Screening of *Isochrysis* strains and utilization of a two-stage outdoor cultivation strategy for algal biomass and lipid production

Zheng Sun [a,b,c], Hehong Wei [d], Zhi-gang Zhou [a,b,c], Muthupandian Ashokkumar [e], Jin Liu [d*]

[a] *International Research Center for Marine Biosciences, Ministry of Science and Technology, Shanghai Ocean University, Shanghai, 201306 China*

[b] *Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources, Ministry of Education, Shanghai Ocean University, Shanghai, 201306 China*

[c] *National Demonstration Center for Experimental Fisheries Science Education, Shanghai Ocean University, Shanghai, 201306 China*

[d] *Institute for Food and Bioresource Engineering, Department of Energy and Resources Engineering and BIC-ESAT, College of Engineering, Peking University, Beijing 100871, China*

[e] *School of Chemistry, University of Melbourne, VIC 3010, Australia*

* To whom corresponding should be addressed:

Tel.: 86-10-62766391; E-mail: gjinliu@pku.edu.cn

Abstract

Isochrysis is genus of marine algae without cell wall and capable of accumulating lipids. In this study, the lipid production potential of *Isochrysis* was assessed by comparing fifteen *Isochrysis* strains with respect to their growth rate, lipid production and fatty acid profiles. Three best strains were selected (lipid productivity: 103.0~121.7 mg L^{-1} day^{-1}) and their lipid-producing capacities were further examined under different controlled parameters, *e.g.*, growth phase, medium nutrient and light intensity in laboratory cultures. Furthermore, the three *Isochrysis* strains were monitored in outdoor panel photobioreactors with various initial cell densities and optical paths, and the strain CS177 demonstrated the superior potential for outdoor cultivation. A two-stage semi-continuous strategy for CS177 was subsequently developed, where high productivities of biomass (1.1 g L^{-1} day^{-1}) and lipid (0.35 g L^{-1} day^{-1}) were achieved. This is a comprehensive study to evaluate the lipid-producing capability of *Isochrysis* strains under both indoor and outdoor conditions. Results of the present work lay a solid foundation for the physiological and biochemical responses of *Isochrysis* to various conditions, shedding light on the future utilization of this cell wall-lacking marine alga for biofuel production.

Key Words: *Isochrysis*, Screening, Biofuel feedstock, Two-stage outdoor cultivation

HIGHLIGHTS

- Fifteen *Isochrysis* strains were screened for lipid production
- Key biological and engineering parameters were optimized for indoor /outdoor cultures
- A two-stage semi-continuous strategy was developed
- High-quality oil production and robustness in outdoor cultivation were demonstrated

Introduction

The ever-increasing consumption of energy worldwide and the concerns over global warming call for alternatives to fossil fuels. The exploitation of the available sources for biodiesel started from the food crops to woody crops, agricultural residues or waste, and now has come to microalgae Compared with higher plants, microalgae grow much faster and are able to produce a great variety of lipids [1]. Furthermore, microalgae can be adapted to grow in a broad range of environmental conditions, so that they do not compete with food for arable lands [2]. As such, microalgae have been hailed as the solution to the global energy crisis, though many challenges remain to be addressed.

To move the algal biodiesel from promise to reality, the selection of appropriate algal strains is the first and most fundamental step. Through literature search, the green algae (*Chlorophyta*) and diatoms (*Bacillariophyta*) have been the most commonly-studied ones regarding the lipid-producing capability [1, 3-5]. The present work is focused on *Isochrysis*, a genus of marine flagellated algae. There are several reasons that *Isochrysis* could be a promising biodiesel feedstock: 1) it belongs to marine algae and is suitable for growing with seawater, thus avoiding the competition for freshwater; 2) it has a fast growth rate for biomass production [6]; 3) *Isochrysis* has no cell walls, so that the oil extraction from *Isochrysis* is much easier than other algae, which can vastly enhance the economics of down-stream processing for biodiesel production; 4) it has high concentrations of docosahexaencic acid (DHA, 22: 6n-3), a valuable omega-3 polyunsaturated fatty acid with significant nutritional and

healthy benefits [7, 8]. . Therefore, it is highly possible to develop the integrated production of oil and value-added products from *Isochrysis*, which is crucial for the commercialization of microalgae-based biodiesel; 5) from a biorefinery point of view, the residual biomass after oil extraction can be used as feeds for aquaculture applications [9].

The main objective of this study is to select a high-performance *Isochrysis* strain and optimize several key biological and engineering parameters for the improved lipid production. Fifteen *Isochrysis* strains were used for comparative analysis with respect to their growth, lipid content, lipid productivity and fatty acid profile. The best strain CS177, which showed the highest lipid productivity and most suitable fatty acid composition, was investigated by subjecting to several nutritional and environmental factors. Its biomass and oil production potential was further evaluated in outdoor panel photobioreactors (PBRs) by manipulating the initial cell density and length of optical path. Furthermore, a two-stage semi-continuous cultivation strategy, which is suitable for outdoor algal growth under sunlight, was developed to promote the biomass and lipid production. The biomass and lipid productivities achieved are higher or comparable to previous reports of algal strains, shedding light on the further exploration of this cell wall-lacking marine alga for lipid production in pilot-scale PBRs or open ponds.

Materials and methods

Isochrysis strains and maintenance

Fifteen *Isochrysis* strains were used in the present study, including CCMP 355, 463, 715, 1244, 1324, 1611 from Provosoli-Guillard National Center for Culture of Marine Phytoplankton, UTEX 987, 1292, 2307 from the Culture Collection of Algae at the University of Texas, CCAP 927/1, 927/12, 927/14 from Culture Collection of Algae and Protozoa, CS 177, 254 from Australian National Algae Culture Collection and LABI 1100 maintained at Peking University, China. All strains were maintained in 250-mL Erlenmeyer flasks containing 100 mL F/2 medium and 25 g L^{-1} sea salt (Central Aquatics, Franklin, WI, USA). Cultures were kept at 22°C with continuous illumination of a low light (20 μmol m^{-2} s^{-1}). Cultures were shaken by hand once a day.

Strain screening in 100-mL bubble column photobioreactors

Algal cultures in flasks were inoculated into column photobioreactors (PBRs, internal diameter = 3.0 cm) containing 100 mL modified F/2 medium (200 mg L^{-1} N and 9 mg L^{-1} P) and grew at 22°C aerated with 1.5% CO_2 enriched air (compressed air and CO_2 are mixed at a ratio of 100:1.5) and illuminated with continuous light of 60 μmol m^{-2} s^{-1}. The cultures in late exponential growth phase were inoculated into new column PBRs with a starting cell density of 0.1 g L^{-1}, cultured to late exponential growth phase, and harvested for the analysis of growth and lipids.

CCMP1244, CCMP1324 and CS177 exhibited the comparably high growth and lipid productivity and were therefore selected for further study. Their growth and lipid production were investigated in response to different growth phases, nitrate and phosphate concentrations, and light intensities. Unless otherwise stated, the basal

130 culture conditions were the same as those used for strain screening (*i.e.*, 200 mg L^{-1} N,
131 9 mg L^{-1} P, light intensity of 60 μmol m^{-2} s^{-1}, salinity of 25 g L^{-1}).

132 **Outdoor cultures in flat-plate PBRs**

133 Cultures of the three strains were conducted in the outdoor panel PBRs arranged in a
134 South-North orientation in summer in Shanghai, China (latitude 31° 140' N, longitude
135 121° 290' E). The PBRs are 140-cm high and 120-cm long. Compressed air was
136 bubbled through a perforated plastic tube running at the bottom of the reactors to mix
137 algal suspension. A stainless iron tube-based thermo-exchanger was placed in each
138 PBR to prevent the culture temperature from exceeding 30°C. During the night, the
139 cooling system was turned off to allow the cultures to follow the ambient temperature.
140 Seed cultures were maintained in 20-L indoor panel PBRs with continuous
141 illumination of 100 μmol m^{-2} s^{-1} to late exponential growth phase and inoculated into
142 outdoor panel PBRs at 6:00 PM (consider this day as day 0). Cell samples were
143 collected every day at 6:00 PM for analyses.

144 CS177 showed the best growth performance and was further investigated. To optimize
145 the outdoor growth and lipid production, PBRs with various internal widths being 1.8,
146 3.5, and 7.0 cm (corresponding to the optical path lengths of the reactor) were
147 employed. The CS177 seeds were inoculated either at the same initial volumetric cell
148 density (0.6 g L^{-1}) or at the same initial areal cell density (21 g m^{-2}).

149 Semi-continuous culture experiments were also performed. Cells were first inoculated
150 in a 7.0-cm panel PBR with a low cell density of 0.3 g L^{-1} and allowed to grow for 2
151 days to reach a high cell density of ~1.2 g L^{-1}, which were then transferred to a 1.8-cm

PBR for rapid biomass and lipid production. Semi-continuous cultivation was deployed in the thin PBR, with a harvest of 3/4 cultures per 3 days.

Growth measurements

The optical density (OD) of culture was measured at 750 nm with a 1.5 cm light path cuvette in a HACH DR 2700 spectrophotometer. Culture suspension (5-10 mL) was filtered through a pre-dried Whatman GF/C filter paper (1.2 μm pore size) and washed twice with 10 mL 0.5 M NH_4HCO_3. Cells on the filter paper discs were dried at 100°C in an oven until constant weight and were subsequently cooled to room temperature in a desiccator before weighing. Samples were ashed at 500°C for 2 hours in a muffle furnace to obtain ash-free dry weight (AFDW). Biomass productivity was calculated on an AFDW basis.

Nitrate and phosphate measurements

Algal suspensions were centrifuged at 3,800 g for 5 min and the supernatants were collected to measure the residual nitrate-N and phosphate-P by using a Quickchem 8500 (Lachat, Loveland, Colorado, USA) according to the instructions provided by the manufacturer.

Lipid and fatty acid analyses

Cells were centrifuged at 3,800 g for 5 min. The pellet was lyophilized for fatty acid analysis. Lipids were extracted from lyophilized algal samples with a solvent mixture of chloroform, methanol and water (2:1:0.75, by volume) according to a modified Folch procedure [10]. The extracts were dried under nitrogen gas, and then weighed. Dry lipid extracts were re-suspended in chloroform for immediate use or stored at

-20°C under nitrogen for later use.

Fatty acid methyl esters (FAMEs) were prepared by direct transmethylation of lipids with sulphuric acid in methanol [10]. The FAMES were separated by GC-MS using the Agilent 7890 capillary gas chromatograph equipped with a 5975 C mass spectrometry detector and a HP-88 capillary column (60 m x 0.25 mm) (Agilent Technologies). 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). DHA in samples was quantified by using DHA standard (Sigma, St. Louis, MO, USA).

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 Ramos et al. [11] and Hoekman et al. [12].

Statistical analyses

All data were obtained by using at least two 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 $p < 0.05$.

196

197 Results and discussion

198 Screening of *Isochrysis* strains for lipid production

199 Fifteen *Isochrysis* strains were screened with respect to cell density, lipid content and
200 lipid productivity. As indicated by Fig. 1A, the cell density ranged from 0.65 to 3.99 g
201 L^{-1}, suggesting that the growth potential of *Isochrysis* is strain-dependent. The three
202 fastest growing strains were CCMP1324, CCMP1244 and CS177, whose cell
203 densities within a 10-day cultivation reached 3.51, 3.69 and 3.99 g L^{-1}, respectively.
204 As for the biomass productivities, the three were all above 0.3 g L^{-1} day^{-1}. This is
205 superior to a vast number of microalgae, as the biomass productivity of many algal
206 strains lie between 0.1 and 0.3 g L^{-1} day^{-1} [13]. The lipid content of *Isochrysis* varied
207 across strains in the range of 19.8-30.5% of dry weight, and CS177 accumulated the
208 highest level of lipids (30.5%, Fig. 1B). High lipid productivity is another desirable
209 characteristic of an algal strain for biodiesel production. Green microalgae usually
210 produce large amounts of lipids within a relatively short period, whose average lipid
211 productivity was about 70 mg L^{-1} day^{-1}, much better than that of other algae (~30 mg
212 L^{-1} day^{-1}) [13]. In the present study, the lipid productivity of several *Isochrysis* strains
213 has exceeded this value, with the highest (CS177) being 122 mg $L^{-1}day^{-1}$ (Fig. 1C).
214 The characteristics of fatty acids of *Isochrysis* strains were also examined, because
215 they determine, to a great extent, the key properties of biodiesel. As indicated by
216 Table 1, the fatty acid composition among the fifteen strains was quite similar, mainly
217 consisting of myristic acid (C14:0), palmitic acid (C16:0), oleic acid (C18:1),

218 stearidonic acid (C18:4) and docosahexaenoic acid (C22:6). On the other hand, the
219 level of individual fatty acids varied greatly, for example, C14:0 ranging from 9.3 to
220 30.8%, C18:1 from 21.5 to 34.0%, and C22:6 from 5.3 to 17.7% of total fatty acids.
221 Compared with saturated fatty esters, the unsaturated ones have sound
222 low-temperature properties to prevent the solidification of oil; on the other hand, their
223 oxidative stability is much poorer [14]. To reach a compromise between oxidative
224 stability and cold-flow properties, a high proportion of oleic acid (C18: 1) ester is
225 preferred [15]. In the present study, all *Isochrysis* strains contained a high level of
226 C18:1 (~20-30%), indicating they are the suitable feedstock for biodiesel.
227 [Table 1 near here]
228 We further evaluated the biodiesel properties of *Isochrysis*-derived oils, including
229 kinematic viscosity, specific gravity, cloud point, cetane number, iodine value and
230 higher heating value. As shown in Table 2, the oils from most *Isochrysis* strains meet
231 the specification established by US (ASTM D6751) and Europe (EN 14214) standards.
232 Based on these results, three strains demonstrating the highest potential for oil
233 production, namely CCMP1244, CCMP1324 and CS177 were selected for further
234 investigation.
235 [Table 2 near here]
236 **Lipid and fatty acid profiles of *Isochrysis* strains as affected by growth phases**
237 During algal cultivation, the accumulation of metabolites may vary greatly depending
238 upon the growth phases of the culture. In the present study, the lipid contents and fatty
239 acid profiles of the three *Isochrysis* strains were analyzed under different growth

phases, i.e. early exponential (EE), late exponential (LE), and late stationary (LS). As shown in Fig. 2A, all three strains showed just a slight increase of total lipids when cells entered stationary growth phase, and this observation is consistent with previous reports on *Isochrysis* [6]. The total fatty acid (TFA) profile, on the other hand, showed distinct patterns among the three strains (Fig. 2B-D). In the EE stage, CS177 exhibited the highest relative abundance of C14:0 (37.2%) but the lowest C22:6 (5.1%). When cultured to LE and LS stages, all three strains showed a considerable decrease in C14:0 with a concomitant increase in C18:1.

Growth and lipid production as affected by medium nutrients

Nitrogen (N) is the second main component of algal biomass after carbon. As an essential macronutrient for algae, the concentration of N greatly influences the intracellular lipid accumulation. N limitation /starvation is usually associated with the enhanced synthesis of lipids, in particular neutral lipids [16, 17]. It could be because that when the nitrogen gets limited or exhausted, carbon uptake continues and will be consequently accumulated within algal cells as lipids. On the other hand, a low concentration of N limits the algal growth. Therefore, the optimization of N contents is of great importance to maximize the lipid accumulation whilst maintaining a proper algal growth. In the present study, four concentrations of nitrate-N (25 - 200 mg L^{-1}) were tested. As shown in Fig. 3A, when the initial N concentration ranged from 25 to 100 mg L^{-1}, all three strains could efficiently utilize nitrate-N with a closely complete consumption at the end of culture period (Fig. 3A). The final cell density was positively dependent on the initial N concentration and reached the maximum with an

initial N concentration of 100 mg L^{-1} (Fig. 3B). Further increase in N concentration to 200 mg L^{-1} gave no beneficial effect on cell density (Fig. 3B) and there were substantial amounts of unconsumed N (Fig. 3A), indicating that other factors than N became limiting in the batch cultures. The lipid content of *Isochrysis* strains showed a slight difference in response to the initial N concentration and was promoted slightly by lower N concentrations (Fig. 3C), differing a lot from other algae such as *Chlorella* and *Nannochloropsis* strains in which the lipid content was influenced considerably by N limitation /starvation [18, 19]. This may be explained by the fact that *Isochrysis* strains utilize the recycled membrane lipids rather than the *de novo* synthesized fatty acyls for neutral lipid synthesis upon N stress [20]. Similarly, the lipid productivity of *Isochrysis* was in a N concentration-dependent manner, which reached the maximum at 100-200 mg L^{-1} N (Fig. 3D). Given the huge diversity of microalgae, nitrogen limitation may not always be linked to lipid accumulation. For example, the diatoms *Achnanthes brevipes* and *Tetraselmis* spp. accumulate carbohydrates rather than lipids upon nitrogen starvation [21, 22], and their enhanced lipid synthesis was found to be associated with silicate limitation [23].

Phosphorus (P) is another important nutritional factor that is involved in the energy transfer of the algal cells as well as in the syntheses of phospholipids and nucleic acids. According to previous reports, the algal response to the P starvation is species-dependent [24, 25]. In this study, we found higher P concentrations promoted biomass accumulation within the tested P concentrations (Fig. 3F), yet the effect was less prominent compared with N concentrations (Fig. 3B), suggesting that N is more

284 critical for algal growth than P. Similar to N, P concentrations just influenced the lipid
285 content slightly (Fig. 3G). Therefore, the lipid productivity of *Isochrysis* was less
286 affected by P than N and reached the highest value with 4.5-9.0 mg L^{-1} P (Fig. 3H).

287 **Growth and lipid production as affected by light intensities**

288 Aside from the cultivation medium composition, light intensity represents a critical
289 environmental factor to influence the algal growth and lipid synthesis. Four light
290 intensities including 30, 60, 120 and 240 μmol m^{-2} s^{-1} were tested (Fig. 4). When the
291 light intensity increased from 30 to 60 μmol m^{-2} s^{-1}, a typical light-dependent growth
292 was observed in batch cultures of *Isochrysis* strains leading to the enhanced cell
293 density (Fig. 4A). With a further increase in light intensity to 120 μmol m^{-2} s^{-1}, no
294 apparent impact on cell density (Fig. 4A) was observed, indicating the light saturation
295 might be reached. A further increase to 240 μmol m^{-2} s^{-1} led to reduced amounts of
296 algal cells, suggesting the photoinhibition has occurred which results in impaired algal
297 growth and considerably attenuated cell density (Fig. 4A). Light intensity exhibited
298 slight impact on lipid content (Fig. 4B), similar to N and P concentrations (Fig. 3C
299 and 3G). Accordingly, the optimal lipid production of *Isochrysis* strains was achieved
300 with light intensity of 60-120 μmol m^{-2} s^{-1} under the tested laboratory cultivation
301 conditions (Fig. 4C).

302 **Lipid production in outdoor panel PBRs**

303 Since the outdoor culture conditions are much less controlled than the laboratory
304 cultures, some algal strains with satisfactory indoor growth may not be suitable for
305 outdoor cultivation. In this regard, the outdoor growth performance of the three

306 *Isochrysis* strains were evaluated in panel PBRs, which offer a large surface
307 area-to-volume ratio for efficient light utilization and biomass production [26]. As
308 shown in Table 3, the biomass and lipid productivity of CS177 were significantly
309 higher than other two, indicating its superior potential for the outdoor cultivation.
310 [Table 3 near here]
311 According to indoor results, CS177 suffered photoinhibition and growth deterioration
312 when the light intensity reached 240 μmol m^{-2} s^{-1}. This imposes a challenge for the
313 outdoor growth of CS177 because the peak solar irradiation can reach as high as
314 2,500 μmol m^{-2} s^{-1} in summer, which may cause severe photosynthesis inhibition or
315 even photooxidative damage to the algal cells. To avoid the ill-effect of strong solar
316 irradiance and achieve a high efficiency of conversion of light energy, the adjustment
317 of the optical path of PBRs is a feasible solution. As a key parameter of engineering
318 design, the length of optical path has a remarkable effect on the production of cell
319 mass as well as algae-derived metabolites [27-29]. In this study, three light paths of
320 1.8, 3.5, and 7.0 cm were examined in the panel PBRs with an initial inoculation cell
321 density of 0.6 g L^{-1}, corresponding to the areal cell densities of 10.8, 21.0 and 42.0 g
322 m^{-2} (vertical surface), respectively. CS177 in the 1.8-cm PBR gave the lowest
323 volumetric biomass productivity (0.27 g L^{-1} day^{-1}, Table 4 and Fig. 5A) and lowest
324 areal biomass productivity (5.2 g m^{-2} day^{-1}). The poor productivities might arise from
325 photoinhibition as the cultures were subjected to the highest per cell light intensity. In
326 contrast, the highest volumetric biomass productivity (0.57 g L^{-1} day^{-1}) was achieved
327 in the 3.5-cm panel PBR while the highest areal biomass productivity (29.9 g m^{-2}

328 day^{-1}) was achieved in the 7.0-cm PBR (Table 4 and Fig. 5A). Similarly, the highest
329 volumetric lipid productivity (0.18 g L^{-1} day^{-1}) was achieved in the 3.5-cm PBR,
330 whereas the highest areal lipid productivity (9.0 g m^{-2} day^{-1}) was obtained in the
331 7.0-cm PBR (Table 4).
332 The highest areal biomass and lipid productivities obtained in the 7.0-cm PBR might
333 be due to the longest PBR light path or the highest starting areal cell density. To
334 confirm this, an identical areal cell density of 21.0 g m^{-2} was introduced to 1.8, 3.5,
335 and 7.0-cm panel PBRs. As indicated by Table 4, the biomass and lipid productivities
336 were positively dependent on the light path length of panel PBRs and reached the
337 highest values (30.9 g m^{-2} day^{-1} and 8.9 g m^{-2} day^{-1}, respectively) in the 7.0-cm panel
338 PBR. However, from a volumetric productivity standpoint, the biomass and lipid
339 productivities were negatively affected by the length of panel PBRs and reached their
340 maximum (0.88 and 0.28 g L^{-1} day^{-1}, respectively) in the 1.8 cm-PBR (Table 4 and
341 Fig. 5B).
342
343 [Table 4 near here]
344 **Enhanced lipid production by a two-stage semi-continuous culture strategy**
345 The biomass productivity may vary depending upon growth phases of the culture. As
346 indicated by the batch culture in Fig. 5, the maximum biomass productivity occurred
347 on days 2-4 regardless of the optical path length of PBRs and initial cell densities
348 tested. In order to avoid the ill-effect of solar light and sustain maximum lipid
349 production, a two-stage semi-continuous culture strategy was developed. A seed

culture was firstly prepared in stage I. Since the cell density of initial inoculation should not be too high, cells were firstly inoculated at a low concentration of 0.3 g L^{-1} in a 7.0-cm panel PBR. A high cell density (~ 1.2 g L^{-1}) was reached after growing for 2 days, which served as the seed culture. In stage II, the seed culture was subsequently transferred to a 1.8-cm panel PBR in a semi-continuous mode for rapid biomass and lipid production. As shown in Fig. 6, the semi-continuous cultures sustained a stable production of biomass and lipid. The daily productivities of biomass and lipid were maintained at ca. 1.1 and 0.35 g L^{-1} day^{-1}, much higher than that in batch cultures.

Finally, the overall performance of *Isochrysis* CS177 was compared with some previously reported algae: not only from *Isochrysis*, but also from *Chlorella*, a genus known as a good candidate for oil production in biodiesel application. As shown in Table 5, the lipid-producing capacity of *Isochrysis* CS177 was comparable to or higher than many of these strains.

[Table 5 near here]

Conclusions

In this study, the biomass accumulation, lipid production and fatty acid profiles of fifteen *Isochrysis* strains were comprehensively evaluated. CS177 showed the best overall performance, from which the oil derived meets the specification established by U.S. and European standards. Considering its rapid growth, robustness in outdoor cultivation, lack of cell wall and the ability to accumulate value-added products, CS177 has the potential to be a promising candidate for oil production in biodiesel

application .

Abbreviations:

AFDW: ash-free dry weight; DHA: docosahexaencic acid; EE: early exponential; FAMEs: fatty acid methyl esters; LE: late exponential; LS: late stationary; N: nitrogen; P: phosphorus; PBR: photobioreactor; TFA: total fatty acid.

Funding:

This work was financially supported by ‘Young Eastern Scholar Program’ at Shanghai Institutions of Higher Learning (QD2015047), a start-up grant from the National Youth Thousand Talents Program, National Natural Science Foundation of China (31501493) and the Special Project of Marine Renewable Energy from the State Oceanic Administration (SHME2011SW02).

Consent for publication:

All authors approved the manuscript.

Conflict of Interest:

The authors indicate no potential conflicts of interest.

References

1. Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., & Darzins, A. (2008). Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *Plant Journal*, *54*, 621-639.
2. Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnology Advances*, *25*, 294-306.
3. Liu, J., & Hu, Q. (2013). *Chlorella*-Industrial production of biomass and chemicals. In: A. Richmond, Q Hu (Ed.), *Handbook of microalgal cultures* (2nd ed., pp. 327-338). New Jersey: Wiley Blackwell.
4. Li, Y., Horsman, M., Wu, N., Lan, C. Q., & Dubois-Calero, N. (2008). Biofuels from microalgae. *Biotechnology Progress*, *24*, 815-820.
5. Rodolfi, L., Zittelli, G. C., Bassi, N., Padovani, G., Biondi, N., Bonini, G., & Tredici, M. R. (2009). Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnology and Bioengineering*, *102*, 100-112.
6. Liu, J., Sommerfeld, M., & Hu, Q. (2013). Screening and characterization of *Isochrysis* strains and optimization of culture conditions for docosahexaenoic acid production. *Applied Microbiology and Biotechnology*, *97*, 4785-4798.
7. Devos, M., Poisson, L., Ergan, F., & Pencreac'h, G. (2006). Enzymatic hydrolysis of phospholipids from *Isochrysis galbana* for docosahexaenoic acid enrichment. *Enzyme and Microbial Technology*, *39*, 548-554.
8. Sun, Z., Chen, Y., Mao, X., & Liu, J. (2017). Physiological and biochemical

changes reveal differential patterns of docosahexaenoic acid partitioning in two marine algal strains of *Isochrysis*. *Marine Drugs*, *15*, 357.

9. Williams, P. J. B., & Laurens, L. M. L. (2010). Microalgae as biodiesel & biomass feedstocks: Review & analysis of the biochemistry, energetics & economics. *Energy and Environmental Science*, *3*, 554-590.

10. Christie, W. W. (2003). *Lipid analysis: isolation, separation, identification and structural analysis of lipids*. 3rd ed. Bridgwater: The Oily Press.

11. Ramos, M. J., Fernandez, C. M., Casas, A., Rodriguez, L., & Perez, A. (2009). Influence of fatty acid composition of raw materials on biodiesel properties. *Bioresource Technology*, *100*, 261-268.

12. Hoekmana, S. K., Broch, A., Robbins, C., Ceniceros, E., & Natarajan, M. (2012). Review of biodiesel composition, properties and specifications, *Renewable and Sustainable Energy Reviews*, *16*, 143-169.

13. Sun, Z., Liu, J., & Zhou, Z. (2016). Algae for biofuels: an emerging feedstock. In: R. Luque (Ed.), *Handbook of biofuels' production: processes and technologies*. (2nd ed., pp. 673-698). Cambridge: Woodhead Publishing, Elsevier.

14. Knothe, G. (2008). "Designer" biodiesel: optimizing fatty ester composition to improve fuel properties. *Energy & Fuels*, *22*, 1358-1364.

15. Knothe, G. (2009). Improving biodiesel fuel properties by modifying fatty ester composition. *Energy & Environmental Science*, *2*, 759-766.

16. Hsieh, C. H., & Wu, W. T. (2009). Cultivation of microalgae for oil production with a cultivation strategy of urea limitation. *Bioresource Technology*, *100*,

438 3921-3926.
439 17. Lacour, T., Sciandra, A., Talec, A., Mayzaud, P., & Bernard, O. (2012). Neutral
440 lipid and carbohydrate productivities as a response to nitrogen starus in *Isochrysis*
441 sp. (T-ISO; *Haptophyceae*): starvation versus limitation. *Journal of Phycology*, *48*,
442 647-656.
443 18. Converti, A., Casazza, A. A., Ortiz, E. Y., Perego, P., & Borghi, M. D. (2009).
444 Effect of temperature and nitrogen concentration on the growth and lipid content of
445 *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production.
446 *Chemical Engineering and Processing*, *48*, 1146-1151.
447 19. Sun, Z., Zhou, Z., Gerken, H., Chen, F., & Liu, J. (2015). Screening and
448 characterization of oleaginous *Chlorella* strains and exploration of *Chlorella*
449 *protothecoides* for oil production. *Bioresource Technology*, *184*, 53-62.
450 20. Liu, J., Sun, Z., & Chen, F. (2013). Heterotrophic production of microalgal oils.
451 In: A. Pandey, D. J. Lee, Y. Chisti, C. R. Soccol (Ed.), *Biofuels from algae* (pp.
452 111-142). Amsterdam: Elsevier.
453 21. Gladue, R., & Maxey, J. (1994). Microalgal feeds for aquaculture. *Journal of*
454 *Applied Phycology*, *6*, 131-141.
455 22. Guerrini, F., Cangini, M., Boni, L., Trost, P., & Pistocchi, R. (2000). Metabolic
456 responses of the diatom *Achnanthes brevipes (Bacillariophyceae)* to nutrient
457 limitation. *Journal of Phycology*, *36*, 882-890.
458 23. Wen, Z. Y., & Chen, F. (2000). Heterotrophic production of eicosapentaenoid acid
459 by the diatom *Nitzschia laevis*: effects of silicate and glucose. *Journal of Industrial*

460 *Microbiology and Biotechnology*, *25*, 218-224.
461 24. Khozin-Goldberg, I., & Cohen, Z. (2006). The effect of phosphate starvation on
462 the lipid and fatty acid composition of the fresh water eustigmatophyte *Monodus*
463 *subterraneus*. *Phytochemistry*, *67*, 696-701.
464 25. Yongmanitchai, W., & Ward, O. P. (1991). Growth of and omega-3 fatty acid
465 production by *Phaeodactylum tricornutum* under different culture conditions.
466 *Applied and Environmental Microbiology*, *57*, 419-425.
467 26. Ugwu, C. U., Aoyagi, H., & Uchiyama, H. (2008). Photobioreactors for mass
468 cultivation of algae. *Bioresource Technology*, *99*, 4021-4028.
469 27. Hu, Q., & Richmond, A. (1994). Optimizing the population density in *Isochrysis*
470 *galbana* grown outdoors in a glass column photobioreactor. *Journal of Applied*
471 *Phycology*, *6*, 391-396.
472 28. Hu, Q., Guterman, H., & Richmond, A. (1996). A flat inclined modular
473 photobioreactor for outdoor mass cultivation of photoautotrophs. *Biotechnology*
474 *and Bioengineering*, *51*, 51-60.
475 29. Richmond, A. (2003). Biological principles of mass cultivation. In A. Richmond
476 (Ed.), *Handbook of microalgal culture: biotechnology and applied phycology* (pp.
477 125-177). New Jersey: Wiley-Blackwell.
478 30. Molina Grima, E., Sánchez Pérez, J. A., García Sánchez, J. L., García Camacho,
479 F., & López Alonso, D. (1992). EPA from *Isochrysis galbana*. Growth conditions
480 and productivity. *Process Biochemistry*, *27*, 299-305.
481 31. Molina Grima, E., Sánchez Pérez, J. A., García Camacho, F., García Sánchez, J.

L., Acién Fernández, F. G., & López Alonso, D. (1994). Outdoor culture of *Isochrysis galbana* ALII-4 in a closed tubular photobioreactor. *Journal of Biotechnology*, *37*, 159-166.

32. Huerlimann, R., De Nys, R., & Heimann, K. (2010). Growth, lipid content, productivity and fatty acid composition of tropical microalgae for scale-up production. *Biotechnology and Bioengineering*, *107*, 245-257.

33. Feng, D., Chen, Z., Xue, S., & Zhang, W. (2011). Increased lipid production of the marine oleaginous microalgae *Isochrysis zhangjiangensis (Chrysophyta)* by nitrogen supplement. *Bioresource Technology*, *102*, 6710-6716.

34. Moheimani, N. (2013). Long-term outdoor growth and lipid productivity of *Tetraselmis suecica*, *Dunaliella tertiolecta* and *Chlorella* sp. *(Chlorophyta)* in bag photobioreactors. *Journal of Applied Phycology*, *25*, 167-176.

35. Zhou, X., Xia, L., Ge, H., Zhang, D., & Hu, C. (2013). Feasibility of biodiesel production by microalgae *Chlorella* sp. (FACHB-1748) under outdoor conditions. *Bioresource Technology*, *138*, 131-135.

36. Guccione, A., Biondi, N., Sampietro, G., Rodolfi, L., Bassi, N., & Tredici, M. (2014). *Chlorella* for protein and biofuels: from strain selection to outdoor cultivation in a green wall panel photobioreactor. *Biotechnology for Biofuels*, *7*, 84.

37. Breuer, G., Lamers, P. P., Martens, D. E., Draaisma, R. B., & Wijffels, R. H. (2012). The impact of nitrogen starvation on the dynamics of triacylglycerol accumulation in nine microalgae strains. *Bioresource Technology*, *124*, 217-226.

38. Munkel, R., Schmid-Staiger, U., Werner, A., & Hirth, T. (2013). Optimization of

outdoor cultivation in flat panel airlift reactors for lipid production by *Chlorella vulgaris*. *Biotechnology and Bioengineering*, *110*, 2882-2893.

Figure Captions

Fig. 1. The cell density (A), lipid content (B), and lipid productivity (C) of fifteen *Isochrysis* strains. Data were obtained from cells grown for 10 days with continuous illumination of 60 μM $s^{-1}m^{-2}$.

Fig. 2. Lipid content (A) and fatty acid profile of CCMP1244 (B), CCMP1324 (C) and CS177 (D) as affected by growth phase – early exponential (EE, white column), late exponential (LE, grey column) and late stationary (LS, black column).

Fig. 3. Growth and lipid production as affected by various starting concentrations of N (A-D) or P (E-H). The initial N concentrations used were 25, 50, 100 and 200 mg L^{-1}; the initial P concentrations were 1.1, 2.3, 4.5 and 9.0 mg L^{-1}.

Fig. 4. The cell density (A), lipid content (B) and lipid productivity (C) as affected by various light intensities of 30, 60, 120 and 240 μM $s^{-1}m^{-2}$.

Fig. 5. Biomass yield of CS177 in outdoor panel PBRs as affected by various optical paths of 1.8 cm (square), 3.8 cm (circle) and 7.0 cm (triangle). (A): the initial volumetric cell density was fixed at 0.6 g L^{-1}, (B): the initial areal cell density was fixed at 21 g m^{-2}.

Fig. 6. A two-stage culture strategy for enhanced and stable lipid production. (A): cell density, (B) lipid content, (C): biomass /lipid productivity. Cells were first inoculated in a 7.0-cm panel PBR with a low cell density of 0.3 g L^{-1} and allowed to grow for 2 days to reach a high cell density of about 1.2 g L^{-1}, which were then transferred to a 1.8-cm panel PBR for rapid biomass and lipid production. Semi-continuous cultivation was deployed in the thin PBR, with a harvest of ¾ cultures per 3 days.

Table 1 Fatty acid profiles of the fifteen *Isochrysis* strains cultured for 10 days. Data were expressed as percentage of total fatty acids (%).

Strain	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C18:4	C22:0	C22:6
CCMP 355	30.8 ± 1.6	17.9 ± 1.0	1.2 ± 0.1	1.1 ± 0.1	28.1 ± 1.6	2.7 ±0.2	1.6 ±0.1	3.2 ± 0.1	-	8.3 ± 0.5
CCMP 463	9.3 ± 0.5	20.3 ± 0.7	1.5 ± 0.1	2.9 ± 0.2	32.1 ± 1.8	4.0 ±0.2	3.7 ±0.2	12.1 ± 0.5	1.5 ± 0.1	10.6 ± 0.4
CCMP 715	24.2 ± 1.4	16.3 ± 0.5	1.4 ± 0.0	1.8 ± 0.1	28.1 ± 1.3	3.5 ±0.2	1.6 ±0.1	6.5 ±0.3	-	10.5 ± 0.5
CCMP 1244	11.4 ± 0.4	16.3 ± 0.9	2.8 ± 0.2	1.4 ± 0.1	21.5 ± 1.1	2.2 ±0.1	4.1 ± 0.2	19.8 ± 1.1	0.9 ± 0.1	15.5 ± 0.8
CCMP 1324	10.3 ± 0.5	16.8 ± 0.8	1.4 ± 0.1	1.8 ± 0.1	29.2 ± 1.0	4.7 ±0.3	4.2 ± 0.2	15.5 ± 0.8	1.0 ± 0.0	12.2 ± 0.7
CCMP 1611	25.8 ± 1.3	15.4 ± 0.6	0.9 ± 0.0	2.2 ± 0.1	34.0 ± 2.0	5.7 ±0.3	1.7 ± 0.1	4.4 ± 0.2	-	6.1 ± 0.2
UTEX 987	22.3 ± 1.2	14.5 ± 0.3	0.7 ± 0.0	1.1 ± 0.1	28.5 ± 1.2	6.1 ±0.3	6.5 ± 0.3	5.1 ± 0.3	-	9.5 ± 0.5
UTEX 1292	16.6 ± 0.9	14.4 ± 0.8	1.1 ± 0.1	1.4 ± 0.1	31.4 ± 1.6	7.1 ±0.4	3.4 ± 0.2	5.9 ± 0.3	-	11.3 ± 0.6
UTEX 2307	26.4 ± 0.8	15.0 ± 0.7	1.3 ± 0.0	0.8 ± 0.0	30.0 ± 1.2	5.0 ±0.2	2.3 ± 0.1	8.7 ± 0.4		9.4 ± 0.4
CS177	25.0 ± 1.1	16.1 ± 0.9	1.6 ± 0.1	0.5 ± 0.0	27.3 ± 1.7	9.8 ±0.6	3.3 ± 0.2	9.6 ±0.3	0.2 ± 0.0	5.3 ± 0.3
CS254	23.5 ± 1.0	15.8 ± 0.6	0.8 ± 0.0	1.4 ± 0.1	31.3 ± 1.5	5.0 ±0.3	4.4 ± 0.2	4.3 ± 0.1	-	8.3 ±0.3
CCAP 927/1	21.9 ± 0.9	15.9 ± 0.8	0.7 ± 0.1	1.0 ± 0.0	29.5 ± 1.6	5.4 ±0.2	4.0 ± 0.2	4.6 ±0.2	-	11.1 ± 0.6
CCAP 927/12	19.0 ± 0.8	16.2 ± 0.7	1.4 ± 0.1	2.2 ± 0.2	33.8 ± 1.3	4.0 ±0.2	2.1 ± 0.1	6.0 ± 0.3	0.8 ±0.0	9.0 ± 0.3
CCAP 927/14	12.9 ± 0.7	15.8 ± 0.6	1.6 ± 0.1	0.8 ± 0.0	23.2 ± 1.0	3.0 ±0.1	3.8 ± 0.2	16.2 ± 0.7	1.1 ± 0.1	17.7 ± 1.0
LABI 1100	25.6 ± 1.1	15.6 ± 0.7	0.6 ± 0.0	2.5 ± 0.2	30.9 ± 1.2	5.3 ±0.3	2.3 ±0.1	6.5 ± 0.3	1.4 ± 0.1	8.0 ± 0.4

-, below detection level

Table 2 The predicted properties of biodiesel from *Isochrysis* strains

	Kinematic viscosity 40 °C (mm^2 s^{-1})	Specific gravity (kg L^{-1})	Cloud point (°C)	Cetane number	Iodine value (g I_2/100 g)	Higher heating value (MJ/kg)
CCMP 355	4.56	0.88	6.33	56.06	88.78	40.33
CCMP 463	4.17	0.88	-1.97	51.91	135.02	41.43
CCMP 715	4.38	0.88	2.61	54.20	109.53	40.83
CCMP 1244	3.86	0.88	-8.49	48.65	171.32	42.29
CCMP 1324	4.02	0.88	-5.11	50.34	152.50	41.84
CCMP 1611	4.54	0.88	5.91	55.84	91.13	40.39
UTEX 987	4.34	0.88	1.57	53.68	115.29	40.96
UTEX 1292	4.27	0.88	0.17	52.98	123.09	41.15
UTEX 2307	4.33	0.88	1.39	53.59	116.32	40.99
CS 177	4.39	0.88	2.82	54.30	108.32	40.80
CS 254	4.43	0.88	3.65	54.72	103.71	40.69
CCAP 927/1	4.33	0.88	1.53	53.66	115.51	40.97
CCAP 927/12	4.40	0.88	3.03	54.41	107.15	40.77
CCAP 927/14	3.86	0.88	-8.42	48.69	170.94	42.28
LABI 1100	4.43	0.88	3.61	54.70	103.94	40.69
Biodiesel	4-5	0.87-0.89		44-55		38-41
ASTM D6751	1.9-6.0	0.85-0.90		Min 47		
EN 14214	3.5-5.0			Min 51	Max 120	

Table 3 Biomass and lipid productivities of three *Isochrysis* strains grown in an outdoor panel PBR.

	Cell density ($g\ L^{-1}$)	Biomass productivity ($g\ L^{-1}\ day^{-1}$)	Lipid productivity ($mg\ L^{-1}\ day^{-1}$)
CCMP 1244	3.8 ± 0.2^{ab}	0.53 ± 0.03^{ab}	135 ± 9^{a}
CCMP 1324	3.5 ± 0.2^{a}	0.48 ± 0.03^{ac}	140 ± 10^{a}
CS 177	4.2 ± 0.3^{b}	0.60 ± 0.03^{b}	180 ± 11^{b}

Cells were harvested after 6-day cultivation for analysis. Values in each column followed by the same letter are not significantly different ($P>0.05$).

Table 4 Biomass and lipid productivities of CS177 grown in an outdoor panel PBR.

	Volumetric cell density ($g\ L^{-1}$)		Areal cell density[1] ($g\ m^{-2}$)		Volumetric productivity ($g\ L^{-1}\ day^{-1}$)		Areal productivity[1] ($g\ m^{-2}\ day^{-1}$)	
Light path (cm)	Initial	Final	Initial	Final	Biomass	Lipid	Biomass	Lipid
Initial volumetric cell density=0.6 $g\ L^{-1}$								
1.8	0.6	2.2 ± 0.1^{a}	10.8	42.4 ± 1.9^{a}	0.27 ± 0.02^{a}	0.08 ± 0.00^{a}	5.2 ± 0.3^{a}	1.5 ± 0.1^{a}
3.5	0.6	4.0 ± 0.2^{b}	21.0	152.4 ± 7.7^{b}	0.57 ± 0.04^{b}	0.18 ± 0.01^{b}	21.6 ± 1.4^{b}	6.7 ± 0.3^{b}
7.0	0.6	3.0 ± 0.2^{c}	42.0	225.0 ± 14.3^{c}	0.39 ± 0.02^{c}	0.12 ± 0.01^{c}	29.9 ± 1.6^{c}	9.0 ± 0.6^{c}
Initial areal cell density =21.0 $g\ m^{-2}$								
1.8	1.2	6.5 ± 0.3^{a}	~21.0	123.1 ± 6.3^{a}	0.88 ± 0.05^{a}	0.28 ± 0.02^{a}	16.7 ± 1.1^{a}	5.3 ± 0.3^{a}
3.5	0.6	4.3 ± 0.2^{b}	21.0	163.0 ± 8.1^{b}	0.62 ± 0.03^{b}	0.19 ± 0.01^{b}	24.0 ± 1.4^{b}	7.2 ± 0.4^{b}
7.0	0.3	2.7 ± 0.1^{c}	21.0	208.2 ± 9.2^{c}	0.41 ± 0.03^{c}	0.12 ± 0.01^{c}	30.9 ± 2.2^{c}	8.9 ± 0.6^{c}

[1] 'Area' refers to the vertical surface of the PBR.

Cells were harvested after 6-day cultivation for analysis. Values in each column within the same experiment followed by the same letter are not significantly different ($P>0.05$).

Table 5 Photoautotrophic lipid production of *Isochrysis* CS177 in comparison with previously reported microalgae

Algal strain	Culture conditions	Biomass productivity ($g\ L^{-1}\ day^{-1}$)	Lipid content (% DW)	Lipid productivity ($mg\ L^{-1}\ day^{-1}$)	References
Isochrysis sp.	I, 100-mL column	0.41	29.9	123	This study
	O, 10-L panel PBR (batch)	0.88	31.9	281	
	O, 10-L panel PBR (semi-continuous)	1.1	33.3	350	
Isochrysis galbana	I, 1-L flasks	0.31	22.0	68	[30]
	O, 50-L tubular PBR	0.32			[31]
Isochrysis sp.	I, 250-mL flask	0.17	22.4	38	[5]
Isochrysis sp.	I, 15-L Carboy	0.09	23.5	21	[32]
Isochrysis zhangjiangensis	I, 600-mL column	0.34	40.9	141	[33]
Isochrysis sp.	I, 0.5-5-L flask	0.15	51.5	78	[6]
Chlorella protothecoides	I, 100-mL column	0.57	48.3	280	[19]
	O, 50-L panel PBR	0.87	48.9	340	
Chlorella sp.	O, 120-L polyethylene bags	0.24	34.6	83	[34]
Chlorella sp.	O, 70-L tube PBRs	0.15	43.3	34	[35]
Chlorella sp.	I, 300-mL glass tubes	0.5	50.8	250	[36]
	O, 10-L panel PBRs	0.6	26.6	160	
Chlorella vulgaris	I, 250-mL flasks	0.49	35.4	170	[37]
Chlorella vulgaris	O, 30-L panel PBRs	0.67	44.6	390	[38]
Chlorella zofingiensis	I, 250-mL flasks	0.67	44.8	301	[37]

I: Indoor; O: Outdoor