Performance of LED with mixed wavelengths or two-phase culture on the growth and lipid accumulation of *Chlorella pyrenoidosa*

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Abstract: *Chlorella pyrenoidosa*, a type of lipid-rich green algae, features broad prospects for application in such fields as healthy foods, biodiesel and so on. The light-utilizing efficiency of the cells is a critical factor that influences the biomass and lipid contents of photoautotrophic microalgae. Inconsistent illumination wavelengths hinder microalgal growth. The patterns about the impacts of mixed light emitting diode (LED) wavelengths or two-phase culture over the growth and lipid accumulation of *Chlorella pyrenoidosa* were reported. Among the different LED wavelengths (white, purple, blue, green, yellow and red) at the light intensity of 200 μmol/m^2·s tested, red and green gave maximum biomass and lipid contents, respectively. Based on the discovery, two-phase (red was illuminated for 12 d in the first phase, and then shifted to green light for 8 d in the second phase, R→G) or mixed LED (R:G=3:7 or R:G=7:3) culture protocol was adopted for the high lipid-accumulation of *Chlorella pyrenoidosa*. The results indicated that the lipid contents of *Chlorella pyrenoidosa* treated with two-phase (R→G) or mixed LED culture was significantly higher than that of white light with the same intensity (p<0.05), and the highest lipid-accumulation rate was 26.37 mg/L·d in the two-phase culture. Fatty acid (FA) analysis showed that 13 types FAs were detected and unsaturated FAs were over 50% (w/w). 26.8%-27.7% (w/w) palmitic acid (C16:0) was the major saturated FA, while the largest proportions of monounsaturated FA and polyunsaturated FA were oleic acid (C18:1) and linoleic acid (C18:2), respectively. Additionally, although no difference in the FA composition of *Chlorella pyrenoidosa* treated with different protocols was found, the absolute content did differ significantly, coinciding with that of the total lipids. Furthermore, the ratio of unsaturated FAs in *Chlorella pyrenoidosa* was significantly increased under the mixed LED (R:G=7:3) (p<0.05).
Keywords: *Chlorella pyrenoidosa*, mixed LED wavelength, two-phase, biomass, lipid, fatty acid
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1 Introduction

Microalgae is a type of lower aquatic (or terrestrial, aerial and symbiotic) microbes that are commercially exploited for the production of high-quality and high value-added organic compounds e.g. lipids, pigments. Microalgae are normally unicellular or colonial and are capable of photosynthesis. Moreover, microalgae come with fast-growing cells and short growth periods without being subject to the effects of season and environmental conditions, and cultivation of microalgae can reduce the competition for arable land and fresh water as well[1]. Under certain conditions, many microalgae are able to accumulate a large number of triglycerides that are able to produce lower glycerol alcohol esters via catalytic reactions with lower alcohols, hence microalgae are recognized as a raw material for alternative renewable energy that countries around the world are actively pursuing[2]. Some microalgae are also rich in functional lipids (e.g. docosahexaenoic acid, eicosapentaenoic acid, arachidonic acid, etc.) that are extensively adopted in the production of foods, medicine, fodder, and so on[3]. More importantly, the photosynthetic efficiency of microalgae is 10-50 times that of higher plants. Approximately 1.83 t of CO_2 can be fixed in every 1 t of microalgae biomass production, which not only provides microalgae with the carbon source they need for growth, but also facilitates environmental protection, and alleviates the greenhouse effects[4].

The light source is one of the major factors that impact the growth and reproduction of microalgae. Even in a closed photobioreactor, microalgae culture demands the supplementary of artificial lights to make up for the limitations of sunlight, thereby improving the growth rate of microalgae as well as its lipid-accumulation[5]. The efficient cultivation of microalgae needs controllable lights. Traditional lights are primarily fluorescent, which means that most of the energy transforms into

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thermal energy, thus increasing the burden of the temperature control system. Light-emitting diode (LED) has huge potential for application in scaled commercial production of microalgae with energy-efficiency, high stability, multiple colors, high brightness and so on[6,7]. In recent years, some of the experimental work conducted on the impact of different LED wavelengths over the biomass and lipid contents of microalgae has been carried out[8]. Ra et al.[9] found that the most appropriate mixture ratios for biomass were red:blue=1:1 at 100 μmol/m^2·s, and green light at the same intensity was more preferable for the lipid-accmulation. Studies by Kim et al.[10] demonstrated that when cultivated under monochromatic LED light qualities (purple, blue, green, yellow and red), the optimal lights for improving the biomass and lipid contents of *C. Vulgaris* were red light and green light, repectively. Shih *et al.* compared the impact of cultivation under different LED light qualities (red, orange, yellow, green, blue, purple and white) over the biomass and lipid productivity of *Cyclotella cryptica*. It is found that blue light was the optimal one for promoting growth, while yellow light was more preferable for lipid-accumulation. And blue-yellow combination could lift the lipid contents of *Cyclotella cryptica* by four times[11]. Das et al.[12] reported that the biomass of *Nannochloropsis* sp. treated with different LED wavelengths was ranked as the order of blue, white, green, red. The largest yield of fatty acid methyl ester reached 112.0 mg/L cultivated under blue light. Severes et al.[13] studied the effect of mixed cultivation of *C. Vulgaris* under different LED light qualities and intensities, the results showed that 500 lx of blue-red combination gave maximum biomass and LED of red light 220 lx wavelength doubled the lipid dry weight. In conclusion, these studies demonstrate that different light wavelengths including red, blue, green, etc. hold varying influences over the growth rate and lipid accumulation of microalgae depending upon their species and growth stages.

Chlorella pyrenoidosa, a type of lipid-rich green algae, is approved in 2012 as New Resource Food by the National Health Commission of China for highly nutritional, safe and non-toxic. It also has received considerable attention in a broad range of fields, including nutrition & health care, cosmetics, and food[14]. The key issue of its industrialization is how to improve the lipid production of *Chlorella pyrenoidosa* and reduce its production cost. Previous studies demonstrate that light source has great effects on the biomass and intracellular lipid transformation of microalgae. However, to the best of our knowledge, little data of LED light wavelengths stresses are available in the literature on lipid accumulation influence on *Chlorella pyrenoidosa*, let alone, the mixed or two-phase lighting strategies that are optimized for its high lipid-accumulation. Thus, *Chlorella pyrenoidosa*, a type of representative lipid-producing microalgae, is used as the research objects in this study. LED lights with different spectroscopy (white, purple, blue, green, yellow and red) are used to investigate their influences on the dynamic changes of growth patterns and lipid transformation. On this basis, two culture strategies including two-phase and mixed LED lights culture are adopted for comparative studies on their impact on the biomass and lipid contents of *Chlorella pyrenoidosa*. More specifically, in the mixed lights culture protocol, the intensity ratio of the optimal lights is set as 3:7 or 7:3[15]. For the design of two-phase culture, LED light wavelengths were set followed the principles below: during the stage of steady growth, a lighting exposure program that could help the microalgae enter the exponential phase (first phase) was carried out; and when the growth of microalgae reaches to the stationary phase, optimized lighting schemes that are conducive to lipid synthesis are adopted (second phase), thereby promoting the growth and improves the transformation efficiency of lipid in *Chlorella pyrenoidosa*[10]. The effects of two protocols on the fatty acid composition of intracellular lipids were also determined.

2 Materials and methods

2.1 Microalgae strains and culture conditions

Chlorella pyrenoidosa (FACHB-9) were purchased from the Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB), China, and were cultivated using BG11 culture medium (formula provided by FACHB) in a smart artificial climate chest (PRX-450C, Shanghai Joyn Electronic Co., Ltd., China). Stock culture conditions were as follows: temperature of 25°C; humidity of 60%; the light intensity of (100±10) μmol/m^2·s (OHSP350P, Hangzhou HOPOO Optoelectronics Technology Co., Ltd., Hangzhou, China); light-dark cycle (the light period from 8:00 am to 8:00 pm; the dark period from 8:00 pm onward); and artificial intermittent shaking thrice a day at 8:00 am, 2:00 pm and 8:00 pm. After a period of steady cultivation for 5 d, *Chlorella pyrenoidosa* were diluted using fresh BG11 culture medium to approximately 0.4-0.5 of OD_{630} for experiment purposes.

2.2 Experimental procedure

Six LED light qualities (OPPLE Lighting Co., Ltd., Zhongshan, China) with the light intensity of 200 μmol/m^2·s including white light (380-760 nm), purple light (400-410 nm), blue light (460-470 nm), green light (525-550 nm), yellow light (590-600 nm) and red light (620-630 nm) were chosen for the cultivation of *Chlorella pyrenoidosa*. The cultivation conditions were identical to those during steady cultivation, and the microalgae were shaken every three hours during lighting. Exactly 50 mL of evenly mixed liquid suspension culture of *Chlorella pyrenoidosa* were taken every other day for determining the biomass and lipid contents. Treatments lasted for 12 consecutive days.

The optimal LED wavelengths for the growth and lipid synthesis of *Chlorella pyrenoidosa* were determined respectively according to the changes in the biomass and lipid contents. On this basis, two lighting culture strategies for high lipid-accumulation which were described well in the literatures—two-phase and mixed lights culture[16], were employed to evaluate the impact over the lipid yield of *Chlorella pyrenoidosa*. Specifically, a mixture LED wavelengths consisted of the optimal monochromatic LED for biomass and lipid-accumulation of *Chlorella pyrenoidosa* were set with respective light intensities ratio of 7:3 or 3:7. The two-phase culture strategy included the first phase allowing high biomass production using the optimal light wavelength for growth improvement of *Chlorella pyrenoidosa* for 11 d. Then, the optimal light wavelength that suitable for lipid synthesis of *Chlorella pyrenoidosa* in the second phase culture was carried out for 9 d to accumulate high lipids. A control experiment was carried out using white LED light at the same light intensity (200 μmol/m^2·s) and operating conditions.

2.3 Biomass determination

The suspension of *Chlorella pyrenoidosa* was centrifuged for 5 min at 3000 r/min in the centrifuge tube. Then, the collected microalgae were washed by ultrapure water twice to eliminate the effect of the original BG11 solute. Finally, the microalgae so obtained were dried for 72 h using a Labconco vacuum freeze-dryer (Labconco Co., Kansas City, MO, USA). The

biomass (mg) of *Chlorella pyrenoidosa* was evaluated by gravimetric analysis[17].

2.4 Lipid contents determination

Exactly 40.0-50.0 mg of freeze-dried microalgae were suspended in 5 mL precooled petroleum ether (30°C-60°C, Sinopharm Group Co., Ltd., Shanghai, China), and went through thorough homogenate using the glass homogenizer under ice-water bath. Then, the extract solution was treated by an ultrasonic cell disruptor (JY92-IIDN, Ningbo Xinyi Ultrasonic Equipment Co., Ltd., China) under ice-water bath at 270 W, 5 min, 2 s on/1 s off for further ultrasonic extraction. Next, the extracted liquid was transferred to the polypropylene centrifuge tube and centrifuged for 5 min at 4000 r/min. The supernatant was collected and the residue was subjected to a repeated extraction. All of the extracts were transferred into a 25 mL round-bottom flask for nitrogen blow-off till dry. Total lipids (mg/g dw) quantified by gravimetric method[18].

2.5 Fatty acid measurement

2.5.1 Methyl esterification of fatty acid

The fatty acid (FA) composition of intracellular lipids was analyzed as fatty acid methyl esters (FAMEs) via a transesterification method with slight modifications[19]. Nonadecanoic acid (C19:0) of internal standard (1.0 mg/mL, dissolved with *n*-hexane) that weighs 20% of the lipid and 2 mL of sodium hydroxide-methanol solution (0.5 mol/L) were added into the flask that contained the lipid. The samples were refluxed in a water bath of 75°C and continued for another 30 min after the fat droplets disappear. Afterward, 2.5 mL of boron trifluoride-methanol solution with a concentration of 13%-15% (Aladdin chemistry Co., Ltd., Shanghai, China) was added and kept 3 min of continued boiling. The reaction solution was transferred to glass tubes with plus and cooled prior to extraction of FAMEs using hexane. Finally, FAMEs extract was subjected to filtering with 0.22 μm filter membrane before analysis by gas chromatograph-mass spectrometry (GC-MS).

2.5.2 GC-MS analysis

FAMEs analysis was performed using an Agilent 7890A gas chromatograph fitted with 5975C mass spectrometry with silica capillary column (Agilent HP-5MS, 30.0 m×0.25 mm×0.5 μm, Agilent Technologies, Inc. USA). The column temperature adjustments were as follows: the initial temperature was 90°C, followed by an increase to 240°C at a rate of 5°C/min, which was subsequently maintained for 15 min, then to a final temperature of 300°C at 10°C/min, holding for 5 min. Both injector and MS detector temperatures were set to 250°C. FAMEs were identified by comparing their retention times against those of authentic standards. The absolute FA contents were calculated by the peak area ratio of FA constituents and the internal standard (nonadecanoic acid).

2.6 Statistical analysis

Each experiment was carried out in triplicate. The data was processed using Excel, with results shown as mean±SD. One-way analysis of variance (ANOVA) analysis and Duncan's multiple range test (p<0.05) was performed using SPSS Software (ver. 17.0; SPSS Inc., Chicago, USA).

3 Results and discussion

3.1 Effects of different light wavelengths on the biomass and lipid contents of *Chlorella pyrenoidosa*

Table 1 and Figure 1a show the changes in biomass and lipid contents of *Chlorella pyrenoidosa* cultivated under different LED wavelengths (white, purple, blue, green, yellow and red). Among wavelengths, red LED produced the highest biomass of *Chlorella pyrenoidosa*, achieving 2076.3 mg/L at the end stage of cultivation (D13). *Chlorella pyrenoidosa* exposed to red LED entered the exponential phase from day 3 and reached the stationary phase of growth on the 11th day. The fastest growth rate could be identified during days 5-7, reaching 181.5 mg/L·d. From day 7 onwards, the impact of red LED on improving the biomass of *Chlorella pyrenoidosa* was significantly greater than that of the other treatments (p<0.05), which coincided with the conclusions reported by Matthijs[20] and Yan[21]. The possible explanation was that the main photosynthetic pigment of *Chlorella pyrenoidosa* — chlorophyll strongly absorbs red light[22]. Meanwhile, it had been reported that red light promoted the cell division of microalgae[23]. The impacts of LED wavelengths on the biomass of *Chlorella pyrenoidosa* ranked as red, yellow, white, purple, green (blue).

The changes of lipid contents of *Chlorella pyrenoidosa* cultivated under different light qualities are presented in Table 2 and Figure 1b. The lipid-accumulation entered into a period of rapid growth from day 5, and the impacts of different LED wavelengths on lipid contents exhibited significant differences. The illumination with green LED wavelength for 6 days (D7) led to higher microalgae lipid production than those of other single LED wavelengths and white LED. This might be due to the reason that the green wavelength was more suitable for the synthesis of chlorophyll, thus facilitating lipid accumulation[24]. Moreover, another study indicated that green light could penetrate into the plant canopy more effectively, as a result in promoting the absorption of green light by microalgae[25]. Similar results were obtained for *Nannochloropsis* sp.[26]. The fastest lipid-accumulation rate was achieved under green LED during days 5-7, reaching 17.18 mg/g/d, and the highest lipid yield achieved was 192.03 mg/g dw at the end stage of cultivation (D13). The effects of LED wavelengths on the lipid-accumulation ranked order as green, blue, white, red, yellow, purple.

Table 1 Effects of different light wavelengths on the biomass of *Chlorella pyrenoidosa* (mg/L)

Time	White	Purple	Red	Yellow	Blue	Green
D1	1039.3±28.0^{a}	1039.3±28.0^{a}	1039.3±28.0^{a}	1039.3±28.0^{a}	1039.3±28.0^{a}	1039.3±28.0^{a}
D3	1119.3±32.1^{a}	1145.9±18.0^{a}	1131.1±13.5^{a}	1137.8±41.6^{a}	1147.4±31.9^{a}	1150.4±22.8^{a}
D5	1321.5±9.3^{b}	1342.2±7.7^{ab}	1362.2±6.7^{a}	1363.7±12.6^{a}	1344.4±14.6^{ab}	1327.4±35.4^{b}
D7	1576.3±3.4^{c}	1556.3±11.4^{c}	1725.1±53.6^{a}	1647.4±28.1^{b}	1561.5±17.3^{c}	1540.0±8.0^{c}
D9	1768.1±24.5^{c}	1725.9±21.4^{cd}	1946.7±15.6^{a}	1829.6±22.8^{b}	1700.7±44.5^{d}	1718.5±17.3^{d}
D11	1890.4±85.7^{bc}	1838.5±30.2^{bc}	2051.1±94.0^{a}	1959.3±25.3^{ab}	1754.1±110.2^{c}	1741.5±120.5^{c}
D13	1919.3±72.4^{bc}	1852.5±66.6^{cd}	2076.3±66.9^{a}	1980.7±53.0^{ab}	1764.4±64.4^{d}	1778.5±88.2^{d}

Note: Different lowercase letters in the same row indicate significant differences, p<0.05.

Table 2 Effects of different light wavelengths on the lipid-accumulation of *Chlorella pyrenoidosa* (mg/g dw)

Time	White	Purple	Red	Yellow	Blue	Green
D1	82.1±3.5^{a}	82.1±3.5^{a}	82.1±3.5^{a}	82.1±3.5^{a}	82.1±3.5^{a}	82.1±3.5^{a}
D3	89.6±13.7^{a}	84.1±2.8^{a}	89.1±4.2^{a}	83.6±14.1^{a}	84.0±6.4^{a}	85.0±3.8^{a}
D5	107.6±3.5^{ab}	94.9±4.7^{cd}	100.6±5.2^{bd}	93.4±5.7^{d}	106.3±11.3^{abc}	116.0±4.9^{a}
D7	127.7±2.4^{bc}	109.4±11.5^{d}	116.3±5.2^{cd}	110.4±6.8^{d}	134.6±14.3^{b}	150.3±5.8^{a}
D9	140.4±6.6^{c}	123.0±4.7^{d}	133.6±9.2^{cd}	128.7±9.4^{cd}	155.7±5.4^{b}	177.8±7.4^{b}
D11	143.5±9.1^{c}	125.3±7.7^{d}	139.5±6.3^{c}	133.1±7.3^{cd}	164.1±5.8^{b}	186.2±4.9^{a}
D13	150.1±12.1^{c}	129.8±5.6^{e}	143.0±5.2^{cd}	135.8±5.8^{de}	169.0±5.8^{b}	192.0±5.5^{a}

Note: Different lowercase letters in the same row indicate significant differences, p<0.05.

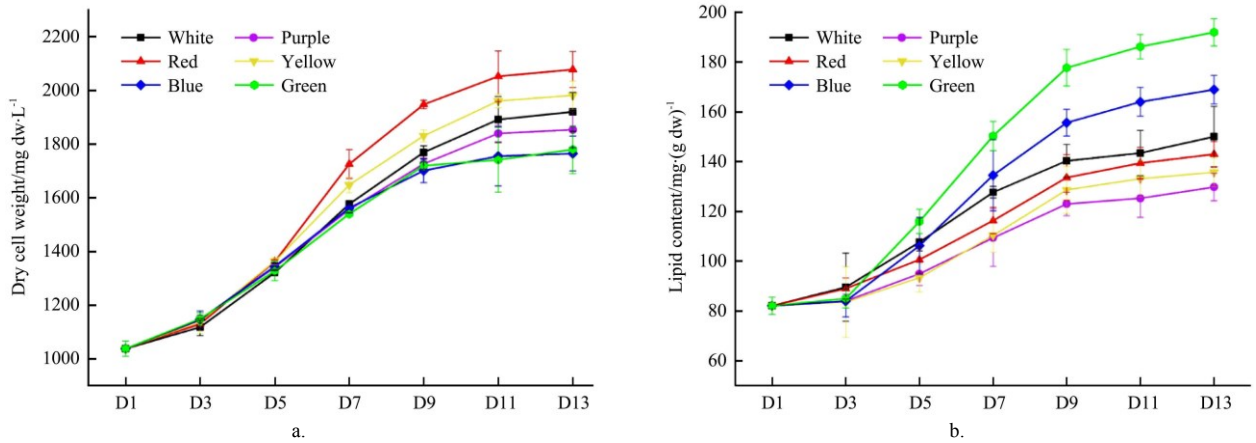


Figure 1 Effects of different light qualities on the biomass (a) and lipid (b) of *Chlorella pyrenoidosa*

The above results demonstrated that the optimal light wavelengths for improving the growth and lipid accumulation of *Chlorella pyrenoidosa* did not coincide. Hence, the two methods that were well reported i.e. two-phase and mixed lights culture[16] were adopted for the high lipid-accumulation of *Chlorella pyrenoidosa*. The intensities of mixed LED lights were set at R:G=7:3 (mixed lights 1, ML1) and 3:7 (mixed lights 2, ML2). In two-phase culture protocol, because the periods that *Chlorella pyrenoidosa* reached the stationary phase of growth under red light and fast-proliferating phase of lipid exposed to green light were 11 d and 9 d, respectively, the culture time of two-phase protocol was set at 20 d, i.e. *Chlorella pyrenoidosa* was cultivated using red light for 11 d in the first phase, and then green light for 9 d in the second phase. The cultivation conditions were the same as above, and *Chlorella pyrenoidosa* cultured under the white light of the same intensity (200 μmol/m^2·s) was used as the control group.

3.2 Effects of two-phase or mixed lights culture protocol on the biomass and lipid of *Chlorella pyrenoidosa*

The changes of biomass and lipid contents of *Chlorella pyrenoidosa* cultivated by mixed lights (red and green) or two-phase (R→G) are presented in Table 3, Table 4 and Figure 2. In the mixture wavelengths treatment, the biomass of *Chlorella pyrenoidosa* treated with ML2 (R:G=7:3) and ML1 (R:G=3:7) was significantly higher than white light from the 9th day and 13th day, respectively (p<0.05). Moreover, the lipid contents at day 17 of exposure to ML1 were significantly higher than those cultivated under white light (p<0.05). In addition, little difference was found in terms of impact over biomass between the two mixed lights, but the lipid contents of *Chlorella pyrenoidosa* cultivated under ML1 was significantly higher than ML2 (p<0.05) from the 17th day, which might be attributable to the specific selection of light wavelengths by the photosynthetic pigments of the microalgae[27].

In the two-phase culture, red light treatment facilitated the rapid reproduction of *Chlorella pyrenoidosa* in the first phase, but the lipid contents were relatively low. Previous studies had reported that higher growth rates by the LED wavelength stress condition were usually accompanied by lower lipid contents, which led to increased biomass and decreased lipid productivity[28]. In the second phase, the green LED could enhance the performance of lipid accumulation with the highest lipid contents achieving 389.61 mg/L on the 21st day, which was significantly higher than those of white LED and ML2 (p<0.05). The impacts of these protocols on promoting lipid contents ranked order as two-phase (389.61 mg/L), ML1 (372.43 mg/L), ML2 (349.92 mg/L), white light (258.95 mg/L). It should be noted that, since the ML2 maintained the maximum growth rate of *Chlorella pyrenoidosa* comparing to the monochrome red LED light, it is speculated that the lipid yield should be higher conducted by the two-phase strategy if ML2 is used instead of red light in the first phase. Taken together, we conclude that that two-phase protocol is more suitable than mixed LED light culture for the purpose of improving lipid-accumulation of *Chlorella pyrenoidosa*.

Table 3 Effects of two-phase or mixed lights culture on the biomass of *Chlorella pyrenoidosa* (mg/L)

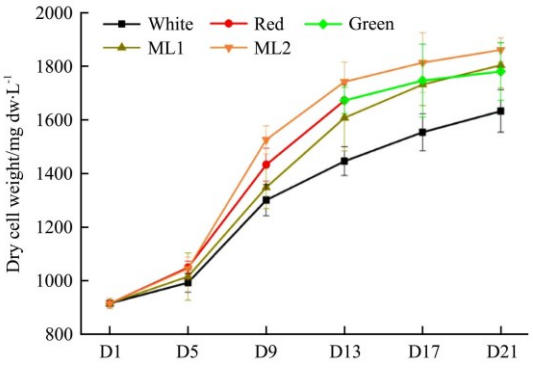
Time	White	Two-phase (R→G)	ML1	ML2
D1	915.3±18.9^{a}	915.3±18.9^{a}	915.3±18.9^{a}	915.3±18.9^{a}
D5	992.6±35.2^{a}	1049.3±23.1^{a}	1015.3±88.1^{a}	1045.3±43.1^{a}
D9	1300.6±58.5^{c}	1432.6±61.6^{ab}	1348.0±79.1^{bc}	1525.9±52.3^{a}
D13	1446.0±53.7^{b}	1672.6±49.0^{a}	1608.1±123.4^{a}	1742.1±74.1^{a}
D17	1554.0±68.2^{b}	1746.6±136.4^{ab}	1732.0±78.7^{a}	1814.0±111.3^{a}
D21	1632.6±78.8^{b}	1780.6±107.5^{ab}	1804.1±85.1^{a}	1861.3±45.1^{a}

Note: ML1 and ML2 represent mixed lights 1 with R:G=3:7 and mixed lights 2 with R:G=7:3, respectively; different lowercase letters in the same row indicate significant differences, p<0.05.

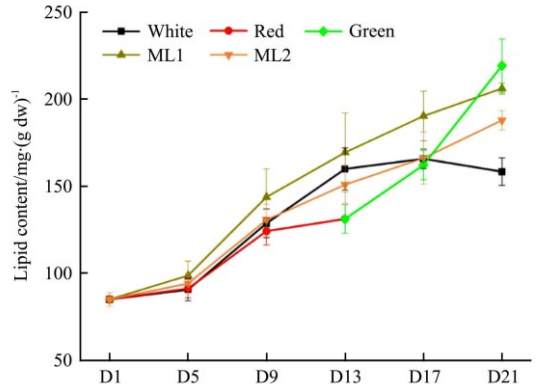
Table 4 Effects of two-phase or mixed lights culture on the lipid accumulation of *Chlorella pyrenoidosa* (mg/g dw)

Time	White	Two-phase (R→G)	ML1	ML2
D1	84.9±3.9[a]	84.9±3.9[a]	84.9±3.9[a]	84.9±3.9[a]
D5	90.7±6.4[a]	91.2±5.3[a]	98.7±8.3[a]	94.2±5.4[a]
D9	128.7±8.2[a]	124.2±7.8[a]	143.9±16.2[a]	130.7±8.9[a]
D13	160.1±12.1[a]	131.3±8.1[b]	169.5±22.6[a]	150.9±10.5[ab]
D17	165.9±5.5[b]	162.2±8.4[b]	190.5±14.2[a]	166.3±15.0[b]
D21	158.4±7.8[c]	219.4±15.4[a]	206.3±3.2[a]	188.1±5.5[b]

Note: ML1 and ML2 represent mixed lights 1 with R:G=3:7 and mixed lights 2 with R:G=7:3, respectively; different lowercase letters in the same row indicate significant differences, $p<0.05$.



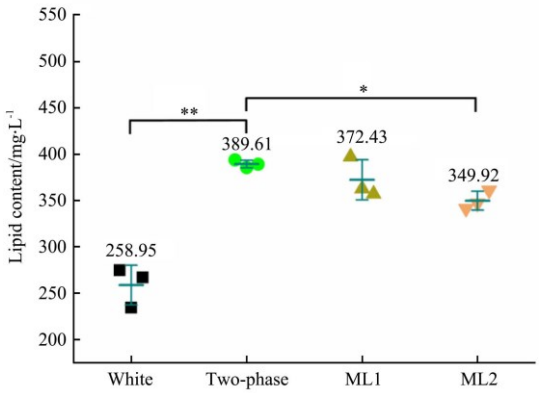
a.



b.

Note: ML1 and ML2 represent mixed lights 1 with R:G=3:7 and mixed lights 2 with R:G=7:3, respectively; two-phase culture included first phase illuminated by red and second phase by green

Figure 2 Effects of two-phase or mixed lights culture on the biomass (a) and lipid (b) of *Chlorella pyrenoidosa*



Note: ML1 and ML2 represent mixed lights 1 with R:G=3:7 and mixed lights 2 with R:G=7:3, respectively; * means significant differences, $p<0.05$; **, $p<0.01$.

Figure 3 Lipid contents of suspension of *Chlorella pyrenoidosa* after cultivating for 20 d (D21) by two-phase or mixed lights culture protocol

3.3 Effects of two-phase or mixed lights culture protocol on fatty acid composition and content of *Chlorella pyrenoidosa*

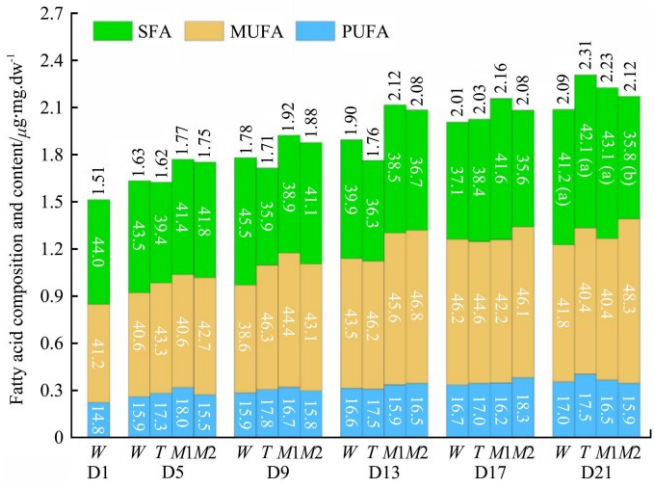
The fatty acid (FA) composition and content of intracellular lipids of *Chlorella pyrenoidosa* treated with different lighting culture strategies for 20 d are shown in Table 5 and Figure 4. Thirteen types of FAs including 6 saturated (SFAs; C14:0, C16:0, C17:0, C18:0, C20:0, and C22:0), 3 monounsaturated (MUFAs; C16:1, C18:1 and C20:1) and 4 polyunsaturated FAs (PUFAs; C16:4, C18:2, C18:3, and C18:4) were found in *Chlorella pyrenoidosa*, and over half total fatty acids was unsaturated FAs (UFAs) account for 56.8%-65.8% (w/w). Among these FAs, 26.8%-27.7% (w/w) palmitic acid (C16:0) was predominant form observed in the lipids, while oleic acid (C18:1) and linoleic acid (C18:2) were the major MUFA and PUFA, respectively. Moreover, docosanoic acid (C22:0) was the lowest FA at less than 1% (w/w) of the total FAs. These results are consistent with those of Wu et al.[29]. Heptadecanoic acid (C17:0) of odd-numbered carbon was detected in *Chlorella pyrenoidosa*, hence, *n*-nonadecanoic acid (C19:0) was selected as the internal standard for quantification. Additionally, the intracellular FA compositions of *Chlorella pyrenoidosa* exhibited no difference among the treatments exposed to different wavelengths, but the absolute FA contents varied significantly with the trends coincided with the changes of the total lipids. It was interesting to note that treatment by ML2 (R:G=7:3) led to a decline in the proportion of SFAs of *Chlorella pyrenoidosa*. At the end stage of cultivation (D21), the proportion (w/w) of SFAs decreased from 44.0% to 35.8% (w/w), significantly lower than other treatments ($p<0.05$), as shown in Figure 4. The possible reason is that ML2 stress in the cultivation of *Chlorella pyrenoidosa* leads to the promotion of cell division and growth, which induces more synthesis of membrane fractions (primarily UFAs)[30]. The same phenomenon could be

Table 5 Effects of two-phase or mixed lights culture on the composition and content of fatty acid (D21) of *Chlorella pyrenoidosa* (μg/g dw)

Fatty acids	White	Two-phase culture (R→G)	ML1	ML2
C14:0	33.02±8.76	34.61±6.82	38.95±12.91	29.46±4.63
C16:0	559.09±124.38	633.32±143.73	616.95±51.21	586.90±125.66
C16:1	55.26±8.70	68.29±18.88	60.57±8.43	77.16±29.13
C16:4	53.98±4.72	75.19±5.03	43.76±3.48	63.67±22.20
C17:0	30.95±11.48	33.40±24.73	30.77±11.23	16.51±8.04
C18:0	208.5±20.48	231.18±67.08	242.05±74.03	120.76±5.12
C18:1	770.17±138.57	800.05±39.22	789.28±89.89	933.44±43.13
C18:2	203.72±35.25	221.05±70.71	227.37±10.11	200.30±43.13
C18:3	27.19±8.77	25.14±22.64	26.06±7.23	16.43±4.08
C18:4	69.3±4.40	82.19±33.32	70.11±5.09	63.88±4.19
C20:0	21.38±13.94	30.91±27.43	22.26±12.65	14.15±9.30
C20:1	47.13±17.26	62.69±39.88	49.03±18.37	37.84±19.81
C22:0	8.55±1.72	9.29±4.54	10.08±1.97	10.04±4.52
SFA	861.52±139.90	972.75±266.78	961.09±108.05	777.84±113.41
MUFA	872.56±130.01	931.04±86.09	898.89±71.61	1048.45±98.63
PUFA	354.21±38.08	403.57±130.74	367.31±14.73	344.30±68.97
TUFA	1226.78±164.93	1334.61±214.33	1266.21±74.10	1392.75±164.99
TFA	2088.30±303.54	2307.37±472.77	2227.30±92.64	2170.60±274.10

Note: SFA, MUFA, PUFA, TUFA and TFA stand for saturated fatty acid, monounsaturated fatty acid, polyunsaturated fatty acid, total unsaturated fatty acid and total fatty acid, respectively; ML1 and ML2 represent mixed lights 1 with R:G=3:7 and mixed lights 2 with R:G=7:3, respectively; two-phase culture included the first phase illuminated by red and second phase by green.

observed from the first phase (exposure to red light wavelength, D13) of two-phase culture (Figure 4). It was implied that ML2 and red light could contribute to the UFAs-accumulation of *Chlorella pyrenoidosa*.



Note: *W*, *T*, *M*1 and *M*2 represent white light, two-phase culture, mixed lights 1, and mixed lights 2, respectively; SFA, MUFA and PUFA stand for saturated fatty acid, monounsaturated fatty acid and polyunsaturated fatty acid; the figures in the bar chart are the proportions of SFA, MUFA and PUFA; and the different letters in the bar chart mean significant differences, $p<0.05$.

Figure 4 Changes of fatty acid composition of *Chlorella pyrenoidosa* treated with two-phase or mixed lights culture for 20 d (D21)

4 Conclusions

Light wavelength is an essential parameter for the growth of photoautotrophic microalgae. Through photosynthesis, the microalgae are able to convert H_2O, CO_2 and inorganic salts into organic matter, and to release oxygen. Since natural lighting face limits of either scarcity of light intensity during monsoons or photoinhibition during hot summers. Therefore, the light-utilizing efficiency of the microalgae cells is challenged. Recently, artificial supplementary lighting is currently a global focus in the scaled cultivation of microalgae for its cheap, durable, reliable and highly efficient. However, the wavelengths absorbed by microalgae differ depending on the species and cultivation conditions which are reflected in the process of photosynthesis for photoautotrophic microalgal growth and lipid production by microalgae. In this study, firstly, the impacts on the biomass and lipid of *Chlorella pyrenoidosa* exposure to six types of light qualities (white, purple, blue, green, yellow and red) were screened, and it was determined that red light was the optimal for improving growth, and green light for lipid-accumulation. Next, the two-phase or mixed lights culture methods, which had been reported to be conducive to lipid-accumulation of microalgae, were applied for evaluating the effects of the two lighting protocols on lipid yield and fatty acid composition of *Chlorella pyrenoidosa*. The results indicated that *Chlorella pyrenoidosa* cultivated by two-phase culture process yielded the highest lipid contents of 389.61 mg/L. Fatty acids (FAs) analysis of intracellular lipids showed that a total of 13 types of FAs were identified in *Chlorella pyrenoidosa*, of which over 50% (w/w) were unsaturated fatty acids. Palmitic acid (C16:0) was the major FA in *Chlorella pyrenoidosa* accounting for 26.8%-27.7% (w/w) of the total FA content. Meanwhile, no significant differences in FA composition were found between treatments of two-phase and mixed lights, but the contents varied significantly and exhibited a similar trend with that of the total lipids. Furthermore, it was also found that the mixed lights (R:G=7:3) and red light contributed to the proportion of UFAs in *Chlorella pyrenoidosa*. Overall, the two-phase culture protocol could be used as a simple and convenient method for enhancing the accumulation of lipid without changing the culture medium from one photobioreactor.

This study provides a systematic analysis of the impacts of different light wavelengths as well as two-phase and mixed lights culture over the growth and lipid accumulation of *Chlorella pyrenoidosa*, so as to provide some references on developing optimized artificial supplementary lighting protocols for industrial production of *Chlorella pyrenoidosa*. However, there are a large number of factors that influence the microalgal growth and lipids, we only investigated the impacts of different illumination protocols at a single light intensity of 200 μmol/m^2·s over microalgal biology of *Chlorella pyrenoidosa*. Meanwhile, the period of cultivation was relatively long due to the photoautotrophic growth of *Chlorella pyrenoidosa*. In future studies, the effects of light-related culture factors including light intensity, light wavelengths combined with nutritional stress, heterotrophic culture, etc. on biomass and lipid-accumulation of *Chlorella pyrenoidosa* should be explored to further optimize the cultivation strategy for efficient and high-quality production of lipids by *Chlorella pyrenoidosa*.

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