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Exploring an isolate of the oleaginous alga *Micractinium inermum* for lipid production: molecular characterization and physiochemical analysis under multiple growth conditions

Meicheng Shi[1] · Hehong Wei[1] · Qiuyi Chen[1] · Xiaofei Wang[1] · Wenguang Zhou[2] · Jin Liu[1]

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Abstract

Molecular characterization based on the internal transcribed spacer 2 (ITS2) sequence identified *Micractinium inermum* JL1, a local isolate of green alga capable of growing robustly under photoautotrophic, mixotrophic, and heterotrophic culture modes. Physiochemical analyses of the alga revealed great variations in growth, lipid content, oleic acid abundance, and productivities of lipids and triacylglycerol (TAG) under various culture conditions including nitrogen concentrations, phosphorus concentrations, light intensities, salinity levels, and glucose concentrations with or without light illumination. The carbon shunt from starch and to a lesser extent from membrane lipids likely contributed to the TAG accumulation. Noteworthily, salt supplementation promoted TAG content and productivity by ~ 88% and ~ 55%, respectively. The alga could accumulate ~ 52% lipids per dry weight and achieved a high lipid productivity of 0.68 g L^{-1} day^{-1}. C18:1 percentage, a biodiesel quality indicator, reached ~ 50%, and correlated well with TAG contents in a positive manner, demonstrating the feasibility of using C18:1 percentage for TAG quantification thereby avoiding time- and labor-intensive analysis. These results together indicate the potential of *M. inermum* JL1 as a lipid producer for future exploration.

Keywords Algae · Chlorophyta · Lipid · Oleic acid · Triacylglycerol · Biodiesel

Introduction

Microalgal lipids are considered as the next-generation biodiesel feedstock and have attracted increasing attention during recent decades (Chisti 2007; Wijffels and Barbosa 2010; Mallick et al. 2016; Zhou et al. 2017). Efforts have been made to increase production economics of algal biodiesel by strain selection (Breuer et al. 2012; Pribyl et al. 2012; Sun et al. 2015), trait improvement through genetic engineering (Radakovits et al. 2010; Wei et al. 2017; Xin et al. 2017), exploration of next-generation culture systems (Singh and Sharma 2012), co-production with value-added products (Liu et al. 2016a), integration with bio-mitigation of flue gas and waste water (Sforza et al. 2014; Zhou et al. 2017), and development of state-of-the-art downstream processes (Suali and Sarbatly 2012; Kim et al. 2013). It is expected that a promising production strain for biodiesel should have a fast growth rate for high cell density, synthesize abundant oils, and perform well in downstream processes (Wijffels and Barbosa 2010).

Algal growth, lipid content, and fatty acid composition, the key parameters for evaluating algal biodiesel production potential, are culture condition-dependent and can be greatly influenced by a variety of environmental factors and nutrients (Breuer et al. 2012; Sun et al. 2015; Hirai et al. 2016; Ho et al. 2017; Sun et al. 2018). Light is an indispensable factor for the photoautotrophic growth of algae. There is a saturation light intensity for optimal algal growth, below which the light becomes insufficient, leading to the photo limitation-associated slow growth; when exceeding the saturation value, accumulation of storage compounds such as lipids may occur resulting in changes in fatty acid composition as well (Liu et al. 2013; Sun et al. 2015, 2018). Salinity is another important environmental factor affecting algal growth and salt stress has the potential to induce lipid synthesis in algae (Hirai et al. 2016; Ho et al. 2017). Among the nutrients, carbon is the most prominent element of algae and accounts for approximately 50% of the biomass. Algae can grow photoautotrophically by

✉ Jin Liu
gjinliu@pku.edu.cn

[1] Laboratory for Algae Biotechnology and Innovation, College of Engineering, Peking University, Beijing 100871, China

[2] School of Resources, Environmental and Chemical Engineering and MOE Biomass Engineering Research Center, Nanchang University, Nanchang, China

utilizing carbon dioxide or heterotrophically with organic carbon such as sugar to achieve high cell density (Liu et al. 2011, 2014a; Sirisansaneeyakul et al. 2011; Breuer et al. 2012). Nitrogen and phosphorus represent the additional important nutrients and their availability impacts algal growth and lipid production considerably (Breuer et al. 2012; Liu et al. 2013; Sun et al. 2015, 2018). Understanding the effect of these factors on algal growth, lipid synthesis, and fatty acid composition helps facilitate the optimization of culture conditions for improved lipid production (Hu et al. 2008). Although the effect of individual factors mentioned above on algal growth and lipid profiles has been well studied in many studies, a comprehensive evaluation of those factors in a single study has been rarely done.

We have isolated several algal strains from a local freshwater area, one of which was capable of growing robustly under photoautotrophic, mixotrophic, and heterotrophic culture modes, and showed better performance with respect to lipid production by an initial screening. In the present study, the sequence of internal transcribed spacer 2 (ITS2) of the alga was firstly obtained and sequenced for phylogenetic analysis, indicative of it being a *Micractinium inermum* strain (referred to as *M. inermum* JL1 thereafter). There have been several reports about using *M. inermum* strains for lipid production (Smith et al. 2015; Park et al. 2017, 2018), but they missed the information about triacylglycerol (TAG) and fatty acid profiles. In order to further characterize *M. inermum* JL1, detailed physiological and biochemical analyses of *M. inermum* JL1 were conducted regarding growth, lipid content, oleic acid abundance, and productivities of lipids and TAG under various photoautotrophic culture conditions including nitrogen concentrations, phosphorus concentrations, light intensities, and salinity levels. Furthermore, the effect of glucose concentrations on growth and lipid production of *M. inermum* JL1 was examined, either with light or in the dark. Finally, we discuss implications of our results and future exploration of this oleaginous alga for production uses.

Materials and methods

Algal strain and maintenance

The algal strain *Micractinium inermum* JL1 was isolated from a local water area of Beijing, China (N 39° 59′ 38″, E 116° 18′ 17″), and maintained on agar plate of the modified BG-11 medium (100 mg L^{-1} of nitrogen in the form of nitrate). Briefly, 10 mL of liquid BG-11 was inoculated with cells from agar plate 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 μmol photons m^{-2} s^{-1}. The cells were then inoculated at 10% (*v*/*v*) into culture columns provided with illumination of 80 μmol photons m^{-2} s^{-1} and aeration with 1.5% CO_2-enriched air, grown to late exponential phase and used as seed cultures for subsequent experiments.

PCR amplification of ITS2 and phylogenetic analysis

The ITS2 fragment of the algal strain was PCR amplified according to procedures described by Liu et al. (2014b), using the following primers: forward, AGGAGAAGTCGTAA CAAGGT and reverse, TCCTCCGCTTATTGATATGC. The DNA amplicon was purified and sequenced (NCBI accession no. MG692443). The ITS2 and other algal homologous ITS2 sequences were aligned by ClustalX 2.1 (Larkin et al. 2007). Phylogenetic tree was constructed using MEGA4 software (Tamura et al. 2007).

Algal growth in bubble columns or in the flasks supplemented with glucose

Algal cultures were grown at 25 °C in 250 mL modified BG-11 medium in columns (3-cm inner diameter) aerated with 1.5% CO_2 enriched air and illuminated with continuous light at 80 μmol photons m^{-2} s^{-1}. The cultures at late exponential growth phase were used as inocula for investigating the algal growth and oil production in response to different nitrogen concentrations, phosphorus concentrations, light intensities, and salinity. The culture conditions were the same as the above-mentioned except where otherwise indicated. For the nitrogen concentration experiment, nitrogen concentrations were 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, and 14.26 mg L^{-1}. For the light intensity experiment, light intensities were 40, 80, 160, 320, and 640 μmol photon m^{-2} s^{-1}. For the salinity level experiment, the NaCl concentrations were 0, 0.1, 0.2, and 0.4 M.

For glucose supplementation, the cultures were grown in the flasks with orbital shaking at 150 rpm and supplemented with different glucose concentrations of 0, 5, 10, 20, 30, and 40 g L^{-1}, with or without 80 μmol photons m^{-2} s^{-1} continuous light.

Analytical methods

Cell samples were centrifuged at 3800×*g* for 5 min, resuspended in distilled water (three times), and filtered through a pre-dried Whatman GF/C filter paper (1.2 μm pore size). The algal cells on the filter paper discs were dried at 100 °C in a vacuum oven overnight to reach constant weight and were cooled to room temperature in a desiccator before weighting.

For biochemical analysis, cell samples were centrifuged as above, washed three 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). Starch was determined with a Starch Assay Kit (Sigma-Aldrich, USA). The staining of algal cells with the fluorescence dye Bodipy for microscopic observation was conducted following Liu et al. (2013). Total lipids were analyzed gravimetrically after extraction with chloroform–methanol (2:1) 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, USA) using a solvent system of petroleum ether/diethyl ether/acetic acid (70:30:1, by volume). Lipids were visualized by brief exposure to iodine vapor and were scratched off for transmethylation and quantification by gas chromatograph mass spectrometry.

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 with an Agilent 7890 capillary gas chromatograph equipped with a 5975 C mass spectrometry detector and a HP-88 capillary column (60 m × 0.25 mm) (Agilent Technologies, USA). The temperature program consisted of an initial hold at 100 °C for 5 min, ramping at 3.5 °C min^{-1} to 240 °C, and a final hold at 240 °C for 5 min. The injector was kept as 250 °C with an injection volume of 2 μL in a splitless mode. The flow rate of the carrier gas (helium) was 1.5 mL min^{-1}, and the ionization energy was 70 eV (EI, full scan mode). FAMEs were identified by chromatographic comparison with authentic standards (Sigma). The quantities of individual FAMEs were calculated from the peak areas on the chromatogram using heptadecanoic acid as the internal standard.

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., USA). Statistical significances were achieved when $p < 0.05$.

Results

Molecular and biochemical characterization of *M. inermum* JL1

Identification of an algal isolate typically relies on molecular approaches based on the sequences of 18S rRNA and internal transcribed spacer (ITS) (Krienitz et al. 2004). The ITS2 fragment of our algal isolate was PCR amplified using the primers described by Liu et al. (2014b) and subjected for sequencing, with the sequence deposited in GenBank (Accession No. MG692443). Phylogenetic tree analysis of ITS2 sequences of different origin suggested that this alga is likely a *M. inermum* strain (Fig. 1a). In batch culture, the alga grew fast and entered stationary growth phase at day 8, with a biomass concentration of 4.3 g L^{-1} (Fig. 1b). Lipid content remained relatively constant and started to increase gradually at day 4 (Fig. 1b). TAG, the neutral storage lipid, showed a basic level during the first 4 days and then a sharp increase to 20% of dry weight (DW) at the end of culture period (Fig. 1b), presumably due to the consumption of medium nutrients (e.g., nitrogen and phosphorus). The accumulation of neutral lipids during late culture period was also evidenced by the staining of the cells with the fluorescent dye Bodipy (Fig. 1c). Starch, another storage compound, exhibited a different accumulation pattern from lipid: reaching the maximum at day 8 (early stationary growth phase) and declining thereafter (Fig. 1b). Protein, on the other hand, showed a gradual decrease in response to culture time (Fig. 1b).

As the composition and structure of fatty acid esters determine important properties of biodiesel, such as cetane number, viscosity, cold flow, and oxidative stability, the fatty acid profiles of *M. inermum* JL1 total lipids on different days were compared. The fatty acid composition consisted mainly of C16:0, C18:1, C18:2, and C18:3, which together accounted for over 85% of the total fatty acids regardless of the culture time (days 4, 8, and 12) in batch culture (Table 1). As the culture time increased, saturated fatty acids, namely C16:0 and C18:0, showed little change, polyunsaturated fatty acids (PUFAs), C18:3 in particular, exhibited a considerable decrease, while C18:1, the predominant monounsaturated fatty acid (MUFA), increased greatly from 16.9 to 38.7%. It has been suggested that C18:1 ester can act as a balance between oxidative stability and low-temperature properties and thus serve as an indicator for biodiesel quality of microalgal oils (Knothe 2009). In this regard, old cultures of *M. inermum* JL1, which accumulated more C18:1 at the expense of PUFAs, are suitable for biodiesel production.

Growth and lipid production as affected by different nitrogen concentrations

Nitrogen (N) is an essential nutrient for algal growth and can greatly influence lipid production. Here, we employed nitrate as the nitrogen source to examine the effect of nitrogen concentrations (12.5–200 mg L^{-1} N) on *M. inermum* JL1 growth and lipid production (Fig. 2). It is obvious that lower N concentrations (12.5 and 25 mg L^{-1} N) impaired algal growth during late growth period, while higher N concentrations (50–200 mg L^{-1} N) had little effect (Fig. 2a). The initial N consumption rate appeared almost identical under all the tested N concentrations, and the N in the culture medium was exhausted within 4 days except under initial N concentration of 200 mg L^{-1} N (Fig. 2b). N concentration showed a clear negative correlation with lipid accumulation: the contents of both total lipids and TAG reached the maximum at the lowest N concentration of 12.5 mg L^{-1} N (Fig. 2c). Similar to lipid

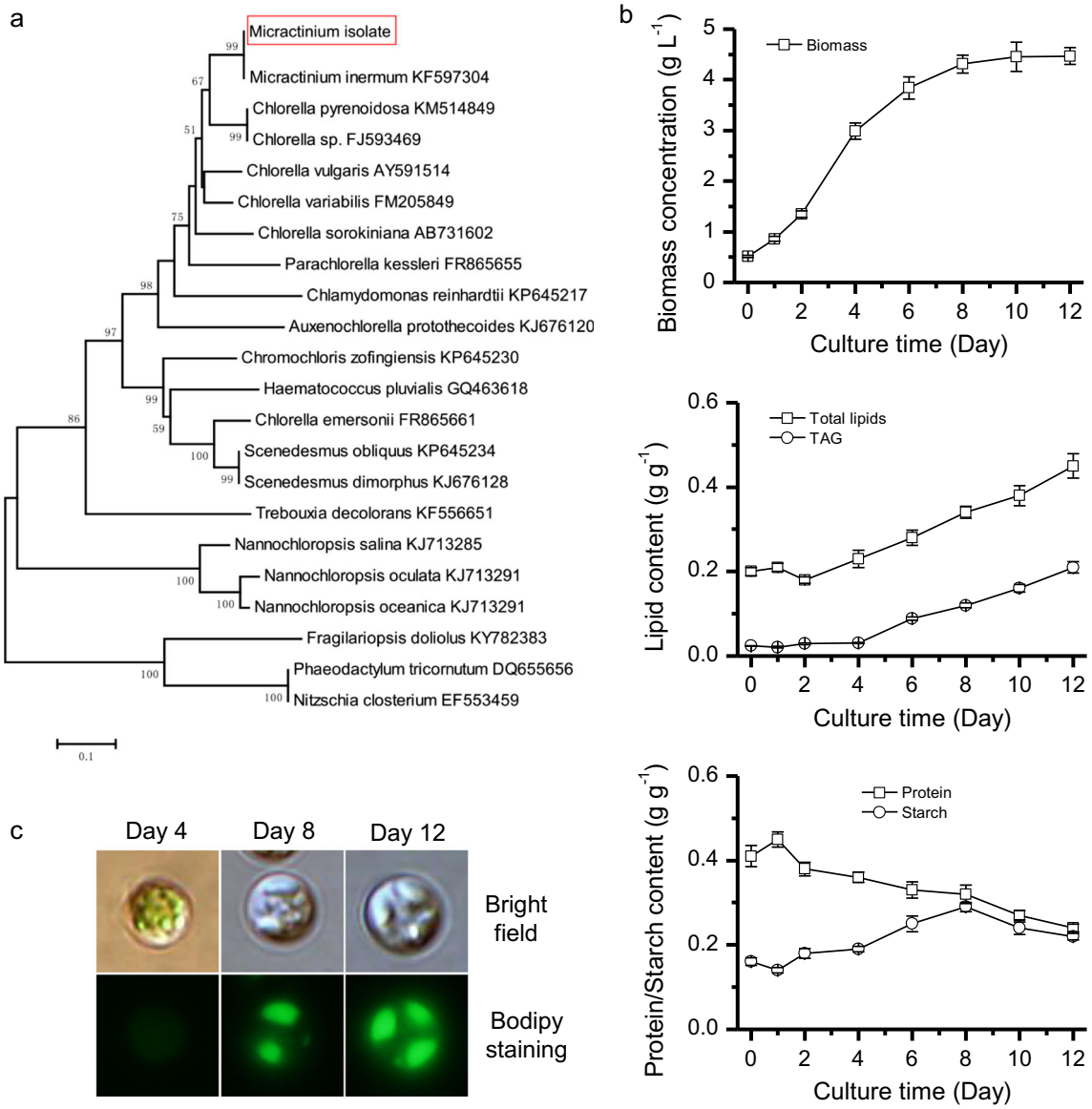

Fig. 1 Molecular and biochemical characterization of *M. inermum* JL1. **a** Phylogenetic tree analysis of ITS2 sequences from *M. inermum* JL1 and other algae. The neighbor-joining method was used to reconstruct the cladogram, with the bootstrap value (obtained from 1000 replicates) shown on each node. The scale bar 0.1 represents 10% divergence, calculated as the estimated number of replacement. **b** Time course of biomass and contents of lipids, protein, and starch. **c** Bodipy staining of *M. inermum* JL1 cells under different culture days. **b** Data are expressed as mean ± SD ($n = 3$). The algal cells were cultured at 25 °C with the BG-11 medium (100 mg L^{-1} N) in columns provided with illumination of 80 μmol photons m^{-2} s^{-1} and aeration of 1.5% CO_2 enriched air

content (Fig. 2c), the oleic acid percentage in both total lipids and TAG were negatively related to N concentration and reached the higher value (~ 50%) under lower N concentrations (Fig. 2d). As the dual effect of N concentration, the maximum lipid productivity was not achieved at the lowest N concentration but instead at 50 mg L^{-1} N (Fig. 2e).

Growth and lipid production as affected by different phosphorous concentrations

Phosphorus (P) concentration had a similar effect to N concentration on the growth of *M. inermum* JL1: within the tested P concentrations of 0.89–14.26 mg L^{-1}, the higher the P concentration, the greater biomass produced at the end of culture period (Fig. 2f). Obviously, the consumption of P by the algal cells depended on the initial P concentrations in the medium: the higher the initial P concentration, the greater the P consumption rate, particularly within the first 4 days of cultivation (Fig. 2g). Similar to N concentration, P concentration showed a negative correlation with lipid content (Fig. 2h) and oleic acid percentage (Fig. 2i). Taking the combination of biomass concentration and lipid content into account, the maximum lipid productivity was achieved at relatively high P

Table 1 Fatty acid profiles of *M. inermum* JL1 on different culture days

Fatty acids[a]	Day 4	Day 8	Day 12
C16:0	22.9 ± 1.4	22.8 ± 0.9	24.2 ± 1.3
C16:1	2.1 ± 0.1	2.4 ± 0.2	2.8 ± 0.2
C16:2	6.2 ± 0.2	4.3 ± 0.3	3.1 ± 0.2
C16:3	3.9 ± 0.3	3.7 ± 0.1	3.0 ± 0.1
C16:4	1.6 ± 0.1	1.1 ± 0.1	0.9 ± 0.1
C18:0	2.4 ± 0.2	2.3 ± 0.1	2.6 ± 0.1
C18:1	16.9 ± 0.9	28.5 ± 2.2	38.7 ± 1.9
C18:2	29.5 ± 1.1	26.4 ± 1.6	17.8 ± 1.2
C18:3	14.4 ± 1.6	8.6 ± 0.9	6.5 ± 0.4
MUFA	18.9 ± 1.0	30.8 ± 2.2	41.5 ± 2.1
PUFA	55.7 ± 3.3	44.1 ± 2.7	31.3 ± 1.9
UFA	74.7 ± 4.3	74.9 ± 5.0	72.7 ± 4.0
DUS[b]	1.5 ± 0.1	1.3 ± 0.1	1.2 ± 0.1

[a] Data are expressed as percentage of total fatty acids (%);

[b] DUS (∇/mol), the degree of fatty acid unsaturation = [1.0 (% monoenes) + 2.0 (% dienes) + 3.0 (% trienes) + 4.0 (% tetraenes)]/100. Data are mean ± SD (n = 3). The culture conditions are the same as stated in Fig. 1

MUFA monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids, *UFA* unsaturated fatty acid

concentration, namely, 7.13 mg L^{-1} P for TAG and 14.26 mg L^{-1} P for total lipids (Fig. 2j).

Growth and lipid production as affected by different light intensities

Aside from medium nutrients, environmental factors such as light intensity and salinity level play key roles in algal growth and lipid profile. Here, we tested a broad range of light intensities of 40 to 640 μmol photons m^{-2} s^{-1} (Fig. 3a–d). Obviously, within the range of 40 to 160 μmol photons m^{-2} s^{-1}, a light intensity-dependent growth response was observed, leading to highest growth rate and final biomass concentration with the light intensity of 160 μmol photons m^{-2} s^{-1} (Fig. 3a). Further increase of light intensity to 320 μmol photons m^{-2} s^{-1} resulted in a decline in biomass concentration (Fig. 3a), indicating the occurrence of photoinhibition. Light intensity also affected the lipid accumulation in *M. inermum* JL1 considerably: within the range of 40 to 320 μmol photons m^{-2} s^{-1}, the higher the light intensity, the greater content of both total lipids and TAG; further increase of the light intensity to 640 μmol photons m^{-2} s^{-1}; however, attenuated lipid accumulation slightly (Fig. 3b). Similarly, C18:1 accumulation was positively dependent on light intensities (Fig. 3c). The results were consistent with previous reports where high light benefited the accumulation of lipids including C18:3 in *Chlorella* cells (Liu et al. 2012a; Sun et al. 2015). As for the productivity, the maximum value of both total lipids and TAG was achieved under the light intensity of 320 μmol photons m^{-2} s^{-1} (Fig. 3d).

Growth and lipid production as affected by different salinity levels

Freshwater algae generally cannot survive with marine water, but are able to grow under low salinity levels, accompanied by growth retardation and cellular biochemical changes (Hirai et al. 2016; Ho et al. 2017). Within the tested salt concentrations of 0.1 to 0.4 M, the algal growth was negatively related with salt concentration: a slight decline in biomass under 0.1 M salt and a considerable decline (40%) in biomass under 0.4 M salt at the end of batch culture (Fig. 3e). By contrast, the accumulation of lipids was promoted by salt addition, for example, 32% more total lipids and 88% more TAG produced in the presence of 0.2 M salt (Fig. 3f). Noteworthily, compared to 0.2 M salt, 0.4 M salt led to an obvious decrease of both total lipids and TAG (Fig. 3f), possibly due to the high salinity-associated impairment in lipid synthesis. Salt addition also promoted the synthesis of C18:1, ~ 42% more than the control (Fig. 3g). As for the lipid productivity, 0.2 M salt was best, in particular for TAG which was 55% higher than the control (Fig. 3h).

Growth and lipid production as affected by different glucose concentrations

Many algal strains are able to utilize glucose for growth and lipid production (Liu et al. 2014a), as they have a hexose/H^+ symport system to transport glucose into cytosol for metabolism (Tanner 2000). We firstly tested the effect of glucose on growth and lipid production of *M. inermum* JL1 under light (Fig. 4a–d). The addition of glucose greatly promoted biomass concentration particularly during the early culture days, e.g., reaching 6.0 g L^{-1} compared to 1.5 g L^{-1} without glucose supplementation on day 2 (Fig. 4a). It is worth noting that high glucose concentration, e.g., over 20 g L^{-1}, inhibited algal growth, leading to a considerable lower biomass concentration (7.0 g L^{-1} compared to 10.0 g L^{-1} under 20 g L^{-1} glucose) at the end of culture period (Fig. 4a). In addition to promoting biomass accumulation, glucose addition induced lipid synthesis (Fig. 4b) and C18:1 percentage (Fig. 4c). The maximum productivities of total lipids and TAG were achieved under 20 g L^{-1} glucose, being 0.48 and 0.21 g L^{-1} day^{-1}, respectively (Fig. 4d).

We also tested the effect of glucose on growth and lipid production of dark-grown *M. inermum* JL1 (Fig. 4e–h). Unlike the inhibitory effect of high glucose concentrations (over 20 g L^{-1}) in the light-grown cultures (Fig. 4a), the final biomass concentration correlated positively with glucose concentration used in the dark-grown cultures (Fig. 4e), indicating a lower glucose utilization efficiency of light-grown cultures

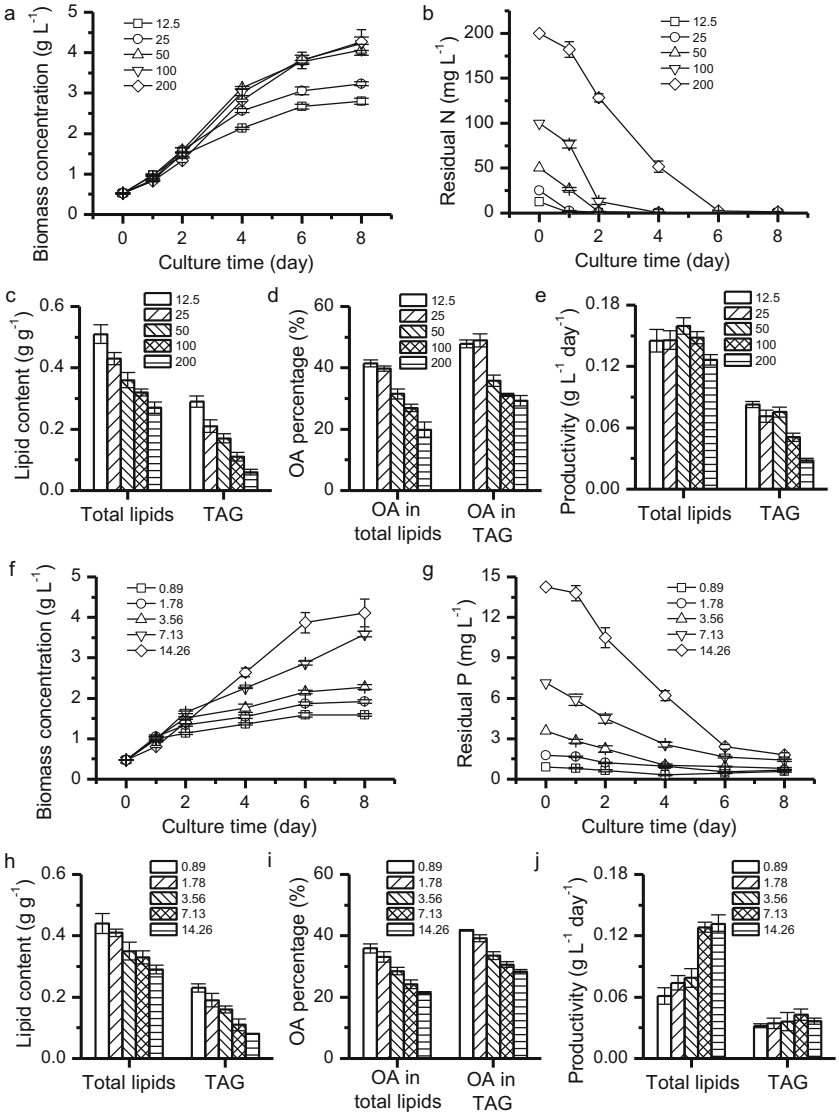

Fig. 2 Growth and lipid production of *M. inermum* JL1 as affected by different concentrations of N (**a**–**e**) and P (**f**–**k**). **a**, **b** Time course of biomass concentration (**a**) and residual N in the medium (**b**) under different N concentrations (12.5–200 mg L^{-1}). **c**–**e** Content (**c**), OA percentage (**d**), and productivity (**e**) of total lipids and TAG for algal cells on day 8 under different N concentrations. **f**, **g** Time course of biomass concentration (**f**) and residual P in the medium (**g**) under different P concentrations (0.89–14.28 mg L^{-1}). **h**–**j** Content (**h**), OA percentage (**i**), and productivity (**j**) of total lipids and TAG for algal cells on day 8 under different P concentrations. Data are expressed as mean ± SD ($n = 3$). The algal cells were cultured at the same condition as in Fig. 1 except that different N concentrations and P concentrations were used for N and P experiments, respectively. OA, oleic acid (C18:1)

under high glucose concentrations. This is an interesting finding and remains to be further studied for clarification. Similar to light-grown cultures, glucose concentration exhibited a positive correlation with lipid content (Fig. 4f) and C18:1 percentage (Fig. 4g) in dark-grown cultures. The maximum lipid productivity of 0.68 g L^{-1} day^{-1} (Fig. 4h) was achieved when supplemented with 30 g L^{-1} glucose, being 42% higher than that achieved under mixotrophic conditions.

It has long been suggested that photosynthesis and heterotrophic respiration in algae can act non-competitively in mixotrophic growth, and thus mixotrophic cell growth should equate to the sum of photoautotrophic and heterotrophic growth (Endo et al. 1977). In the present study, the mixotrophic biomass yield of *M. inermum* JL1 exceeded the sum of photoautotrophic and heterotrophic biomass yield during the early culture period on days 1–2 (Table 2). This is likely due to the so-called synergistic effect, which happens particularly for mixotrophic cultures without aeration: the metabolism of organic carbon provides an endogenic source of CO_2 to promote photosynthesis, which in turn supplies enough O_2 for respiration and enhances organic carbon metabolism (Smith et al. 2015). Intriguingly, this is not the case for *M. inermum* JL1 during the late culture period; instead, the mixotrophic biomass yield became lower than the sum of photoautotrophic and heterotrophic biomass yield, particularly under high glucose concentrations of 30 and 40 g L^{-1} (days 6–8, Table 2). Probably, the N availability, which is much less during the late culture period due to the consumption, affects the photosynthesis and thus the biomass yield. It is also possible that the respiration rate differs between these two culture stages, contributing to the difference in glucose metabolism and thus the growth. Moreover, the high C/N ratio under high glucose concentrations may have an antagonistic effect on the mixotrophic growth, particularly during the late culture period. Further investigations are needed to elucidate this interesting phenomenon.

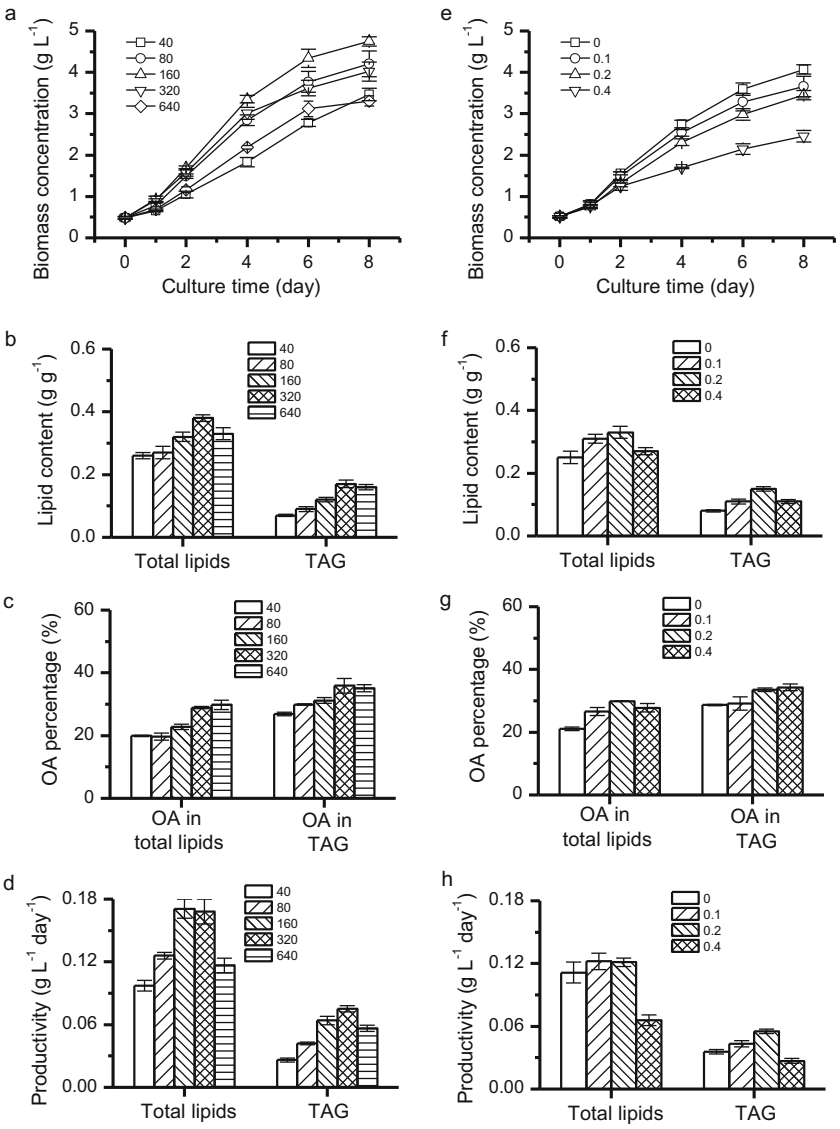

Fig. 3 Growth and lipid production of *M. inermum* JL1 as affected by different light intensities (**a**–**d**) and salinity levels (**e**–**h**). **a** Time course of biomass concentration under different light intensities (40–640 μmol photons m^{-2} s^{-1}). **b**–**d** Content (**b**), OA percentage (**c**), and productivity (**d**) of total lipids and TAG for algal cells on day 8 under different light intensities. **e** Time course of biomass concentration under different salinity levels (0–0.4 M). **f**–**h** Content (**f**), OA percentage (**g**), and productivity (**h**) of total lipids and TAG for algal cells on day 8 under different salinity levels. Data are expressed as mean ± SD ($n = 3$). The algal cells were cultured at the same condition as in Fig. 1 except that different light intensities and salinity levels were used for light intensity and salinity level experiments, respectively. OA, oleic acid (C18:1)

TAG accumulates at the expense of polar lipids and starch

Algae tend to synthesize less polar lipids when TAG accumulates (Hu et al. 2008). In the present study, TAG content showed a negative correlation ($R^2 = 0.60$) with polar lipid content (Fig. 5a), suggesting the contribution of recycling of polar lipids to TAG biosynthesis (Liu et al. 2016a, b; Ma et al. 2016; Wei et al. 2017). However, the contribution is minor considering the low slope (− 0.26, Fig. 5a).

Micractinium inermum JL1 accumulates starch in addition to TAG as a carbon and energy-storing compound within the cells (Fig. 1b). When plotting TAG content and starch content, an obvious negative correlation was observed ($R^2 = 0.83$) (Fig. 5b), suggesting that TAG increased at the expense of starch under induction conditions. Given that TAG and starch share common precursors for biosynthesis, they may be interconvertible. This has been suggested by several reports that starch accumulates earlier than TAG and declines lately to supply carbon precursors for continuing TAG synthesis in green algae (Msanne et al. 2012; Recht et al. 2012; Zhu et al. 2015).

Discussion

The green alga *M. inermum* has been used for lipid production in several studies (Smith et al. 2015; Park et al. 2017, 2018). Nevertheless, the TAG and fatty acid profiles of this alga are less documented particularly in response to various culture parameters, which is important to optimize lipid production for biodiesel production. In the present study, we isolated a new *M. inermum* strain and characterized its growth and lipid variation under photoautotrophic conditions with different

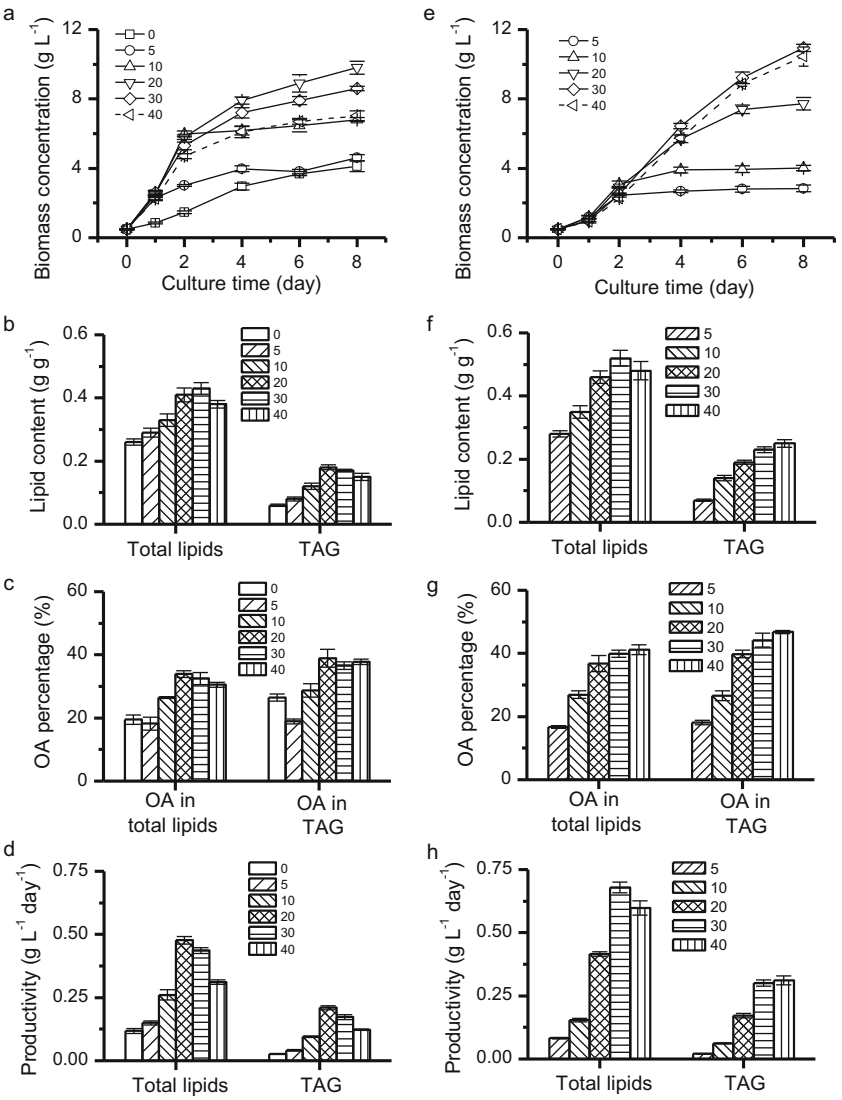

Fig. 4 Growth and lipid production of *M. inermum* JL1 as affected by different glucose concentrations with (**a**–**d**) or without (**e**–**h**) light illumination. **a** Time course of biomass concentration with light illumination under different glucose concentrations (0–40 g L^{-1}). **b**–**d** Content (**b**), OA percentage (**c**), and productivity (**d**) of total lipids and TAG for algal cells on day 8 with light illumination (80 μmol photons m^{-2} s^{-1}) under different glucose concentrations. **e** Time course of biomass concentration without light illumination under different glucose concentrations (5–40 g L^{-1}). **f**–**h** Content (**f**), OA percentage (**g**), and productivity (**h**) of total lipids and TAG for algal cells on day 8 with light illumination under different glucose concentrations. Data are expressed as mean ± SD ($n = 3$). The algal cells were cultured at 25 °C with BG-11 medium (100 mg L^{-1} N) in flasks supplemented with various glucose concentrations. OA = oleic acid (C18:1)

parameters including nitrogen concentrations, phosphorus concentrations, light intensities, and salinity levels (Figs. 2 and 3). Based on the results, a set of culture parameters (50 mg L^{-1} N, 7.13 mg L^{-1} P, 160 μmol photons m^{-2} s^{-1}, and 0.1 M salt), which each promoted lipid accumulation yet without severe growth impairment (Figs. 2 and 3), were tested to serve as an attempt to increase productivity (Table 3). There have been many reports in which microalgae particularly *Chlorella* strains were employed to examine lipid production (Table 4). The photoautotrophic lipid productivity (0.27 g L^{-1} day^{-1}) for *M. inermum* JL1 is much higher than the previous report of *M. inermum*, and higher or comparable to that for *Chlorella* strains except for the study of *C. vulgaris* by Pribyl et al. (2012). The further improvement in photoautotrophic lipid productivity may be achieved by more systematical optimization of environmental factors and nutrients. Metabolic engineering may also represent a feasible approach toward maximizing the inherent lipid production capacity of this alga if genetic tools are available in future, e.g., by redirecting carbon flux from starch to lipids via suppressing ADP-glucose pyrophosphorylase (Li et al. 2010; de Jaeger et al. 2014; Vonlanthen et al. 2015), and pulling fatty acids to TAG by overexpressing diacylglycerol acyltransferase genes (Wei et al. 2017; Xin et al. 2017). Moreover, *M. inermum* JL1 grows fast using CO_2 as the carbon source and is capable of removing N and P quickly from the culture medium under photoautotrophic conditions (Fig. 2), enabling it to be a promising strain for the integrated production of biodiesel with bio-mitigation of flue gas and wastewater (Zhou et al. 2017).

It is worth noting that *M. inermum* JL1 is capable of growing robustly heterotrophically using glucose as the carbon source (Fig. 4). The heterotrophic lipid productivity (0.68 g L^{-1} day^{-1}) for *M. inermum* JL1 is considerably greater than the photoautotrophic one. Furthermore, the well-developed fermentation technologies, e.g., fed-batch

Table 2 Comparison of biomass yield under various culture modes

Culture time (day)	Biomass yield (g L^{-1}) 5 g L^{-1} glucose			Biomass yield (g L^{-1}) 10 g L^{-1} glucose			Biomass yield (g L^{-1}) 20 g L^{-1} glucose			Biomass yield (g L^{-1}) 30 g L^{-1} glucose			Biomass yield (g L^{-1}) 40 g L^{-1} glucose		
	M	H	H + P	M	H	H + P	M	H	H + P	M	H	H + P	M	H	H + P
1	1.8 ± 0.1a	0.7 ± 0.1b	1.1 ± 0.1c	2.0 ± 0.1a	0.7 ± 0.1b	1.1 ± 0.1c	2.1 ± 0.1a	0.5 ± 0.1b	0.9 ± 0.1c	2.1 ± 0.1a	0.5 ± 0.1b	0.9 ± 0.1c	1.8 ± 0.1a	0.4 ± 0.0b	0.8 ± 0.1c
2	2.5 ± 0.1a	2.0 ± 0.1b	3.0 ± 0.2c	5.5 ± 0.1a	2.6 ± 0.2b	3.6 ± 0.2c	5.3 ± 0.1a	2.4 ± 0.1b	3.4 ± 0.1c	4.8 ± 0.2a	2.0 ± 0.1b	3.0 ± 0.1c	4.2 ± 0.1a	1.8 ± 0.1b	2.8 ± 0.2c
4	3.5 ± 0.1a	2.2 ± 0.1b	4.7 ± 0.2c	5.7 ± 0.2a	3.4 ± 0.1b	5.9 ± 0.3a	7.4 ± 0.2a	5.2 ± 0.2b	7.7 ± 0.4a	6.7 ± 0.3a	6.0 ± 0.1a	8.4 ± 0.3b	5.6 ± 0.3a	5.3 ± 0.2a	7.7 ± 0.3b
6	3.3 ± 0.1a	2.3 ± 0.1b	5.5 ± 0.3c	6.0 ± 0.3a	3.5 ± 0.2b	6.7 ± 0.3c	8.4 ± 0.5a	6.9 ± 0.3b	10.1 ± 0.5c	7.4 ± 0.2a	8.7 ± 0.4b	12.0 ± 0.5c	6.2 ± 0.2a	8.4 ± 0.2b	11.6 ± 0.4c
8	4.1 ± 0.2a	2.6 ± 0.2b	6.0 ± 0.4c	6.3 ± 0.1a	3.5 ± 0.1b	7.2 ± 0.4c	9.3 ± 0.4a	7.2 ± 0.3b	10.9 ± 0.7c	8.1 ± 0.2a	10.5 ± 0.3b	14.1 ± 0.6c	6.6 ± 0.3a	10.0 ± 0.5b	13.6 ± 0.8c

M biomass yield under mixotrophic conditions, *H* biomass yield under heterotrophic, *H* + *P* sum of biomass yield under heterotrophic and photoautotrophic (0 g L^{-1} glucose) conditions

Biomass yield (day_x) = [$Y(day_x) - Y(day_0)$], x indicates day number while Y indicates biomass concentration; data were calculated from Fig. 4. Values in each row within the same glucose concentration followed by different letters are significantly different ($P < 0.05$), based on one-way analysis of variance and Tukey's honest significant difference test. Data are mean ± SD ($n = 3$)

cultivation, continuous cultivation, and continuous cultivation with cell recycling (Liu et al. 2014a), can be applied to *M. inermum* JL1 for achieving much higher yield and productivity. However, glucose is used as the carbon source for growth, adding cost to heterotrophic production. To bring down the production cost, cheaper sugar sources such as molasses are potential substitutes for glucose (Liu et al. 2012b), which is worthy of future exploration for *M. inermum* JL1. Another issue associated with heterotrophic production is the low conversion efficiency of glucose to biomass, which was below 40% for *M. inermum* JL1 in our case (Fig. 4) and much lower than the theoretical threshold as suggested by Xiong et al. (2010). Thus, increase the conversion efficiency has the potential to bring down the heterotrophic production cost of this alga.

In addition to lipid productivity (Griffiths and Harrison 2009), fatty acid profile is of importance for evaluating the potential of an alga for biodiesel production because it determines the key properties of biodiesel (Knothe 2009). Among the fatty acids, C18:1 is believed to be beneficial to biodiesel quality: the higher the C18:1 percentage, the better the biodiesel quality. *Micractinium inermum* JL1 is

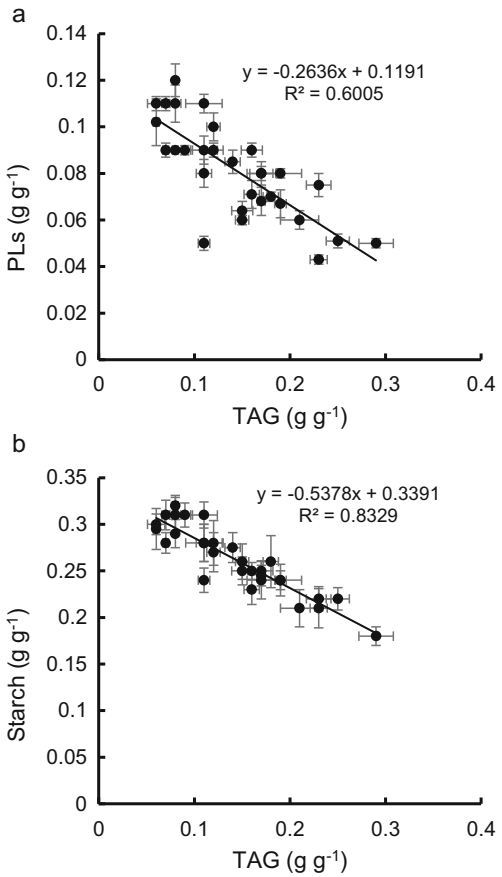

Fig. 5 Plotting of TAG and PLs (**a**) and of TAG and starch (**b**) in *M. inermum* JL1 under different culture conditions. The data used for plotting are from the 8-day cultures in Figs. 2, 3, 4. Data are expressed as mean ± SD ($n = 3$). PLs = polar lipids

Table 3 The production of biomass and lipids by *M. inermum* JL1 under the conditions of 50 mg L^{-1} N, 7.13 mg L^{-1} P, 160 μmol photons m^{-2} s^{-1}, and 0.1 M salt

Culture time (day)	Content (g g^{-1})		Productivity (g L^{-1} day^{-1})[a]		
	Total lipids	TAG	Biomass	Total lipids	TAG
1	0.23 ± 0.01a	0.05 ± 0.00a	0.42 ± 0.01a	0.10 ± 0.00a	0.02 ± 0.00a
2	0.31 ± 0.02b	0.09 ± 0.01b	0.59 ± 0.03b	0.17 ± 0.01b	0.05 ± 0.00b
3	0.40 ± 0.02c	0.14 ± 0.01c	0.75 ± 0.04c	0.25 ± 0.01c	0.08 ± 0.01c
4	0.45 ± 0.02cd	0.16 ± 0.01cd	0.75 ± 0.03c	0.27 ± 0.02c	0.09 ± 0.01c
6	0.49 ± 0.01d	0.19 ± 0.01e	0.53 ± 0.02d	0.20 ± 0.01b	0.07 ± 0.00c
8	0.45 ± 0.02cd	0.18 ± 0.02de	0.41 ± 0.01a	0.17 ± 0.01b	0.05 ± 0.00b

Values in each column followed by different letters are significantly different ($p < 0.05$), based on one-way analysis of variance and Tukey's honest significant difference test

[a] Productivity (day_x) = [$Y(day_x) - Y(day_0)$]/x, x indicates day number while Y indicates biomass, total lipids, or TAG. Data are mean ± SD ($n = 3$)

capable of accumulating C18:1 up to 50%, indicative of its potential as a producer for high-quality biodiesel. Moreover, the relationship between lipid content and C18:1 percentage was examined by plotting the values under different culture conditions (Figs. 2, 3, 4). Obviously, there was a good positive correlation between C18:1 percentage and lipid content ($R^2 = 0.87$) (Fig. 6a). Among the algal lipids, TAG, the most energy-dense lipid class, is considered as an ideal precursor for making biofuels (Hu et al. 2008). When plotting TAG content and C18:1 percentage, a better positive correlation ($R^2 = 0.92$) was observed (Fig. 6b). These results suggest that C18:1 percentage could be used for calculating lipid content in *M. inermum* JL1 as long as a standard curve is established. Thus, the fatty acid determination by GC-MS has dual effect, quantifying fatty acid composition and at the same time lipid content based on the correlation between C18:1 percentage and lipid content. This helps saving time and labor as the analysis of lipids particular TAG requires time-consuming procedures such as lipid extraction, TLC separation, recovery, and transesterification while C18:1 analysis can be done only by direct biomass transesterification prior to GC-MS.

In conclusion, *M. inermum* JL1 can grow robustly and accumulate substantial amount of lipids including TAG. The alga could accumulate ~52% lipids per dry weight and achieved a high lipid productivity of 0.68 g L^{-1} day^{-1}, higher than or comparable to previous reports. The C18:1 percentage, a biodiesel quality indicator, reached ~50% and correlated well with both total lipids and TAG contents in a positive manner, demonstrating the feasibility of using C18:1 percentage for fast lipid quantification. Our results help understand

Table 4 Lipid production of *M. inermum* JL1 in comparison with previously reported green algal strains

Algal strain	Biomass productivity (g L^{-1} day^{-1})	Lipid content (% DW)	Lipid productivity (mg L^{-1} day^{-1})	References
M. inermum JL1	0.75	45.0	270	This study
	1.16[a]	41.0[a]	480[a]	
	1.30[b]	52.0[b]	680[b]	
M. inermum NLP-F014	0.84	27.5	115	Park et al. 2018
Chlorella zofingiensis	1.04[c]	45.5[c]	473[c]	Liu et al. 2016a
Chlorella protothecoides	0.57	48.3	280	Sun et al. 2015
Chlorella sp.	0.5	50.8	250	Guccione et al. 2014
Chlorella vulgaris	0.49	35.4	170	Breuer et al. 2012
Chorella vulgaris	1.05	57.3	604	Pribyl et al. 2012
Chlorella zofingiensis	0.67	44.8	301	Breuer et al. 2012
Chlorella protothecoides	0.41	16.8	69	Sirisansaneeyakul et al. 2011

Unless otherwise indicated, the data were obtained from photoautotrophic cultures

[a] Data were from 8-day mixotrophic cultures in Fig. 4

[b] Data were from 8-day heterotrophic cultures in Fig. 4

[c] Data were from semi-continuous cultures

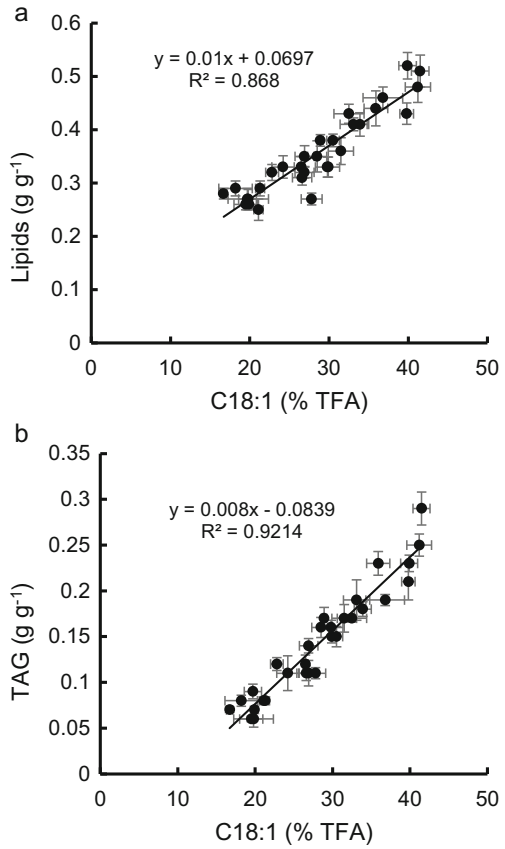

Fig. 6 Plotting of C18:1 abundance and lipid content (**a**) and of C18:1 abundance and TAG content (**b**) in *M. inermum* JL1 under different culture conditions. The data used for plotting are from the 8-day cultures in Figs. 2, 3, 4. Data are expressed as mean ± SD ($n = 3$)

the growth and production properties of *M. inermum* JL1 and provide insights into future exploration of this alga for biodiesel production.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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