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Improving high carbon dioxide tolerance and carbon dioxide fixation capability of *Chlorella* sp. by adaptive laboratory evolution

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ABSTRACT

CO_2 capture by microalgae is a promising method to reduce greenhouse gas emissions. It is critical to construct a highly efficient way to obtain a microalgal strain tolerant to high CO_2 concentrations with high CO_2 fixation capability. In this study, two evolved *Chlorella* sp. strains, AE10 and AE20 were obtained after 31 cycles of adaptive laboratory evolution (ALE) under 10% and 20% CO_2, respectively. Both of them grew rapidly in 30% CO_2 and the maximal biomass concentration of AE10 was 3.68 ±0.08 g/L, which was 1.22 and 2.94 times to those of AE20 and original strain, respectively. The chlorophyll contents of AE10 and AE20 were significantly higher than those of the original one under 1 - 30% CO_2. The influences of ALE process on biochemical compositions of *Chlorella* cells were also investigated. This study proved that ALE was an effective approach to improve high CO_2 tolerance of *Chlorella* sp.

Keywords

Adaptive laboratory evolution; High CO_2 concentration tolerance; *Chlorella sp.*;CO_2 fixation

1. Introduction

CO_2 concentration in the atmosphere is currently less than 0.04% (v/v) but the amount is still increasing annually due to the utilizations of fossil fuels in the industrial processes. If it is more than one threshold level, it will lead to serious effects for climate change and environmental protection. Although there are lots of discussion

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and speculation on the relationship between CO_2 concentration and environmental issues, a majority agrees upon the urgent need to reduce greenhouse gas emission including CO_2, CH_4 and others through chemical, physical and biological capture technology (Farrelly et al., 2013). Plants have the ability to fix CO_2 through photosynthesis and microalgae are the most efficient ones among them. At the same time, the algal biomass could be converted into many useful products, including feeds, food and biofuels. Therefore, microalgae CO_2 capture technology could fulfill multiple purposes, such as environmental remediation, raw material production and energy supply, etc.

During the evolution process, CO_2 Concentration Mechanism (CCM) is established in microalgae so they can live under low CO_2 concentration conditions with high photosynthesis efficiency (Zhao and Su, 2014). In general, the most suitable CO_2 concentration for microalgae is about 1-10 % (Anjos et al., 2013; Chiu et al., 2011; Fulke et al., 2010; Ramanan et al., 2010). Higher CO_2 concentration will lead to negative effect for microalgae growth (Tang et al., 2011) while the typical CO_2 concentrations of flue gas or other exhaust gas sources are in the range of 10-30% (Kumar et al., 2014; Praveenkumar et al., 2014; Van den Hende et al., 2012). It is very important to obtain a high CO_2 concentration tolerant strain of microalgae for CO_2 capture. Most studies used strains screened from hot springs (Sakai et al., 1995; Seckbach et al., 1970) or other natural environment. Mutations by chemical or physical (Cheng et al., 2013) methods are possible. Adaptive laboratory evolution (ALE) is utilized for bacteria (Lu et al., 2012), yeast (Gu et al., 2014; Wang et al., 2014) and microalgae (Fu et al., 2012; Fu et al., 2013; Perrineau et al., 2014; Tillich et

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al., 2014; Wichuk et al., 2014; Yu et al., 2013) to enhance their metabolic phenotype or special capabilities to tolerant stress conditions. In general, ALE is performed under optimal conditions (Yu et al., 2013) or stress conditions. It is proved that ALE is a powerful tool to improve strain properties of microalgae. High light intensity (Fu et al., 2012; Fu et al., 2013; Wichuk et al., 2014), high salt concentration (Perrineau et al., 2014), high temperature (Tillich et al., 2014) and others are typical environmental stresses in ALE.

Chlorella is widely regarded as a candidate for CO_2 capture and biodiesel production in large scale. The strain improvement of *Chlorella* is essential for biochemistry, metabolic engineering and process development in the field of microalgae biotechnology. In this study, a new approach based on ALE was proposed in order to improve tolerance to high CO_2 concentration and enhance CO_2 fixation capability of a *Chlorella.* sp.. During the ALE process, 10% and 20% CO_2 were selected as environmental stresses. Detailed adaptation process and the related analysis were presented.

2. Materials and Methods

2.1. Experimental organism and growth conditions

The freshwater *Chlorella* sp. was obtained from a water sample collected locally (Shanghai). It was maintained in BG11 medium. All experiments were carried out at 28±0.5℃ in bubble column photobioreactors (PBRs), which were cylindrical with a

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height of 40cm, diameter of 4.5cm, and a working volume of 300±5ml in a temperature-control system. Input gas was 0.2 L/min of CO_2-enriched air. Different CO_2 concentrations were used in the experiments. White LEDs were used for the continuous illumination during the cultivations on one side of the PBRs. The average light intensity was around 95μmol·m^{-2}·s^{-1}, measured by a LI-250 Light Meter with a quantum sensor (SR.NO.Q49770 of QUANTUM, LI-COR, USA) on the inner surface of each PBR. The initial cell density in the culture was controlled around OD_{750} of 0.1 (approximately 0.02 g DCW/L).

2.2. Adaptive laboratory evolution under high CO_2 concentrations

ALE was performed in a special culture system to make *Chlorella* sp. remaining in the exponential growth phase. For each new cycle, the initial cell density was controlled at OD_{750} of 0.1 by diluting the culture with fresh BG11 medium. The original *Chlorella* sp. strain was cultivated under 1% CO_2. At the middle of the exponential growth phase, the algae were inoculated in two groups of PBRs supplied with 10% and 20% CO_2 for the evolution, respectively. At the beginning of the ALE process, the algae cells grew with a long period of lag phase under these high CO_2 concentrations. Therefore, the first cycle was conducted for 7 days to gain enough *Chlorella* cells for the next adaption cycle. The cycles from the second one were set at 3 days. The ALE experiment was sustained for 31cycles (97 days) to obtain two end strains. One evolved in 10% CO_2 in ALE process was denoted as the end strain AE10 and another one was denoted as the end strain AE20 which was evolved in 20% CO_2 in ALE process.

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2 2.3. Cultivation of obtained end strains after ALE in 1-30% CO_2

3 In order to keep all of the algae cells in a similar growing status, the original strain,
4 the end strain AE10 and the end strain AE20 were cultivated under 1%, 10% and 20%
5 CO_2 conditions for 4 days, respectively. Then, these three kinds of *Chlorella* sp.
6 strains were all inoculated to fresh BG11 medium and evaluated under 1%, 10%, 20%
7 and 30% CO_2 for 11 days. The cell growth rate was measured by the method
8 described in our previous study (Yu et al., 2013). During the cultivation, 10ml of the
9 samples was filtered using a pre-dried and pre-weighed mixed cellulose membrane
10 (pore size, 0.45 μ m), washed with deionized water, dried for 24h at 105□, cooled in a
11 desiccator and then weighted again. 15 ml of culture was collected in a clean glass
12 tube, and the pH of the sample was measured by Five Easy pH meter (METTLER
13 TOLEDO) immediately.

14

15 2.4. Biomass component analysis

16 2.4.1 Sample preparation for cell composition analysis

17 At the end of the cultivation, 0.5ml of sample was centrifuged at 13,400 rpm for
18 10min in a 2ml tube, the supernatant was discarded and 1ml of deionized water was
19 added to wash the pellet using vortex for 5-10 seconds. The sample was then
20 centrifuged at 13,400 rpm for 10min and the supernatant was discarded. The pellet
21 was then dried with vacuum freeze-drying for 24h. The 2ml tube containing sample
22 was then stored in darkness at -20℃ until analysis.

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2.4.2 Total carbohydrate analysis

1.8ml 1M H_2SO_4 was added to the sample, mixed well by vortexing, heated in a water bath at 95℃ for 2 h, cooled to room temperature and centrifuged at 13,400 rpm for 10min. The supernatant was used for carbohydrate measurement by phenol-sulfuric acid method (Dubois et al., 1956). 0.4 ml of the supernatant or standard glucose solution was added to 0.2ml of 5% phenol solution in a 2ml tube. Subsequently, 1ml of concentrated sulfuric acid was added rapidly and mixed well by vortexing immediately. After cooling the tube for 30min at room temperature for color development, 200 µl of the solution was pipetted into the bottom of a 96-well microplate (Greiner Bio-One, 655101) and absorbance was measured at 490nm by iMark™ microplate reader (BIO-RAD).

2.4.3 Total protein analysis

The protein solution was prepared as described by Pruvost et al. (Pruvost et al., 2011), after suitable modification. 1ml 1N NaOH was added to the sample, mixed well by vortexing, and heated in a water bath at 95℃ for 10 min. After alkaline lyses, 0.5ml 1.6 N HCl was added for neutralization. Then the samples were cooled to room temperature and centrifuged at 13,400 rpm for 10min. The supernatant was used for protein measurement by the Lowry method (Lowry et al., 1951). BSA was used as protein standard.

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2.4.4 Total lipid analysis

The lipid content was measured by the colorimetric sulfo-phospho-vanillin (SPV) method as described by Mishre et al. (Mishra et al., 2014) with some modifications. The standard lipid stocks were prepared by dissolving 10 mg corn oil (Sigma-Aldrich) in 10ml chloroform. Different amounts of standard stocks containing 50-500 μg lipids were added to the bottom of 2ml tube. The solvent was dried under a nitrogen evaporator at 30℃.

Subsequently, 100 μl deionized water was added to the 2 ml tube containing a known amount of algal biomass or corn oil. 1.4ml of concentrated sulfuric acid was added and mixed well by vortexing, Then the mixture was heated for 10min at 100℃, cooled in an ice bath for 5min and mixed again. 100μl of the mixture was pipetted into the bottom of microplate wells. Background absorbance at 530nm is measured. 100ul of vanillin-phosphoric acid reagent (1.2mg vanillin per ml 68% phosphoric acid) was added to each well for color development for 20min and the absorbance was measured at 530nm.

2.4.5 Pigment analysis

The pigments were extracted and calculated as described in Pruvost et al. (Pruvost et al., 2011). The absorbances at 480, 652, 665 and 750 nm were measured by HACH DR2800 in a glass cell with a path length of 1 cm.

2.5 Statistics

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All experiments were replicated three times and the results were expressed as mean±standard deviation. A Student's t test was used to assess statistical significance. A P value of less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Growth profile of *Chlorella* sp. strains during ALE

The original *Chlorella* sp. strain was cultivated under 1% CO_2. When transferring the strain growing in 1% CO_2 to 10% or 20% CO_2, a long lag phase was needed to adapt to the environmental stress. The first cycle of ALE was conducted for 7 days. At the end of the first cycle, the biomass concentration under 10% and 20% CO_2 was 0.74±0.04 g/L and 0.87±0.09 g/L, respectively. After the first cycle, the strain began to tolerate these stress conditions. Then the following cycles of the ALE were adjusted to 3 days because the cells can grow under these conditions.

As shown in Fig. 1, the phenotypes of the strains evolved in 10% and 20% CO_2 fluctuated strongly during the ALE process. This phenomenon is similar to the previous study of Yu et al. (2013). In spite of the fluctuation, the growth rate tended to be increased during the whole ALE process. Comparing with the early five cycles (from 2 to 6), the average growth rate of strains cultivated in 10% and 20% CO_2 for the end five cycles (from 27 to 31) increased 1.33 and 1.47 times.

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The growth rate of microalgae under 10% CO_2 during the 31 cycles of ALE is higher than that under 20% CO_2. Previous studies have shown that microalgae such as *Chlamydomonas reinhardtii* (Yu et al., 2013), *Chlorella vulgaris* (Fu et al., 2012), and *Dunaliella salina* (Fu et al., 2013) require a great length of time to obtain a stable improved phenotype by ALE. In order to further enhance the growth characteristics under 20% CO_2, the ALE process should be conducted much longer. Our study demonstrates that the appropriate environmental stress should be set, for improving the phenotype of microalgae in a severe environment by ALE. In the further study, the physiological parameters and gene expressions will be investigated similar to the rapid response to high salt concentration by *C. reinhardtii* (Perrineau et al., 2014). It will be helpful to increase the efficiency of ALE and obtain much more understanding of the ALE mechanism.

3.2. Growth of *Chlorella* sp strains under different CO_2

After 31 cycles of ALE, the end strain AE10 and the end strain AE20 tolerant to high CO_2 were obtained. The growth rates of the original strain, the end strain AE10 and the end strain AE20 under 1%, 10%, 20% and 30% CO_2, respectively, are shown in Fig. 2. The growth rates of the original strain in 1% CO_2 at day 3, 5 and 7 are significantly higher than those in 10% CO_2. The biomass dry weight of the original strain at day 11 was decreased from 3.12 g/L under 1% CO_2 to1.71 g/L under 20% CO_2 and 1.25 g/L under 30% CO_2, respectively. Less than 10% CO_2 is suitable for the cultivation of the original strain. More than 10% CO_2 leads to negative effect for its growth.

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Fig. 2 clearly shows that the obtained end strains after ALE could grow rapidly with 1~30% CO_2 without obvious inhibition. Compared with the original strain, the growth profiles of the end strains were similar under 1~30% CO_2. Under all the conditions, the maximum and minimum biomass concentrations of the ending strain AE10 on day 11 was 4.06±0.04 g/L with 1% CO_2 and 3.68±0.08 g/L with 30% CO_2, respectively. Both of them were higher than the maximum biomass concentrations of the ending strain AE20 and the original strain. This indicates that 10% CO_2 is a better environmental stress than 20% CO_2 during the ALE process, for the sake of obtaining an algal strain which can grow faster under high CO_2 concentration.

The initial pHs are related to CO_2 concentration and they are 6.4, 5.6, 5.4, and 5.2 under 1%, 10%, 20% and 30% CO_2, respectively. In the cultivation process, pH is increased shown in Fig.2. The growth patterns for the original strain, the end strain AE10 and the end strain AE20 are similar when they were cultivated under 1% and 10% CO_2. The pH of the original strain is always lower than those of the end strain AE10 and AE20 when they were cultivated under 20% and 30% CO_2. Under both CO_2 concentrations, the end strains maintained high CO_2 fixation rates so the pHs in media were higher than that of the original strain. Controlling pH in media will help microalgae to tolerate high concentration of CO_2 (Han et al., 2013). In further studies, pH control experiments will be investigated for the end strains.

3.3. Pigments' content of *Chlorella* sp. strains cultivated under different CO_2 concentrations

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The pigments in *Chlorella* sp. are consisted of chlorophyll a (chl-a), chlorophyll b (chl-b) and several kinds of carotenoids, which are vital in photosynthesis. In order to explain the difference of the growth status between the original strain and the end strains (AE10 and AE20), we measured the pigments' content of these strains. The results are shown in Fig. 3.

Considering the pigments' contents from different cultivation time as a function of CO_2 concentrations, we found that the maximum of chl-a, chl-b and carotenoids content of the original strain decreased as the increase of CO_2 concentration from 1% to 30%, individually. For the end strain AE10, the pigments' contents were almost identical under the CO_2 concentration from 1% to 20%, and decreased in 30% CO_2 concentration. However, the pigments' contents almost remain steady value under 1% - 30% CO_2 for the end strain AE20.

Considering the pigments' contents under different CO_2 concentrations as a function of the cultivation time, we found that the pigments' contents were increased during the initial 3 days and decreased later for the original strain cultivated in 1% CO_2, the end strain AE10 and AE20 cultivated in all levels of CO_2 concentrations. This might be caused by the change of nitrogen uptake rate, which had been shown in the previous studies (Lv et al., 2010; Zheng et al., 2012). For the original strain, the pigments' contents were slightly increased in the initial 9 days under 10% CO_2, and decreased in the initial 3 days and slightly increased later under 20% and 30% CO_2, respectively. These might be caused by the combination of the inhibition of high CO_2 concentration and promotion of sufficient nitrogen sources.

In conclusion, the pigments' contents of the end strain AE10 and AE20 were obviously higher than those of the original strains. Higher chlorophyll content of *C.*

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reinhardtii after 1000 generation evolved under 0.105 % CO_2 was also proposed (Collins and Bell, 2004). Compared with the original strain cultivated under 1% CO_2, the maximum contents of chl-a, chl-b, and carotenoids in the end strain AE10 cultivated in the same CO_2 concentration were enhanced 49%, 48% and 46%, respectively. Comparing the two end strains, we found the pigments' contents in end strain AE10 were higher than those in end strain AE20 cultivated in 1%, 10% and 20% CO_2, respectively. While cultivated in 30% CO_2, the results were reverse during the initial 3 day. Then the pigments' contents tended to be identical in these adapted strains in the later cultivation time. This phenomenon is correlated to the growth type in the Fig. 2.

Meanwhile, the chl-a/chl-b ratio in the end strain AE10 was slightly higher than that in the end strain AE20. Chlorophyll and some carotenoids can absorb light energy, and then collected energy should be passed on to chl-a which is the reaction center of the antenna array of core proteins. Much more light energy can be absorbed due to the increase contents of pigments. The increase of chl-a/chl-b ratio may indicate the improvement of light energy conversion efficiency of the end strain AE10.

3.4. Biochemical composition of *Chlorella* sp. strains under different CO_2 concentrations

The contents of total carbohydrates, proteins and lipids in the original strain, the end strain AE10 and the end strain AE20 cultivated under 1%, 10%, 20% and 30% CO_2 on day 11 were measured, respectively. The effects of the different CO_2 concentrations and algal strain status on the biochemical compositions of microalgae are illustrated in Fig. 4.

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Compared with the cultivation under 1% CO_2, the carbohydrates content of the original strain was increased by 30.7%, 13.2% and 20.9% under 10%, 20% and 30% CO_2, respectively. For the end strain AE10, there is no significant effect on carbohydrates content with the elevated CO_2 concentration from 1% to 20%. While it was cultivated under 30% CO_2, in which a little inhibition of growth was found, the carbohydrates content was significantly increased by 29.6% compared with that cultivated in 10% CO_2. For the end strain AE20, the carbohydrates content of the cultures under 10% and 20% CO_2 were almost identical, which were both significantly higher than that cultivated in 1% CO_2 and lower than that cultivated in 30% CO_2. These results indicate that, the proper high CO_2 level could promote the formation of carbohydrates in these *Chlorella* sp. strains. Compared with the carbohydrate contents of the original strain and the end strain AE10, those in the end strain AE20 are lower under all the four CO_2 concentrations. The metabolism of microalgae in ALE process leads to the decrease of carbohydrate formation under this severely high CO_2 concentration (20%). Moreover, our study indicates that the evolution under the appropriately high CO_2 concentration (10%) might lead to the stable production of carbohydrates stable in a wide range of CO_2 concentration (from 1% to 20%), since the strain had adapted very well to this high CO_2 concentration with only a slight inhibition.

For the original strain, the CO_2 concentration has a significant influence on the protein content. The protein content was increased from 26.1±1.0% to 30.5±0.5% and 32.9±1.7% as the CO_2 concentration increased from 1% to 20% and 30%, respectively,

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while 1-10% CO_2 concentration did not significantly affect the protein content. For the end strain AE10 and AE20, the varied CO_2 concentrations from 1% to 30% had no significant effects on the protein content. Compared to the original strain cultivated in 1% CO_2, the protein content of the end strain AE10 cultivated in 10% CO_2 almost remained unchanged, and the protein content of the end strain AE20 cultivated in 20% CO_2 was significantly increased by more than 10%. These results indicate that the evolution under severely high CO_2 concentration (20%) could promote the production of proteins. The extra proteins might be used to adjust the metabolic reactions for adapting to the severe environment stress.

For each of the three strains, the lipid content was significantly affected by the CO_2 concentration. As shown in Fig.4, the formation of lipid tended to be inhibited by the increase of CO_2 concentration. After the evolution in 10% and 20% CO_2 for 97 days, the lipid contents of the end strains AE10 and AE20 were both enhanced under all CO_2 concentrations compared with those of the original strain. Furthermore, the highest lipid content was 20.9±1.7% obtained by the strain which was evolved under the severely high CO_2 concentration (20%) and cultivated in the lowest CO_2 concentration (1%), which was 1.27 and 1.60 times to that in the original strain cultivated in 1% and 30% CO_2, respectively.

4. Conclusions

In this study, ALE was introduced as an effective method for obtaining high CO_2 tolerant *Chlorella* sp. strains. An appropriate stress should be critical to improve the growth phenotype of *Chlorella* under 1-30% CO_2 concentration in ALE. The improvement of the growth phenotype might be corresponding to the increase of

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pigments' contents and the suitable chl-a/chl-b ratio. For the obtained end strains, their carbohydrate contents were increased when the CO_2 concentration was increased and the lipid contents had a negative trend. There is no significant change between the protein content and CO_2 concentration for each of the end strains.

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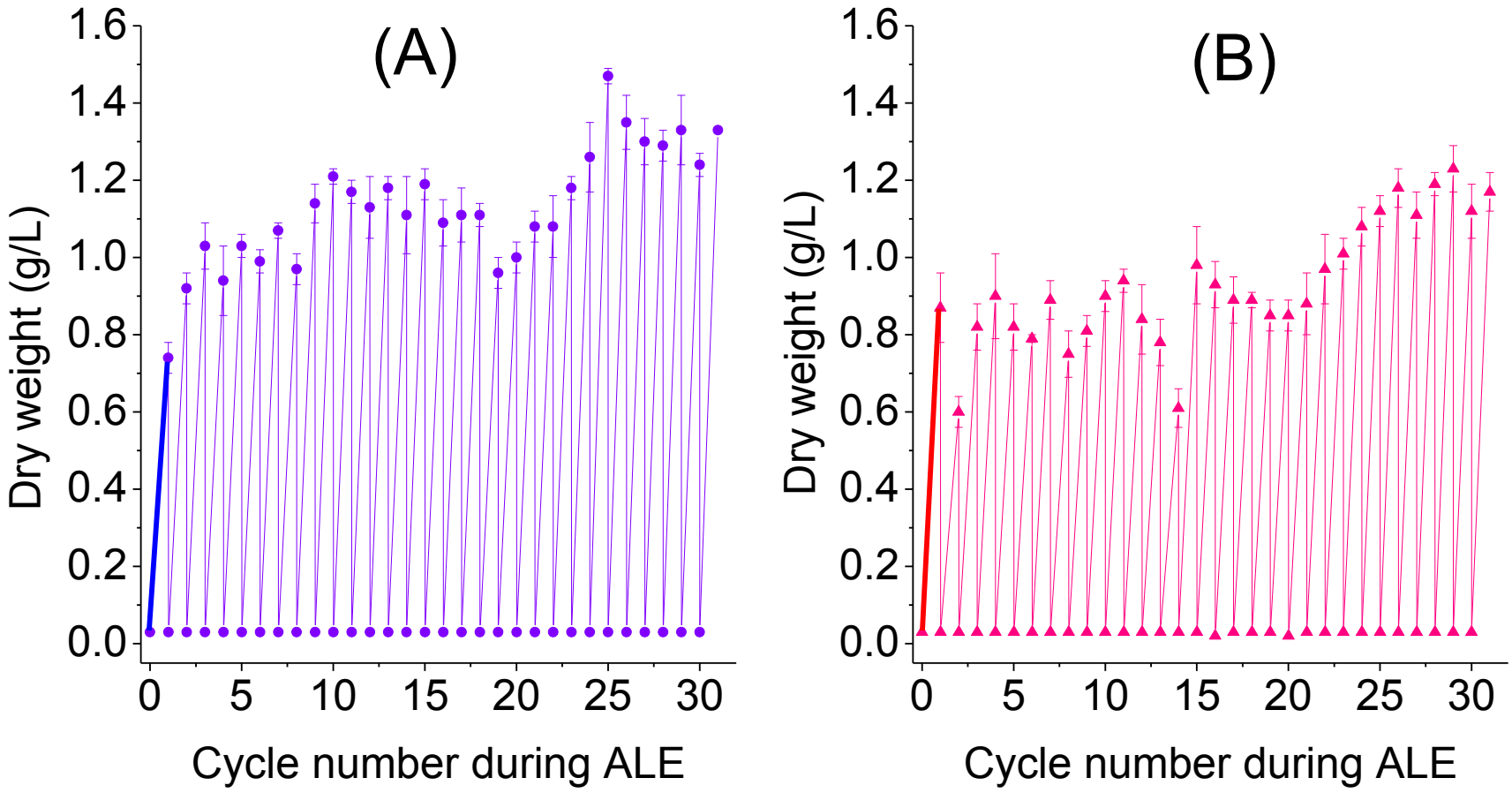
Figure Captions

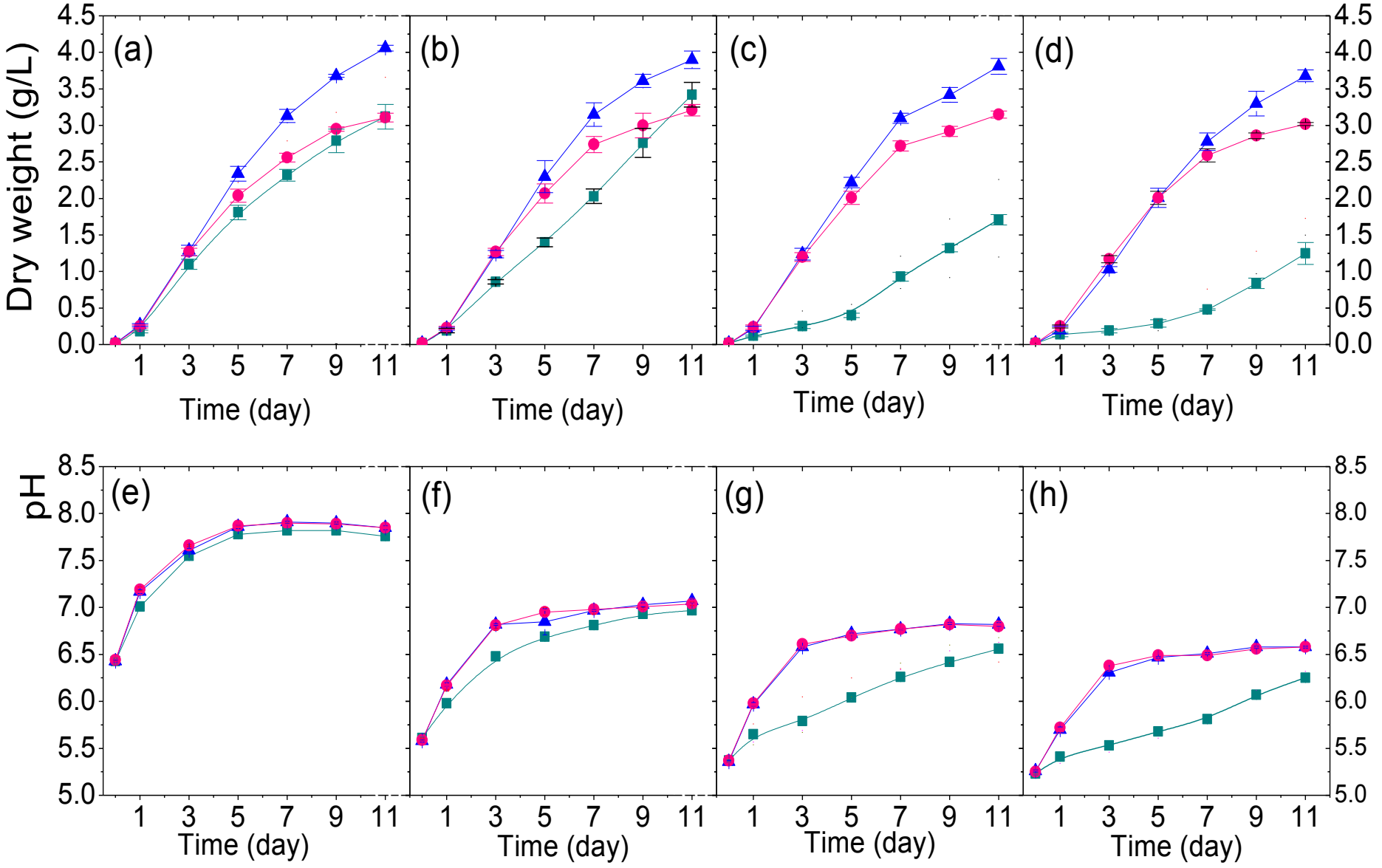
Fig. 1. Growth profile of *Chlorella* sp. strains under 10% CO_2 (A) and 20% CO_2 (B) from 1 to 31 cycles of ALE. The first cycle consists of 7 days, and the cycles from the second one consist of 3 days. For each new cycle, initial cell density in cultivation was diluted to OD_{750} of 0.1 with fresh BG11 medium.

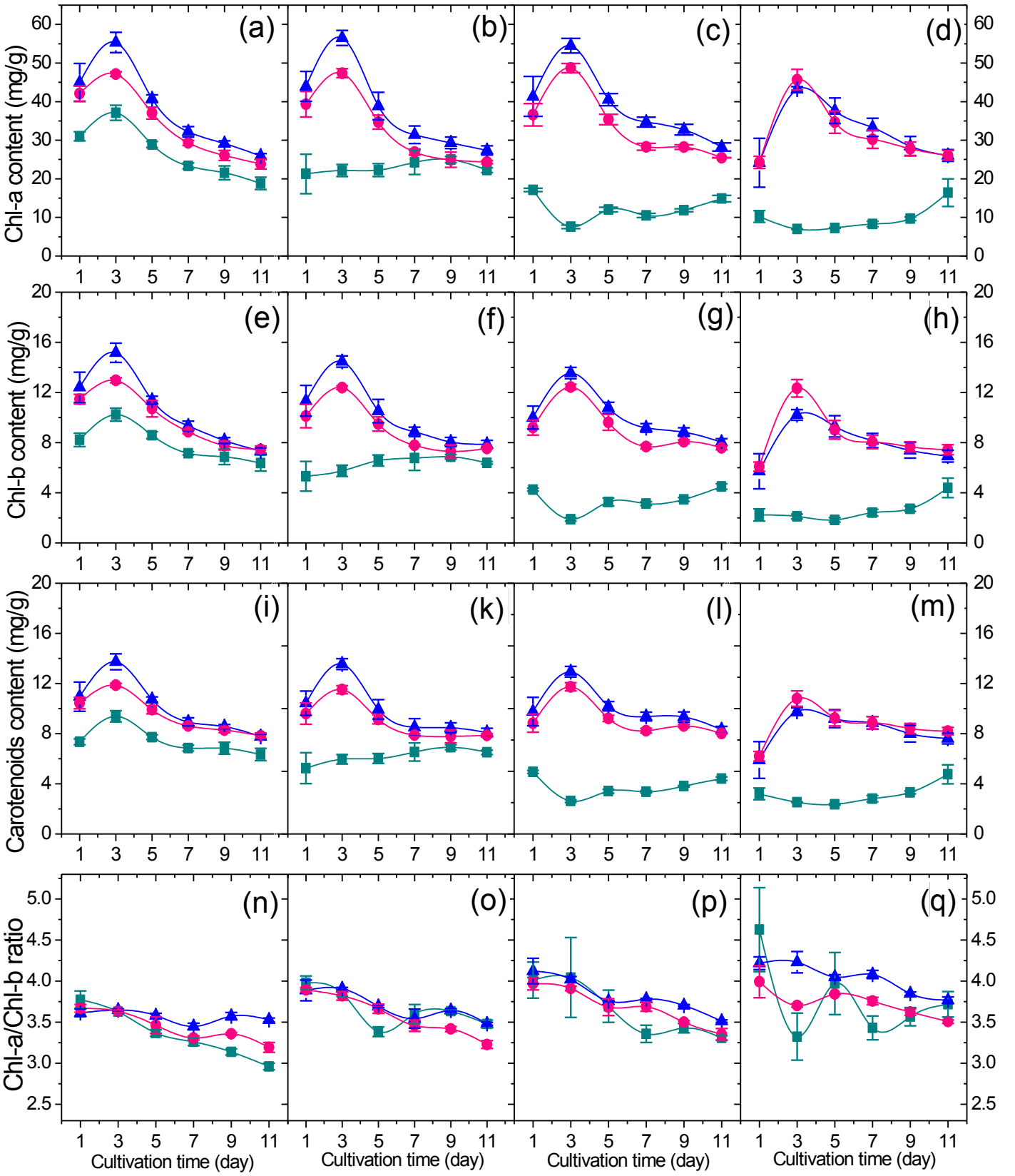
Fig.2. Growth profiles (a, b, c and d) and pH variation (e, f, g, and h) plots of the original strain (■), end strain AE10 (▲), end strain AE20 (●) of *C*. sp. under 1% (a, e), 10% (b, f), 20% (c, g) and 30% (d, h) CO_2 during the whole cultivation process.

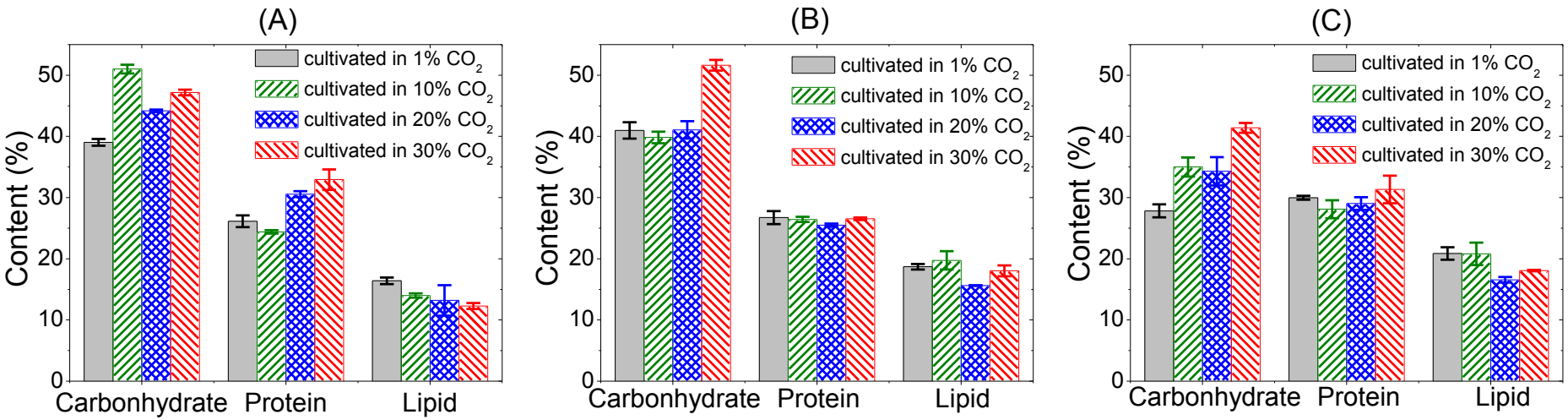
Fig.3. Changes of *chl*-a (a, b, c and d), *chl*-b (e, f, g and h), and carotenoids contents (i, k, l and m) and *chl*-a/*chl*-b ratio (n, o, p and q) in original strain (■), end strain AE10 (▲) and end strain AE20 (●) of *C*. sp. under 1% (a, e, i and n), 10% (b, f, k and o), 20% (c, g, l and p) and 30% (d, h, m and q) CO_2 during the 11-day batch cultivation.

Fig.4. Biochemical compositions of the original *C*. sp. strain (A), end strain AE10 (B) and end strain AE20 (C) under 1%, 10%, 20% and 30% CO_2 at the end of batch cultivation.

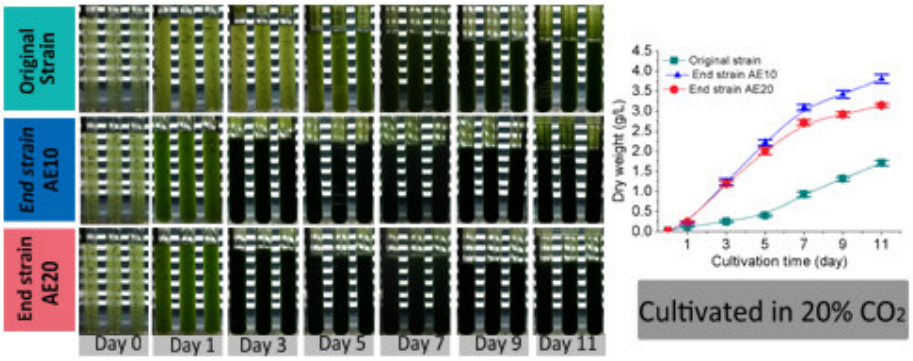

(A)
(B)
Dry weight (g/L)
Cycle number during ALE
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
0
5
10
15
20
25
30


Dry weight (g/L)
pH
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
Time (day)
Time (day)
Time (day)
Time (day)
Time (day)
Time (day)
Time (day)
Time (day)


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(k)
(l)
(m)
(n)
(o)
(p)
(q)
Chl-a content (mg/g)
Chl-b content (mg/g)
Carotenoids content (mg/g)
Chl-a/Chl-b ratio
Cultivation time (day)
Cultivation time (day)
Cultivation time (day)
Cultivation time (day)


(A)
(B)
(C)
Content (%)
0
10
20
30
40
50
cultivated in 1% CO2
cultivated in 10% CO2
cultivated in 20% CO2
cultivated in 30% CO2
Carbonhydrate
Protein
Lipid

1



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Highlights

- Microalgae strains tolerance to 20-30% CO_2 were constructed by adaptive evolution
- High growth rates in 1-30% CO_2 were obtained after adaptive evolution in 10% CO_2
- 10% CO_2 is better than 20% CO2 as environmental stress in adaptive evolution
- Chlorophyll and carotenoid concentrations in 10-30% CO_2 were improved

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