RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Simultaneous enhancement of microalgae biomass growth and lipid accumulation under continuous aeration with 15% CO_2

Yun Huang[1,2], Jun Cheng[1]*, Hongxiang Lu[1], Rui Huang[1], Junhu Zhou[1], Kefa Cen[1]

[1]State Key Laboratory of Clean Energy Utilization, Zhejiang University, Hangzhou 310027, China

[2] Key laboratory of Low-grade Energy Utilization Technologies and Systems, Ministry of Education, Chongqing University, Chongqing, 400044, China

Abstract

To overcome the opposing trends in biomass yield and lipid accumulation, *Chlorella* PY-ZU1 cultures were continuously aerated with 15% CO_2 to simultaneously enhance biomass yield (2.78 g L^{-1}) and lipid content (47.04%). Microalgal cells consumed almost all the nitrate in the culture after 1 day to synthesize 24 mg L^{-1} chlorophyll, which supported a peak growth rate of 0.675 g L^{-1} d^{-1}. Meanwhile, increased expression of key enzymes related to lipid synthesis (e.g., acetyl coenzyme A) enhanced lipid productivity to 192.10 mg L^{-1} d^{-1}. During the growth process, Carbon content of the dried biomass increased from 47.00% to 56.02% while nitrogen content decreased from 6.36% to 1.99%. The unsaturated fatty acids decreased and saturated fatty acids increased, thus improving the anti-oxidation stability of microalgal biodiesel.

Keywords: microalgae, CO_2 fixation, lipid, biomass, nitrogen deprivation

* Corresponding author: Prof. Dr. Jun Cheng, State Key Laboratory of Clean Energy Utilization, Zhejiang University, Hangzhou 310027, China. Tel.: +86 571 87952889; fax: +86 571 87951616. E-mail: juncheng@zju.edu.cn

1. Introduction

The combustion of fossil fuels has created serious concern because of global greenhouse gases (GHGs) accumulation and their effects on world economies and human habitats. Therefore, studies should be performed to increase the availability of renewable energy that decreases the emission of GHGs. Microalgae-based biofuels are expected to play a major role in solving these problems because microalgae show high efficiency in using solar energy and have a high growth rate. Moreover, cultivation of microalgae for producing biofuels is an environmentally sustainable method for microalgae can transform CO_2 to organic substances. [1–2]

Microalgal biodiesel production would based on a high lipid content in their cells [1–3]. Several growth conditions such as nutrient ratio, light intensity, cultivation temperature, and pH influence lipid content in microalgal cells [4]. The most common and effective method to increase lipid content in microalgal cells is to expose these cells to stressful conditions such as nitrogen limitation and starvation or high salinity. Illman et al. reported that lipid content in *Chlorella emersonii*, *C. minutissima*, *C. vulgaris*, and *C. pyrenoidosa* was 29%, 31%, 18%, and 11%, respectively, under regular nitrogen concentrations but increased to 63%, 57%, 40%, and 23%, respectively, under nitrogen-limitation conditions [5]. Takagi et al. increased lipid content in *Dunaliella* cells from 60% to 70% by increasing seawater salinity from standard 0.5 M NaCl to 1.5 M NaCl [6]. Total lipid yield by microalgae is equal to the lipid content multiplied by biomass yield. Although the above conditions effectively increase lipid content in microalgal cells, these conditions significantly reduce the production of microalgal biomass [7–8]. For example, Wu and Miao increased lipid content in *C. pyrenoidosa* cells from 28.58% to 54.49% by decreasing nitrogen concentration from the standard 15 mM to 0 mM (nitrogen starvation); however, this

decrease in nitrogen concentration significantly decreased biomass production from 2.01 to 0.42 g L^{-1}, which ultimately decreased total lipid production by 60.16% [9]. Therefore, in order to increase biofuel production, microalgae cultivation methods that can enhance lipid content but without biomass yield decrease should be developed.

Mujtaba et al. adopted a two-step method to increase lipid content in microalgal cells. In this method, *C. vulgaris* cells were first cultivated in a nitrogen-rich medium to achieve microalgal biomass of 1.87 g L^{-1}, after which the cells were transferred to a nitrogen-free medium for 24 h to enrich their lipid concentration to 53%. However, after transferring to the nitrogen-free medium, microalgal biomass decreased from 1.87 to 1.6 g L^{-1} [10]. In another study, Jiang et al. first cultivated *Nannochloropsis* cells in nitrogen-rich medium supplemented with 15% CO_2 as the carbon source to achieve microalgal biomass of 0.71 g L^{-1}; the cells were then cultured in a nitrogen-free medium under high light intensity. In this study, microalgal biomass and lipid concentration increased to 2.23 g L^{-1} and 59.9%, respectively[11]. Although the aforementioned two-step methods effectively increased microalgal lipid production, the correct timing of transferring algal cells from nitrogen-enriched medium into nitrogen-free medium and the appropriate cultivation time in nitrogen-free medium should be established to achieve optimum lipid productivity. These data gaps make the two-step methods complicated and unmanageable. Chiu et al. adopted a semi-continuous approach in which the medium of microalgal cell suspension was replaced periodically with the same amount of fresh culture medium[12]. The results of this study showed that microalgal biomass and lipid content reached approximately 1.4 g L^{-1} and 41.2%, respectively, after 12 days of cultivation when three-fifth of the medium of microalgal cell suspension was replaced by fresh culture medium every 3 days.

However, replacing the culture medium periodically is associated with several drawbacks such as higher cultivation cost, nutrient loss, and complicated operation, which prohibit large-scale cultivation and production.

CO_2 is the one of the most important nutrient for microalgal growth, and high concentrations of CO_2 promote lipid synthesis during microalgal growth[12–14]. However, limited studies have been performed to assess the effect of high CO_2 concentrations in promoting lipid synthesis during microalgal growth. The present study employed a new one-step method for promoting lipid accumulation and microalgal growth simultaneously using CO_2. In this study, the preponderant *C. pyrenoidosa* was cultivated in standard complete Brostol's solution that was continuously aerated with 15% CO_2 in nine-stage sequential bioreactors (BRs). The high concentration of inorganic carbon dissolved in the culture medium simultaneously promoted microalgal biomass and lipid production. Biomass elements and components were measured during microalgal growth to determine the synthesis and internal transformation of organic components, including proteins, sugars, and lipids. In addition, gene expressions of key enzymes related to lipid synthesis (e.g., acetyl coenzyme A) were measured to determine the effect of CO_2 on biomass growth and lipid accumulation.

2. Materials and methods

2.1 Strains and media

Chlorella PY-ZU1, a highly CO_2-tolerant and fast-growing microalgal species, was used in the present study. This strain was obtained by γ irradiation and high CO_2 domestication from *Chlorella pyrenoidosa*[15]. The cells were maintained in Brostol's solution (also known as soil extract, SE)[15–16], containing 0.25 g of $NaNO_3$, 0.075 g of $K_2HPO_4 \cdot 3H_2O$, 0.075 g of $MgSO_4 \cdot 7H_2O$,

0.025 g of $CaCl_2$•$2H_2O$, 0.175 g of KH_2PO_4, 0.025 g of NaCl, 40 mL of soil extract, 0.005 g of $FeCl_3$•$6H_2O$, 1 mL of Fe-EDTA, and 1 mL of A5 solution in 958 mL of deionized water.

2.2 Cultivation of microalgae under continuous aeration with 15% CO_2

All the experiments were performed in an artificial greenhouse at 27 °C (The optimal temperature for *Chlorella* PY-ZU1). Initial biomass concentration was maintained at 0.2 g L^{-1}. Microalgae were cultivated in nine-stage sequential BRs that were connected to cylindrical BRs (160 mm × Φ56 mm; 300-mL working volume) one by one. CO_2 (15%, which is the concentration of CO_2 in flue gas from most coal-fired power plants) was bubbled into the BR via a pipe at a rate of 30 mL min^{-1} to achieve the optimal molar ratio of nitrogen to carbon of 0.17 for lipid production[16]. Initial pH was adjusted to 6.5 by using 0.1 M HCl and 0.1 M NaOH. During incubation, light intensity of 6,000 Lux was applied on the surface of the BR by using four cool white lights and two plant lights (TLD 36W; Philips) fixed above the BR, and the pH of culture would be reached a stable level of 7.0 by aeration of 15% CO_2.

2.3 Analysis of biomass and lipid compositions

During cultivation, 10 mL samples were dewatered by centrifugation (Beckman Avanti J26-XP, USA) at 8,500 rpm for 10 min and dried at 70 °C for 24 h to obtain dry biomass. This biomass was used for compositional analysis.

Lipid samples dissolved in hexane were mixed with an internal standard (C19:0) and analyzed by a gas chromatograph (Agilent 7890A, USA) equipped with a HP-INNOWAX column (30 m × 320 μm × 0.25 μm, USA) and a FID detector. Operating conditions were as follows: He of carrier gas, 250 °C of injection temperature, and 250 °C of detector temperature.

The oven temperature was maintained at 150 °C for the initial 1 min and then rise to 200 °C at a heating rate of 15 °C/min that controlled by temperature programmer. Subsequently, the temperature was increased at 2 °C/min to 250 °C and maintained at 250 °C for the final 5 min. The components in Lipid samples were identified by comparing their retention times with those of the standards.[17] Biomass elements were determined using an elemental analyzer (Flash EA1112, USA). Total carbohydrate concentration was determined by performing high-performance liquid chromatography (Waters 2695, USA), and total proteins and amino acid concentrations were determined using automatic Kjeldahl nitrogen analyzer (Kjeltec Foss8400, Denmark) and automatic amino acid analyzer (Hitachi L-8900, Japan), respectively.

2.4 Analysis of differentially expressed genes of *Chlorella* PY-ZU1 under continuous aeration with 15% CO_2 and air

In the logarithmic phase of the culture, *Chlorella* PY-ZU1 strains that cultivated in 15% CO_2 and air were collected by centrifugation for extracting DNA. The gene encoding full-length 18s rDNA was amplified to obtain the algal genome by using the protocol performed in Cheng's study[8]. The following primers were used for 18s rDNA amplification: 18s-F, AACCTGGTTGATCCTGCCAGT, and 18s-R, TGATCCTTCTGCAGGTTCACCT. The gene was inserted into a cloning vector pMD19-T. Next, positive results were selected for sequencing. For cDNA library construction and Illumina sequencing, total RNA was extracted using TRIzol reagent (Invitrogen). mRNA was separated using magnetic sand method and was cleaved to synthesize double-stranded cDNA and fill to plane. A was added at the 3′ terminal end, and index connection was linked using TruSeq™ RNA Sample Preparation Kit. The target strip was enriched using polymerase chain reaction (PCR; 15 cycles) and was recycled using 2% agarose

gel. TBS380 (Picogreen) was used for definite quantitative determination, and bridge amplification was conducted for cBot cluster generation. HiSeq 2000 sequencing platform was used for 2*100 bp sequencing test. Sequence assembly and annotation were similar to those used in Cheng's study[18].

2.5 Calculation of biomass and lipid productivity

Biomass concentration (g L^{-1}) was calculated from the dry microalgal biomass weight produced per liter of culture. Absolute growth productivity (AGP, g L^{-1} d^{-1}) was calculated using Eq. (1):

$$AGP = \frac{M_1 - M_2}{t_1 - t_2} \quad (1)$$

where M_1 is the biomass concentration at time t_1 and M_2 is the biomass concentration at time t_2.

The CO_2 fixation rate was calculated from the biomass concentration and carbon element content using Eq. (2):

$$CO_2 \text{ fixation rate} = \frac{W_1 \times C_1\% - W_2 \times C_2\%}{t_1 - t_2} \times \frac{M_{CO2}}{M_C} \quad (2)$$

where C_1 is the biomass carbon element content at time t_1, C_2 is the content at time t_2, and M is the molar mass.

Absolute lipid productivity (AGP, g L^{-1} d^{-1}) was calculated using Eq. (3):

$$ALP = \frac{M_1 L_1 - M_2 L_2}{t_1 - t_2} \quad (3)$$

where L_1 is the lipid content at time t_1 and M_2 is the lipid content at time t_2.

3. Results and discussion

3.1 Biomass yield and lipid content of microalgae cultivated with 15% CO_2

The biomass concentration and lipid content of *Chlorella* PY-ZU1 continuously aerated with 15% CO_2 simultaneously increased to 2.78 g L^{-1} and 47.04%, respectively (Fig. 1); these values are 119.23% and 132.08% higher than those obtained for *Chlorella* PY-ZU1 cultivated under continuous air flow (1.30 g L^{-1} and 18.95%, respectively). Lipid productivity increased by 408.8% under the same conditions. Lipid production and productivity reached maximum values of 1.31 g L^{-1} [Fig. 2(a)] and 192.10 mg L^{-1} d^{-1} [Fig. 2(b)], respectively.

Excessive consumption of sodium nitrate on the first day of cultivation caused biomass production and CO_2 fixation rates to peak at 0.675 g L^{-1} d^{-1} and 1.223 g L^{-1} d^{-1}, respectively, on the second day; however, these rates declined subsequently as the nutrients depleted. Rapid nitrate consumption during the first two days of cultivation induced nitrogen deprivation in the culture medium. Some reports indicate that nitrogen deprivation results in the accumulation of large amounts of lipids in the form of triacylglycerols[5, 19] and that this accumulation reaches maximum levels 2–3 days after nitrogen exhaustion[20]. In the present study, lipid accumulation by *Chlorella* PY-ZU1 showed a similar pattern. Lipid productivity reached 192.10 mg L^{-1} d^{-1} after 4 days of cultivation. Thereafter, as the rate of lipid production decreased, lipid concentration increased continuously over the next 8 days of cultivation. Although nitrogen starvation is the most effective method to enhance lipid concentration in microalgae[8], but not to increase biomass yield[21]. In the present study, lipid content and biomass yield increased simultaneously under high CO_2 concentrations.

Table 1 lists several methods to increase lipid production of *C. pyrenoidosa*. Tang et al. cultivated *C. pyrenoidosa* SJTU-2 in different CO_2 concentrations (from 0.03% to 50%) for lipid production. These microalgae showed the best lipid-producing capacity at 10% CO_2; the

maximum biomass concentration and lipid productivity under this condition were 1.55 g L^{-1} and 26.85 mg L^{-1} d^{-1}, respectively.[14] Han et al. increased lipid productivity of *C. pyrenoidosa* to 82.50 mg L^{-1} d^{-1} by semi-continuously cultivating the microalgae under nitrogen limitation condition.[13] Wang et al. achieved lipid productivity of 134.0 mg L^{-1} d^{-1} by optimizing the growth culture.[22] Wen et al. achieved higher lipid productivity of 144.93 mg L^{-1} d^{-1} by introducing nitrate in a chemostat culture of *C. pyrenoidosa* XQ-20044 at a specific rate of 2.41 mmol g^{-1} d^{-1}. [23] In the present study, biomass production and lipid concentration of *Chlorella* PY-ZU1 increased simultaneously under continuous aeration with 15% CO_2. Lipid productivity peaked at 192.10 mg L^{-1} d^{-1}, indicating the feasibility and significant advantage of using one-step method for microalgal lipid production.

3.2 Analysis of improvements in biomass and lipid productivity by 15% CO_2

Simultaneous biomass and lipid production under continuous aeration with 15% CO_2 is mainly associated with nutrient consumption, chlorophyll synthesis, and gene expression. Aeration with 15% CO_2 supplies adequate concentration of inorganic carbon (approximately 17.41 mM) to microalgal cultures[16]. Abundance of organic carbon accelerates nitrogen metabolism[12, 24], leading to nitrate nitrogen depletion by microalgae after 1 day of cultivation. This nitrate nitrogen is converted to chlorophyll (Fig. 3) and proteins. Synthesis of chlorophyll and proteins supplies enough sites and enzymes for microalgal photosynthesis, which rapidly increases biomass concentration (2.71 g L^{-1}) from days 1–5. In the succeeding days, because of nitrogen deficiency, the microalgae consume the chlorophyll that they have produced as a nitrogen source to synthesize organic substances, thus guaranteeing steady biomass production.

In addition, nitrogen deprivation promoted lipid production. Thus, biomass and lipid production increase simultaneously with cultivation time.

Gene expression profiling of microalgae treated with 15% CO_2 and air was performed as Cheng's study[18]. Microalgal genes related to cell growth, such as, nitrogen metabolism-related genes (E1.7.1.1), carbon metabolism-related genes (EC:5.3.16), chloroplast synthesis-related genes (E1.1.1.39, EC:1.1.1.37, EC:1.1.1.37, and EC:2.7.9.1), and mitochondria synthesis-related genes (EC2.6.1.2, E1.1.1.40, and EC2.6.1.1), showed higher expression in the presence of 15% CO_2 than in the presence of air (Table 2). Upregulation of nitrate reductase increased the conversion of nitrate to ammonia for DNA replication. Upregulation of ribulose-5P improved carbon fixation during photosynthesis. These results indicated that the abovementioned genes worked together to promote microalgal growth[18]. Upregulation of nitrogen metabolism makes the culture go to nitrogen-deficiency stage in advance. Thus, cultivation in 15% CO_2 guarantees microalgal growth (peak at 0.675 g L^{-1} d^{-1}) and CO_2 fixation (peak at 1.223 g L^{-1} d^{-1}). While the stress induced by nitrogen deprivation results in high lipid productivity (peak at 192.10 mg L^{-1} d^{-1}).

Acetyl coenzyme A, an important intermediate substrate, is common to the synthesis of carbohydrates, lipids, and proteins.[25] Quantitative PCR testing indicates that in microalgae, expression of the gene encoding acetyl coenzyme A is higher during cultivation in 15% CO_2 than during cultivation under air (Fig. 4). Thus, acetyl coenzyme A is not only a precursor of fatty acids and energy compounds such as ketone bodies but also a rate-limiting enzyme in fatty acid synthesis. Enhancements in the expression of acetyl coenzyme A generation-related genes may promote biomass yield and lipid content simultaneously of *Chlorella* PY-ZU1.[26]

3.3 Characteristics of microalgal biomass and microalgae-based biodiesel

Results of elemental analysis of microalgal biomass cultivated under continuous aeration with 15% CO_2 are shown in Table 3. Elemental carbon (C) and hydrogen (H) concentrations increased with cultivation time from the initial values of 45.48% and 6.82%, respectively, to the final values of 56.02% and 8.56%, respectively. These increases are mainly attributed to the abundance of dissolved carbon and water in the culture, which supplied C and H to the microalgae. In addition, higher heating value of the biomass increased (from 17.90 to 25.19 MJ kg^{-1}) as the carbon concentration increased.[5, 27] Because lipids are the main energy carriers in microalgal cells, increase in the heating value indicated that the metabolic pathway favored lipid accumulation.

Lipids from *C. pyrenoidosa* biomass are rich in C16–C18, indicating the potential use of *C. pyrenoidosa* biomass in producing biodiesel[28]. Although the composition of microalgal lipids generally depends on the algal species and cultivation conditions[8, 14, 19, 29–31], in the present study, the lipid composition depended on nitrogen deprivation caused by CO_2 aeration. The four most abundant lipid components were C16:0 (22.27%–34.56% of the total biodiesel content), C18:1 (5.28%–20.77% of the total biodiesel content), C18:2 (15.87%–23.14% of the total biodiesel content), and C18:3 (13.91%–21.80% of the total biodiesel content; Fig. 5). Concentrations of polyunsaturated fatty acid methyl esters (C16:2, C16:3, C18:2, and C18:3) in the biomass-based biodiesel decreased with cultivation time while those of saturated and partially saturated methyl esters, especially C18:1, increased from 5.28% to 20.77% with cultivation time. Decrease in the concentrations of polyunsaturated fatty acid methyl esters and increase in the concentrations of

saturated and partially saturated methyl esters may help improve the antioxidative stability of microalgal biodiesel.

3.4 Dynamic changes in the organic composition of microalgal biomass cultivated under continuous aeration with 15% CO_2

Previous studies[14, 19] on the response of lipid metabolism to environmental stress indicate that carbon elements are distributed in energy-rich materials such as lipids and carbohydrates under nitrogen deprivation conditions at the expense of protein and peptide consumption[20] and that specific transformation depends on the algal species under study[32]. Similarly, under nitrogen deficiency, proteins in *C. pyrenoidosa* were mainly converted to lipids (Fig. 6). After depletion of nitrates in the culture medium, protein concentration in the microalgal biomass decreased from 39.07% to 18.96%; in contrast, carbohydrate concentration decreased minimally during cultivation. Phosphoenolpyruvate (PEP) generated by glycolysis is usually delivered to plasmids as an acetyl coenzyme A precursor[33–34], thereby supplying materials for lipid synthesis. Expression of PEP carboxylase, which catalyzes the conversion of PEP to oxaloacetic acid, decreases under nitrogen deprivation, which in turn may restrain the competitive effects of PEP reaction on fatty acid synthesis [4] and result in metabolic flow responsible for lipid accumulation. In general, decrease in both carbohydrate and protein concentrations increased microalgal lipid concentration to 47.04%.

Analysis of amino acid compositions shown in Table 4 demonstrates that >10 types of amino acids were present in *Chlorella* PY-ZU1 biomass, including eight essential amino acids (accounting for 43.48%–54.04% of the total amino acid concentration). Thus, *Chlorella* PY-ZU1 biomass can be a valuable raw material for producing medicines. In the presence of adequate

nitrogen, protein concentration of the microbial biomass was as high as 43.35%; under nitrogen deprivation, protein concentration decreased to 8.69% and lipid concentration increased to 47.04%. These results indicated that high CO_2 concentrations could efficiently promote inter-transformation of organic material in microalgal cells and enhance biomass and lipid production. Thus, continuous aeration with 15% CO_2 presents a simple and efficient one-step approach for lipid production by microalgal cells. Moreover, the microalgal biomass may be used comprehensively according to its organic composition. For example, biomass with high protein or lipid conent may be used to produce high-performance health products or biodiesel, respectively.

4. Conclusions

Continuous aeration with 15% CO_2 induced nitrogen deprivation during *Chlorella* PY-ZU1 cultivation, thus simultaneously promoting biomass (2.78 g L^{-1}) and lipid (47.04%) production. Aeration with 15% CO_2 supplies adequate amount of inorganic carbon (approximately 17.41 mM) required by microalgae and upregulates the expression of some microalgal genes related to nitrogen metabolism, chloroplast synthesis, and carbon fixation. Upregulation of nitrogen metabolism makes the culture nitrogen deficient. Thus, aeration with 15% CO_2 can guarantee microalgal growth (peak at 0.675 g L^{-1} d^{-1}) and CO_2 fixation (peak at 1.223 g L^{-1} d^{-1}). Moreover, the stress induced by nitrogen deprivation leads to high lipid productivity (peak at 192.10 mg L^{-1} d^{-1}). Thus, cultivation of *Chlorella* PY-ZU1 under high CO_2 concentration promotes simultaneous biomass and lipids production.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (51176163,

51476141), National High Technology R&D Program of China (2012AA050101), Zhejiang Provincial Natural Science Foundation of China (LR14E060002).

References

1. S. Vidyashankar, K. Deviprasad, V. S. Chauhan, G.A. Ravishankar, R. Sarada, *Bioresource Technology*, 2013, **144**, 28–37.
2. Yoo C, Jun SY, Lee JY, Ahn CY, Oh HM, *Bioresource Technology*, 2010,**101**, 71–4.
3. S. Basu, AS Roy, K. Mohanty, AK. Ghoshal, *Bioresource Technology*, 2013, **143**, 369–77.
4. N.M.D. Courchesne, A. Parisien, B. Wang, C.Q.Lan, *Journal of Biotechnology*, 2009, **141**, 31–41.
5. A.M. Illman, A.H. Scragg, S.W. Shales, *Enzyme Microbiology Technology*, 2000, **27**, 631–5
6. M. Takagi, T. Yoshida , *Journal of bioscience bioengineering*, 2006, **101**, 223–6.
7. Q. Lin, J. Lin, *Bioresource Technology*, 2011, **102**, 1615–21.
8. A. Widjaja, C.C. Chien, Y.H. Ju, *Journal of Taiwan Institute Chemical*, 2009, **40**, 13–20.
9. H.Q. Wu, X.L. Miao, *Bioresource Technology*, 2014, **170**, 421–7.
10. G. Mujtaba, W. Choi, C.G. Lee, K. Lee, *Bioresource Technology*, 2012, **123**, 279–83.
11. L. Jiang, S. Luo, X. Fan, Z. Yang, R. Guo, *Applied Energy*, 2011, **88**, 3336–41.
12. S.Y. Chiu, C.Y. Kao, M.T. Tsai, S.C. Ong, C.H. Chen, C.S. Lin, *Bioresource Technology*, 2009, **100**, 833–8.
13. F.F. Han, J.K. Huang, Y.G. Li, W.L. Wang, M.X. Wan, G.M. Shen, J. Wang, *Bioresource Technology*, 2013, **136**, 418–24.
14. D. Tang, W. Han, P. Li, X. Miao, J. Zhong, *Bioresource Technology*, 2011, **102**, 3071–6.
15. J. Cheng, Y. Huang, J. Feng, J. Sun, J. Zhou, K. Cen, *Bioresource Technology*, 2013, **136**, 496–501.
16. J. Cheng, Y. Huang, J. Feng, J. Sun, J. Zhou, K. Cen, *Bioresource Technology*, 2013, **144**, 321–7.
17. J Cheng, R Huang, T Li, J Zhou, K Cen. *Bioresource Technology*, 2014, **170**, 69–75.
18. R.L. Cheng, J. Feng, B.X. Zhang, Y. Huang, J. Cheng, C.X. Zhang, *Bioenergy Research*, 2014, **7**, 192–205.
19. S. Mandal, N. Mallick, *Applied Microbiology Biotechnology*, 2009, **84**, 281–91.
20. M. Siaut, S. Cuine, C. Cagnon, B. Fessler, M. Nguyen, P. Carrier, A. Beyly, F. Beisson, C. Triantaphylides, Y. Li-Beisson, G. Peltier, *BMC Biotechnology*, 2011, **11**, 7.
21. C. Weldy, M. Huesemann, *DOE Journal of underg Research*, 2007, **7**, 115–22.
22. W. Wang, F. Han, Y. Li, Y. Wu, J. Wang, R. Pan, G. Shen, *Bioresource Technology*, 2014, **170**, 395–403.
23. X. Wen, Y. Geng, Y.G. Li, *Bioresource Technology*, 2014, **161**, 297–303.
24. M. Picardo, de Medeiros, O. Araújo, R.M.Chaloub, *Bioresource Technology*, 2013, **143**,

242–50.

25. O. Maestri, F. Joset, *Molecular Microbiology*, 2000, **37**, 828–38.
26. S.W. Ragsdale, H.G.Wood, *Critical Reviews in Biochemistry and Molecular Biology*, 1991, **26**, 261–300.
27. P. Biller, R. Riley, A. Ross, *Bioresource Technology*, 2011, **102**, 4841–8.
28. G. Knothe, *Energy and Fuel*, 2008, **22**, 1358–64.
29. J. Liu, J. Huang, Z. Sun, Y. Zhong, Y. Jiang, F. Chen, *Bioresource Technology*, 2011, **102**, 106–10.
30. A.M. Lizzul, P. Hellier, S. Purton, F. Baganz, N. Ladommatos, L. Campos, *Bioresource Technology*, 2014, **151**, 12–8.
31. J.M. Lv, L.H. Cheng, X.H. Xu, L. Zhang, H.L. Chen, *Bioresource Technology*, 2010, **101**, 6797–804.
32. S.H. Ho, C.Y. Chen, J.S Chang, *Bioresource Technology*, 2012, **113**, 244–52.
33. S. Baud, S. Wuillème, B. Dubreucq, A. De Almeida, C. Vuagnat, L. Lepiniec, M. Miquel, *Plant Journal*, 2007, **52**, 405–19.
34. J. Schwender, J.B. Ohlrogge, *Plant Physiology*, 2002, **130**, 347–61.

List of Figures and Tables:

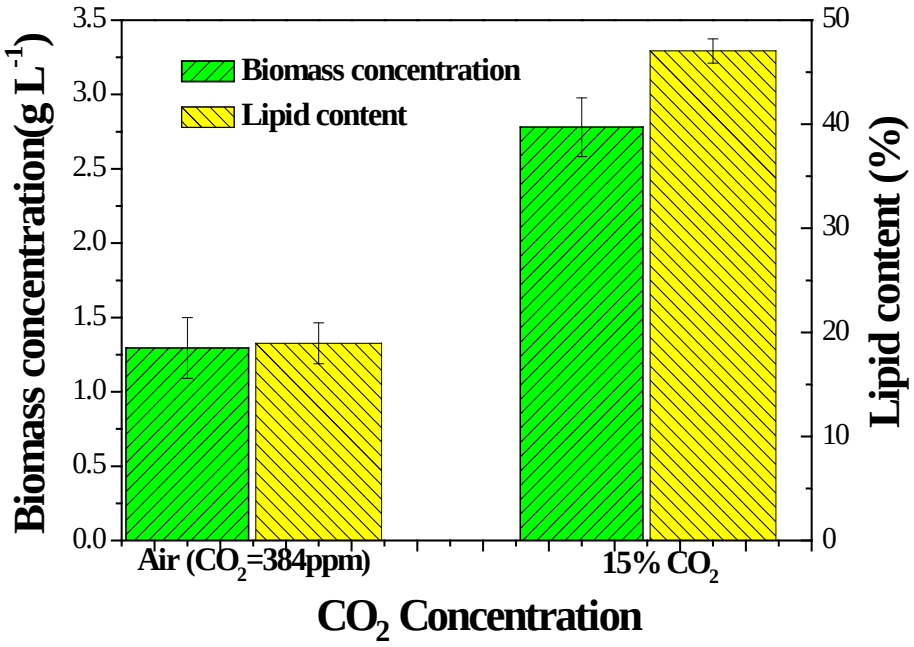
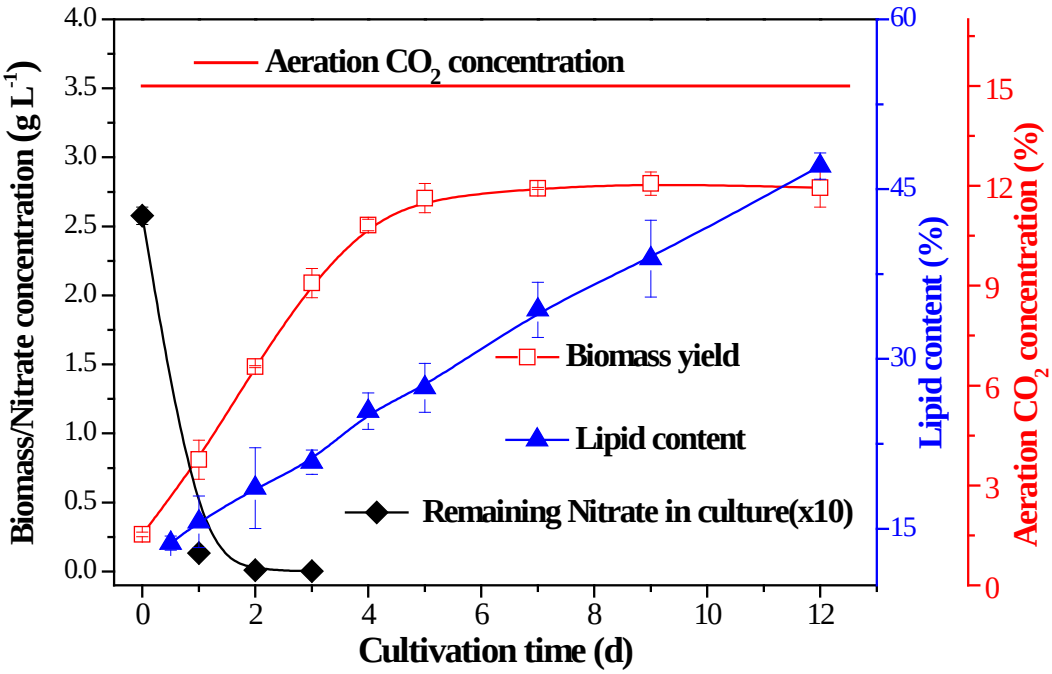
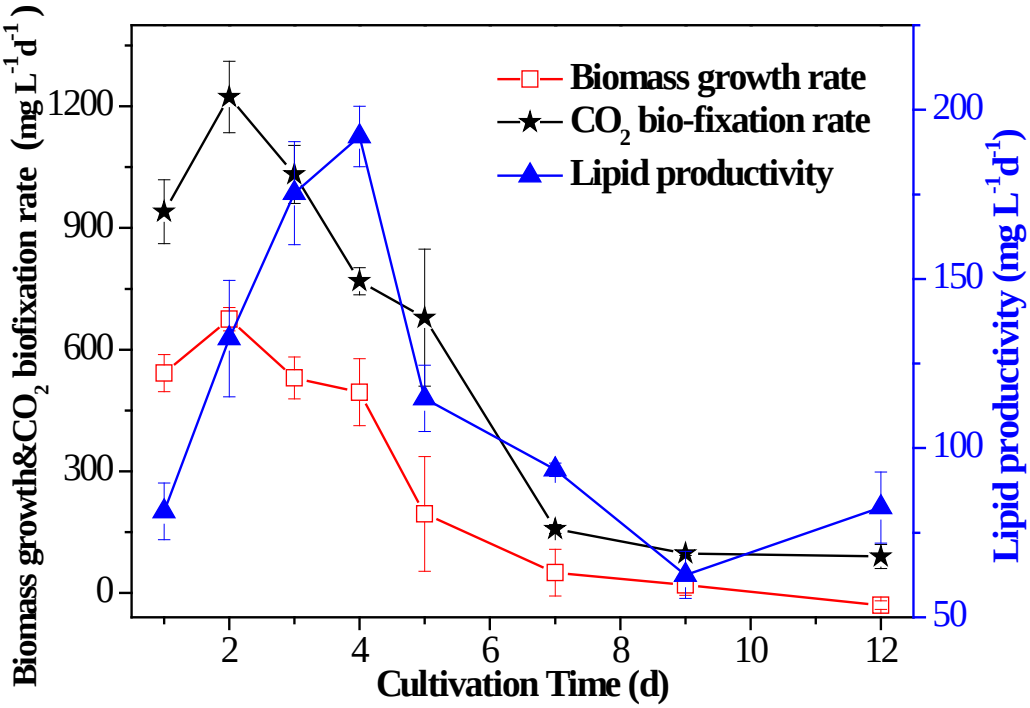


Fig.1 Comparison of biomass yield and lipid content of *Chlorella* PY-ZU1 cultivated under air and 15% (v/v) CO_2.



(a) Biomass growth and lipid content with nitrate consumption under 15% CO_2



(b) Biomass and lipid productivity

Fig.2 Biomass and lipid production curves of *Chlorella* PY-ZU1 cultivated under continuous aeration with 15% CO_2. (a) Biomass growth and lipid content with nitrate consumption; (b) Biomass and lipid productivity.

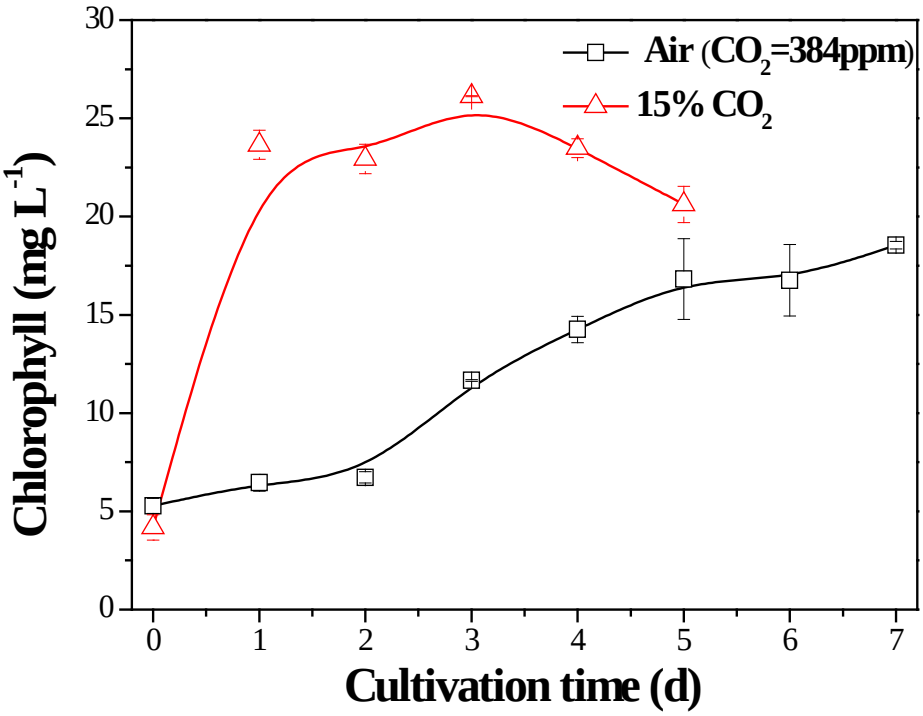


Fig.3 Chlorophyll synthesis curves of *Chlorella* PY-ZU1 cultivated under continuous aeration with air and 15% CO_2.

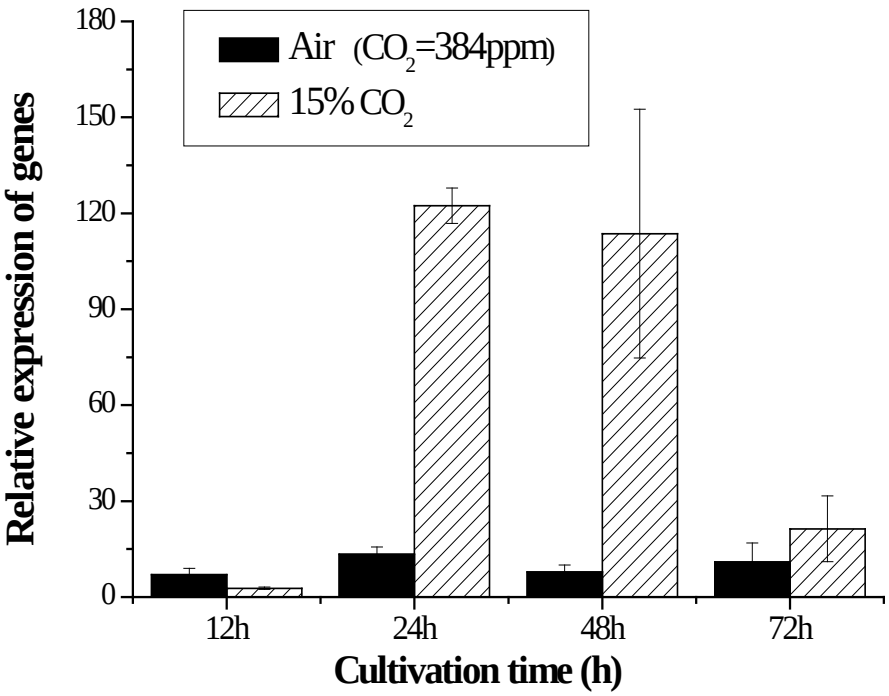


Fig.4 Relative expressions of acetyl coenzyme carboxylase genes in *Chlorella* PY-ZU1 cultivated under continuous aeration with air and 15% CO_2.

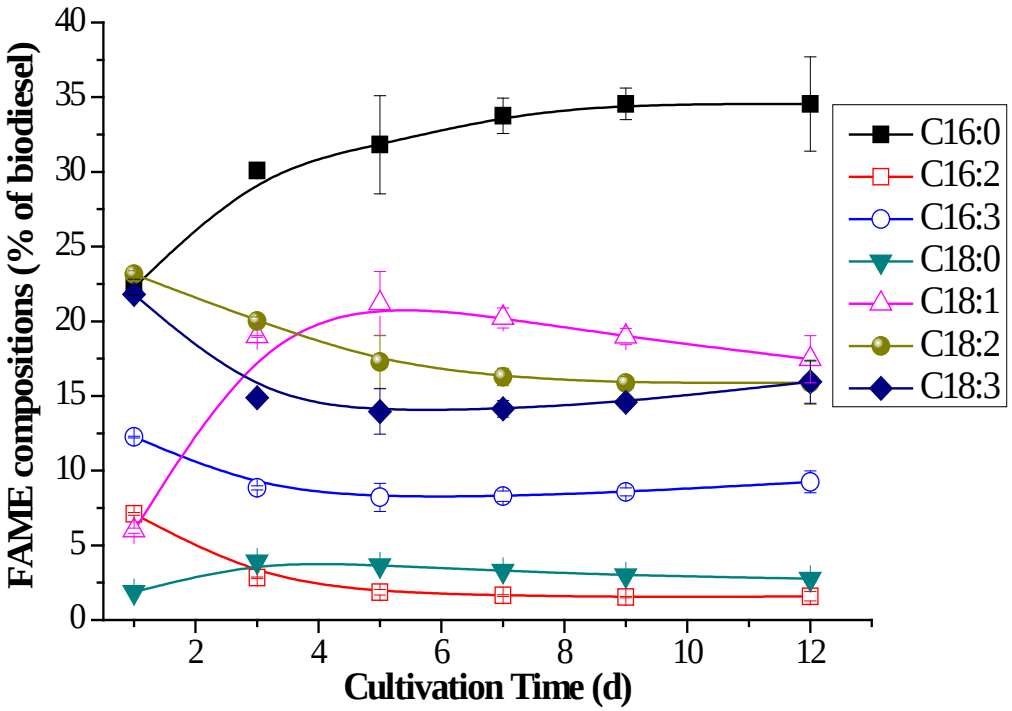


Fig.5 Dynamic changes in the composition of fatty acid methyl esters in the biodiesel obtained from microalgal biomass cultivated under continuous aeration with 15% CO_2.

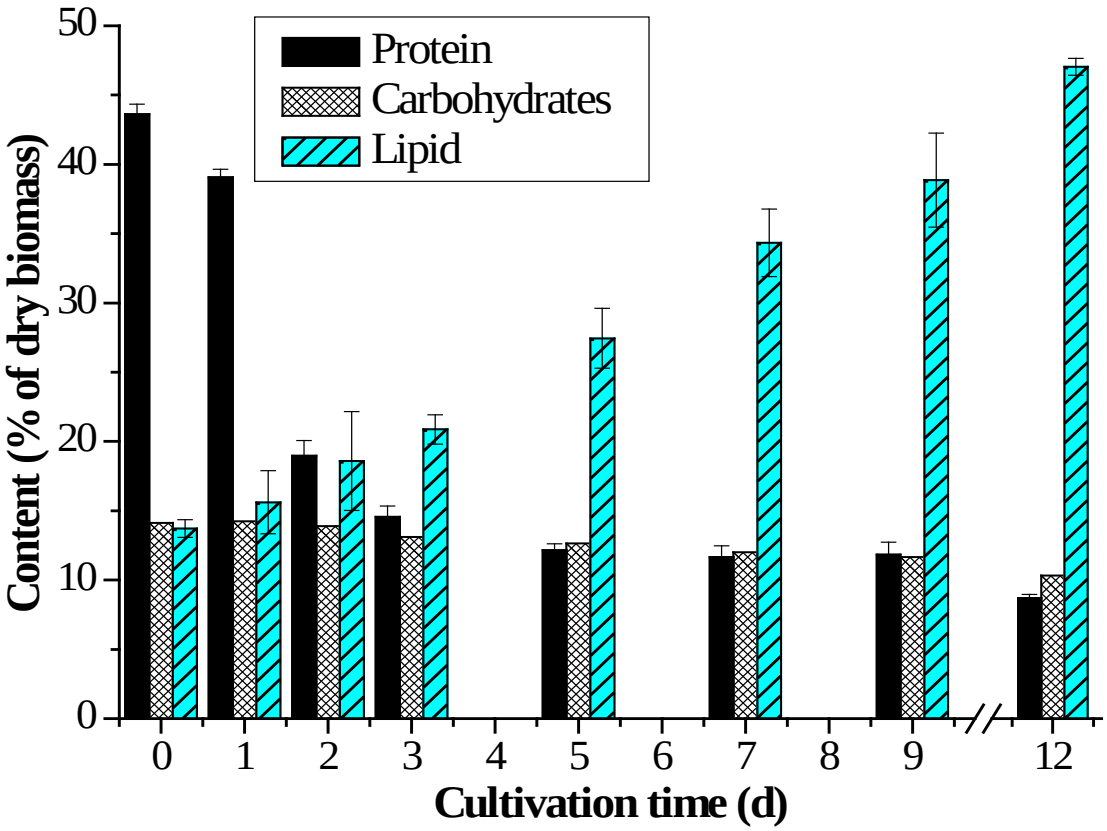


Fig.6 Dynamic changes in organic compositions of microalgal biomass cultivated under continuous aeration with 15% CO_2.

Table 1. Comparison of lipid productivity of *C. pyrenoidosa* cultivated under different cultivation conditions.

Species	Cultivation	CO_2 conc. (%)	Biomass conc. (g L^{-1})	Biomass productivity (g L^{-1} d^{-1})	Lipid content (%)	Lipid productivity (mg L^{-1} d^{-1})	Reference
C. pyrenoidosa SJTU-2	with different CO_2	10	1.55	0.144	24.25	34.92*	Tang et al., 2011
C. pyrenoidosa	nitrogen limitation	100	1.41	0.222 [a]	30.9	82.50	Han et al., 2013
C. pyrenoidosa	growth culture optimization	2	1.77	—	27.8	134.0	Wang et al., 2014
C.pyrenoidosa XQ-20044	inputting nitrate into chemostat culture at a certain rate	1	NA	0.414	34.69	144.93	Wen et al., 2014
C. pyrenoidosa PY-ZU1	with a continuous flow of 15% CO_2	15	2.71	0.675	47.04	192.10	This study

[a] calculated based on the reported data.

Table 2. Comparison of gene expressions related to cell growth and lipid synthesis in *Chlorella* PY-ZU1 cultivated under continuous aeration with 15% CO_2 and air.

KEGG Gene Name[a]	**Description**	**Function**	**Relative expression of genes**	
			15% CO_2	**Air**
E1.7.1.1	Nitrate reductase Nitrite reductase	Nitratre conversion	2100	124.61
E1.4.1.4	glutamate dehydrogenase	glutamate decomposition	92.69	284.66
GLT1	glutamate synthase, NADH-dependent		251.17	251.97
E4.2.1.1	beta- carbonic anhydrase cynT	inorganic carbon Conversion	97.8	343.77
EC:5.3.16	Ribulose-5P	CO_2 fixation	39	5190
E1.1.1.39	malic enzyme	chloroplast synthesis	13.72	1.68
EC:1.1.1.37	malate Dehydrogenase		263.85	43.61
EC:2.7.9.1	Pyruvate orthophosphate dikinase (PPDK)		931.96	8257.03
EC2.6.1.2	glutamate pyruvate transaminase (GPT), ALT	Mitochondria synthesis	86.87	57.42
E1.1.1.40	Malic enzyme (NADPME) MaeB		60.32	51.97
EC2.6.1.1	serum glutamic-oxaloacetic transaminase GOT1		24.84	54.5

[a] KEGG is the abbreviation of "Kyoto Encyclopedia of Genes and Genomes".

Table 3. Elemental compositions and heating values of microalgal biomass cultivated under continuous aeration with 15% CO_2.

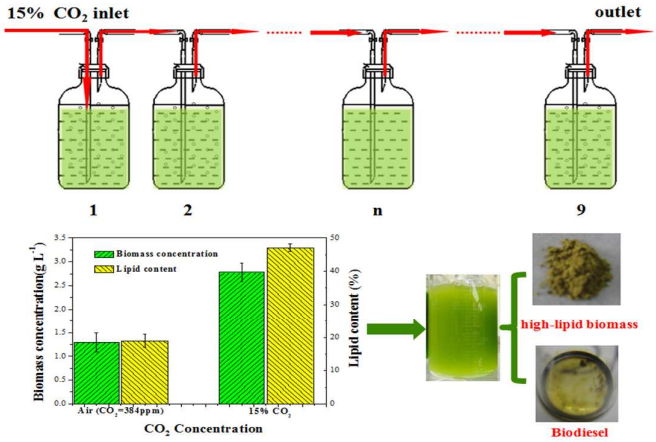
Cultivation time (d)	Elemental compositions (%)				C/N (M/M)	Higher Heating value (MJ/Kg) [b]	Biomass Formula
	N	C	H	O [a]			
1	6.36	47.95	6.88	38.81	8.80	19.10	$CH_{1.721}O_{0.607}N_{0.114}$
2	3.16	47.00	7.07	42.77	17.35	18.35	$CH_{1.805}O_{0.683}N_{0.0576}$
3	2.28	47.80	7.23	42.69	24.46	18.86	$CH_{1.815}O_{0.669}N_{0.0409}$
4	2.14	48.52	7.37	41.97	26.45	19.43	$CH_{1.823}O_{0.648}N_{0.0378}$
5	1.95	50.16	7.65	40.24	30.01	20.70	$CH_{1.830}O_{0.602}N_{0.0333}$
7	2.00	51.78	7.84	38.38	30.21	21.85	$CH_{1.817}O_{0.556}N_{0.0331}$
9	2.05	53.09	8.08	36.78	30.21	22.92	$CH_{1.826}O_{0.520}N_{0.0330}$
12	1.99	56.02	8.56	33.44	32.91	25.19	$CH_{1.834}O_{0.447}N_{0.0304}$

[a] The oxygen contents were calculated from mass balance of biomass.

[b] The higher heating value (HHV) was estimated with the Dulong formula [27].
HHV(MJ/kg)=0.338C+1.428(H-O/8)+0.095S

Table 4. Main amino acid compositions of microalgal biomass cultivated under continuous aeration with 15% CO_2.

Cultivation time (d)		1	3	5	7	9	12
Total amino acids (TAA, % of dry biomass)		38.91	13.73	11.91	10.62	10.75	8.65
Essential amino acids (EAA, % of dry biomass)		16.60	6.59	5.88	5.52	5.61	4.67
EAA/TAA (%)		42.67	48.03	49.31	51.98	52.20	54.04
Compositions of EAA	Valine	12.75	7.79	7.56	8.57	8.37	9.25
	Leucine	5.22	13.33	13.94	14.78	14.88	18.15
	Lysine	5.96	6.34	5.88	6.21	5.95	3.70
	Isoleucine	5.22	7.50	8.48	9.32	9.21	10.40
	Threonine	4.34	2.69	2.77	2.92	2.98	2.89
	PhenylalaninePhe	4.27	4.15	3.95	4.05	3.81	2.66
	Methionine	2.31	2.48	2.52	2.54	2.70	2.31
Compositions of NEAA	Glutamic acid	13.47	10.63	10.33	9.32	9.30	8.79
	Aspartic acid	7.68	6.55	6.72	5.37	5.77	4.86
	Alanine	5.99	5.61	5.54	5.37	5.02	6.36
	Glycine	4.03	3.06	3.69	4.14	3.91	5.09
	Arginine	5.99	8.96	8.48	9.32	9.12	7.63



Continuous aeration with 15% CO_2 induced nitrogen deprivation during *Chlorella* PY-ZU1 cultivation, thus simultaneously promoting biomass (2.78 g L^{-1}) and lipid (47.04%) production.