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Year: 2018

Version: Publisher's PDF

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Please cite the original version:

Huo, S., Wang, Z., Zhu, S., Shu, Q., Zhu, L., Qin, L., Zhou, W., Feng, P., Zhu, F., Yuan, Z., & Dong, R., (2018). Biomass Accumulation of *Chlorella Zofingiensis* G1 Cultures Grown Outdoors in Photobioreactors. *Frontiers in Energy Research* 8, 1–8. <https://doi.org/10.3389/fenrg.2018.00049>



Biomass Accumulation of *Chlorella Zofingiensis* G1 Cultures Grown Outdoors in Photobioreactors

Shuhao Huo^{1,2,3,4}, Zhongming Wang^{2,3*}, Shunni Zhu^{2,3}, Qing Shu^{2,3}, Liandong Zhu^{2,3,4}, Lei Qin^{2,3}, Weizheng Zhou^{2,3}, Pingzhong Feng^{2,3}, Feifei Zhu¹, Zhenhong Yuan^{2,3} and Renjie Dong⁵

¹ School of Food and Biological Engineering, Jiangsu University, Zhenjiang, China, ² Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences, Guangzhou, China, ³ Key Laboratory of Renewable Energy, Chinese Academy of Sciences, Guangzhou, China, ⁴ Vaasa Energy Institute, University of Vaasa, Yliopistonranta, Vaasa, Finland, ⁵ College of Engineering, China Agricultural University, Beijing, China

OPEN ACCESS

Edited by:

Qiang Wang,
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*Correspondence:

Zhongming Wang
wangzm@ms.giec.ac.cn

Specialty section:

This article was submitted to
Bioenergy and Biofuels,
a section of the journal
Frontiers in Energy Research

Received: 03 April 2018

Accepted: 28 May 2018

Published: 14 June 2018

Citation:

Huo S, Wang Z, Zhu S, Shu Q, Zhu L,
Qin L, Zhou W, Feng P, Zhu F, Yuan Z
and Dong R (2018) Biomass
Accumulation of *Chlorella Zofingiensis*
G1 Cultures Grown Outdoors in
Photobioreactors.
Front. Energy Res. 6:49.
doi: 10.3389/fenrg.2018.00049

The complicated and changeable weather conditions and pest invasion increase difficulties in outdoor microalgae cultivation. In this paper, outdoor microalgae cultivation experiments were investigated in Foshan city, South China. During June and August when the temperature is high, the cooling system-water spray combined shade tarpaulin can effectively reduce the temperature to 4–7°C in the 40 L vertical tubular outdoor photobioreactors. Under 300 mg/L urea and 200 mg/L ammonium bicarbonate conditions, *Chlorella zofingiensis* G1 had the largest biomass accumulation. Addition of acetic acid to adjust the pH of the medium can effectively improve the *C. zofingiensis* G1 biomass in the photobioreactor, which was four times more than that only through air ventilation conditions. The biomass accumulation of *C. zofingiensis* G1 was essentially unchanged during night time.

Keywords: *Chlorella zofingiensis*, outdoor cultivation, photobioreactor, biomass cumulation, cooling system

INTRODUCTION

The lack of robust microalgae strains and the limitations on the cultivation conditions are two important factors that cause the exorbitant microalgae production cost. Due to the cost input into nutritive salts such as chemical fertilizers and energy consumption for microalgae harvesting, the microalgal biodiesel has not been successfully applied to commercial production yet (Sheehan et al., 1998; Chisti, 2007; Wang et al., 2015). Utilizing the sunlight resource to magnify the cultivation of microalgae under outdoor condition is an effective way to reduce the cost of microalgal biodiesel production. The current studies on energy microalgae are mainly carried out at indoor labs as there are many difficulties in outdoor cultivation (Miao and Wu, 2004; Xiong et al., 2008; Sebestyén et al., 2016). The main difficulties for sustainable outdoor cultivation of microalgae are pollutions from fungi, protozoa, bacteria and other hybrid algae and complex and changeable weather conditions (Rodolfi et al., 2009; Ouyang et al., 2010; Huo et al., 2011; Wang et al., 2018). Under these adverse conditions, the chosen strains must have a relatively strong ability in accumulating oil, and more importantly, the algal strain should have a very strong adaptability to the external environment. Because *Chlorella* have broader adaptability and better survivability to external environment, there are more studies about its cultivation in outdoor raceway pond (Bhowmick et al., 2014; Lu et al., 2015).

Some reports show that *Chlorella zofingiensis* can adapt to various cultivating conditions and it has a fast growth rate which can reach 0.769 d^{-1} (Rise et al., 1994; Feng et al., 2011). The oil content of *C. zofingiensis* accounted for 52% of the dry weight under indoor heterotrophic conditions (Lin and Chen, 1994; Liu et al., 2011). Now the cultivation of *C. zofingiensis* is mainly focused on the production of astaxanthin (Sheehan et al., 1998; Orosa et al., 2000; Ip et al., 2004; Ip and Chen, 2005). The studies about outdoor cultivation of *C. zofingiensis* aiming for bioenergy production are still relatively limited. Feng et al reported that the oil content of *C. zofingiensis* accounted for 65.8% of its dry weight under nitrogen deficiency condition when transferred from indoor to 60 L outdoor flat bio-reactor. Because the pollution was not obvious, the algal cells can adapt to outdoor environment very quickly (Feng et al., 2011). Up to now, there are few reports on the outdoor cultivation of *C. zofingiensis* during high temperature season and the pH regulation through acetic acid addition. This paper attempted to cultivate *C. zofingiensis* G1 using a self-made 40 L vertical tubular outdoor photobioreactor and study its feasibility for outdoor cultivation, and also investigate the impacts of different nitrogen sources and the impacts of adjusting pH by adding acetic acid (HAc) on the growth of *C. zofingiensis* G1.

MATERIALS AND METHODS

Microalgal Strain and Culture Conditions

C. zofingiensis G1 was observed at Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences (Guangzhou, Guangdong, China). BG11 medium (Rippka et al., 1979) was used. About 100 mL pre-culture broths mentioned above were inoculated into a vertical tubular photobioreactor containing 1.0 L medium. The vertical tubular photobioreactor consisted of glass tubes of 70.0 cm heights and 5.0 cm outside diameters. Light was supplied by cool white fluorescent lamps at the single side of the photobioreactor [light intensity: $200 \pm 50 \mu\text{E}/(\text{m}^2 \text{ s})$]. Aeration and mixing were achieved by the sparging air enriched with 6.0% 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 and Controls, Inc., Orange-burg, NY, USA). The temperature of the culture media was $25 \pm 1^\circ\text{C}$ regulated by the room air conditioner (Gree Electric Appliances Inc., Zhuhai, Guangdong, China). After 6 days of cultivation, the cultures were used for outdoor experiment.

The outdoor microalgae cultivation experiment in this paper was carried out in Foshan, Guangdong, China, which belongs to subtropical and south subtropical oceanic monsoon climate areas. The work was carried out between June and August, which was the most scorching season of a year in South China region. The experiment used 40 L vertical tubular outdoor photobioreactor ($8.7 \text{ cm} \times 160 \text{ cm} = \text{diameter} \times \text{height}$) to cultivate the above mentioned *C. zofingiensis* G1. The reactor was composed by organic glass base and glass tube, having an organic glass cover on the top and a vent-hole in the base (Figure 1). Before the cultivation start, the internal walls should be washed with cleanser and clean water. The water used during the test was

local mountain spring water, whose components could be found in Table 1. The water quality could basically meet the required elements for microalgae growth, and it contained comparatively more Si, Mg, and Ca.

The following nutritive salts was replenished during the experiment: 300 mg/L of urea ($\text{CO}(\text{NH}_2)_2$), 60 mg/L of KH_2PO_4 , 60 mg/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1 mg/L of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. 3 mL of NaClO solution was added at dusk (18:00 local time) to make the final concentration of available chlorine reach 7.5 ppm and disinfect overnight. The air compressor was used in the whole cultivation process to ensure constant ventilation and avoid the sedimentation of *C. zofingiensis* G1. The pH, temperature and light intensity of the culture condition was tested and recorded four times a day (8:00, 11:00, 14:00, 17:00), and the pH was adjusted to 7.5 ± 0.3 by using acetic acid and the quantity of acetic acid added was recorded every time. Samples of each tube should be taken at 17:00 every day to test the OD_{680} value.



FIGURE 1 | 40 L vertical tubular outdoor photobioreactor for *Chlorella zofingiensis* G1 cultivation.

TABLE 1 | The quality of mountain spring water for microalgae outdoor cultivation.

| Element | Content ($\mu\text{g/L}$) |
|---------|-----------------------------|
| Mn | 5.99 ± 3.46 |
| Fe | 22.37 ± 17.52 |
| P | 128.28 ± 1.50 |
| Si | 3481.00 ± 9.90 |
| Na | 28.27 ± 0.64 |
| K | 160.05 ± 6.12 |
| Mg | 1174.60 ± 9.33 |
| Zn | 26.84 ± 4.02 |
| Ca | 1979.60 ± 12.16 |
| Mo | n.d. |
| Cu | n.d. |

n.d., not detected.

Chemical Fertilizer and Reagents

The urea was produced by Sichuan Meifeng Agricultural Chemical Co., Ltd, the NH_4HCO_3 was produced by Hunan Yizhang Chemical Fertilizer Co., Ltd, the KH_2PO_4 was produced by Wuhan Nanqing Science & Technology Development Co., Ltd, the $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was bought at Guangzhou Chemical Co., Ltd, the $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was produced by Guangzhou Chemical Reagent Factory, and the acetic acid whose purity exceeds 99% was produced by Jiangsu Sopo (Group) Chemical Co., Ltd.

Sunshade Net and Atomization Spray System

The top of vertical tubular outdoor photobioreactor was covered with a sunshade net. The sunshade net was made of high density polyethylene fire-retardant materials, with a shading rate of 40–55%. The water pressure of the atomization spray was 250 KPa, its water flow rate was 9.0 L/h and its flow distance was

1.5 m. The equipment was used for observing the sun block and temperature reduction effects of the cooling system - water spray combined shade tarpaulin on the growth of *C. zofingiensis* G1 under outdoor environment of the most scorching season in South China.

RESULTS AND DISCUSSION

Sun Block and Temperature Reduction Effects of Cooling System – Water Spray Combined Shade Tarpaulin

If microalgae was cultivated under high temperature environment, the CO_2 fixation rate would be restrained and the O_2 release rate would be limited (Kanno and Uyama, 2005). In June-August of South China, it was very hot and the temperature can exceed 40°C . As the algae cells absorb light, the temperature in the vertical tubular outdoor photobioreactor would be even higher than 45°C , which would greatly limit the algae

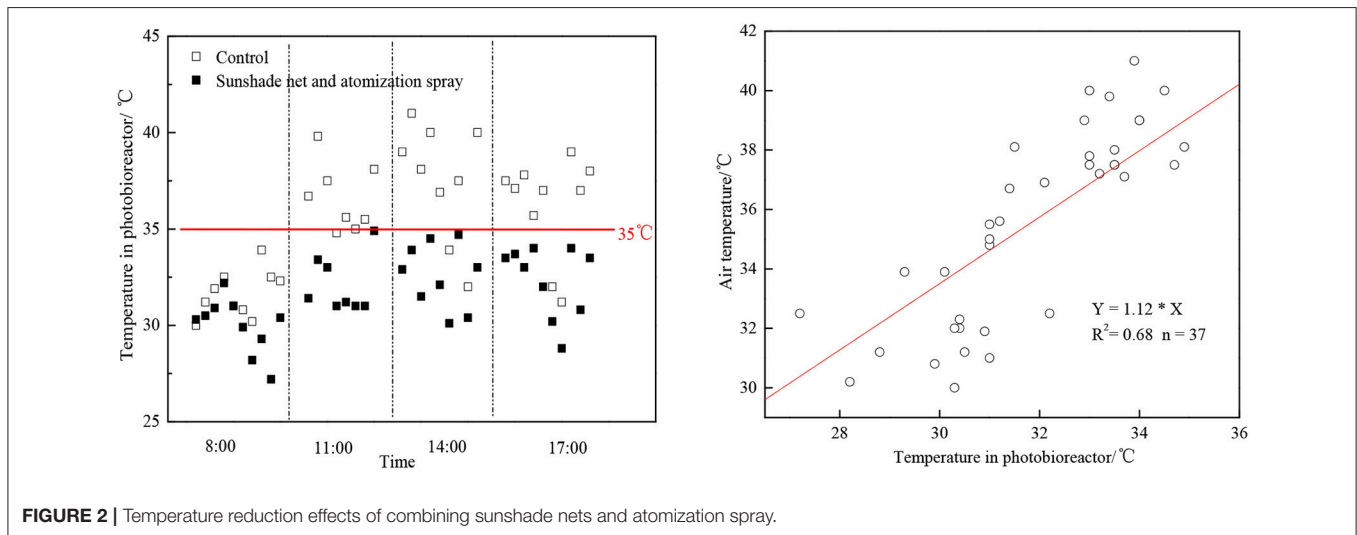


FIGURE 2 | Temperature reduction effects of combining sunshade nets and atomization spray.

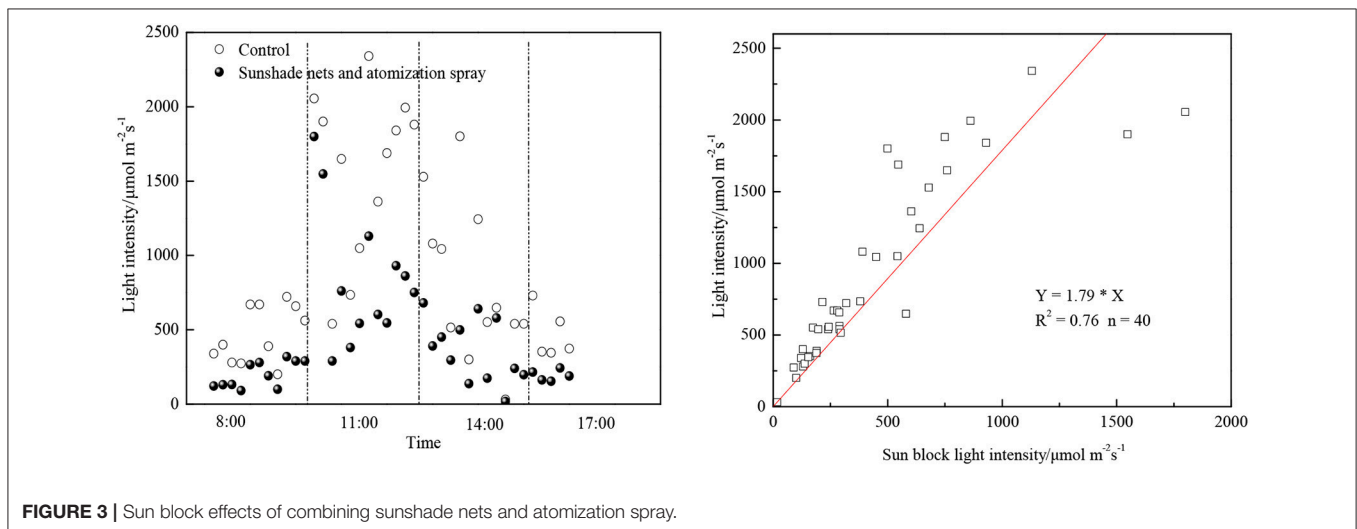


FIGURE 3 | Sun block effects of combining sunshade nets and atomization spray.

growth rate. The combination of polyethylene materials and atomization spray can reduce the photobioreactor temperature effectively at noon by 4–7°C (Figure 2). There was a linear relation between the column temperature after reduction and the external temperature (as Figure 2 shows). The temperature in the morning was relatively low itself, so the cooling effect was not as obvious as at noon. This combining method can keep the photobioreactor temperature below 35°C, therefore the growing rate of algae would not be limited.

While reducing the photobioreactor temperature effectively, the sunshade net could also reduce the light intensity. The actual shaded effects were shown as Figure 3. The angle of sun incidence changed during day time, as a result the actual shaded effects were changing which caused the sunlight to be in-homogeneous during the algae cultivation cycle. Excessively strong sunlight would result in the photo-inhibition phenomenon, which would affect the efficiency of photosynthesis or even cause cell oxidation. As was shown in Figure 3, the light intensity at sunny noon could exceed 2,000 μmol/ (m²•s), while the proper light intensity range for most microalgae was between 36 and 180 μmol/ (m²•s) (Feng et al., 2011). There was also a linear relation between the sun light intensity after block and the external light intensity (as Figure 3 shows). It is suggested that the proper light intensity range was between 100 and 1,600 μmol/ (m²•s) and the light saturation point was around 500 μmol/ (m²•s) (Rise

et al., 1994). The appropriate light intensity of *C. zofingiensis* XQ–200419 was found at 150 μmol/ (m²•s), and it could reach its maximum growth speed when the concentration of KNO₃ reached 0.5 mg/mL. Constant increase of sunlight or KNO₃ concentration would limit the split and growth of algae (Rise et al., 1994). In the initial stage of experiment, the sunshade net could reduce the light intensity to some extent which could avoid the photo-inhibition phenomenon in the early growth stage of algae, and would not affect the necessary light intensity needed for photosynthesis after the microalgae concentration increase.

Confirmation of Cheap Nitrogenous Fertilizer

Among all the fertilizers used in the cultivation of microalgae, the consumption of nitrogenous fertilizer was the highest. The experiment studied the use of cheap nitrogenous fertilizer, urea and ammonium bicarbonate replacing traditional nitrate nitrogen such as potassium nitrate on the growth of *C. zofingiensis* G1. The results were shown in Figure 4, and the weather conditions during cultivation were shown as Table 2. When the concentration of urea and NH₄HCO₃ are 300 and 200 mg/L, respectively, it grew slowly in the first 2 days after inoculation but it had the biggest algae biomass cumulation 4 days later.

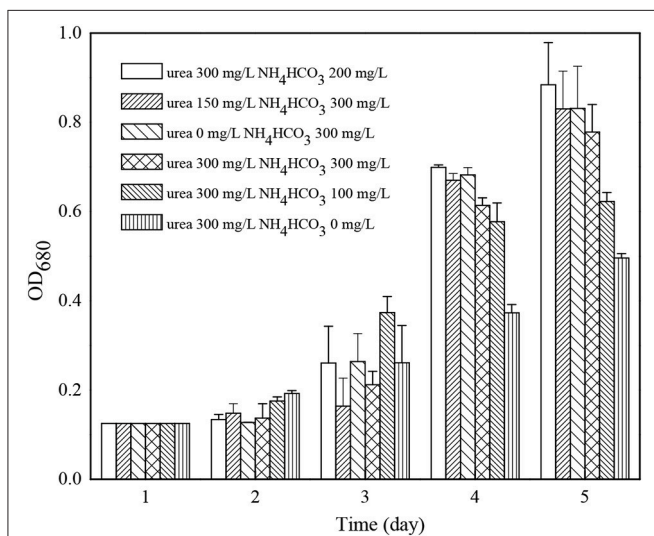


FIGURE 4 | The effects of different nitrogenous fertilizer on the growth of *Chlorella zofingiensis* G1.

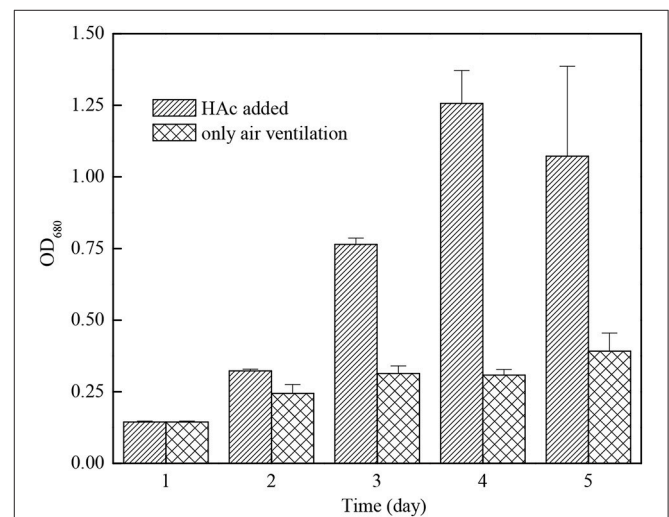
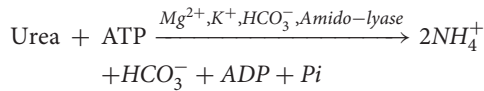


FIGURE 5 | The growth effect of *Chlorella zofingiensis* G1 under air ventilation only or HAc addition.

TABLE 2 | Weather conditions of microalgae cultivation.

| Cultivation time | 1st day | 2nd day | 3rd day | 4th day | 5th day |
|--|---------------|---------------|---------------|---------------|---------------|
| Temperature in photobioreactor/°C | 34.0 ± 4.9 | 33.9 ± 3.4 | 31.9 ± 3.5 | 31.3 ± 1.5 | 33.5 ± 2.0 |
| Sun light ^a /μmol/(m ² •s) | 254.8 ± 196.4 | 216.8 ± 210.1 | 329.8 ± 226.5 | 472.0 ± 200.7 | 489.8 ± 230.7 |
| Weather | Rain to sunny | Overcast | Cloudy | Sunny | Sunny |

C. zofingiensis utilized the reduced nitrogen such as ammonia nitrogen and urea better than nitrate nitrogen (Yun et al., 1996; Converti et al., 2006). As a nitrogen source, the nitrate nitrogen is reduced to sub-nitrite nitrogen using nitrate reductase first, and then would be reduced to ammoniacal nitrogen using nitrite reductase again before it was absorbed and utilized by microalgae cell (Converti et al., 2006). Under alkaline conditions, the urea is decomposed into ammonia by algal metabolism (Torre et al., 2003), so it could be absorbed. In the chlorophyceae, the urea was catalyzed and degraded by amido lyase, and the amidogen produced during the process would enter the nitrogen metabolism of amino acid. The reaction equation was as follows (Lee, 2008):



Walker reported that urea was the best nitrogen nutrition to get the highest microalgae biomass accumulation (Walker, 1953). High concentration urea would not have toxic restrains to the algal cell as ammonia nitrogen and nitrate nitrogen, but

excessively high concentration would lead to the denaturation of protein. During the use of urea, the pH had no big changes. Ammonium bicarbonate was a physiological and neutral nitrogen fertilizer, and Xun et al found that *Prorocentrum donghaiense* more compatible to the use of NH_4^+ than urea (Xu et al., 2012). When the concentration of NH_4HCO_3 reached 200 mg/L, it showed restrains on the algal growth. When the concentration of NH_4HCO_3 reached 300 mg/L, which meant that the concentration of NH_4^+ reached 68.1 mg/L, the restrains were very obvious. The growth rate was still very slow on the second day, and became quick on the third day. The restrains of the ammonia nitrogen concentration to algae are closely related to the cultivating conditions (Tarn and Wong, 1996). It is reported that when the concentration of ammonia nitrogen exceeded 2 mM (~36 mg/L) and pH exceeded 8.0, it would restrain the photosynthesis of *Scenedesmus obliquus* and affect the growth of algal cells (Abeliovich and Azov, 1976). There were also similar reports on the cultivation of *Scenedesmus* sp. in an anaerobic digestion effluent, showing that the concentration of ammonia nitrogen would not have restrains to the growth of algae when it was 100 mg/L, but would reduce 70% of the algal growth when the concentration of ammonium was in the

TABLE 3 | The fluctuation of irradiance and air temperature of each day under outdoor conditions with only air ventilation or HAC addition.

| Cultivation time | 1st day | 2nd day | 3rd day | 4th day |
|-----------------------------------|---------------|---------------|---------------|--------------------|
| Temperature in photobioreactor/°C | 28.9 ± 6.1 | 29.7 ± 6.4 | 30.1 ± 6.7 | 30.2 ± 5.4 |
| Sun light*/(μmol/(m²•s)) | 315.8 ± 215.1 | 283.8 ± 204.9 | 292.0 ± 238.7 | 239.5 ± 212.6 |
| Weather | Sunny | Cloudy | Sunny | Cloudy to overcast |

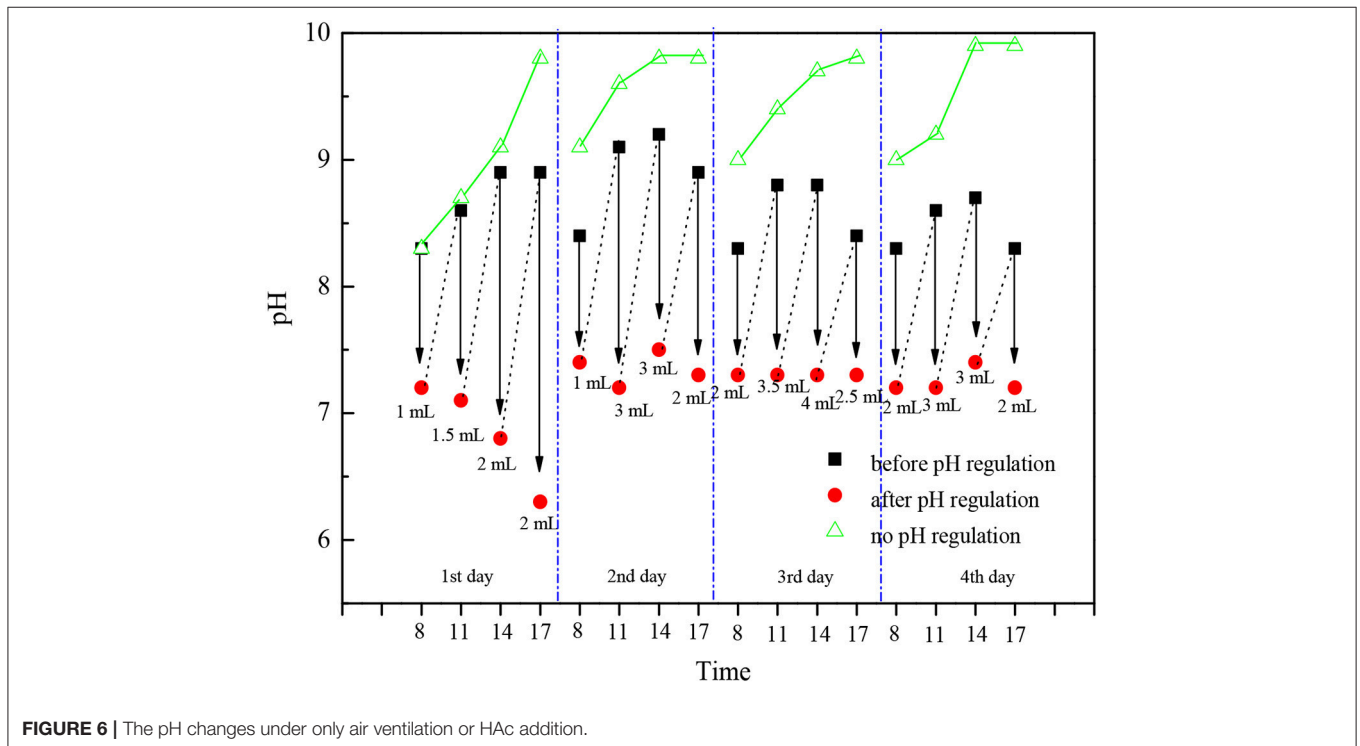


FIGURE 6 | The pH changes under only air ventilation or HAC addition.

range of 200–500 mg/L (Park et al., 2010). The quantity of free ammonium NH_3 and free ammonia depends on the pH value of the solution. Under low pH conditions, the alga cell might not be restrained by free ammonia, but the increase of free ammonium under high pH values above 9.0 would restrain the growth of alga cell very obviously (Konig et al., 1987). The ammonium would be totally ionized when the pH value was lower than 8.0 under 25°C and almost half ammonium would not be ionized when the pH value was 9. With the increase of temperature, the toxicity of ammonium would increase. Although the pH of cultivated solution was adjusted, and it would have restrains to the growth of *C. zofingiensis* G1 when the pH of the culture medium of the solution increases to 9.0 after 3 h (Wurts, 2003). We could see from the results that the concentration of urea had no obvious impact on *C. zofingiensis* G1 growth under this concentration range.

Effects of Ventilating and Adding Acetic Acid on Growth of Algae

Figure 5 indicated that the growth rate of *C. zofingiensis* G1 can be increased effectively by adding acetic acid. On the third day of outdoor cultivation, the concentration of *C. zofingiensis* G1 after adding acetic acid was 4.1 times of that in the case of ventilating only. The conclusion confirmed to the results of Lin, who found that the *C. pyrenoidosa* had the fastest growing speed when adding acetic acid and in-letting CO_2 at the same time (Lin and Chen, 1994).

As **Table 3** shows, during the cultivation period, the average temperature in the photobioreactor and light intensity had no big changes until the fourth day when the light intensity decreased because of cloudy weather, so the growth rate of the group adding acetic acid slowed down. The light intensity reached its maximum [450–561 $\mu\text{mol}/(\text{m}^2\cdot\text{s})$] at 11:00 a.m. every day, and it was comparatively low in the morning and afternoon. The temperature in photobioreactor was comparatively low at 21.2–23.5°C at 8:00 a.m., and it would increase to 34.2–36.9°C gradually with the increase of light intensity in this season. As

what had shown on **Figure 6** that if we did not adjust the pH value, then it would increase gradually from 8:00 a.m. because of the photosynthesis of *C. zofingiensis* G1, and the rate of increase slowed down to 9.7–9.9 in the afternoon. There was no photosynthesis in the night time, and the respiration and release of CO_2 would cause the pH value to drop back to 8.9–9.0. It is reported that the increase of pH would lead to the precipitation of phosphate and the blowing off of ammonia nitrogen (Gonzalez et al., 2008). We could not add too much acetic acid when the concentration of microalgae was low in the early stage of cultivation, otherwise it would lead to low pH value and produce foams during the cultivation, which was not good to the growth of algae. As the increase of microalgal concentration, we needed to increase the quantity of acetic acid. The temperature, light intensity and photosynthesis were comparatively strong at 11:00 and 14:00 every day, we needed to add more acid to adjust pH value. The adding of acetic acid could increase the growth rate and biomass accumulation of microalgae obviously, but adding acetic acid could not achieve this effect in the night time because there was no light.

Under the condition of adjusting pH by adding acetic acid, *C. zofingiensis* G1 not only utilized the sunlight to fix the CO_2 in the daytime, but also utilize the organic carbon source such as acetic acid by mixotrophic cultivation. Under the condition of no light at night, the algae were heterotrophic cultivation, the rate of metabolism slowed down and the temperature was between 26 and 32°C (**Figures 7, 8**). A small quantity of acetic acid would be burned off, the pH value would increase within 3 h, the changes of biomass concentration was limited, and at the same time the respiration would consume the saccharides and other organics of microalgae, which would cause the decrease of the biomass accumulation of *C. zofingiensis* G1. The concentration of microalgae biomass of this experiment had no obvious changes, however Torzillo et al used tubular outdoor photobioreactor to cultivate *Spirulina platensis* in Florence, Italy showed that the rate loss of biomass at night is about 7.6% under

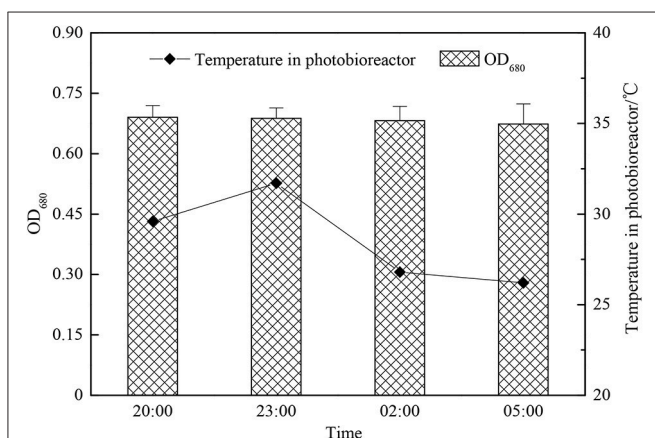


FIGURE 7 | The growth characteristic of *Chlorella zofingiensis* during the night time.

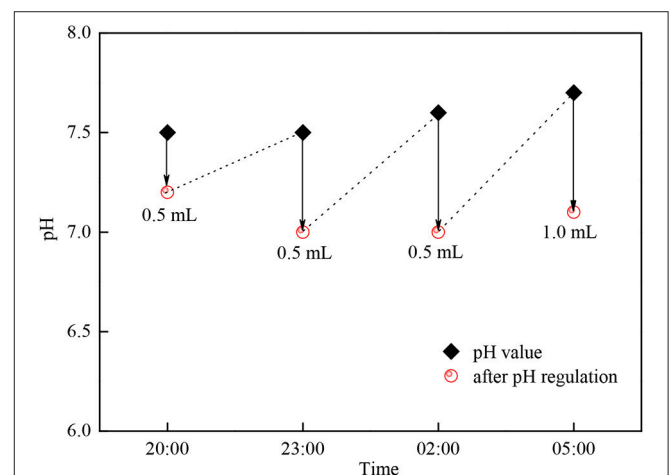


FIGURE 8 | The pH adjustment of *Chlorella zofingiensis* G1 during the night time.

25°C, and the rate loss was 5.0% under 35°C (Torzillo et al., 1991).

CONCLUSION

Adopting sunshade net and atomization spray combined system can effectively reduce the photobioreactor temperature in the scorching summer of sub-tropical South China areas. When the concentration urea and NH_4HCO_3 were 300 and 200 mg/L respectively, the concentration of *C. zofingiensis* G1 reached its maximum. *C. zofingiensis* G1 had the comparatively strong adapting ability in outdoor photobioreactor. The biomass accumulation of *C. zofingiensis* G1 by adding acetic acid was four times more than that only through air ventilation conditions. The biomass accumulation of *C. zofingiensis* G1 at night was not obvious changed by adding acetic acid.

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AUTHOR CONTRIBUTIONS

SH wrote the main part of the paper and together with QS, LZ, LQ, WZ, and PF performed the experiments. ZW, RD, and ZY conceived the experiments. SZ and FZ revised the paper.

ACKNOWLEDGMENTS

This research was funded by the National Natural Science Foundation of China (21506084), the China Postdoctoral Science Foundation (2015T80502), the Senior Talent Scientific Research Initial Funding Project of Jiangsu University (14JDG024). A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD) and the Training Project of the Young Core Instructor of Jiangsu University.

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- Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
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