Bioresource Technology 270 (2018) 80–87

Contents lists available at ScienceDirect

Bioresource Technology

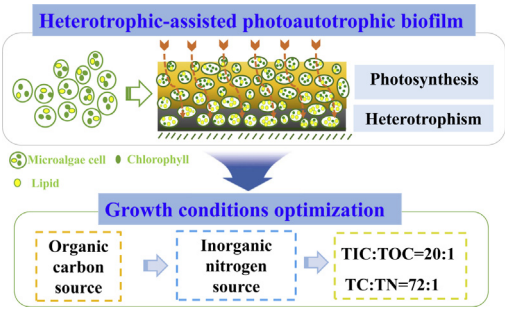
journal homepage: www.elsevier.com/locate/biortech

Optimizing culture conditions for heterotrophic-assisted photoautotrophic biofilm growth of *Chlorella vulgaris* to simultaneously improve microalgae biomass and lipid productivity

Yangli Ye[a,b], Yun Huang[a,b,*], Ao Xia[a,b], Qian Fu[a,b], Qiang Liao[a,b], Weida Zeng[a,b], Yaping Zheng[a,b], Xun Zhu[a,b]

[a] Key Laboratory of Low-Grade Energy Utilization Technologies and Systems, Chongqing University, Ministry of Education, Chongqing 400044, China
[b] Institute of Engineering Thermophysics, School of Energy and Power Engineering, Chongqing University, Chongqing 400044, China

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Microalgae biofilm
Heterotrophic-assisted photoautotrophic
Light penetration
Nutrients optimization
Lipid production

ABSTRACT

In order to solve the technical bottleneck that the biomass yield and lipid accumulation cannot be increased simultaneously during microalgae growth, a heterotrophic-assisted photoautotrophic biofilm (HAPB) growth mode of *Chlorella vulgaris* was constructed. The light penetration capability of the microalgae biofilm formed through heterotrophic-assisted photoautotrophic growth was 64% stronger than that formed by photoautotrophic growth. Due to the different demands of autotrophic and heterotrophic growth of microalgae, the nutrient environment and growth conditions were optimized to fully utilize the advantages and potentials of the HAPB culture model. An optimized molar ratio of total inorganic carbon (CO_2) to total organic carbon (glucose) (20:1) and a molar ratio of total carbon to total nitrogen (72:1) were obtained. The maximum specific growth rate of *Chlorella vulgaris* increased by 78% compared to that before optimization. Meanwhile, the lipid content and yield increased by 120% and 147%, respectively, up to 47.53% and 41.95 g m^{-2}.

1. Introduction

In the past few decades, although petroleum fuel has generated social progress and economic prosperity, it has also caused a double crisis of energy shortage and environmental pollution, as a result, countries worldwide are looking for an environmentally friendly alternative fuel, such as biodiesel (Hasan and Rahman, 2017). Biodiesel made from natural plant-derived fats and acid monoesters is a clean,

* Corresponding author.
E-mail address: yunhuang@cqu.edu.cn (Y. Huang).

https://doi.org/10.1016/j.biortech.2018.08.116
Received 5 August 2018; Received in revised form 27 August 2018; Accepted 28 August 2018
Available online 30 August 2018
0960-8524/

renewable, safe, and low-cost green energy source (Aghbashlo and Demirbas, 2016; Hill et al., 2006). Compared with other feedstocks for biodiesel production, such as rapeseed and soybeans, microalgae has many advantages, including its short cultivation cycle and high biomass and lipid production efficiency. (Sun et al., 2018; Wei et al., 2018)

Cultivation of microalgae is an important part of the microalgae biodiesel production, which is of great significance for reducing cost and large-scale production. Biofilm cultivation of microalgae refers to a bio-enhanced technology that uses physical or chemical means to limit or locate free-living algal cells with certain activity in a certain space and keep them biologically active. The removal of large amounts of water leads to more complete illumination, lower transport resistance of CO_2 and nutrients, better mass transfer, and higher photosynthetic efficiency, which can significantly increase biomass production. (Berner et al., 2014; Gross et al., 2015; Schnurr and Allen, 2015; Wang et al., 2017). As a result, it is an economically viable and extremely promising cultivation technology.

Up to now, biofilm cultivation of microalgae has mostly depended on photosynthetic autotrophic growth (Huang et al., 2016; Zheng et al., 2017). That is, microalgae use H_2O, CO_2, and simple inorganic nutrients to synthesize substances and carry out metabolism and self-propagation under light conditions. In this culture mode, the thickness of the biofilm is very thin in the initial growth stage, resulting in more sufficient light and less resistance to transport of CO_2 and nutrients. However, the thickness of biofilm becomes thicker and thicker with the increasing density of biomass, resulting the weakening of the light penetration characteristic in the microalgae biofilm. Algae cells in the light-dead area continuously degrade their organic matter and perform dark respiration, which makes the growth of biofilm hysteretic and the biomass yield declining. Furthermore, the uniqueness of photosynthesis and growth metabolism of microalgae makes the lipid content of the photoautotrophic biofilm inferior. In general, the lipid content of this fast-growing photosynthetic microalgae biomass is lower than 13.42%. (Hulatt and Thomas, 2011) To sum up, large-scale degradation of biomass in the late growth stage and poor lipid content make it difficult to achieve large-scale microalgae biodiesel production. Nonetheless, microalgae cells can also degrade organic matter in a non-lighting condition for heterotrophic growth. That is to say, microalgae cells in the upper layer of biofilm with light can synthesize biomass quickly by photoautotrophic metabolism, while the cells in the bottom without light can use organic substrates to synthesize lipids by heterotrophic metabolism. What's more, heterotrophic growth of microalgae can significantly increase the lipid content. (Song and Pei, 2018). In conclusion, a heterotrophic metabolic growth model can be combined to construct a heterotrophic-assisted photoautotrophic biofilm cultivation system. Moreover, the research on the growth characteristic of heterotrophic-assisted photoautotrophic biofilm, especially the lipid content, will effectively promote the development and application of microalgae biomass to produce biodiesel, which has important economic and social value. However, due to the different cultivation methods of autotrophic growth and heterotrophic metabolism of microalgae, the demands on culture conditions and the nutrient environment are quite different (Morales-Sanchez et al., 2015). In general, heterotrophic microalgae cells metabolize and secrete a large amount of acidic substances, which will reduce the pH of the culture, thereby resulting in an acidic environment and affecting normal photosynthetic autotrophic growth in the presence of heterotrophic growth (Perez-Garcia et al., 2011). Although some scholars have conducted similar research on microalgal suspension cultures (Zhan et al., 2017), studies on simultaneous photosynthetic autotrophic and heterotrophic growth of algal cells in microalgae biofilm have not yet been reported. Hence, for the sake of giving full play to the advantages and potentials of numerous growth modes of microalgae cells, it is necessary to determine a nutrient environment and growth conditions suitable for this culture mode. In addition, the medium is a direct substance for the growth of algae, and its nutrient composition and content play an important role in the growth, development, reproduction, and other life activities of the algal cells (Costa et al., 2018) In particular, the morphology and content of carbon and nitrogen sources in the medium are crucial for the growth, metabolism, and synthesis of chlorophyll and lipids in algal cells (Leite et al., 2016; Li et al., 2018). Glucose is used as an organic carbon source which can be recovered from the enormous amounts of organic residues occur globally in the form of straw, wood, green biomass, food waste, feces, manure, etc. Therefore, the cost and economic benefits of microalgae cultivation are rarely considered. Even if it is applied to engineering practice, the problem of high cost of organic carbon sources can also be solved by using the organic waste. (Pleissner and Rumpold, 2018).

In this study, to promote the metabolism and growth of microalgae cells and lipid productivity in the HAPB cultivation system, the morphology and content of organic and inorganic substances were studied in order to determine the optimal nutrients for simultaneous biomass and lipid accumulation. This may provide technical support and experimental guidance for the large-scale cultivation and industrial application of microalgae biomass.

2. Materials and methods

2.1. Microorganism and culture medium

The freshwater microalgae *Chlorella vulgaris* FACHB-31 and the *Scenedesmus obliquus* FACHB-417 used in this study were purchased from the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). Both algae cells were maintained in Blue-Green medium (BG11) (Huang et al., 2016) at 25 °C under a light intensity of 100 μmol m^{-2} s^{-1}.

2.2. Heterotrophic-assisted photoautotrophic biofilm growth system and operation

The complete experimental system consisted of a culture component, gas distribution component, and illumination component. Rectangular boxes (300 × 60 × 50 mm, L × W × H) made of polymethyl methacrylate (PMMA) were used as bioreactors for microalgae biofilms that were placed on the surface of polyurethane porous polymer materials after suction filtration, which were covered by glass plates (Fig. 1a). The core component, a thin and porous polymer material, acts as a support for the immobilization of microalgae by self-adhesion. The functional characteristic of this element is to separate cells from the bulk of the culture medium while allowing transport of water, dissolved nutrients, and gases by diffusion and by evaporative flux directed towards the biofilm surface. Parallel fluorescent lamps were fixed above the PMMA chamber as lights sources to provide continuous illumination for algal growth. The distance between the lamps and the top surface of the PMMA chamber could be adjusted to produce different light intensities on the top cover surface of the chamber. What needs to be emphasized is that different light intensities are used only when testing the light penetration characteristic of biofilm, and the intensity of light (maintained at 100 μmol m^{-2} s^{-1}.) remains unchanged in the optimization process of nutrients for biofilm growth. A miniport was fixed on the left side of the glass chamber. It was connected to a compressed CO_2-enriched air supplier at a rate of 100 mL min^{-1}. If not specially stated, the concentrations of enriched gases were 5% CO_2 (v/v) and 19% O_2 (v/v). All the experiments were conducted at a temperature of 25 ± 1 °C. The biomass concentration of algae precultured for 5 days was measured using the gravimetric method (Ji et al., 2014). Specifically, the sampled microalgae suspension was centrifuged and dried in an oven under 105 °C overnight to obtain the biomass concentration. Afterwards, according to the microalgae biofilm inoculation area (10 g m^{-2}), a certain volume of precultured algal fluid was measured with a pipette and then filtered through a vacuum filter to form an initial microalgae biofilm area

density. Assuming that the effective inoculation area of the filter membrane was A (m^2), the formula for calculating the initial biofilm area density X_0 is as shown in Eq. (1):

$$X_0 = \frac{C_0 V_0}{1000A} \quad (1)$$

注意氧气和二氧化碳具体的传输方向

The HAPB culture system was a complex biochemical reaction system with multiple components and multiphase transfer. In this system, the gas mixture of CO_2 and O_2 and the nutrient solution were placed on both sides of the porous medium, in which the gas directly contacted with the algal cells and diffused into the porous network structure of the biofilm, thereby participating in the photosynthetic autotrophic and heterotrophic growth of the algae cells. Meanwhile, an organic carbon source required for heterotrophic nutrition was predissolved in the culture fluid to form a stable liquid film on the surface of the polyurethane porous polymer material, Then, under the combined action of evaporation driving force and capillary force, the medium was diffusely transferred to the polyurethane porous material to achieve a certain degree of liquid saturation. Finally, synergistic growth of microalgae biofilms was realized by means of the diffusion effect of the microporous membrane. Last, O_2 produced by photosynthesis and CO_2 released by heterotrophic metabolism were transferred in opposite directions and biochemically transformed in the porous media structure composed of microalgal cells. Carbon/nitrogen source types and concentrations in the culture were determined, as listed in Table 1. Then, in order to investigate the applicability of the optimized nutrient solution, growth experiments of microalgae were performed using *C. vulgaris* and *Scenedesmus obliquus*, respectively. Among them, the molar ratio of total inorganic carbon (TIC) to total organic carbon (TOC) refers to the molar ratio of the amount of CO_2 supplied to the initial supply of glucose and the calculation of the amount of CO_2 is the total volume of carbon dioxide gas (the total volume of gas used multiplied by the concentration of CO_2) divided by the molar volume of gas. The ratio of total carbon (TC) to total nitrogen (TN) refers to the molar ratio of the amount of carbon source (total amount of CO_2 and glucose) to the initial supply of inorganic nitrogen source (sodium nitrate).

2.3. Analytical methods

2.3.1. Determination of light penetration characteristics

Algal mud concentrated by centrifugation was washed with first grade deionized water and then poured into transparent PMMA cubes (50 × 50 × 30 mm) to form microalgae biofilms with different biomass densities (5, 10, 20, 40, 60, 70, 80, 90, 100, and 120 g m^{-2}). The incident light intensities of 90, 130, 160, and 200 μmol m^{-2} s^{-1} were selected by adjusting the distance between the cube and fluorescent lamps and the number of fluorescent Then, a photosynthetic quantum sensor (Li-250A, Li-Cor, Lincoln, NE, USA) was placed underneath each cube and tightly attached to the PMMA sheet to measure the penetrated photon flux density. In order to eliminate the influence of glass, the output light intensity of the biofilm was added to the light intensity loss that penetrated the PMMA.

Table 1
Optimal design of experimental conditions based on BG11 medium.

		Concentration (mM)			
Carbon source	Glucose	16	33	49	65
	Sucrose		33		
	Sodium acetate		33		
Concentration (mM)	(Concentration of glucose is 49 mM)				
Nitrogen source	Sodium nitrate	10	14	17	21
	Sodium nitrite		14		
	Urea		14		
	Ammonium chloride		14		

2.3.2. Determination of microalgae growth

The biofilm was washed down from the supporting membrane followed by centrifuging and drying to constant weight (W, g) at 105 °C to calculate the areal density of the biofilm. Biomass density (X, g m^{-2}) and specific growth rate (μ, h^{-1}) of the microalgae biofilm were calculated using Eqs. (2) and (3), respectively, as follows:

$$X = \frac{1.5W}{0.001254} \quad (2)$$

$$\mu = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \quad (3)$$

where 0.001254 represents the area of land occupied by the biofilm (m^2). X_2 (g m^{-2}) is the microalgae biofilm density at time t_2 (h) and X_1 (g m^{-2}) represents the microalgae biofilm density at time t_1 (h).

Chlorophyll content was determined according to the methods described by Wellburn (1994). In addition, the procedure for measuring the maximum light quantum efficiency of the PSII in the microalgae intracellular photosystem was the same as that described by Ryan et al. (2004).

Lipids contained in the microalgae cells were extracted, and the lipid content was determined gravimetrically by employing the direct transesterification method (Liao et al., 2017). Percentage (ω, %) and yield (L, g m^{-2}) of lipid were calculated using Eqs. (4) and (5), respectively, as follows:

$$\omega = \frac{W_1}{W_0} \times 100\% \quad (4)$$

$$L = X_t \times \omega \quad (5)$$

where W_0 (g) and W_1 (g) are the dry weight of microalgae and lipid, respectively, and X_t refers to the final microalgae biofilm area density (g m^{-2}).

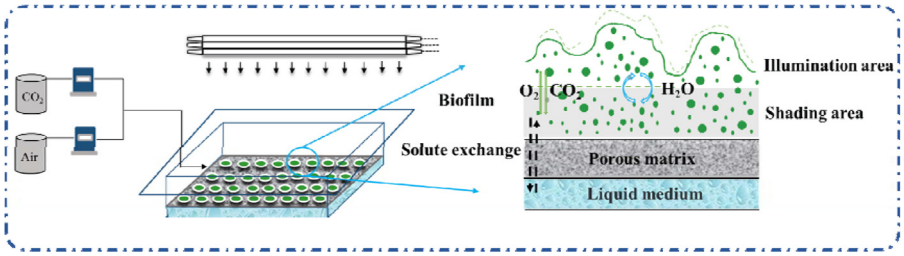
2.3.3. Observation of microscopic morphology of the biofilm

Biofilm fixation and dehydration steps for electron microscope scanning were determined according to the methods described by Little et al. (1991). At 100 times magnification, multiple microalgae cells were uniformly selected in five fields of view to measure the diameter using an optical microscope (BX63, OLYMPUS), and the average value was taken as the cell diameter in this state. The dehydrated and fixed microalgae biofilm was placed on the microscope optical platform. The observations were subjected to tomography and 3D reconstruction using a mobile NanoFocus 3D confocal microscope (μsurf custom) to recover the biofilm morphology.

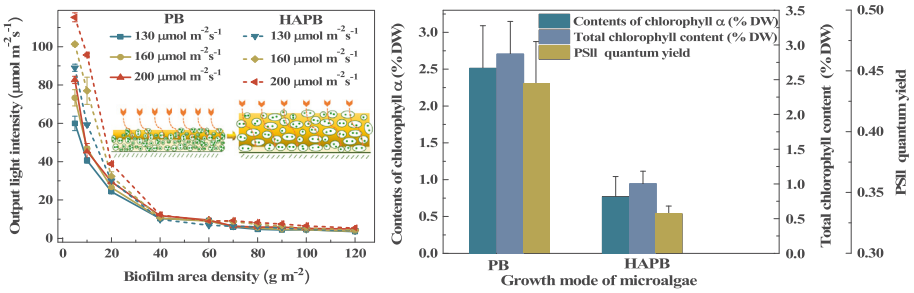
3. Results and discussion

3.1. Comparison of light penetration characteristics in the microalgae biofilm

Light penetration characteristics in the HAPB and PB are shown in Fig. 1b. Light penetration characteristics in the two types of biofilm were clearly different, especially when the biofilm area was lower than 40 g m^{-2}. Specifically, in the case of incident light intensity of 130 μmol m^{-2} s^{-1}, the emitted light intensity of the PB and HAPB reached 60 μmol m^{-2} s^{-1} and 89 μmol m^{-2} s^{-1}, respectively, when the biofilm area density was 5 g m^{-2}. In other words, the effective light penetration capability in the HAPB was 48% stronger than that in the PB. Similarly, when the incident light intensity was 160 μmol m^{-2} s^{-1}, the emitted light intensity of the PB and HAPB reached 47 μmol m^{-2} s^{-1} and 77 μmol m^{-2} s^{-1}, respectively, with a biofilm area density of 10 g m^{-2}, the emitted light intensity from the HAPB increased by 64% compared to that from PB. There was a similar pattern of change when the light intensity was 200 μmol m^{-2} s^{-1}.



(a) Schematic of the cultivation system of the HAPB and material transfer process



(b) Light penetration characteristics. (c) Chlorophyll content and PSII quantum yield

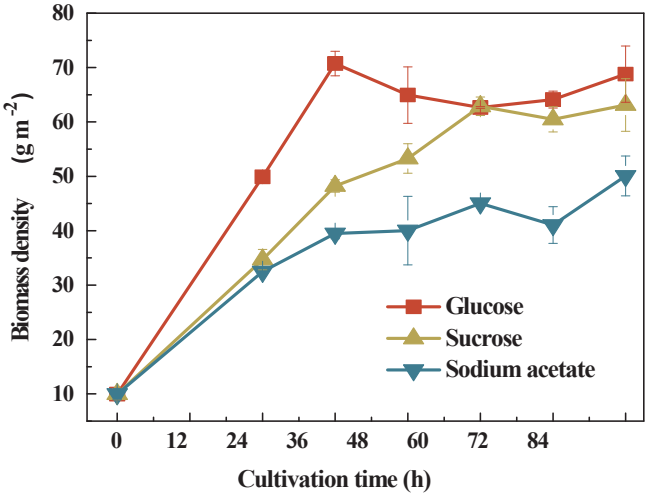
Fig. 1. (a) Schematic of the cultivation system of the HAPB and material transfer process and comparison of (b) light penetration characteristics and (c) chlorophyll content and PSII quantum yield under PB and HAPB.

The above results demonstrated that the HAPB had better light penetration characteristics. Moreover, the better the light penetration performance, the faster the autotrophic growth of microalgae cells and the more lipid accumulation (He et al., 2018). To better explain this experimental phenomenon, the diameter and pigment content of microalgae cells in these two culture methods were compared. The average diameter of microalgae cells cultured with heterotrophic assisted photoautotrophy reached 6 μm, which was 36% higher than that of microalgae cells cultured with photosynthetic autotrophy (average diameter size only reached 4.4 μm). Then, the pigment content of the microalgae cells in both cultures was compared. The chlorophyll α content of cells cultured using photosynthetic autotrophy reached 2.51% of the dry weight of the cells, and the total chlorophyll content reached 2.87%. Conversely, the chlorophyll α content of cells cultured with heterotrophic-assisted photosynthetic autotrophy accounted for 0.77% of the cell dry weight, and the total chlorophyll content only accounted for 1% (Fig. 1). This experimental phenomenon might explain the stronger light penetration ability of the cells cultured with heterotrophic-assisted photosynthetic autotrophy from two aspects. On the one hand, the shape of the microalgae cells was ellipsoidal, and a larger cell diameter means looser biofilm structure, higher porosity, and more gaps, which may lead to greater light penetration. On the other hand, the lower chlorophyll content of cells meant that along the direction of the light path, cells in the upper layer of the biofilm might absorb less photons, and more photons have more chances to penetrate the biofilm (Wang et al., 2015), which leads to stronger photosynthesis inside the biofilm.

3.2. Optimization of morphology and content of carbon source for heterotrophic microalgae-assisted photoautotrophic biofilm

With the increase of culture time, the biomass of microalgae biofilm gradually accumulated, it showed an increasing trend first and then tended to be stabled. The highest biomass yield of microalgae biofilm with glucose as a carbon source was 70.75 g m^{-2}, which was 13% higher than that of the sucrose test group and 42% higher than that of the sodium acetate test group; this was because the cells of *C. vulgaris* contain a hexose transport system that is induced by glucose or its analogues (3-O-methylglucose or 6-deoxyglucose) (Haass and Tanner, 1974), which can degrade and absorb the sugar and use it for cell division and reproduction. This was also demonstrated by the maximum specific growth rate of 0.067 h^{-1} obtained with glucose as the organic carbon source, which was much higher than the maximum specific growth rate obtained with sucrose (0.052 h^{-1}) and sodium acetate (0.049 h^{-1}) (Fig. 2). In summary, considering that glucose is the most economical carbon source material in industrial production, it was selected as the best form of organic carbon for the growth of microalgae biofilm.

At the initial stage of cell growth, the biomass density of the microalgae biofilm cultured at a lower molar ratio of TIC to TOC rapidly increased from 9.940 g m^{-2} to 45.98 g m^{-2} at the maximum specific growth rate of 0.13 h^{-1} (Fig. 3). Among them, when the molar ratio of TIC to organic TOC was 20:1, the biomass density of the biofilm



Biomass density of the HAPB

Fig. 2. Comparison of biomass density of the HAPB of *Chlorella vulgaris* under different carbon sources (at a concentration of 33 mM).

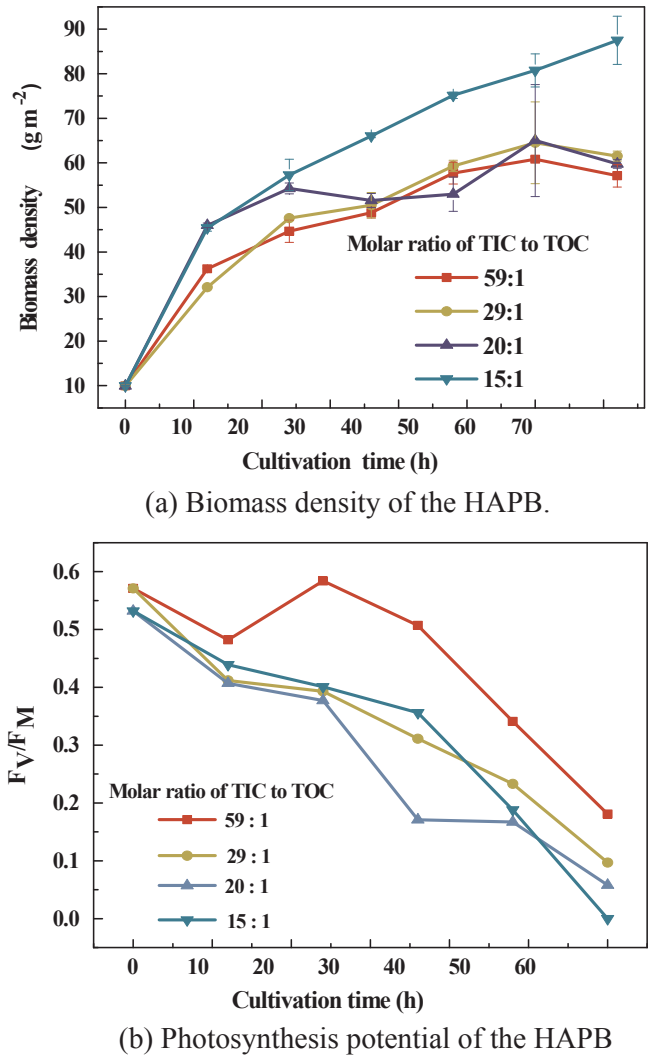


Fig. 3. Comparison of (a) biomass density and (b) photosynthesis potential of the HAPB under different molar ratios of TIC to TOC.

gradually reached a steady state after a period of rapid growth. In addition, it is known from the graph that when the molar ratio of TIC to TOC was higher, the maximum specific growth rate of biofilm and the area density of biomass were lower; this was because the higher the concentration gradient of glucose, the greater the material transport driving force and the better the mass transfer effect, which resulted in the stronger growth of microalgae biofilm (Kong et al., 2013). The effect of the molar ratio of TIC to TOC on the Fv/Fm value of microalgae cells is shown in Fig. 3(b). When the molar ratio of TIC to TOC was relatively high, the heterotrophic growth of cells was weaker and the photosynthesis of cells was stronger, which resulted in a higher photochemical quantum yield of PSII. In conclusion, due to the simultaneous consideration of biological activity, maximum biomass, photosynthetic capacity, and economics of industrial production, the molar ratio of TIC to TOC of 20:1 was chosen as the optimal carbon source concentration for microalgae biofilm cultivation.

3.3. Optimization of morphology and content of nitrogen source for heterotrophic microalgae-assisted photoautotrophic biofilm

The biomass density of biofilm cultured with ammonium chloride was only 25.29 g m^{-2}, and the maximum specific growth rate was 0.036 h^{-1}, which was only half of that of the sodium nitrate group. Less biomass accumulation might have been due to the rapid decrease in the

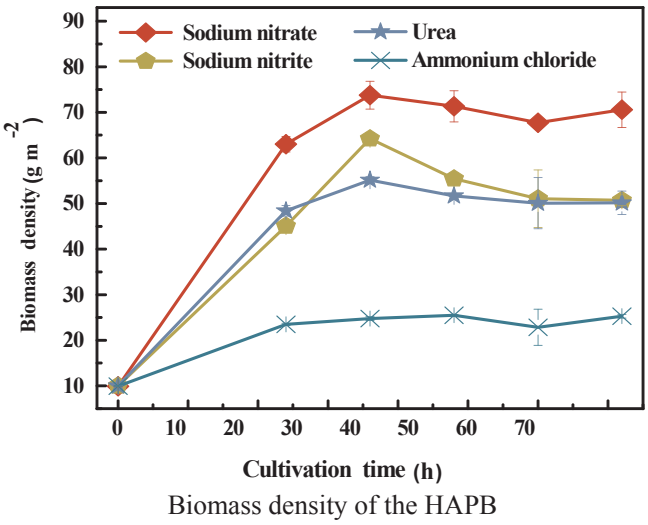


Fig. 4. Comparison of biomass density of HAPB of *Chlorella vulgaris* under different nitrogen sources (at a concentration of 14 mM).

pH of the culture solution caused by the hydrolysis of ammonium ions in the solution, which inhibited the metabolism and reproduction of the algal cells (Ramanna et al., 2014). Biofilm cultured with sodium nitrate rapidly accumulated biomass at the maximum specific growth rate of 0.077 h^{-1}. Then, the biomass density increased from 9.899 g m^{-2} to 63.02 g m^{-2}, and finally stabilized at 73.78 g m^{-2} (Fig. 4). Therefore, from the perspective of biomass accumulation and economics of nitrogen sources, sodium nitrate was selected as the optimal nitrogen source for the growth of HAPB.

As an important part of cellular synthesis of amino acids and other substances, nitrogen sources must be maintained at a certain concentration to support the metabolic demands and reproduction of cells. The effect of the molar ratio of total carbon to total nitrogen on growth of microalgae biofilm is shown in Fig. 5. When the molar ratio of TC to TN was 72:1, the biofilm rapidly accumulated biomass at the maximum specific growth rate of 0.077 h^{-1}, and the final biomass density was also the highest. Nevertheless, when the molar ratio of TC to TN was relatively high, the relatively low concentration of nitrogen sources affected the intracellular tricarboxylic acid cycle and the synthesis of chlorophyll; then, the intracellular photosynthetic system was damaged and the photosynthetic efficiency decreased. These behaviors finally led to cell growth arrest (Young and Beardall, 2003). When the molar ratio of TC to TN was relatively low, the nitrogen source concentration was relatively high, the assimilation rate of intracellular nitrogen sources was much lower than the nutrient supply rate, and ammonium produced by the algal cells over-accumulated and could not be quickly transferred for synthesizing amino acids, which caused cell ammonium poisoning and affected the normal growth of microalgae biofilm (Dai et al., 2014). The experimental results showed that there was an optimal molar ratio of TC to TN for the cultivation of microalgae biofilm, namely, when the molar ratio was 72:1, microalgae cells had the greatest cell viability and underwent vigorous growth, metabolism, and rapid reproduction. The trend of change in chlorophyll *a* content in biofilms cultured with different molar ratios of TC to TN was the same. Over time, the percentage of chlorophyll *a* in the algae decreased. when the molar ratio was 72:1, the rate of decline in chlorophyll *a* content was very high, but the final pigment content was the same as that of the other experimental groups, which indicated a high photoautotrophic growth rate of algal cells.

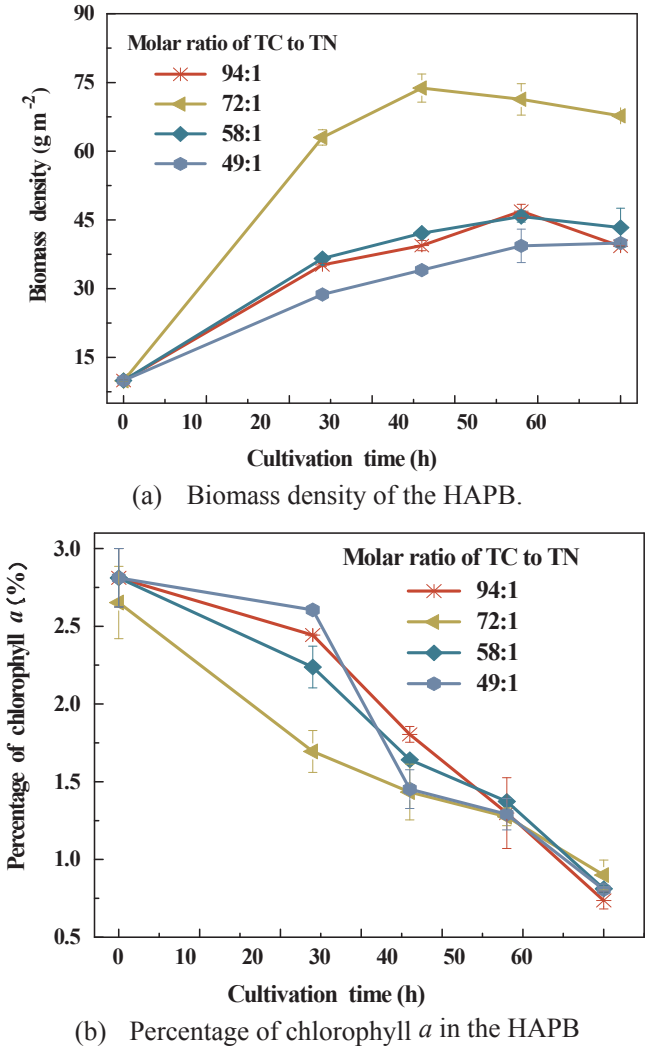
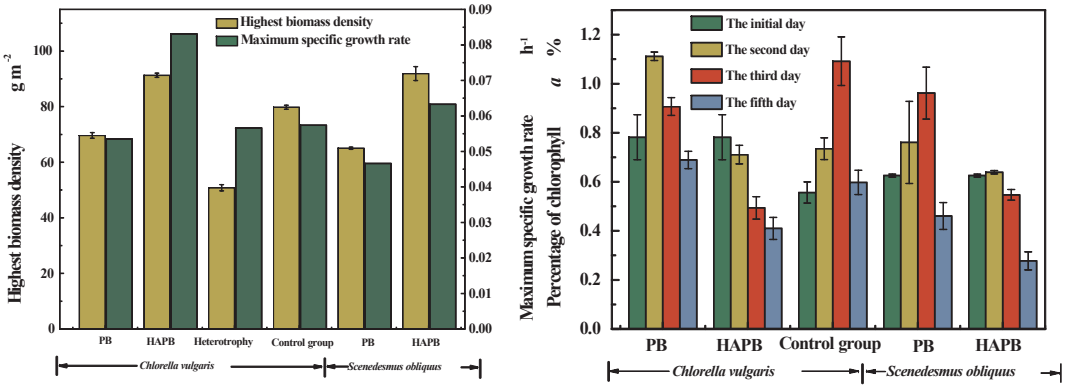


Fig. 5. Comparison of (a) biomass density and (b) percentage of chlorophyll *a* in the HAPB under different molar ratios of TC to TN.

3.4. Heterotrophic-assisted photoautotrophic biofilm growth under the optimized culture conditions

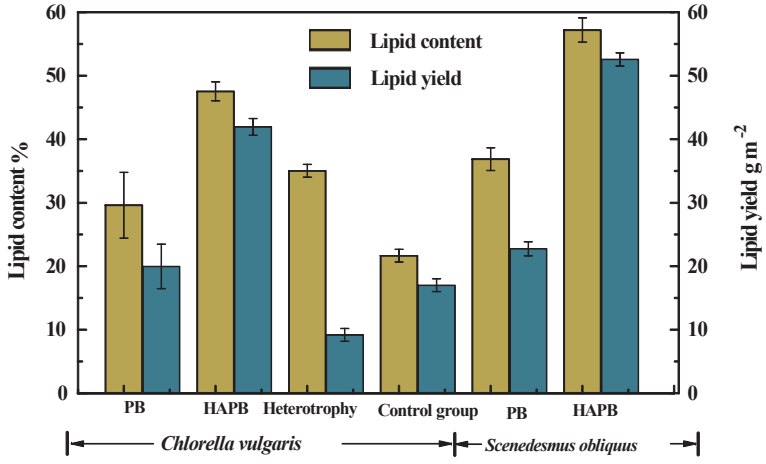
Taking into consideration the effectiveness and applicability of the optimized nutrients, two types of algae, namely *C. vulgaris* and *S. obliquus*, were selected for the experiment. It was found that although the chlorophyll *a* content of the two species of algae was maintained at a relatively high level under photosynthetic growth, the specific growth rate of cells was low and their propagation was slow. In addition, the final biomass densities of *C. vulgaris* and *S. obliquus* only reached 69.63 g m^{-2} and 65.05 g m^{-2}, respectively (Fig. 6). However, if the medium was supplemented with glucose for heterotrophic assisted autotrophic growth, particularly, when the molar ratio of TIC to TOC was 20:1 and the molar ratio of TC to total TN was 72:1, microalgae cells could simultaneously perform strong photosynthetic autotrophic and heterotrophic growth with fast metabolism, good activity, rapid growth, and fertile reproduction capacity under the premise of a suitable external environment (Zhan et al., 2017). We can see that under the condition of heterotrophic growth support, *C. vulgaris* cells were round with a large diameter and dense distribution. In addition, the percentage of chlorophyll *a* in these cells was much lower than that of the cells with photosynthetic autotrophic growth. As a result, it could be inferred that the accumulation of microalgae with a large diameter produced higher porosity. Furthermore, less chlorophyll *a* content in cells resulted in longer and a larger amount of transport pathways of light and nutrients into the biofilm. That is, along the transmission direction perpendicular to the surface of the biofilm, the proportion of microalgae cells that could be effectively irradiated was greater and the photosynthetic efficiency within the biofilm was higher. In addition, the nutrient transfer efficiency increased as the porosity of the microporous structure increased, which resulted in higher mass transfer efficiency of the biochemical reaction (Wan et al., 2011). Therefore, the specific growth rate and the maximum biomass area density of the microalgae biofilm significantly improved. Meanwhile, in order to further clarify the effect of the optimized nutrients, a nutrient combination of BG11 and 33 mM glucose was selected as the growth control group of this experiment, where the molar ratio of TIC to TOC was 29:1 and the molar ratio of TC to TN was 96:1. The experimental results showed that the maximum specific growth rate of the experimental group after nutrient optimization could be as high as 0.08 h^{-1}, which was 78% higher than that of the experimental control group. Meanwhile, the specific growth rate of the heterotrophic growth group was between that of the photosynthetic autotrophic group and the nutrient optimized group at the initial growth stage of the microalgae biofilm, but its highest biomass density was lower than that of both groups; the reason for this phenomenon was that although homogeneously heterotrophic growth in microalgae suspension culture systems could significantly increase the biomass density and lipid production (Perez-Garcia et al., 2011), it might not be suitable for biofilm-membrane culture systems where the dense membrane structure caused too little dissolved oxygen to be present in the system and the heterotrophic metabolism is weak.

From the perspective of lipid content and yield of microalgae, the lipid contents of *c. vulgaris* and *S. obliquus* were 47.53% and 57.19%, which were 60% and 54% higher than that of the photoautotrophic autotrophic group, respectively. This phenomenon was probably attributed to the addition of glucose. Namely, glucose could change the intracellular fat production pathway and promote the accumulation of lipid content (Wan et al., 2011). In addition, the lipid content is much higher than the results of previous scholars. Rincon et al proposed a algal biofilm reactor using glycerol and urea as carbon and nitrogen sources, the total lipid contents were 13 ± 0.02% and 23.91 ± 0.03%, respectively when 2 and 5 g L^{-1} initial glycerol concentrations were used. (Rincon et al., 2017). Zhang et al designed a vertical-algal-biofilm-enhanced raceway pond for wastewater treatment and algal biomass production under different nutrient loading rates, and the lipid content obtained in this paper varied marginally in the range of 18.59–19.37% (Zhang et al., 2018). Miranda et al explored the potentials of natural bioflms, their photosynthetic inhabitants and newly assembled algal/cyanobacterial bioflms as the next generation of bioenergy feedstocks which can grow using wastewaters as a cheap source of key nutrients, and the lipid content varied in the range 6–18% (Miranda et al., 2017). For *C. vulgaris*, the lipid content of its experimental control group was only 21.65%, and the lipid yield of the *C. vulgaris* biofilm was as high as 41.95 g m^{-2}, which was 1.1 and 1.5 times higher than that of the photosynthetic autotrophic group and control group, respectively. A mobile NanoFocus mobile 3D confocal microscope was used to perform 3D reconstruction and measurement analysis of the biofilm to obtain a complete physical appearance of the biofilm surface. As a multi-element porous media system, the biofilm had complex porous channels and surface morphology, which has made its study relatively scarce. In short, this 3D reconstruction technique not only completely recovered the biofilm 3D stereoscopic image by measuring the height of the object, but also effectively realized the qualitative analysis of the intramembrane microstructure. In summary, the optimized nutrients have a significant role in promoting the growth and lipid production of microalgae biofilm, and are of great significance to the production of clean, renewable biodiesel.



(a) Growth situation of microalgae.

(b) Percentage of chlorophyll *a* of microalgae



(c) Lipid yield of the microalgae biofilm

Fig. 6. Comparison of (a) the highest biomass density, maximum specific growth rate, (b) chlorophyll *a* content of the microalgae biofilm, and (c) lipid content and production of microalgae biofilm under different experimental conditions.

4. Conclusions

In this paper, we have proposed a new cultivation mode based on HAPB to simultaneously improve microalgae biomass and lipid productivity. The maximum growth rate and lipid content of biofilm achieved 32 g m^{-2} d^{-1} and 48% by optimizing the composition and proportion of nutrients. Namely, the support of heterotrophy makes the growth of biofilm break through the major bottleneck of previous researches. Although the preliminary objective has been achieved, the theoretical knowledge and experimental mechanism based on biofilm growth are still unclear. Future works will focus on growth model and nutritional transfer to better understand and apply this cultivation mode.

Acknowledgements

The authors are grateful for the financial support provided by the National Science Foundation for Young Scientists of China (No. 51606020), the International Cooperation and Exchange of the National Natural Science Foundation of China (No. 51561145013), and the National Science Foundation for Young Scientists of China (No. 51606021), and the Fundamental Research Funds for the Central Universities (2018CDXYDL0001).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biortech.2018.08.116.

References

Aghbashlo, M., Demirbas, A., 2016. Biodiesel: hopes and dreads. Biofuel Res. J. 3 (2), 379.
Berner, F., Heimann, K., Sheehan, M., 2014. Microalgal biofilms for biomass production. J. Appl. Phycol. 27 (5), 1793–1804.
Costa, S.S., Miranda, A.L., Andrade, B.B., Assis, D.J., Souza, C.O., de Morais, M.G., Costa, J.A.V., Druzian, J.I., 2018. Influence of nitrogen on growth, biomass composition, production, and properties of polyhydroxyalkanoates (PHAs) by microalgae. Int. J. Biol. Macromol. 116, 552–562.
Dai, G.-Z., Qiu, B.-S., Forchhammer, K., 2014. Ammonium tolerance in the cyanobacterium Synechocystis sp. strain PCC 6803 and the role of the psbA multigene family. Plant, Cell Environ. 37, 840–851.
Gross, M., Jarboe, D., Wen, Z., 2015. Biofilm-based algal cultivation systems. Appl. Microbiol. Biotechnol. 99 (14), 5781–5789.
Haass, D., Tanner, W., 1974. Regulation of hexose transport in chlorella vulgaris. Plant. Physiol. 53, 14–20.
Hasan, M.M., Rahman, M.M., 2017. Performance and emission characteristics of biodiesel–diesel blend and environmental and economic impacts of biodiesel production: a review. Renew. Sustain. Energy Rev. 74, 938–948.
He, Q., Yang, H., Hu, C., 2018. Effects of temperature and its combination with high light intensity on lipid production of Monoraphidium dybowskii Y2 from semi-arid desert areas. Bioresour. Technol. 265, 407–414.
Hill, J., Nelson, E., Tilman, D., Polasky, S., Tiffany, D., 2006. Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. Proc. Natl. Acad. Sci. 103 (30), 11206–11210.
Huang, Y., Xiong, W., Liao, Q., Fu, Q., Xia, A., Zhu, X., Sun, Y., 2016. Comparison of Chlorella vulgaris biomass productivity cultivated in biofilm and suspension from the aspect of light transmission and microalgae affinity to carbon dioxide. Bioresour. Technol. 222, 367–373.
Hulatt, C.J., Thomas, D.N., 2011. Energy efficiency of an outdoor microalgal photobioreactor sited at mid-temperate latitude. Bioresour. Technol. 102 (12), 6687–6695.

Ji, B., Zhang, W., Zhang, N., Wang, J., Lutzu, G.A., Liu, T., 2014. Biofilm cultivation of the oleaginous microalgae Pseudochlorococcum sp. Bioprocess Biosyst. Eng. 37 (7), 1369–1375.
Kong, W.-B., Yang, H., Cao, Y.-T., Song, H., Hua, S.-F., Xia, C.-G., 2013. Effect of glycerol and glucose on the enhancement of biomass, lipid and soluble carbohydrate production. Food Technol. Biotechnol. 51 (1), 62–69.
Leite, G.B., Paranjape, K., Hallenbeck, P.C., 2016. Breakfast of champions: fast lipid accumulation by cultures of Chlorella and Scenedesmus induced by xylose. Algal Res. 16, 338–348.
Li, X., Li, W., Zhai, J., Wei, H., 2018. Effect of nitrogen limitation on biochemical composition and photosynthetic performance for fed-batch mixotrophic cultivation of microalga Spirulina platensis. Bioresour. Technol. 263, 555–561.
Liao, Q., Sun, Y., Huang, Y., Xia, A., Fu, Q., Zhu, X., 2017. Simultaneous enhancement of Chlorella vulgaris growth and lipid accumulation through the synergy effect between light and nitrate in a planar waveguide flat-plate photobioreactor. Bioresour. Technol. 243, 528–538.
Little, B., Wagner, P., Ray, R., Pope, R., Cheetz, R.S., 1991. Biofilms: an ESEM evaluation of artifacts introduced during SEM preparation. J. Indust. Microbiol. 8, 213–222.
Miranda, A.F., Ramkumar, N., Andriotis, C., Holtkemeier, T., Yasmin, A., Rochfort, S., Wlodkowic, D., Morrison, P., Roddick, F., Spangenberg, G., Lal, B., Subudhi, S., Mouradov, A., 2017. Applications of microalgal biofilms for wastewater treatment and bioenergy production. Biotechnol. Biofuels 10, 120.
Morales-Sanchez, D., Martinez-Rodriguez, O.A., Kyndt, J., Martinez, A., 2015. Heterotrophic growth of microalgae: metabolic aspects. World J. Microbiol. Biotechnol. 31 (1), 1–9.
Perez-Garcia, O., Escalante, F.M., de-Bashan, L.E., Bashan, Y., 2011. Heterotrophic cultures of microalgae: metabolism and potential products. Water Res. 45 (1), 11–36.
Pleissner, D., Rumpold, B.A., 2018. Utilization of organic residues using heterotrophic microalgae and insects. Waste Manage. 72, 227–239.
Ramanna, L., Guldhe, A., Rawat, I., Bux, F., 2014. The optimization of biomass and lipid yields of Chlorella sorokiniana when using wastewater supplemented with different nitrogen sources. Bioresour. Technol. 168, 127–135.
Rincon, S.M., Romero, H.M., Aframehr, W.M., Beyenal, H., 2017. Biomass production in Chlorella vulgaris biofilm cultivated under mixotrophic growth conditions. Algal Res. 26, 153–160.
Ryan, K.G., Ralph, P., McMinn, A., 2004. Acclimation of Antarctic bottom-ice algal communities to lowered salinities during melting. Polar Biol. 27 (11), 679–686.
Schnurr, P.J., Allen, D.G., 2015. Factors affecting algae biofilm growth and lipid production: a review. Renew. Sustain. Energy Rev. 52, 418–429.
Song, M., Pei, H., 2018. The growth and lipid accumulation of Scenedesmus quadricauda during batch mixotrophic/heterotrophic cultivation using xylose as a carbon source. Bioresour. Technol. 263, 525–531.
Sun, Y., Huang, Y., Liao, Q., Xia, A., Fu, Q., Zhu, X., Fu, J., 2018. Boosting Nannochloropsis oculata growth and lipid accumulation in a lab-scale open raceway pond characterized by improved light distributions employing built-in planar waveguide modules. Bioresour. Technol. 249, 880–889.
Wan, M., Liu, P., Xia, J., Rosenberg, J.N., Oyler, G.A., Betenbaugh, M.J., Nie, Z., Qiu, G., 2011. The effect of mixotrophy on microalgal growth, lipid content, and expression levels of three pathway genes in Chlorella sorokiniana. Appl. Microbiol. Biotechnol. 91, 835–844.
Wang, J., Liu, J., Liu, T., 2015. The difference in effective light penetration may explain the superiority in photosynthetic efficiency of attached cultivation over the conventional open pond for microalgae. Biotechnol. Biofuels 8, 49.
Wang, J., Liu, W., Liu, T., 2017. Biofilm based attached cultivation technology for microalgal biorefineries—A review. Bioresour. Technol. 244, 1245–1253.
Wei, C., Huang, Y., Liao, Q., Fu, Q., Xia, A., Sun, Y., 2018. The kinetics of the polyacrylic superabsorbent polymers swelling in microalgae suspension to concentrate cells density. Bioresour. Technol. 249, 713–719.
Wellburn, A.R., 1994. The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. J. Plant. Physiol. 144 (3), 307–313.
Young, E.B., Beardall, J., 2003. Rapid ammonium- and nitrate-induced perturbations to Chl a fluorescence in nitrogen-stressed Dunaliella. J. Phycol. 39, 332–342.
Zhan, J., Rong, J., Wang, Q., 2017. Mixotrophic cultivation, a preferable microalgae cultivation mode for biomass/bioenergy production, and bioremediation, advances and prospect. Int. J. Hydrogen Energy 42 (12), 8505–8517.
Zhang, Q., Li, X., Guo, D., Ye, T., Xiong, M., Zhu, L., Liu, C., Jin, S., Hu, Z., 2018. Operation of a vertical algal biofilm enhanced raceway pond for nutrient removal and microalgae-based byproducts production under different wastewater loadings. Bioresour. Technol. 253, 323–332.
Zheng, Y., Huang, Y., Liao, Q., Fu, Q., Xia, A., Zhu, X., 2017. Impact of the accumulation and adhesion of released oxygen during Scenedesmus obliquus photosynthesis on biofilm formation and growth. Bioresour. Technol. 244 (Pt 1), 198–205.