Bioresource Technology 121 (2012) 76–82

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Cultivation of *Chlorella zofingiensis* in bench-scale outdoor ponds by regulation of pH using dairy wastewater in winter, South China

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HIGHLIGHTS

- Cultivation of *C. zofingiensis* in dairy wastewater was investigated.
- Algal growth rate and composition were different under CO_2 and HAc regulations.
- It's feasible to cultivate *C. zofingiensis* in outdoor ponds in winter, South China.
- And remarkable content of GABA in *C. zofingiensis* G1 could be obtained.

ARTICLE INFO

Article history:
Received 5 May 2012
Received in revised form 4 July 2012
Accepted 7 July 2012
Available online 16 July 2012

Keywords:
Microalgae
pH adjustment
Wastewater
Biofuel
γ-Aminobutyric acid

ABSTRACT

Cultivation of *Chlorella zofingiensis* and nutrients removal in dairy wastewater were investigated in bench-scale outdoor ponds in winter, South China. The impacts of the two types of pH regulations, 5∼6% CO_2 and acetic acid (HAc) on this process were studied. After 6 days cultivation, the removal rates of total nitrogen (TN) and orthophosphate (PO_4^{3-}) using CO_2 regulation were better than those using HAc. The removal rates of PO_4^{3-} and TN were 97.5% and 51.7%, respectively using CO_2 regulation; 79.6% (TN) and 42.0% (PO_4^{3-}) were obtained using HAc regulation. Higher biomass, protein, sugar content, and stable pH control were found using CO_2 regulation. However, significantly higher lipid content (31.8%) was observed using HAc regulation. The dominant differences of fatty acids were the content of C18:1 and C18:3. The growth characteristics and environmental conditions especially during the typical logarithmic phase were also analyzed.

1. Introduction

Microalgae cultivation for biofuel production has been considered as an important contributor to Greenhouse Gases mitigation and energy security due to its fast growth rates, voracious appetite for CO_2, wide adaptability, and high energy source content (Demirbas and Demirbas, 2010). Intracellular energy source mainly includes lipids and sugars. The lipids, mainly comprising triglycerides can be refined into biodiesel via transesterification reactions. The sugars, mainly composed of cellulose, can be hydrolyzed to reducing sugars for further fermentation to bioethanol. The extracted residual protein could be hydrolyzed into amino acids as high value product for human and animal nutrition which help to improve economics for the whole process (Romero García et al., 2012).

Culture in outdoor ponds is cheaper than in closed photobioreactors. Almost all commercial-scale culture is currently in outdoor ponds. However, it is limited to relatively small number of algae species because of changing temperatures and competition from invasive species in the environmental conditions (Borowitzka, 2005). *Chlorella* has been grown successfully in outdoor ponds due to their robustness, high growth rate. Several studies have also been dedicated to utilizing *Chlorella* (Chinnasamy et al., 2010; De-Bashana et al., 2002; Travieso et al., 2008) even for wastewater applications due to their high tolerance to soluble organic compounds. However, those studies mainly evaluated the qualities of the final effluents. Very few works have determined the changes of lipid, sugar and protein content of algae cell in the wastewater treatment process.

The dynamics of pH in the medium is highly depending on the photosynthesis, the consumption of nitrate, the degradation of the organic acids and the sulfate reduction (Huo et al., 2012). The optimum pH for *Chlorella* growth is around 6.3–7.5 (Borowitzka, 2005; Mayo, 1997). Also dairy wastewater is generally alkaline. In order to achieve the rapid growth of *Chlorella*, the pH of dairy wastewater needs to be adjusted. Attribute to the medium pH as well as the availability of carbon source, CO_2 or HAc has a profound effect on

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http://dx.doi.org/10.1016/j.biortech.2012.07.012

the removal of nitrogen and phosphorus, algal cell growth and intracellular components. The aim of this study is to investigate nitrogen and phosphorus removal, growth characteristics, intracellular components (sugar, lipid and protein) of *Chlorella zofingiensis* G1 cultivated under pH regulations using CO_2 and HAc respectively in outdoor ponds. Because of remarkable cell doubling in logarithmic phase, the growth characteristics and environmental conditions during the typical logarithmic phase (Dec. 9th, 8:00∼Dec. 10th, 8:00) were also analyzed.

In order to achieve this research, bench-scale outdoor ponds using diluted dairy wastewater as medium for algal growth were established in winter, South China. South China mostly belongs to subtropical monsoon climate zone (Tao and Feng, 2000) that with the average temperature not below 10 °C in winter. Between October and March of next year, the cultivation of microalgae outdoor is feasible because of less precipitation, fine weather and sufficient illumination. At the same time, there is less bacteria and protozoa contamination. Temperature cooling down is no need in winter. This research would provided technical support for the full-scale outdoor cultivation using wastewater and also for the sites chosen for microalgal cultivation with similar environmental conditions in other places.

2. Methods

2.1. Microalgal strain and pre-culture conditions

C. zofingiensis G1 was preserved in Guangzhou Institute of Energy Conversion, China. For the cultivation of original culture broths, BG11 medium was used which containing the following components: $NaNO_3$ (1.5 g L^{-1}); $K_2HPO_4{\cdot}3H_2O$ (40 mg L^{-1}); $MgSO_4{\cdot}7H_2O$ (75 mg L^{-1}); $CaCl_2{\cdot}2H_2O$ (36 mg L^{-1}); $NaCO_3$ (20 mg L^{-1}) $FeCl_3{\cdot}6H_2O$ (3.15 mg L^{-1}); citric acid (6 mg L^{-1}) and 1 mL of microelements composed of H_3BO_3 (2.86 mg L^{-1}), $MnCl_2{\cdot}4H_2O$ (1.81 mg L^{-1}), $ZnSO_4{\cdot}7H_2O$ (0.22 mg L^{-1}), $Na_2MoO_4{\cdot}2H_2O$ (0.39 mg L^{-1}), $CuSO_4{\cdot}5H_2O$ (0.08 mg L^{-1}), $Co(NO_3)_2{\cdot}6H_2O$ (0.05 mg L^{-1}) in 1000 mL acidified water which included 1 mL concentrated H_2SO_4 in 1 L distilled water. About 100 mL pre-culture broths mentioned above were inoculated into a vertical tubular photobioreactor containing 1 L medium. The vertical tubular photobioreactor consisted of glass tubes of 70 cm heights and 5.0 cm external diameters. Light was supplied by cool white fluorescent lamps at the single side of the photobioreactor (light intensity: 200 ± 50 μE/(m^2 s)). Aeration and mixing were achieved by the sparging air enriched with 5–6% CO_2 through a glass-filter, which was inserted to the bottom of the reactor and the flow rate of gas was 0.5 vvm regulated by the gas flow meter (Model G, Aalborg Instruments & Controls Inc., NY, USA). The temperature of the culture media was 25 ± 1 °C regulated by the room air conditioner (Gree Electric Appliances Inc, Zhuhai, China).

2.2. Dairy wastewater

Dairy wastewater was collected from the dairy farm of Foshan Aochun Diary Industry Limited Liability Company (Foshan, China) and stored in shady place to avoid exposure to high temperature and sunshine conditions. The characteristics of original dairy wastewater are shown in Table 1. Wastewater was centrifuged for 10 min at 5000 rpm. The supernatant was sterilized in autoclave for 20 min at 121 °C and used for microalgae growth studies.

2.3. Growth conditions using dairy wastewater

The growth medium in this study contained 10% (v/v) dairy wastewater diluted by tap water. The initial optical density (OD_{680}) of culture was controlled between 0.3 and 0.5 when appropriate amount pre-culture broths mentioned above during the logarithmic phase were inoculated. The cultivation was carried out during Dec. 7 to Dec. 12, 2011, in bench-scale plastic ponds on the top floor of Center for Energy and Environmental Education, Chinese Academy of Sciences, located in Sanshui district, Foshan city, China (23°03′N–112°09′E). The weather conditions were shown in Table 2. The ponds were covered with plastic sheets when sporadic rain started. Atmospheric temperature was between 6.2 and 20.8 °C, pressure 101.4 and 102.5 kPa, relative humidity 25–89%, uncertain wind direction, wind speed was between 0.7 and 5.2 m s^{-1}. The time of sunrise and sunset were about 06:58 and 17:43, respectively.

Table 1
Water quality of original wastewater used in the experiments.

Parameters	Concentration
COD (mg L^{-1})	1195 ± 7.0
TN (mg L^{-1})	118.0 ± 2.8
PO_4^{3-} (mg L^{-1})	149.0 ± 2.8
NH_3-N (mg L^{-1})	48 ± 1.5
Total solids (mg L^{-1})	1490 ± 14
pH	8.3 ± 0.2

2.4. Bench-scale outdoor ponds

Outdoor ponds used in this study were made of opaque plastic and were 60 cm wide, 110 cm long and 25 cm deep (Fig. 1). The working volume maintained in outdoor ponds was about 100 L. All the ponds were attached with delivery tubings and air stones and powered by a paddle wheel which was operated at 5 cm depth and rotated at the speed of 20 rpm. Air contained 5–6% CO_2 was bubbled into the ponds or 1–2 mL HAc was added. Stop ventilation and HAc addition during nighttime. After 6 days cultivation, *C. zofingiensis* G1 were harvested.

2.5. Measurement of specific growth rate

Cells were counted using a microscope counting chamber (Refinement Biochemical Reagents Instrument Co. Ltd., Shanghai, China). During exponential growth, the specific growth rate (μ) was calculated with the following equation (Wood et al., 2005):

$$N = N_0 e^{\mu t}$$

$$\mu = \frac{\ln(N_t/N_0)}{t_t - t_0}$$

where N_0 is the population size at the beginning of a time interval t_0, N_t is the population size at the end of the time interval t_t.

2.6. Measurement of dry cell weight

The dry cell weight was measured by filtering an aliquot of the culture suspension through pre-weighed GF/C filters (Whatman, England, UK) under 0.075–0.090 MPa vacuum pressure. After rinsing with 0.5 M ammonium formate, the filters were dried at 105 °C (Yoo et al., 2010).

2.7. Measurement of nitrogen and phosphate content

For water quality measurements, the microalgal culture was centrifuged (16,000 g, 10 min at 4 °C), and the supernatant was filtered through a 0.45-μm membrane. The filtrate was analyzed for the determination of TN, ammonia (NH_3-N) and PO_4^{3-} by a water

Table 2
Weather conditions of microalgae cultivation.

Date	Max irradiance/×1000 lux	Atmospheric temperature/°C	Culture temperature/°C	Weather
Dec. 7, 2011	310 ± 10	18.9–20.8	18.4–23.8	Sporadic rain
Dec. 8, 2011	670 ± 54	12.9–16.0	12.3–18.5	Overcast
Dec. 9, 2011	734 ± 44	9.8–12.5	9.4–16.7	Cloudy
Dec. 10, 2011	1100 ± 100	9.0–14.2	7.4–17.6	Cloudy sunny
Dec. 11, 2011	1150 ± 71	6.2–14.9	4.3–18.5	Sunny
Dec. 12, 2011	1035 ± 92	6.8–17.3	6.3–19.7	Sunny

Fig. 1. Photograph of bench-scale outdoor ponds.

quality analyzer (Hach Co., Colo, US). TN, NH_3-N and PO_4^{3-} were measured according to Method 10071, 10031 and 8180, respectively (Hach, 2008).

2.8. Measurement of total sugar content

The anthrone-sulphuric acid method was applied to quantify the amount of total sugar (Yemm and Willis, 1954). One milliliter of microalgae solution was mixed with 4 mL of 0.2% (w/v) anthrone concentrated sulfuric acid solution. The samples were heated at 100 °C for 10 min. The mixture color turned orange resulting of the interaction between the carbohydrates and the anthrone. The absorbance of orange color was determined under the wavelength of 620 nm.

2.9. Protein content and amino acid composition analysis

Freeze-dried (overnight at −80 °C) samples of *C. zofingiensis* G1 biomass were extracted with 0.5 N NaOH, 80 °C for 20 min on a water baths shaker (150 rpm) (Chu et al., 1996) and the protein concentrations were determined by the dye-binding method (Bradford, 1976). Amino acids concentration of cell protein hydrolysate in the algae freeze-dried samples refer to GB/T 5009.124-2003 (Ministry of Health of China, 2003). Cell free amino acids were analyzed as follow: Proteins in algae freeze-dried samples were separated from cell by sulfosalicylic acid precipitation (6%). Cell free amino acids were analyzed in the supernatant fluid. Those were all conducted with a Hitachi Model 8800 amino acid analyser (Hitachi, Tokyo, Japan).

2.10. Lipid content and fatty acid composition analysis

Bigogno's method (2002) was applied to quantify the amount of total lipid content. Freeze-dried samples of *C. zofingiensis* G1 biomass were extracted with methanol containing 10% DMSO, by warming to 40 °C for 5 min and stirring at 40 °C for another hour. The mixture was centrifuged, the supernatant was removed and the pellet was re-extracted with a mixture of hexane and ether (1:1, v/v). Diethyl ether, hexane and water were added to the combined supernatants, so as to form a ratio of 1:1:1:1 (v/v/v/v). The mixture was shaken and then centrifuged at 100 rpm for 5 min at 35 °C and the upper phase was collected. The water phase was re-extracted twice with a mixture of diethyl ether: hexane (1:1, v/v). The organic phases were combined and evaporated to dryness. The diethyl ether utilized in the extractions and the lipid analysis was peroxide-free and contained 0.01% butylated hydroxytoluene (BHT).

Fatty acid composition analysis was carried out by the saponification reaction with the participation of base catalyst. Then the soap was transformed to fatty acid methyl esters by the direct transesterification method with boron trifluoride-methanol at 100 °C for 15 min (Lepage and Roy, 1984). Fatty acid methyl esters (FAMEs) samples, extracted by 4 ml hexane, were analyzed by a gas chromatograph with FID detector (Shimadzu, GC-2010, Japan). The injector and detector temperatures were assigned to be 300 and 280 °C, respectively. The temperature gradient was programmed from 130 to 180 °C with an increase of 10 °C/min followed by raise to 210 °C with 2 °C/min, and then the temperature was fixed at 210 °C for 3 min (Hsieh and Wu, 2009).

2.11. Measurement of climatic conditions

Light intensity, dissolved oxygen (DO), pH and culture temperature were recorded every 3 h daily. The light intensity was measured by a digital light meter (Smart Sensor, AR-823, Hongkong, China). DO and culture temperature were measured by a portable dissolved oxygen analyzer (Leici, JPB-607A, Shanghai, China). pH was measured by a portable pH analyzer (Yilun, pH-3C, Shanghai, China).

3. Results and discussion

3.1. Growth and biomass accumulation properties in outdoor ponds

Fig. 2 shows the growth characteristics of *C. zofingiensis* G1 using two pH regulations. Immediately after inoculation of the microalgae cells into the medium, biomass amount temporarily remained unchanged in CO_2 regulation and even declined in HAc cultures. The specific growth rate regulated with HAc was negative. On the second day, growth rates both increased significantly and entered into the logarithmic phases. The specific growth rate regulated with HAc was higher than that regulated with CO_2. On the third day, the specific growth rate of two treatments all began to decline. The HAc cultures entered stability period earlier than CO_2 ones with significantly lower specific growth rate. In the growth process, the color of CO_2 regulation culture was greener than the HAc. After 6 days, they both nearly got to the stability period. The maximum cell concentration achieved in HAc regulation culture was 9.05×10^6 cells/mL, which represented 83% of the maximum cell concentration achieved under the condition of CO_2 regulation (1.09×10^7 cells/mL).

3.2. Nitrogen and phosphate removal in bench-scale outdoor ponds

In the growth process, the concentrations of TN, NH_3-N and PO_4^{3-} decreased significantly as shown in Fig. 3. On the first day,

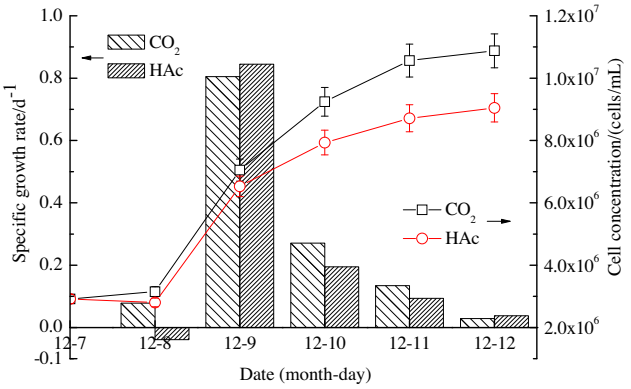

Fig. 2. The growth curves and specific growth rates of *Chlorella zofingiensis* G1 with two different pH adjustments (CO_2: pH control under CO_2 regulation; HAc: pH control under HAc regulation).

although biomass amount did not increase significantly (Fig. 2), all the concentrations of TN, PO_4^{3-} and NH_3-N decreased. When a variety of forms of nitrogen forms exists in the environment, NH_3-N has the priority to be used by algae (Ahmad and Hellebust, 1990; Li et al., 2008). NH_3-N was absorbed and almost depleted completely on the second day. The removal rate of PO_4^{3-} and TN began to increase from next day. Finally, the removal rates of TN in the bench-scale outdoor ponds regulated with HAc and CO_2 reached to 97.5% and 79.6%, respectively. The removal rate of PO_4^{3-} went to 51.7% and 42.0%, respectively. Compared with the two culture regulations, the removal efficiency of TN and PO_4^{3-} with CO_2 regulation was better than that with HAc method.

For freshwater phytoplankton, phosphorus restriction appeared when molar ratio of N and P in the environment was greater than 20:1 and nitrogen was limited when the ratio was less than 10:1 (Schanz and Juan, 1983). Thus, the present ratio of N:P in this study was not optimum. The initial N:P mole ratio was about 8.9, indicating that the algae growth would be nitrogen-limited in senescent phase.

3.3. Environmental conditions during the typical logarithmic phase

Compared with the sunny weather, the light intensity on Dec. 9th was weaker due to clouds. As shown in Fig. 4, the light intensity reached the maximum at 11:00 and weakened dramatically after 17:00. Because of the absorption of light and physiological metabolism of the microalgae cells, the culture temperature was higher than the air temperature during the day time. The fluctuated culture temperature was in the range of 7.4–16.7 °C and reached the maximum at 14:00 some delay compared with the time (11:00) when light intensity reached the maximum. The culture temperature of CO_2 regulation was slightly higher than the HAc about 0.05–0.4 °C (data not shown). Generally, high light intensity was more favorable for *Chlorella*. And the optimum growth temperature of *Chlorella* was about 25–30 °C (Borowitzka, 2005). Due to the low temperature in winter, the growth of *Chlorella* in South China was limited to a certain extent.

3.4. pH regulations during the typical logarithmic phase in bench-scale outdoor ponds

Direct CO_2 sparging into the culture media is good and convenient method for pH control. At the same time it provided CO_2 for high yield in mass algal cultures (Richmond, 2004). As shown in Fig. 5, average culture pH decreased to 6.5–6.7 steadily with 5–6% CO_2 aeration in the day time, while using the HAc adjustment the pH violently increased from 6.6 to 9.5 within 3 h before 11:00. This might be due to the utilization of fatty acids of the wastewater

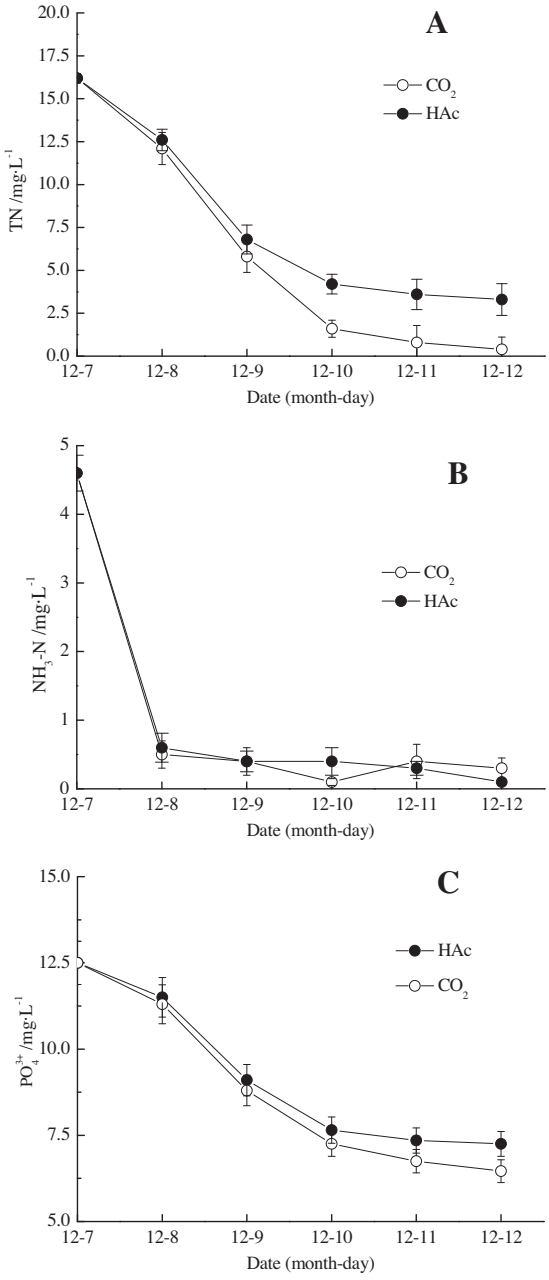

Fig. 3. Removal rate of the nitrogen and phosphate from bench-scale ponds by *Chlorella zofingiensis* G1 (A: Total nitrogen; B: Ammonia; C: Orthophosphate; Data were recorded at 17:00 daily) (CO_2: pH control under CO_2 regulation; HAc: pH control under HAc regulation).

and in excess of self buffering capacity (Danquah et al., 2009). Then HAc was added with the speed of 1–2 mL per hour to control culture pH below 6.5. After 1 h later, the pH rose to 8.9–9.2 again. When the pH reduced to 5.5, a large number of bubbles appeared in the culture indicating that algal cells did not adapt to such low pH. The design of automatic pH feedback device for stable pH is necessary for this regulation. At night, there was no significant difference between two regulations. pH first increased up to 8.1–8.2 gradually and then tended to be stable for the two cases.

3.5. DO changes during the typical logarithmic phase in bench-scale outdoor ponds

Fig. 6 shows that DO increased significantly after sunrise due to the photosynthesis of the algae. DO in the culture under HAc reg-

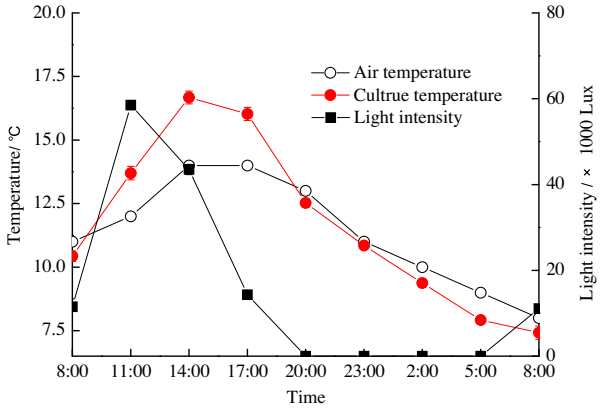

Fig. 4. Climatic conditions changes during the typical logarithmic phase (Culture temperature was an average value of the two regulations).

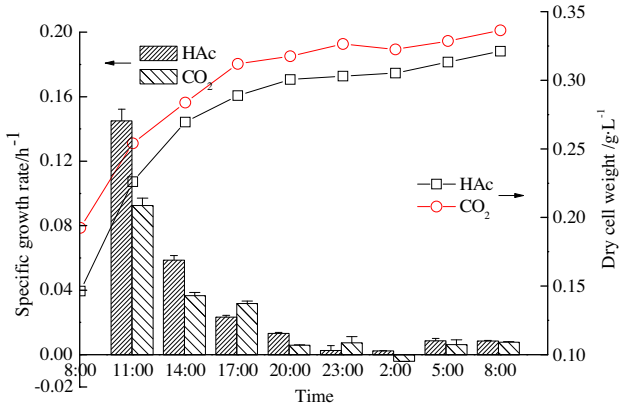

Fig. 7. The growth curve and specific growth rate during the typical logarithmic phase (CO_2: pH control under CO_2 regulation; HAc: pH control under HAc regulation).

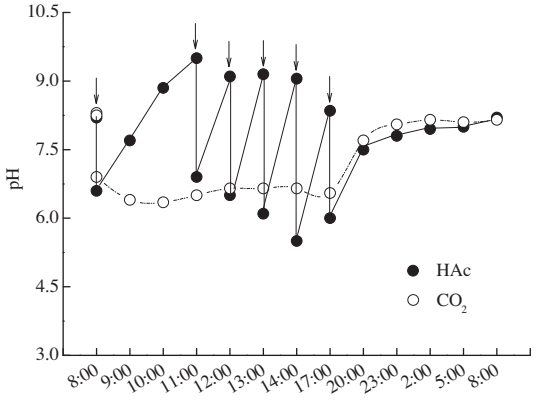

Fig. 5. Culture pH control under 5–6% CO_2 aeration or 1–2 mL HAc added (↓) (CO_2: pH control under CO_2 regulation; HAc: pH control under HAc regulation).

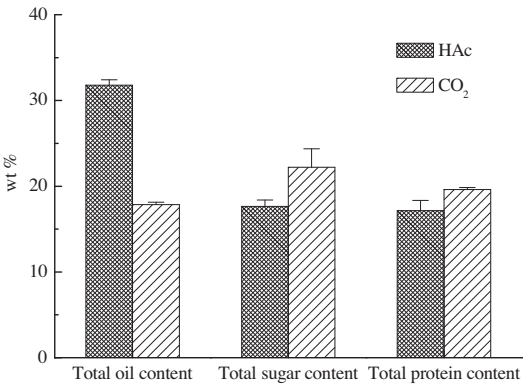

Fig. 8. Total content of sugar, protein and lipid after harvesting (CO_2: pH control under CO_2 regulation; HAc: pH control under HAc regulation).

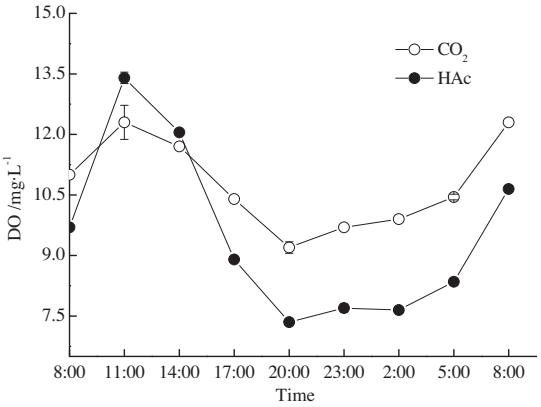

Fig. 6. Culture DO changes under 5–6% CO_2 aeration or 1–2 mL HAc added (CO_2: pH control under CO_2 regulation; HAc: pH control under HAc regulation).

ulation rose faster than that under CO_2. For both regulations, DO concentration got to the highest value at 11:00, and then began to decline because of aerobic respiration and reduction of photosynthesis. After 14:00, DO of CO_2 regulation was higher than HAc addition. DO varied greatly under HAc addition method, while CO_2 adjustment which had a relatively stable DO may had small effect on the cell growth cycle. In the evening, as the temperature decreased gradually, the dissolved oxygen rose slowly (Kalff, 2002). DO reflects a temporary balance of atmospheric dissolved oxygen, release oxygen of microalgal photosynthesis and respiration consumption. It can indicate the metabolism of microalgae in the water environment.

3.6. Growth curve and specific growth rate during the typical logarithmic phase

The carbon sources, daily temperature and light intensity all have impacts on microalgae growth and metabolism. As shown in Fig. 7, within 24 h cloudy weather, the dry mass accumulation was 0.144 g and 0.175 g through CO_2 and HAc regulations, respectively. The amount of biomass accumulated under HAc regulation was higher than that under CO_2 regulation. HAc as a kind of simple organic carbon sources was usually preferred for microalgae (Bhatnagar et al., 2010; Lee and Lee, 2001). Before 11:00 both regulations had the largest specific growth rates due to low DO level, increasing temperature and light intensity. Before 14:00, the specific growth rate of HAc regulation was significantly higher than CO_2 addition. Between 14:00 and 17:00, the specific growth rate of HAc regulation was lower. This may be resulted from HAc addition that made culture pH approaching to be 5.5 and further inhibited algal growth. After 20:00, *Chlorella zofingiens* G1 presented inconspicuous heterotrophic metabolism with declined temperature and light intensity. The growth rate sharply decreased to zero in both regulations. In addition, the growth rate in the CO_2

Table 3
Fatty acid composition (wt.%) of *Chlorella zofingiensis* G1 cells (CO_2: pH control under CO_2 regulation; HAc: pH control under HAc regulation).

	HAc	CO_2
C10:0	0.17 ± 0.24	0.33 ± 0.01
C14:0	0.45 ± 0.02	0.27 ± 0.01
C14:1	0.72 ± 0.03	1.84 ± 0.12
C16:0	25.71 ± 0.55	21.96 ± 0.10
C16:1	2.36 ± 0.21	3.69 ± 0.31
C18:0	2.51 ± 0.14	1.82 ± 0.12
C18:1	26.30 ± 0.05	12.93 ± 0.20
C18:2	18.56 ± 0.33	17.13 ± 0.07
C18:3	18.39 ± 0.10	32.63 ± 0.70
C20:0	0.14 ± 0.03	1.00 ± 0.57
C20:1	0.99 ± 0.11	0.03 ± 0.04
C20:2	0.15 ± 0.02	0.14 ± 0.01

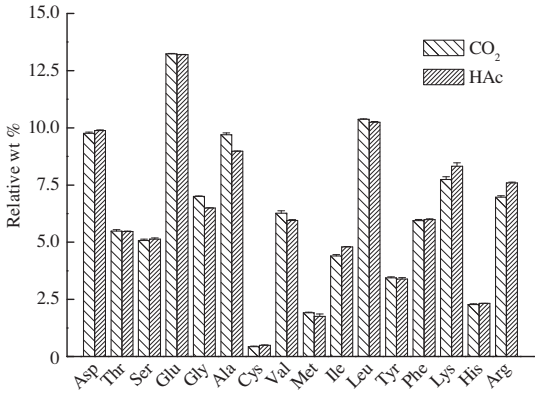

Fig. 9. Relative content of amino acid composition (wt.%) of cell protein hydrolysate of *Chlorella zofingiensis* G1 cells (CO_2: pH control under CO_2 regulation; HAc: pH control under HAc regulation).

regulation was negative after 23:00 and the amount of biomass had a little lost.

3.7. Total content of sugar, protein and lipid after harvesting

It is known that the growth rate of microalgae would slow down under physical or nutritional stresses. Lipids or carbohydrates instead of proteins would be produced using the available nutrients (Rodolfi et al., 2009). In this study, lipid accumulated naturally with nitrogen deficiency resulted from the cultivation. Wang et al. (2009) reported that after 48 h of nitrogen starvation in the presence of HAc, the lipid body content in wild-type increased 15-fold. As shown in Fig. 8, we got the similar result in this study. The culture with HAc regulation had higher total oil content (31.8%) compared to the CO_2 ones (17.9%), by which more sugar accumulated after 6 days cultivation. It is known that the price of liquid CO_2 is about only one third of HAc (about 3500 RMB per ton) in China. Besides, utilization of high concentration CO_2 from industrial flue gas for full-scale algal cultivation is a promising method, not only for CO_2 emission mitigation but also for the low cost production of biomass (Liu et al., 2008).

The fatty acids composition can be significantly changed by the two carbon sources supplementation. Table 3 shows the fatty acids composition in the two different culture conditions. The microalgal lipids contained FAMEs mainly with 16–18 carbons, which are considered suitable for producing biodiesel (Huang et al., 2010). The significant differences of fatty acids were C18:1, and C18:3. The culture with CO_2 regulation has higher C18:3 which is not good for biodiesel stability, but can be used for cardiovascular diseases treatment. The saturated fatty acids were 25.4% and 29.0%, while unsaturated fatty acids were 68.4% and 67.5% with CO_2 and HAc adjustments, respectively. There was no significant difference.

Comparison of the two pH regulations, CO_2 and HAc, the relative content of amino acids of protein hydrolysate did not change significantly as shown in Fig. 9. The differences of carbon sources have not substantially effect on the protein anabolism. But in the CO_2 regulation, the contents of Gly, Ala, Val were a little higher than those in HAc regulation. To the contrary, Ile, Lys, Arg were lower than those in HAc regulation. Compared with HAc addition, the real amino acid content of protein hydrolysate under CO_2 regulation was higher for each composition except Cys (data not shown).

Cell free γ-aminobutyric acid (GABA) is a type of high value product. Results showed that *C. zofingiensis* G1 contains a large number of GABA, while quite low for the other free amino acid (data not shown). Therefore, the purification of GABA was convenient. The content of GABA of freeze dried samples of *C. zofingiensis* was 284.1 mg/g (under CO_2 regulation) and 168.0 mg/g (under HAc regulation). To the best of our knowledge, there is no study report such high content of GABA in *C. zofingiensis*.

4. Conclusion

Using agricultural effluent to cultivate energy microalgae is favorable to wastewater disposal and can lower the cost of the algae production effectively. pH control under CO_2 regulation had many advantages, such as stable pH, low cost with higher content of sugar, protein, C18:3 and GABA. However, with HAc regulation, higher oil content and higher specific growth rate during the typical logarithmic phase can be obtained. It was feasible to cultivate microalgae with those two pH regulations in such climatic conditions.

Acknowledgements

This research was funded by the National Program on Key Basic Research Project of China (973 Program) (2011CB200905), the 12th Five Year Support Plan of the Ministry of Science and Technology, PR China (2011BAD14B03) and the Natural Science Foundation of Guangdong Province, China (No. 10451007006006001).

References

Ahmad, I., Hellebust, J.A., 1990. Regulation of chloroplast development by nitrogen source and growth conditions in a Chlorella protothecoides strain. Plant Physiol. 94, 944–949.
Bhatnagar, A., Bhatnagar, M., Chinnasamy, S., Das, K., 2010. Chlorella minutissima—a promising fuel alga for cultivation in municipal wastewaters. Appl. Biochem. Biotechnol. 161, 523–536.
Bigogno, C., Khozin-Goldberg, I., Boussiba, S., Vonshak, A., Cohen, Z., 2002. Lipid and fatty acid composition of the green oleaginous alga Parietochloris incisa, the richest plant source of arachidonic acid. Phytochemistry 60, 497–503. Biochem. J., 57: 508-514.
Borowitzka, M.A., 2005. Culturing microalgae in outdoor ponds. In: Andersen, R.A. (Ed.), Algal Culturing Techniques. Elsevier Academic Press, Burlington, pp. 205–218.
Bradford, M.M., 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Chinnasamy, S., Bhatnagar, A., Claxton, R., 2010. Biomass and bioenergy production potential of microalgae consortium in open and closed bioreactors using untreated carpet industry effluent as growth medium. Bioresour. Technol. 101, 6751–6760.
Chu, W.L., Phang, S., Goh, S.H., 1996. Environmental effects on growth and biochemical composition of Nitzschia inconspicua Grunow. J. Appl. Phycol. 8, 389–396.
Danquah, M.K., Gladman, B., Moheimani, N., Forde, G.M., 2009. Microalgal growth characteristics and subsequent influence on dewatering efficiency. Chem. Eng. J. 151, 73–78.
De-Bashana, L.E., Moreno, M., Hernandez, J.P., 2002. Removal of ammonium and phosphorus ions from synthetic wastewater by the microalgae Chlorella

vulgaris coimmobilized in alginate beads with the microalgae growth-promoting bacterium Azospirillum brasilense. Water Res. 36, 2941–2948.
Demirbas, A., Demirbas, M.F., 2010. Algae Energy: Algae as a New Source of Biodiesel. Springer-Verlag, London.
Hach, 2008. Procedure Manua. Hach, Loveland, CO.
Hsieh, C., Wu, W., 2009. Cultivation of microalgae for oil production with a cultivation strategy of urea limitation. Bioresour. Technol. 100, 3921–3926.
Huang, G.H., Chen, F., Wei, D., Zhang, X.W., Chen, G., 2010. Biodiesel production by microalgal biotechnology. Appl. Energy 87, 38–46.
Huo, S., Chen, Y., Liu, Y., Zhu, Y., Peng, G., Dong, R., 2012. Experiment on microalgae cultivation in BG11 nutrient solution adding biogas slurry. Trans. CSAE 28, 241–246.
Kalff, J., 2002. Limnology – Inland Water Ecosystems, second ed. Prentice Hall, Inc.
Lee, K., Lee, C.G., 2001. Effect of light/dark cycles on wastewater treatments by microalgae. Biotechnol. Bioprocess Eng. 6, 194–199.
Lepage, G., Roy, C.C., 1984. Improved recovery of fatty acid through direct transesterification without prior extraction or purification. J. Lipid Res. 25, 1391–1396.
Li, X., Hu, H., Gan, K., 2008. Study on the influence of nitrogen on the growth and N, P removal properties of low-nutrient-demanding Scenedesmus dimorphus LXI. Symposium for Pollution Control and Ecological Restoration Techniques of Water Environments in China, 936–948.
Liu, Y., Ruan, R., Kong, Q., Liu, C., Luo, J., Yu, F., 2008. Mass culture of high oil content microalgae on wastewater and power plant flue gases. Chinese J. Bioprocess Eng. 6, 29–33.
Mayo, A.W., 1997. Effects of temperature and pH on the kinetic growth of unialga Chlorella vulgaris cultures containing bacteria. Water Environ. Res. 69, 64–72.
Ministry of Health of China, 2003. Determination of amino acids in foods (GB/T 5009.124-2003). Beijing.
Richmond, A. (Ed.), 2004. Handbook of Microalgal Culture. Blackwell Science Ltd, Oxford, UK.
Rodolfi, L., Zittelli, G.C., Bassi, N., Padovani, G., Biondi, N., Bonini, G., Tredici, M.R., 2009. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in low-cost photobioreactor. Biotechnol. Bioeng. 102, 100–112.
Romero García, J.M., Acién Fernández, F.G., Fernández Sevilla, J.M., 2012. Development of a process for the production of L-amino-acids concentrates from microalgae by enzymatic hydrolysis. Bioresour. Technol.. http://dx.doi.org/10.1016/j.biortech.2012.02.094.
Schanz, F., Juan, H., 1983. Two different methods of evaluating nutrient limitation of periphyton bioassays, using water from the River Rhine and eight of its tributaries. Hydrebiologia 102, 187–195.
Tao, F., Feng, Z., 2000. Terrestrial ecosystem sensitivity to acid deposition in South China. Water Air Soil Poll. 118, 231–244.
Travieso, L., Benítez, F., Sánchez, E., 2008. Performance of a laboratory-scale microalgae pond for secondary treatment of distillerywastewaters. Chem. Biochem. Eng. 22, 467–473.
Wang, Z.T., Ullrich, N., Joo, S., Waffenschmidt, S., Goodenough, U., 2009. Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-Type and starchless Chlamydomonas reinhardtii. Eukaryot. Cell 8, 1856–1868.
Wood, A.M., Everroad, R.C., Wingard, L.M., 2005. Measuring growth rates in microalgal cultures. In: Andersen, R.A. (Ed.), Algal Culturing Techniques. Elsevier Academic Press, Burlington, pp. 269–286.
Yemm, E.W., Willis, A.J., 1954. The estimation of carbohydrates in plant extracts by anthrone. Biochem. J. 57, 508–514.
Yoo, C., Jun, S.Y., Lee, J.Y., Ahn, C.Y., Oh, H.M., 2010. Selection of microalgae for lipid production under high levels carbon dioxide. Bioresour. Technol. 101, S71–S74.