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ORIGINAL PAPER

Biofilm cultivation of the oleaginous microalgae *Pseudochlorococcum* sp.

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Abstract The cultivation of microalgae in biofilm has been a potential way to overcome the shortcoming of conventional algal culture modes of open pond and photobioreactors in liquid suspension. However, the growth characteristics and related effect factors of the biofilm are still far from being understood. In this work, oleaginous microalgae species *Pseudochlorococcum* was cultured in an attached biofilm and influential factors on the growth rate of biofilm were investigated. The results showed that *Pseudochlorococcum* sp. preferred to accumulate more biomass on hydrophilic substrata than on hydrophobic one. The photon flux density of 100 μmol m^{-2} s^{-1} was its light saturation point. The optimal inoculum density was about 3–5 g m^{-2}. The appropriate concentrations of nitrogen, phosphorus in medium and CO_2 in aerated gas were determined as 8.8, 0.22 mmol L^{-1} and 1 %, respectively.

Keywords *Pseudochlorococcum* sp. · Biofilm · Growth rate · Medium composition · Carbon dioxide concentration

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Introduction

With growing concerns on the ultimate shortage of fossil fuel and the environment deterioration due to the greenhouse effect, renewable, sustainable and carbon-neutral biofuels have recently received rising attention [1]. Of all the potential feedstock for biofuels, microalgae are believed to be the only possible feedstock that may significantly replace petroleum-based fuels due to its high productivity potential, less competition with food production and wide adaptability to growth environment when compared with other biomass feedstock options [2–4]. However, though intensive efforts have been made in the past few years on microalgae biofuels [5, 6], none of the microalgae production systems at the commercial scale have been set up due to their low efficiency and high cost of mass cultivation [4, 7]. Currently, the prevailing microalgae culturing devices are open ponds and a variety of closed photobioreactors (PBRs), both of which are based on suspension cultivation. Their most distinguishing feature is that <1 % microalgae cells are suspended in more than 99 % of water. Such a diluted biomass makes the harvesting process cost prohibitive. Another feature is the poor biomass productivity estimated to be <30 g m^{-2} day^{-1} [8, 9] at field level, which is far lower than the theoretical value of 120–150 g m^{-2} day^{-1} [10–12]. Such low biomass productivity means unsustainability of land requirements for massive production of microalgae biomass.

Recently, an improvement in microalgae biomass productivity with the technology of "attached cultivation" has been announced, in which dense algal cells were attached to artificial substratum surface to form a microalgae biofilm. Several types of microalgae such as *Scenedesmus obliquus*, *Botryococcus braunii* SAG 30.81, *Nannochloropsis* OZ-1, and *Cylindrotheca fusiformis* have been validated as being

cultured successfully in biofilm [13, 14]. Some similar cultivation methods investigated by other research groups showed potential advantages in effluent treatments, biomass accumulation, and harvest. Shi et al. [15] proposed a twin-layer system to cultivate *Chlorella vulgaris* and *Scenedesmus rubescens* for the efficient removal of nitrates, phosphates and ammonium from municipal wastewater. Mulbry et al. [16] set up a pilot-scale algal turf scrubber raceway to remove COD and BOD by using filamentous green algae grown in outdoor raceways at different loading rates of raw and anaerobically digested dairy manure effluent. Boelee et al. [17] investigated the capacity of microalgal biofilms for removing both nitrogen and phosphorous from municipal wastewater effluent. Naumann et al. [18] demonstrated the suitability of a new solid-state PBR, based on the twin-layer structure, to cultivate several species of microalgae. Zamalloa et al. [19] established an A/I stage system in which domestic wastewater was subjected to a chemical biological adsorption (A stage), followed by treatment in an innovative roof-installed parallel plate microalgae biofilm reactor for nutrient immobilization (I stage). This system could effectively decrease the concentrations of the total COD, nitrogen, and phosphorous. Johnson and Wen [20] used polystyrene foam as substrata to grow *Chlorella* sp. biofilms with dairy manure wastewater by periodically shaking the foam to be partly immersed into culture medium. A biomass density of 25.65 g m^{-2} and a biomass productivity of 2.57 g m^{-2} day^{-1} were achieved, respectively. Ozkan et al. [21] reported a carpet-like PBR for the cultivation of *B. braunii*. These studies proved that the biofilm cultivation system was long-term stable and basically contamination free and characterized by a low overall energy consumption.

Generally, the solar light intensity is several hundred to thousand μmol m^{-2} s^{-1}, which should be too high for microalgae growth by biofilm type. To avoid the photoinhibition and increased light utilization efficiency, Liu et al. [13] proposed a new strategy of solar light dilution by a bioreactor structure of multiple plates in arrayed style. Biomass growth rate of 50–80 g m^{-2} day^{-1} was obtained with the oleaginous microalga *S. obliquus*, which is 500–700 % higher than that of conventional open ponds under the same climate and light conditions. The cultivation of *B. braunii* also proved the efficiency of this bioreactor [14]. With this design, such algal film bioreactor was thought of as duplicates of single algal film units. Reasonably, the growth of microalgae film on a single substrata surface is the basis of this method and of the derived bioreactor. However, little information about algal biofilm formation and its growth characteristics has been reported so far in the literature to the best of our knowledge.

In this work, a single-layer biofilm reactor was utilized to investigate the effect of different influential factors including the type of substratum, inoculum density, light intensity, nutrient composition of the medium, and the concentration of CO_2 in aerated gas on single algal film growth. For this purpose the oleaginous microalgae species *Pseudochlorococcum* was used as a model species. It is expected that these findings would help the setup of the new cultivation method and then benefit the application of microalgae for biofuels and chemical products.

Materials and methods

Algal strain and inoculum preparation

The strain *Pseudochlorococcum* sp. used in this work was kindly provided by Prof. Qiang Hu from the Arizona State University. The inoculum was cultivated in the autotrophic nutrient medium BG11 [22].

The algal inoculum was obtained by culturing *Pseudochlorococcum* sp. in glass bubbling columns (5 cm in diameter; 58 cm in height, loading 700 mL BG11 culture medium) under light intensity of 90–100 μmol m^{-2} s^{-1} by fluorescent lamps (40 W Cool White, NVC, China), at 25 ± 1 °C for 6 days. 2 % CO_2 (v/v)-enriched compressed air was aerated at 0.2 VVM (volume: volume: min) to mix well the culture and to ensure enough carbon supply.

Photobioreactor structure and culture method

The scheme of the bioreactor is shown in Fig. 1. A glass grid plate, 25 mm in length and 10 mm in height, respectively, was used as a microalgae cultivation system. This plate was placed into a glass chamber (200 × 200 × 100 mm) covered by a glass plate. A miniport aerator pipe (Φ 10 × 16 mm, Shuwu, China) 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 40 mL min^{-1}. If not specially claimed, the concentration of enriched gas was 2 % CO_2 (v/v). Fluorescent lamps were fixed above the glass chamber to continuously provide illumination for algal growth continuously. The distance between the lamps and the top surface of the glass chamber could be adjusted to produce different values of light intensity on the top cover surface of the chamber. All the experiments were carried out at a room temperature of 25 ± 1 °C, and a light intensity of 96 ± 3 μmol m^{-2} s^{-1}, if not specially claimed, was employed.

Agar solid medium was used to provide nutrients and maintain the wettability of the algal biofilm. The preparation steps were as follows: a certain amount of agar powder (gel strength: >700.00 g cm^{-2}, Sinopharm Chemical Reagent Co., Ltd., China) was dissolved in BG11 medium by heating to form a 0.3 % agar medium solution, and then

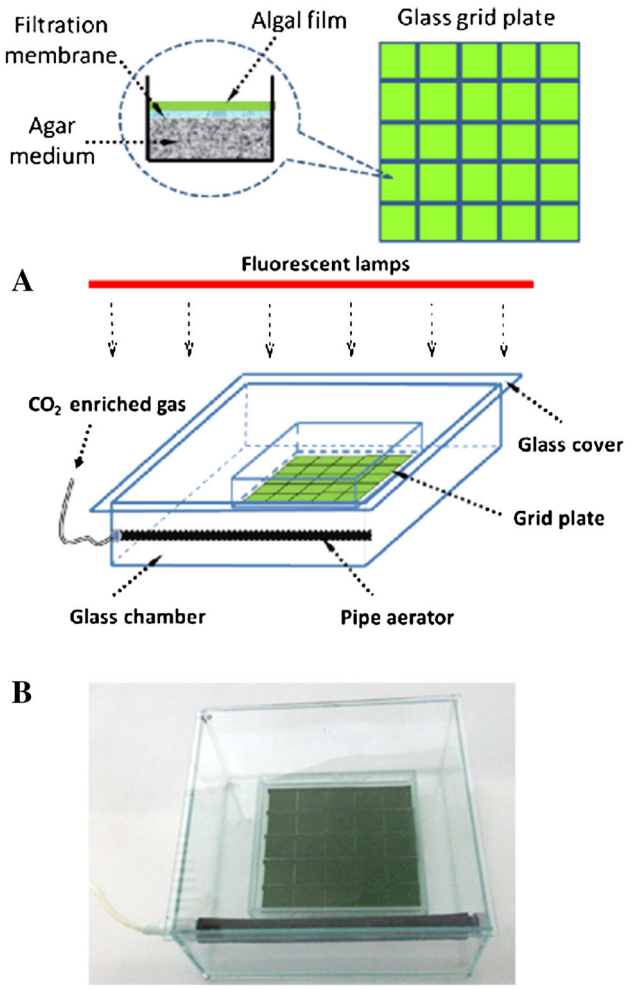

Fig. 1 The biofilm photobioreactor for algal culturing. **a** The schematic photo; **b** the entity photo in operation

the agar solution was poured into a glass grid plate and cooled at room temperature until solidification. To precisely measure the growth rate of algal biofilm, a filtration membrane (diameter 50 mm, pore size 0.45 μm) was used as substrata for algal growth. The inoculated algal biofilm was prepared as follows: a precise volume (V_0, mL) of prepared inoculum medium (the dry biomass concentration, C_0, g L^{-1}, was predetermined by the gravimetric method according to Richmond [23] was evenly vacuum-filtered onto a filtration membrane to form an algal disk with A m^2 footprint. Thus, the initial inoculum density (areal) could be calculated as $X_0 = \frac{C_0 V_0}{1{,}000} \frac{1}{A}$ (g m^{-2}). Besides the experiments to investigate the effect of initial inoculum density on growth, all the other experiments have an initial inoculum density of about 3.0 ± 0.1 g m^{-2}. The algal disk was then cut carefully to 25 × 25 mm square piece, ca. 0.000625 m^2 footprint. Afterward, the algal disk pieces were gently put on the surface of the above agar solid medium. Finally, the inoculated grid plate was inserted into a glass chamber for cultivation.

To detect the effects of hydrophilic/hydrophobic behavior of substrata on the growth of biofilm, eight types of filtration membranes of polypropylene (PP), polytetrafluoroethylene (PTFE), bonded fiberglass (BF), polyethersulfone (PES), JN 6 (Nylon), cellulose acetate (CA), cellulose nitrate (CN), and CA/CN (mixed cellulose) with the same pore size of 0.45 μm were selected from Beijing Haichengshijie Filtration Materials Ltd., China.

Analytical method

The biomass was measured by the gravimetric method. Three pieces of filtration membrane were gently sampled from the glass grid plate with tweezers and each was totally washed out with distilled water from the surface membrane substrata. All the washed solution was collected and filtered by using a pre-weighted 0.45 μm mixed cellulose microfiltration membrane (Xinya, China w_0, g). Washing was executed twice with distilled water to remove all soluble nutrients in the algal pastes. The membrane was then oven-dried overnight at 105 °C and cooled in a desiccator for weighing (w, g) using an analytical scale (XS105DU, METTLER TOLEDO, Switzerland).

The biomass concentration, expressed as biofilm areal density (X, g m^{-2}), was calculated as follows: $X = (w - w_0)/0.000625$, where 0.000625 represents the footprint of each piece of membrane substrata. The biofilm growth rate (RX g m^2 day^{-1}) was calculated as follows: $RX = (X_n - X_0)/n$, where X_n and X_0 are the biofilm areal density on the substrata sample at day n and day 0 (initial inoculation density), respectively, and n represents the time of cultivation (days).

Hydrophilic/hydrophobic behavior of filtration membranes was analyzed by its contact angle (KRUSS-DSA100, Germany).

For the sake of reproducibility, each experimental condition was investigated at least in triplicate.

Results and discussion

The effect of substratum on the growth of microalgae

The biomass areal density of algal biofilm on eight types of filtration membrane after 6 days of cultivation was plotted in Fig. 2. As seen, there are significant effects of the type of substrata on the growth algal film. All the selected membranes had smooth surface with the same pore size of 0.45 μm, but with different hydrophilic/hydrophobic behaviors. The eight membranes could be roughly classified into two types according to their contact angle: hydrophobic ones (PP, PTFE, and BF) which have larger contact angle

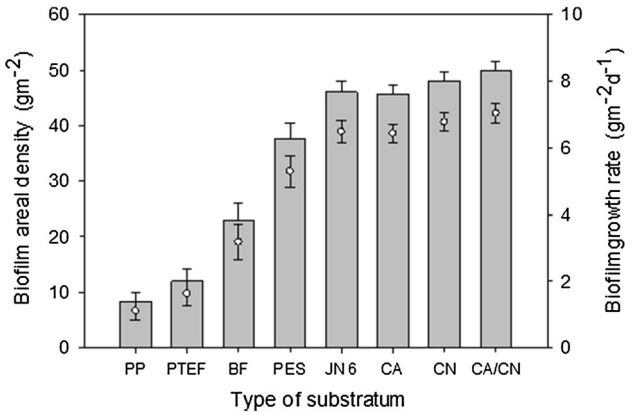

Fig. 2 Influence of the type of substratum on the growth of *Pseudochlorococcum* sp. biofilm. The algal biofilm was cultivated for 6 days under 96 ± 3 μmol m^2 s^{-1} continuous illumination at an initial inoculum density of 3.0 ± 0.1 g m^{-2}. The biofilm growth rate was the average value of the 6 days culture. The data are mean ± standard deviation of three independent experiments

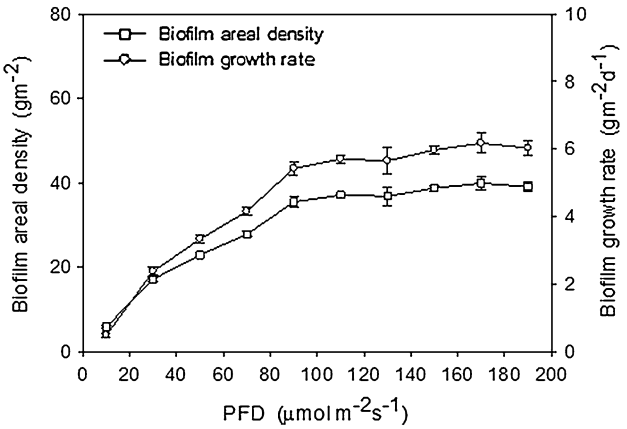

Fig. 3 Influence of light intensity on biofilm areal density and growth rate of *Pseudochlorococcum* sp. The algal biofilm was cultivated for 6 days at an initial inoculum density of 3.0 ± 0.1 g m^{-2}. The biofilm growth rate was the average value of the 6 days culture. The data are mean ± standard deviation of three independent experiments

than 90° and hydrophilic ones (PES, JN 6, CA, CN, and CA/CN) which have spread out ability in seconds. The hydrophobic substrata of PP, PTFE, and BF membrane accumulated less biomass, while the hydrophilic membranes of PES, JN 6, CA, CN, and CA/CN accumulated thicker algal biofilm. It seems that algal biofilm prefers to grow on the surface of hydrophilic substrata. However, it did not mean that the membrane with larger contact angle must have lower growth rate of algae. For example, the biomass density on PTFE and BF membranes is higher than that on PP membrane, and PES membrane accumulated less than that on CA and CN membrane. The effect of substrata on the growth of microalgae is a complicated result of the properties of substrata. Besides the hydrophilic/hydrophobic behavior, the texture of substrata, size of pores, water retention capacity, etc., would also influence the physiology and attachment behaviors of microalgae. Cui and Yuan [24] and Shen et al. [25] have demonstrated that the most influencing factor of the hydrophobic/hydrophilic property of substrata is the attachment capacity or adhering ability of microalgae. In our experiment, by vacuum filtration, all the eight membranes had smooth surface, the same pore size and the same inoculum density. Thus, the difference in the biomass density after 6 days of cultivation is possibly due to differences in water and nutrient supply, since in our work water and nutrient for algal biofilm growth transferred from solid agar medium to the surface of the substrata through membrane diffusion. Hydrophobic substrata hampered the diffusion of water nutrients.

The effect of light intensity

The relationship between light intensity and biomass areal density and biofilm growth rate was investigated with *Pseudochlorococcum* sp. as reported in Fig. 3. In the range of light intensity from 10 to 100 μmol m^{-2} s^{-1}, the growth rate almost linearly increased to about 6 g m^{-2} day^{-1} with the increase of light intensity. This growth is much greater than that in Johnson and Wen [20], Shi et al. [15] and Shen et al. [25]. Beyond 100 μmol m^{-2} s^{-1}, the biofilm growth rate was maintained to be almost the same. This value can be taken as a critical point of photosynthesis efficiency, or roughly as the light saturation point for *Pseudochlorococcum* sp. grown in biofilm. In outdoor cultivation practice, the solar light intensity is in the range from 400 to 2,000 μmol m^{-2} s^{-1}, even if this latter was considered too strong to be well handled by the photosystems and resulted in light inhibition and even photo-bleaching. Thus, the high intensity of sunlight must be "diluted" first to an appropriate level to avoid light damage. The reasonable strategies of light dilution may have two ways of expanding the illuminated surface area of the PBR per unit of land area or providing incident light in a light/dark cycle rather than in a continuous illumination mode, as described by Liu et al. [13].

The effect of inoculum density

In Fig. 4, the changes in biomass areal density and average productivity at different initial inoculum densities after 6 days of cultivation are reported. Both biomass areal density and growth rate were quickly increased when the inoculum density increased from ca. 0.05 to 3–5 g m^{-2}, and the maximum growth rate of about 6 g m^{-2} day^{-1} was obtained at 3–5 g m^{-2} inoculum density. Denser inoculum did not prompt faster growth. Actually, the proliferation of microalgae cells, which thickens the biofilm, is mostly driven by the amount of absorbed photons. The penetration

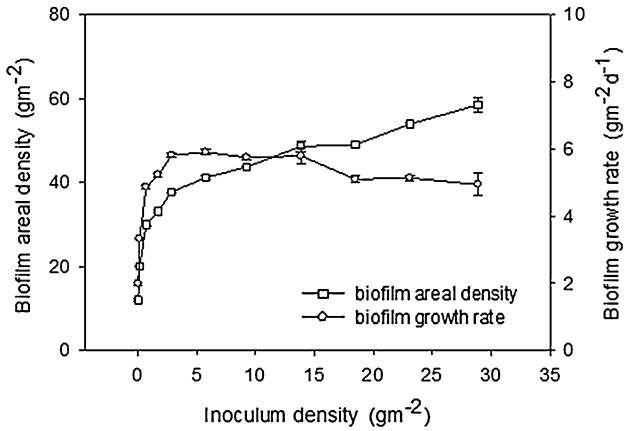

Fig. 4 Influence of initial inoculum density on the growth rate of *Pseudochlorococcum* sp. in biofilm. The algal biofilm was cultivated for 6 days under 96 ± 3 μmol m^2 s^{-1} continuous illumination. The biofilm growth rate was the average value of the 6 days culture. The data are mean ± standard deviation of three independent experiments

path of light in the dense algal biofilm is very short. Our previous work (data not published) has shown that the light penetration path of *Scenedesmus dimorphus* (the cell size is around 3–5 μm, which is roughly equal to that of *Pseudochlorococcum* sp.) biofilm under 100 μmol m^{-2} s^{-1} illumination intensity was only about 15 ± 3 μm, which corresponded to the areal density of about 4–5 g m^{-2}. This means that only the top layer of about 15 ± 3 μm (this layer is dynamically renewed with cultured time because of biofilm thickening by growth) could be illuminated for photosynthetic growth. An inoculum denser than the optimal value, for example 15–30 g m^{-2}, would reasonably result in a more pronounced effect of consumption by respiration of those algal cells below the renewed top layer, leading to a reduction of the average growth rate. On the other hand, the growth rate of algal biofilm was gradually decreased during cultivation (as shown in Fig. 5b) [13]; it may also result from the accumulated thickness of biofilm if the physiological change of algal cell during the cultivation is neglected.

The effect of nitrogen and phosphorous rate

The effect of nitrogen concentration on the growth of *Pseudochlorococcum* sp. biofilm is reported in Fig. 5a. As it can be seen, the nitrogen concentration significantly affected cell growth. When compared with the free nitrogen medium, the addition of a little amount of nitrogen, ca. 3.52 mmol L^{-1} (which corresponds to 1/5 of nitrogen content to standard BG11 medium) caused fast growth of the algal biofilm. In Fig. 5b, the relationship between the average growth rate of the biofilm at the 4th, 8th, and 12th day, respectively, is shown. It can be observed that with the

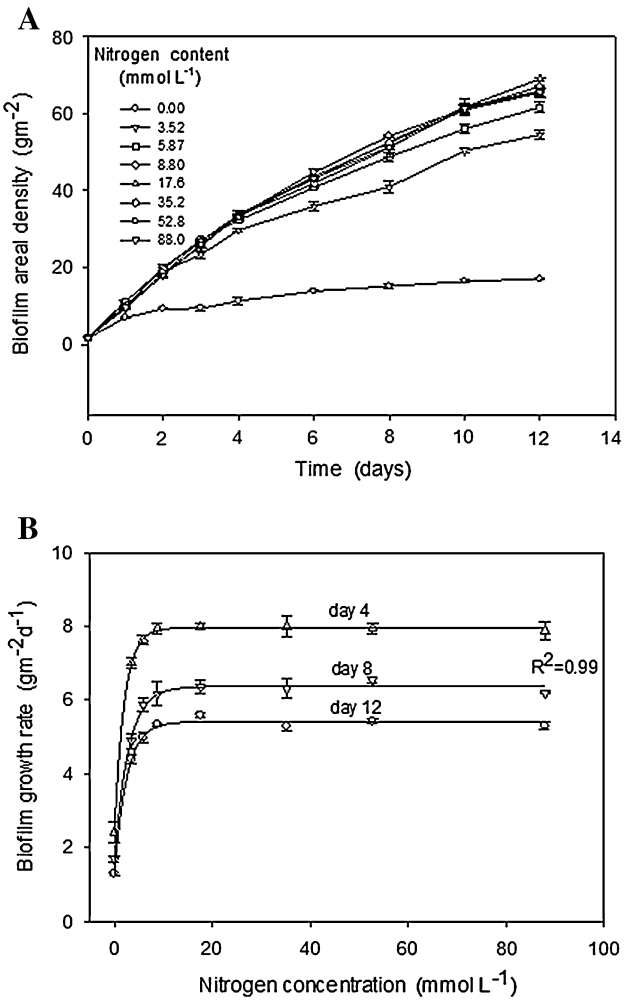

Fig. 5 Influence of nitrogen concentration of the medium on the areal density and growth rate of *Pseudochlorococcum* sp. in biofilm. **a** Biofilm areal density, **b** biofilm growth rate. The algal biofilm was cultivated under 96 ± 3 μmol m^2 s^{-1} continuous illumination at an initial inoculum density of 3.0 ± 0.1 g m^{-2}. The biofilm growth rates were the average values of the 4th day, 8th day, and 12th day, respectively. The data are mean ± standard deviation of three independent experiments

increase in nitrogen concentration, the biofilm growth rates increased steeply to about 5, 6, and 8 g m^{-2} day^{-1}, respectively, when the nitrogen concentration increased in the range from 0 to 8.8 mmol L^{-1} and then leveled off. This means that the nitrogen concentration of 8.8 mmol L^{-1} in the medium (half of the nitrogen concentration to standard BG11 medium) is a critical concentration for the best growth of the algal biofilm. This concentration is a little higher than that with *Aucutodesmus obliquus* culturing in attached biofilm style by Ji et al. [26], in which one-tenth of nitrogen concentration to standard BG11 medium was enough to maintain fast growth and lipid accumulation simultaneously.

Figure 6a shows the effect of phosphorous on the areal density and growth rate of *Pseudochlorococcum* sp. in biofilms. Similar to what was observed for the nitrogen

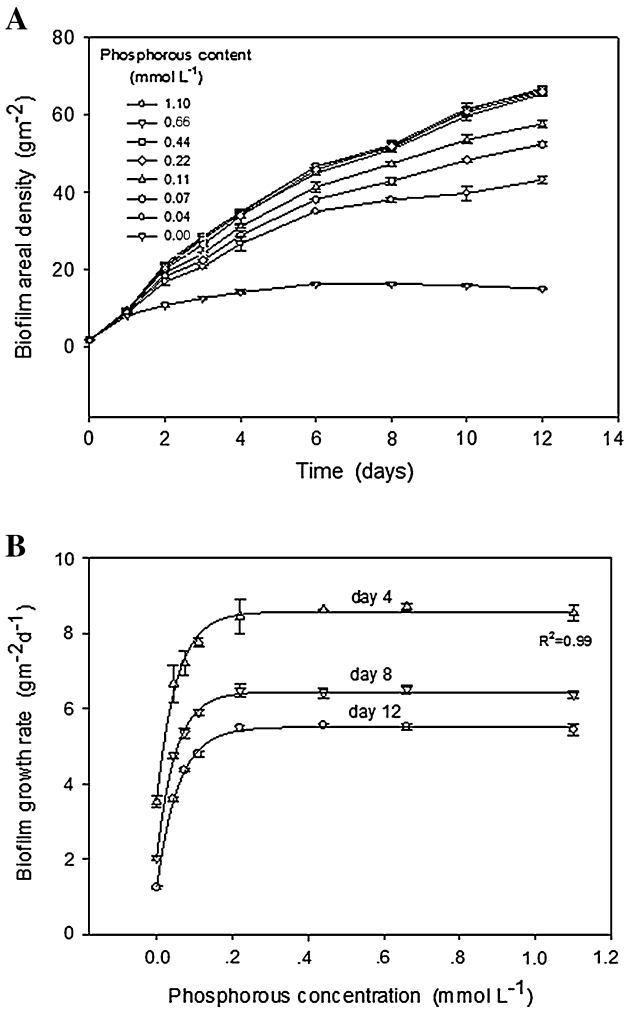

Fig. 6 Influence of phosphorous concentration of the medium on the areal density and growth rate of *Pseudochlorococcum* sp. in biofilm. **a** biofilm areal density, **b** biofilm growth rate. The algal biofilm was cultivated under 96 ± 3 μmol m^2 s^{-1} continuous illumination at an initial inoculum density of 3.0 g m^{-2}. The biofilm growth rates were the average values of the 4th day, 8th day, and 12th day, respectively. The data are mean ± standard deviation of three independent experiments

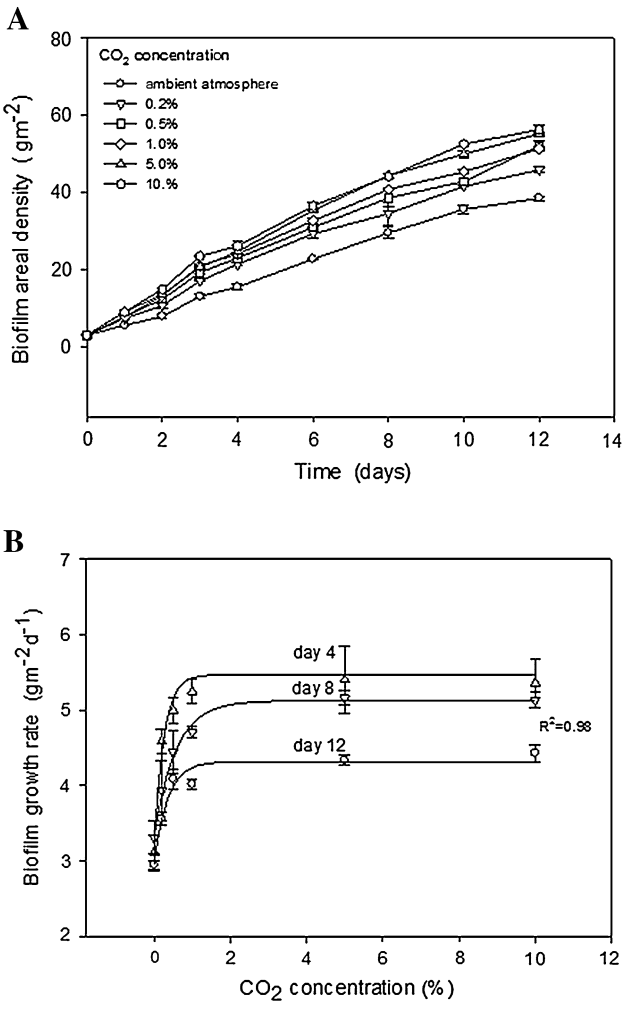

Fig. 7 Effects of carbon dioxide concentration of aerated gas on the growth of *Pseudochlorococcum* sp. in biofilm. The algal biofilm was cultivated under 96 ± 3 μmol m^2 s^{-1} continuous illumination at an initial inocula density of 3.0 ± 0.1 g m^{-2}. The data are mean ± standard deviation of three independent experiments

effect, also the phosphorous concentration of the medium significantly affected cell growth. As it can be seen, when the content of phosphorous was increased from 0 to 0.22 mmol L^{-1}, the growth rate of the biofilm steeply increased (Fig. 6b). After that value, further addition of phosphorous to the medium slightly promoted the growth rate. For this reason, a phosphorous concentration of 0.22 mmol L^{-1} could be considered as the optimal value for the cultivation of *Pseudochlorococcum* sp. in biofilm.

The effect of CO_2 on growth

Different from the conventional suspended culture of microalgae which requires dissolved inorganic carbon resource in open pond or a variety of PBRs, the algal biofilm cultivation mode provided another way of carbon supply for algal photoconversion by transfer through gas–solid contact. The effect of gas CO_2 concentration on the growth of biofilm is plotted in Fig. 7a. It shows that even when atmospheric air was used as a source of CO_2 (of concentration about 0.038 %), the algal biofilm was also able to grow well, though the growth rate was lower than that with CO_2-enriched air. When the CO_2 concentration was gradually increased, there was a gradual increase in the biomass accumulation, indicating that enriched CO_2 gas helped photosynthesis. Figure 7b demonstrates the effects of CO_2 concentration on the biofilm growth rate at different cultivation days. It can be found that there was also a critical value of CO_2 concentration, ca. 1 %. Below that, the growth of biofilm was inhibited by carbon insufficiency, and above

that there was no positive effect on the growth anymore but a lower utilization efficiency of CO_2.

The green alga *Pseudochlorococcum* sp. has the ability to accumulate both lipid and starch in cells. Its neutral lipid and starch content can, respectively, reach maximum levels of 52.2 and 12.2 % in dry weight [27]. So the harvested biomass of *Pseudochlorococcum* sp. can be utilized not only as feedstocks of biofuel, but also for generation of fermentable sugar. Compared with regular suspension cultivation, the culture condition could be more efficiently controlled in biofilm cultivation to obtain the highest productivity of lipid or starch.

Conclusions

In this work, oleaginous microalgae species *Pseudochlorococcum* was cultured in biofilm and the influential factors including the type of substratum, initial inoculum density, light intensity, nutrient composition of the medium, and the concentration of CO_2 in the aerated gas were investigated. More biomass accumulated on the hydrophilic substratum surface, and high light intensity below 100 μmol m^{-2} s^{-1} resulted in a fast growth rate. The optimal inoculum density was found to be about 3–5 g m^{-2}. Higher nitrogen and phosphorous concentrations in the medium and CO_2 concentration in the aerated gas resulted in faster growth rates of the biofilm. The appropriate values of nitrogen, phosphorous, and CO_2 content were found to be 8.8, 0.22 mmol L^{-1} and 1 %, respectively.

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