* CS-41 and found that membrane lipids, glycolipids in particular, underwent significant reduction upon N deficiency (data not shown). But the amount of decreased membrane lipids was only a small portion when compared to the increased TAG, suggesting that N deficiency induced TAG may be mainly from the acyl-CoA dependent pathway. When examining the expression of fatty acid biosynthesis genes, biotin carboxylase (BC) and stearoyl-acyl carrier protein desaturase (SAD), we observed a drastic upregulation of the two genes upon N deficiency (Fig. 3D). BC is a subunit of acetyl-CoA carboxylase (ACCase) which catalyzes the first committed step of de novo fatty acid synthesis, while SAD adds the first double bond to acyl chain to form C18:1 and determines the degree of unsaturation of fatty acids (Liu et al., 2012b). The marked upregulation of the two genes was well coordinated with the increase in total fatty acids and C18:1 (Fig. 3), indicating the possible transcriptional control of fatty acid biosynthesis. Considering that the synthesized fatty acids were incorporated mainly into TAG, the genes coding for enzymes involved in TAG assembly, e.g., acyl-CoA: diacylglycerol acyltransferase (DGAT) and phospholipid: diacylglycerol acyltransferase (PDAT), might be upregulated in *C. prototheecoides* CS-41 under N deficient conditions. This phenomenon has been reported in other microalgae including *Chlamydomonas reinhardtii* (Blaby et al., 2013) and *Nannochloropsis oceanica* (Li et al., 2014). Better understanding of the molecular regulation of lipid metabolism in *C. prototheecoides* CS-41 is yet to be achieved and will facilitate the metabolic engineering of the alga for oil production.

3.4. Effect of phosphorus concentrations on growth and lipid production of C. prototheecoides CS-41

P concentrations had effects similar to N concentrations on the growth of *C. prototheecoides* CS-41: the higher the P concentration, the greater the biomass produced at the end of culture period (Fig. 4A). But the impact of P concentration on growth was much less profound as compared to that of N concentration (Fig. 2D). Maximal cell density was achieved at the concentration of 7.13 mg L^{-1} P and the increase of P concentration to 14.26 mg L^{-1}

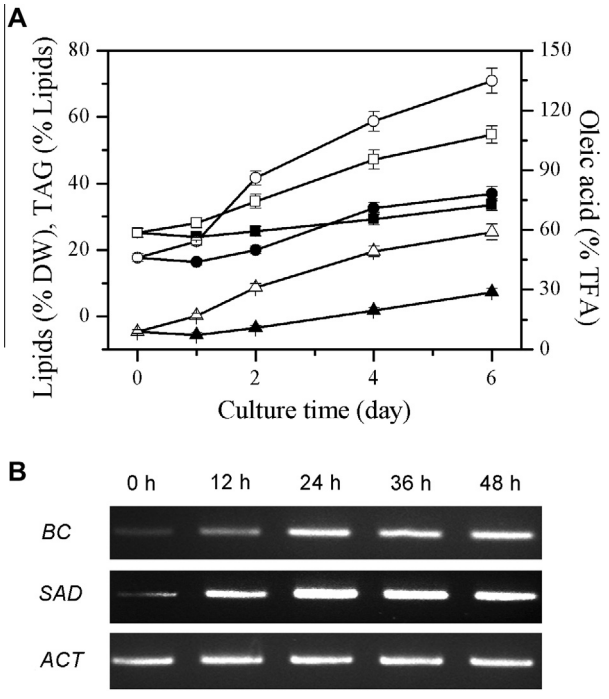

Fig. 3. Time course of lipids (square), TAG (circle), oleic acid (up triangle) (A) and expression of *BC* and *SAD* genes (B) induced by nitrogen deficiency. (open symbols), nitrogen deficient; (filled symbols), nitrogen replete. BC, biotin carboxylase; SAD, stearyl-ACP desaturase; ACT, actin.

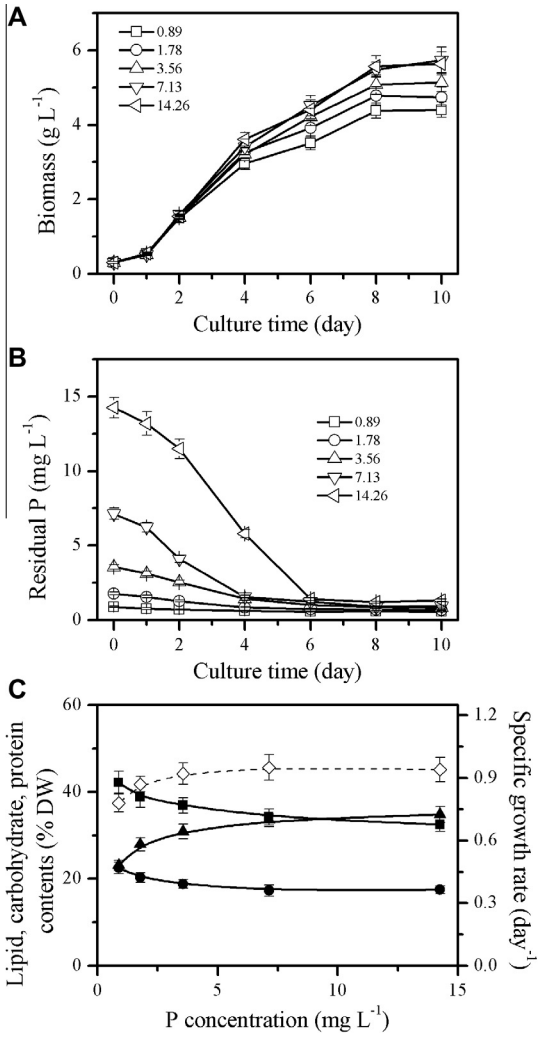

Fig. 4. Biomass (A), phosphorus consumption (B), and biochemical composition (C) of *C. prototheecoides* CS-41 as affected by various P concentrations of 0.89, 1.78, 3.56, 7.13, 14.26 mg L^{-1}. (■), Lipid; (●), carbohydrate; (▲), protein; (◇), specific growth rate. The lipid, carbohydrate, and protein were determined using 10-day cells.

did not further enhance cell density (Fig. 4A), indicating that 7.13 mg L^{-1} P was the optimal concentration for algal growth under the tested culture conditions. There was an obvious luxury consumption of P observed: the cells in the cultures started with 14.26 mg L^{-1} P consumed 13 times more P than that in the cultures containing 0.78 mg L^{-1} P in the first 2 days (Fig. 4B). The algal biochemical composition was also affected by the initial P concentrations, though less prominent as compared to N concentrations (Fig. 4C). Within the tested P concentrations, 3.56–7.13 mg L^{-1} was optimal for the lipid production by *C. protothecoides* CS-41.

3.5. Effect of light intensities on growth and lipid production of C. protothecoides CS-41

Aside from medium nutrients, environmental factors play key roles in algal growth and lipid profile, and we focused on light intensity in this study. As illustrated in Fig. 5, when the intensity increased from 25 to 200 μE m^{-2} s^{-1}, a typical light-dependent growth response was observed, leading to the maximum specific growth rate and final cell density. No increase in biomass was achieved when the light intensity rose to 400 μE m^{-2} s^{-1}, indicating light saturation for photosynthesis by the algal cells. Further increase in light intensity to 800 μE m^{-2} s^{-1}, which greatly exceeds the saturation value, resulted in photoinhibition and thus a decline

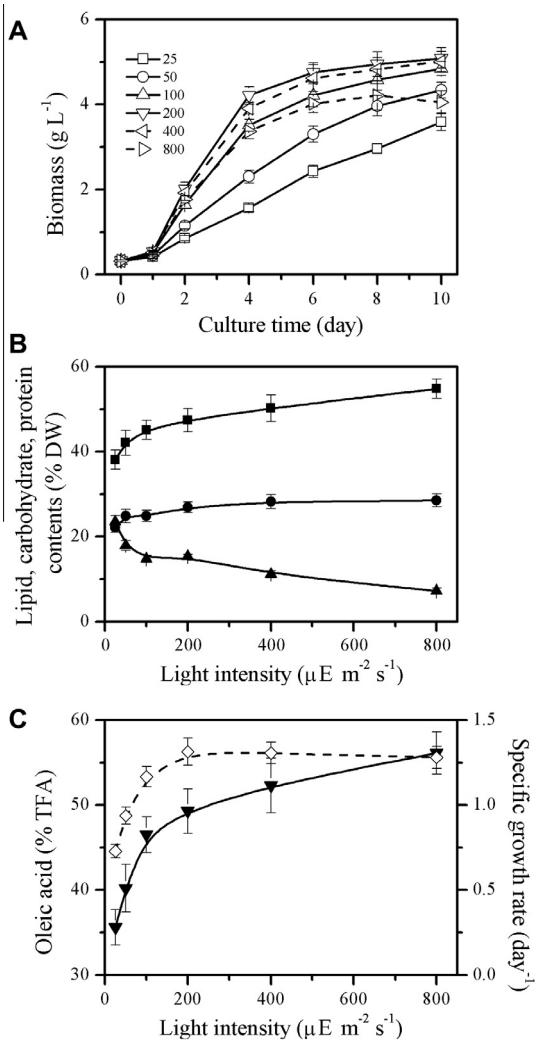

Fig. 5. Growth and biochemical composition of *C. protothecoides* as affected by various light intensities of 25, 50, 100, 200, 400, and 800 μE m^{-2} s^{-1}. (■), Lipid; (●), carbohydrate; (▲), protein; (▼), oleic acid; (◇), specific growth rate. The lipid, carbohydrate, protein and oleic acid were determined using 10-day cells.

in growth and final cell density (Fig. 5A and C). As for biochemical composition, lipids were promoted by high light intensity while proteins by low light intensity; in contrast, the content of carbohydrates remained relatively stable under all tested light intensities (Fig. 5B). Like lipids, the accumulation of C18:1 was positively dependent on light intensities (Fig. 5C). The results were consistent with a previous report where high light benefited the accumulation of lipids including C18:1 in a phototrophic batch culture of *C. zofingiensis* (Liu et al., 2012b).

3.6. Growth and lipid production of C. protothecoides CS-41 in outdoor panel PBRs

As indicated in the above laboratory study, the light intensity exceeding 200 μE m^{-2} s^{-1} (saturation value) exerted deteriorative effect on the growth of *C. protothecoides* CS-41 at the tested initial cell density. This fact may impose a potential challenge for outdoor mass culture of this alga, as the peak solar light irradiation can reach up to 2000 μE m^{-2} s^{-1}. Manipulating the initial cell density (ICD) represents a feasible approach to optimize the light availability to algal cells for growth and lipid production. Four volumetric ICDs of 0.3, 0.6, 1.2, and 2.4 g L^{-1} in the panel PBRs with a 3.5-cm light path, corresponding to areal cell densities of 10.5, 21, 42, and 84 g m^{-2}, respectively were used in this study. As indicated by Fig. 6A, ICDs had distinct impact on the algal growth and the maximum biomass output was achieved with the ICDs of 0.6–1.2 g L^{-1}. The ICD of 0.3 g L^{-1} may be too low to shade the cells from high light intensity, leading to the photo-inhibition of algal cells and thus the severely impaired biomass production. On the other hand, with the high ICD of 2.4 g L^{-1}, the biomass output was also significantly decreased, possibly due to the limited light availability to individual cells for photosynthesis. Low ICDs (corresponding to high light availability per cell) promoted the accumulation of lipids and carbohydrates (Fig. 6B and C), while high ICDs (corresponding to low light availability per cell) favored the protein synthesis (Fig. 6D), similar to the indoor light intensity experiment (Fig. 5). It is worth noting, however, that higher ICDs require more time to prepare the seed culture. Overall, the ICD of 0.6–1.2 g L^{-1} represented the optimal inoculation cell density for maximum lipid production under our outdoor culture conditions (Fig. 6 and Table 3).

Defining a proper light path of panel PBRs represents another means of manipulating the light availability to algal cells in the culture. Three light paths of 1.8, 3.5, and 7.0 cm were tested, with an identical areal ICD of 21 g m^{-2} L^{-1} inoculated. As denoted in Table 3, the longer the light path, the higher the areal biomass and lipid productivities achieved, indicating that greater photosynthesis occurred in PBRs with longer light path. From a volumetric productivity standpoint, however, a reverse relationship was evident, with the shortest light path PBRs giving the highest final cell density (9.7 g L^{-1}) and volumetric productivities of both biomass and lipids (1.25 and 0.59 g L^{-1} day^{-1}, respectively) (Table 3). High biomass density benefits certain downstream processes (e.g., algae harvest and drying) and helps to lower the footprint of water and nutrients. In this context, a two-stage cultivation strategy was proposed for outdoor PBRs: the algae cells were first inoculated in a thick PBR (e.g., 7 cm light path) with a low cell density (e.g., 0.3 g L^{-1}) for biomass production to a certain concentration (e.g., 1.2 g L^{-1}), then transferred to a thin PBR (e.g., 1.8 cm) for further accumulation of biomass and lipids.

3.7. Comparison of lipid production by C. protothecoides CS-41 and other microalgae

There have been many reports of using *Chlorella* for oil production, but only a limited number of attempts were made outdoors.

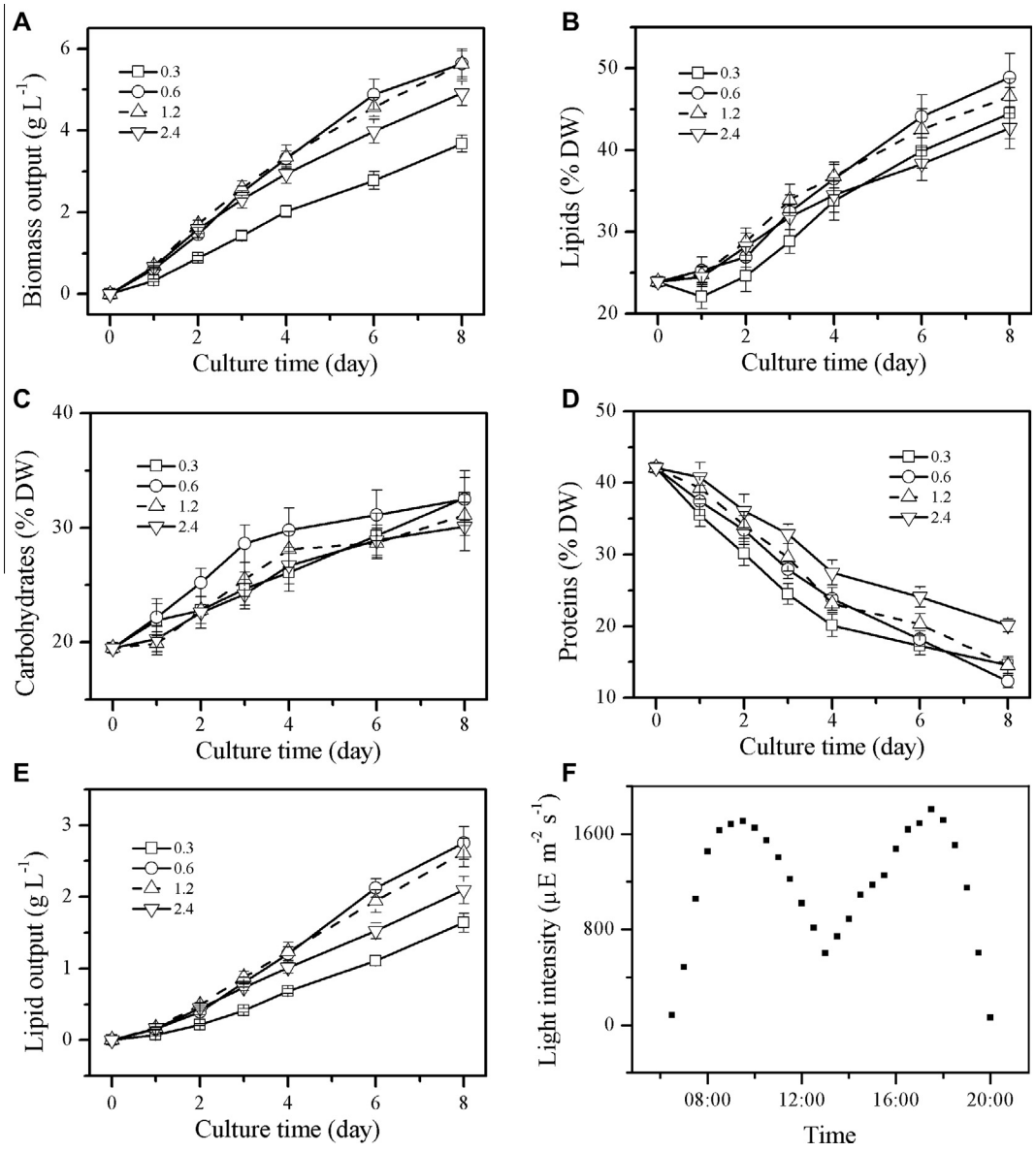

Fig. 6. Biomass output (A), lipids (B), carbohydrates (C), proteins (D), and lipid output (E) of outdoor *C. prototheocoides* as affected by various initial cell densities of 0.3, 0.6, 1.2, and 2.4 g L^{-1}. Cells were cultured in the panel PBRs with 3.5-cm light path. A representative daily light intensity profile was shown in (F).

Table 3
Biomass and lipid productivities of *C. prototheocoides* in outdoor panel PBRs.

Light path (cm)	Starting cell density (g L^{-1})	Final cell density (g L^{-1})	Maximum volumetric productivity (g L^{-1} day^{-1})		Maximum areal productivity (g m^{-2} day^{-1})	
			Biomass	Lipids	Biomass	Lipids
3.5	0.3	3.98 ± 0.22	0.51 ± 0.02	0.21 ± 0.01	17.9 ± 0.7	7.4 ± 0.3
	0.6	6.23 ± 0.29	0.83 ± 0.03	0.34 ± 0.02	29.1 ± 1.1	11.9 ± 0.7
	1.2	6.82 ± 0.38	0.87 ± 0.05	0.33 ± 0.03	30.5 ± 1.7	11.6 ± 0.9
	2.4	7.31 ± 0.39	0.78 ± 0.04	0.26 ± 0.02	27.3 ± 1.4	9.1 ± 0.6
1.8	1.2	9.72 ± 0.58	1.25 ± 0.07	0.59 ± 0.04	22.5 ± 1.3	10.6 ± 0.7
3.5	0.6	6.43 ± 0.32	0.88 ± 0.06	0.36 ± 0.02	30.8 ± 2.1	12.6 ± 0.7
7.0	0.3	4.01 ± 0.25	0.63 ± 0.03	0.23 ± 0.01	44.1 ± 2.2	16.1 ± 0.8

Feng et al. (2011) conducted the cultivation of *C. zofingiensis* in a 60-L panel PBR achieving maximum biomass and lipid productivities of 58.4 and 22.3 mg L^{-1} day^{-1} (or areal productivities of 9.9 and 3.8 g m^{-2} day^{-1}), respectively. Pribyl et al. (2012) investigated the performance of *C. vulgaris* in a 120-L thin layer PBR, with the biomass and lipid productivities being 1.25 and 0.33 g L^{-1} day^{-1} (or areal productivities of 14.2 and 3.75 g m^{-2} day^{-1}), respectively. Later, Munkel et al. (2013) grew another *C. vulgaris* strain in a 30-L panel PBR and the best productivities of biomass and lipids were 0.67 and 0.39 g L^{-1} day^{-1} (or areal productivities of 20.1 and

Table 4
Photoautotrophic oil production of *C. protothecoides* in comparison with previously reported green microalgae.

Algal strain	Culture conditions	Biomass productivity ($g\ L^{-1}\ day^{-1}$)	Lipid content (%DW)	Lipid productivity ($mg\ L^{-1}\ day^{-1}$)	References
C. protothecoides	I, 100-mL columns	0.57	48.3	280	This study
	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	O, 120-L polyethylene bags	0.24	34.6	83	Moheimani (2013)
C. sp	O, 70-L tube PBRs	0.15	43.3	34	Zhou et al. (2013)
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. vulgaris	I, 250-mL flasks	0.22	21.0	44	Ordog et al. (2013)
C. zofingiensis	O, 60-L panel PBRs	0.058	54.5	22	Feng et al. (2011)
C. zofingiensis	I, 250-mL flasks	0.67	44.8	301	Breuer et al. (2012)

$11.7\ g\ m^{-2}\ day^{-1}$), respectively. Very recently, three independent trials using *Chlorella* sp were performed in a 120-L polyethylene bag (Moheimani, 2013), a 70-L tube PBR (Zhou et al., 2013), and a 10-L panel PBR (Guccione et al., 2014), and the biomass and lipid productivities were 240 and $83\ mg\ L^{-1}\ day^{-1}$ (or 36 and $12.5\ g\ m^{-2}\ day^{-1}$), 150 and $34\ mg\ L^{-1}\ day^{-1}$ (or 16.5 and $3.7\ g\ m^{-2}\ day^{-1}$), and 600 and $160\ mg\ L^{-1}\ day^{-1}$ (or 24 and $6.4\ g\ m^{-2}\ day^{-1}$), respectively. In the present study, we achieved high volumetric biomass and lipid productivities of 1.25 and $0.59\ g\ L^{-1}\ day^{-1}$, respectively, or areal biomass and lipid productivities of 44.1 and $16.1\ g\ m^{-2}\ day^{-1}$, respectively, which are higher or comparable to the results obtained in previous reports (Table 4).

C. protothecoides CS-41 consists predominantly of C16 and C18 fatty acids, and C18:1 is the major fatty acid accounting for up to 59% of total fatty acids (Table 2 and Fig. 3), the highest level for any *Chlorella* strain, to the best of our knowledge. It is believed that C18:1 in high proportion benefits the biodiesel quality (Knothe, 2009), indicating that the oil from *C. protothecoides* CS-41 is a good precursor for biodiesel production. In fact, most properties of *C. protothecoides* biodiesel meet the established standards, either by prediction based on the FAME composition (Table 2) or by measurement (Miao and Wu, 2006). In addition to lipids, *C. protothecoides* CS-41 contains substantial amount of carbohydrates and proteins (Fig. 6) and certain high-value products (Liu and Hu, 2013), which can be potentially used as food, feed, and nutraceuticals. *C. protothecoides* also demonstrated a good potential for removing nutrient of N and P (Fig. 2E and 4B) and for wastewater treatment (Sforza et al., 2014).

C. protothecoides has been explored for lipid production, to our best knowledge, under heterotrophic growth conditions using sugars, glucose in particular, as the solo carbon and energy source (Liu et al., 2014). Although heterotrophic *C. protothecoides* is able to achieve high cell density and thus high biomass and lipid productivities, it has intrinsic drawbacks such as low sugar-to-biomass conversion (commonly below 0.5) and relatively high production cost, and thus is less favorable for the production of low-cost commodity oils. In the present study, we performed, for the first time, a comprehensive investigation of indoor and outdoor photoautotrophic *C. protothecoides* CS-41 for oil production, which represents a good starting point for further exploration of this alga in pilot-scale PBRs or open ponds.

4. Conclusions

The growth, lipids, and fatty acids of nine *Chlorella* strains were comparatively assessed and *C. protothecoides* CS-41 demonstrated the greatest lipid production potential. *C. protothecoides* CS-41 accumulated lipids, triacylglycerol, and oleate up to 55% and 39.1% of dry weight, and 59% of total fatty acids, respectively. The best biomass and lipid productivities achieved in outdoor panel PBRs were $1.25\ g\ L^{-1}\ day^{-1}$ and $0.59\ g\ L^{-1}\ day^{-1}$, which are higher than or comparable to the results of previous studies. Taken together, *C. protothecoides* CS-41 represents a promising oleaginous microalga worthy of further exploration for biodiesel use.

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