Comparison of growth, hydrocarbon accumulation and metabolites of *Botryococcus braunii* between attached cultivation and aqueous-suspension cultivation

Cheng Pengfei[1,2*], Wang Yan[3], Yang Qiyong[2], Liu Tianzhong[4]

(1. *School of Water Resources and Hydropower Engineering, Wuhan University, Wuhan 430072, China*; 2. *Poyang Lake Eco-economy Research Center, Jiujiang University, Jiujiang 332005, China*; 3. *E-business Institute, Jiujiang University, Jiujiang 332005, China*; 4. *Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao 266101, China*)

Abstract: *Botryococcus braunii* is a colonial green microalga which can produce extracellular hydrocarbons at a high rate, it is considered as one of the most promising feedstocks for sustainable biofuel production. However, *B. braunii* is generally recognized difficulties for cultivating and has limited amount of substantive scale-up and productivity assessments with conventional aqua-suspended cultivation systems (open pond and varieties of closed photobioreactors). This paper introduces a novel cultivation system based on biofilm technology, which is called "attached cultivation". To investigate the potential of attached cultivation method, attached cultivation of *B. braunii* SAG 807-1 was compared with aqueous-suspension cultivation (flat plate reactors). The growth, hydrocarbon accumulation and metabolites were studied under identical conditions (e.g. temperature, light intensity, CO_2 concentration). The main research results obtained are as follows: compared with conventional aqueous-suspension cultivation, the biomass productivity of *B. braunii* under biofilm attached cultivation was 4.78 g/(m^2·d), which was higher than of 4.43 g/(m^2·d) by aqueous-suspension, and hydrocarbon productivity of the two cultivation methods were 2.52 g/(m^2·d) and 2.37 g/(m^2·d), respectively. The contents of carbohydrate and protein were also similar. This attached cultivation method showed a new model of commercialization for the microalgae-derived biofuels.

Keywords: *Botryococcus braunii*, attached cultivation, aqueous-suspension cultivation

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1 Introduction

The finite fossil fuel depletion and rising global warming issues have strongly motivated researches on renewable bio-fuel production[1-3]. Microalgae, a group of tiny photosynthetic organisms, has been attracting much focus during the past decades due to its potentials in CO_2 mitigation and producing sustainable biofuels and the production of high value products[4-6]. Among this huge diversity of species[7], the green alga *Botryococcus braunii* is a notable one that secreting hydrocarbons under different conditions[8], which is more similar to fossil oil to be converted into oxygen-free fuels[9,10].

However, a cost effective algae cultivation technology that can be scaled up to make a significant contribution in reducing our dependence on foreign oil has not yet to be realized[4,11]. The major challenges for large scale cultivation of *B. braunii* were the slow growth rate and poor benefit in traditional open ponds and kinds

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Biographies: Wang Yan, Master, research interests: Plant ecological process, Email: xinyi_828@163.com; **Yang Qiyong**, Professor, research interests: Environmental Science and Engineering, Email: yqy46901@163.com; **Liu Tianzhong**, professor, research interests: Microalgae biotechnology, Email: liutz@qibebt.ac.cn.

***Corresponding author**: **Cheng Pengfei**, Lecturer, research interests: Microalgae biotechnology, Mailing address: School of Water Resources and Hydropower Engineering, Wuhan University, Wuhan, 430072, China. Tel/Fax: +86-07928312392; Email: pfcheng1792@163.com.

of closed photobioreactors[9,12,13]. These types of system used for cultivating algae suspended in liquid nutrient media. The technologies require in excess of water to cultivate algae oil, and energy intensive dewatering and biomass concentration processes for downstream[4,11,14,15]. Raceway ponds were constructed as artificial ponds having a depth of about 0.3 m[4]. The algae cultivated in these ponds were kept suspended through continuous agitation with a paddlewheel. However, they require energy intensive harvesting and dewatering processes necessary for downstream processing of algae in biorefinery[4]. Another difficulty associated with open systems is the loss of water through evaporation. The most common types of closed photobioreactors reported in the literature are tubular and flat panel types. However, these systems are much more expensive to build and operate than open pond systems and require large amount of auxiliary energy input for cultivation[14]. In such systems, harvesting or removal of microalgae (e.g. by flocculation) is a major cost factor, especially where a limit on suspended solids is imposed by law[16]. In addition, the succession of microalgae in these open ponds cannot be controlled well, further complicating the harvesting process. It is therefore desirable to seek ways to circumvent the removal of microalgae from suspensions.

Research on immobilized algae cultivation systems have been attracting attention as these systems, which could offer the potentials to remove the pollutants (nitrogen and phosphate, especially) in wastewater[17,18]. In pervious researches, we introduced a novel biofilm cultivation system which is called “attached cultivation”. In this system, high density of microalgae paste is attached to a supporting structure that consists of glass plate, filter paper and cellulose acetate/cellulose nitrate membrane to form an artificial “leaf” and multiple of these leaves are vertically inserted into a glass chamber. The “attached cultivation” system is highly efficient in biomass production for many oleaginous microalgae[19]. The biomass productivity and light usage efficiency of “attached cultivated” *B. braunii* are miraculous.

However, there are rarely reports that compared the growth or metabolites of *Botryococcus braunii* between attached cultivation and aqueous-suspension cultivation under identical conditions. This research compared attached cultivation of *B. braunii* SAG 807-1 with aqueous-suspension cultivation. The growth, hydrocarbon accumulation and metabolites of *B. braunii* were studied between attached cultivation and aqueous-suspension cultivation under identical conditions (e.g. temperature, light intensity and CO_2 concentration).

2 Materials and methods

2.1 Algae strain and photobioreactors

The microalgae species *B. braunii* SAG 807-1 was purchased from SAG cultivation collection, University of Göttingen, Germany, and grown in a modified Chu 13 medium[20]. The photobioreactor (PBR) used for aqueous-suspension cultivation experiments with *B. braunii* consisted of flat plate reactor (length 50 cm, width 20 cm, thickness 1 cm, volume 1 L) (Figure 1a). The area occupied by the flat plate reactor was 0.1 m^2, and this area was illuminated by light. The biofilm attached cultivation system applied in this research was similar to that of the immobilized biofilm reactor in Liu et al.[19], which was descripted in detail in Cheng et al.[21] (Figure 1b). In brief, a glass chamber including a glass plate and attached algae biofilm disks were placed on an iron rack with a certain tilt angle against the horizontal plane. The medium was propelled (1.2 L in total, ca. 10 mL/min) by a peristaltic pump (TP12DC 12 V, Guangzhou JU PlasFitting Technology Co., LTD, China) to circulate inside the system. The light intensity measured in the two cultivation bioreactors was (100±10) μmol/(m^2·s) with cold-white fluorescent lamps. Continuous airflow containing 1% CO_2 (v/v) was injected into the glass chamber with a speed of 0.1 vvm to supply carbon source and the temperature inside the glass chamber was (25±2)^oC during the two experiments. Each period of cultivation was maintained of 8 d for all the attached cultivation.

To prepare the inoculum for the two bioreactors, the algae was cultivated with glass bubbling columns (diameter = 0.05 m) for about two weeks (in the middle of exponential phase) and then harvested by centrifugation at 5000 g. Each of these columns

contained 0.7 L of algae broth and was continuously illuminated by cold white fluorescent lamps (NFL28-T5, NVC, China) with light intensity of 60 μmol/(m^2·s). The temperature for algae broth was (25±2)^{o}C during the cultivation. Air bubble that contained 1% CO_2 (v/v) was continuously injected into the bottom of the columns with a speed of 1 vvm to agitate the algae broth as well as supply carbon resource.

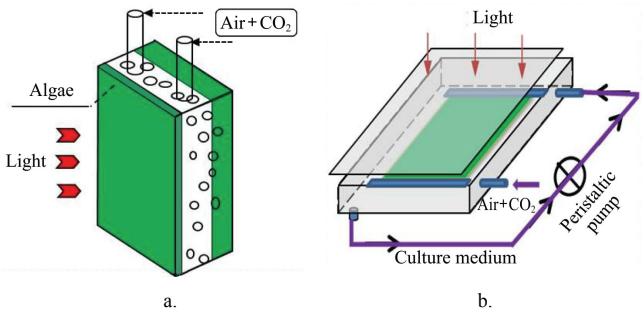

Figure 1 Schematic diagrams of aqueous-suspension and attached cultivation devices

2.2 Growth analysis

The biomass concentration of an "algae disk" (*DW*, g/m^2) was measured with gravimetric method. The cells of "algae disk" were washed down and re-suspended with de-ionized water and then filtered to pre-weighted 0.45 μm GF/C filter membrane (Whatman, England; DW_0). The membrane was oven dried at 105^{o}C for 12 h and then cooled down to room temperature to measure dry weight (DW_1). The *DW* was calculated as follows:

$$DW=(DW_1-DW_0)/0.001 \quad (1)$$

where, 0.001 represents the footprint area of the "algae disk", m^{-2}.

For the experiment with flat plate reactor, a certain volume of algae cells cultivated in aqueous-suspension medium were sampled and then filtered the pre-weighted filter membrane. The biomass density of "algae disk" was considered as identical to that of the cultivation surface.

2.3 Hydrocarbon extraction and analysis

Hydrocarbon was determined according to Sawayama et al.[22] and Cheng et al.[23]. Fifty milligrams of lyophilized cell biomass was homogenized and soaked in n-hexane. The extraction process was repeated several times till the supernatant was colorless and then combined in a pre-weighed glass vial. The crude hydrocarbon extract was dried under gentle flow of nitrogen gas (>99%). The sample of the crude hydrocarbon was then purified by column chromatography on silica gel with n-hexane as an eluent. The residual extracts were fractionated on a same column using chloroform and methanol. As a result, pure hydrocarbon, non-polar lipids and polar lipids were well isolated with reference to their elution with that of the retention times of the internal standard[20,24,25].

2.4 Fatty acid methyl ester (FAME) content analysis

The total lipid of *B. braunii* was measured according to Bligh and Dyer's method and Cheng et al.[23,26]. Then a 0.5 mg sample of purified biodiesel was dissolved in 1 mL heptane containing 50 μg heptadecanoic acid methyl ester ($C_{18}H_{37}COOCH_3$) as internal standard for FAME analysis on a Varian 450GC (Varian Inc., USA) equipped with a flame ionisation detector (FID) and Agilent HP-5 GC Capillary Column (30 m×0.25 mm ×0.25 μm). Nitrogen was used as carrier gas. The injector temperature was set at 280^{o}C with an injection volume of 2 μL under split mode (10:1). The detector temperature was set at 280^{o}C. The individual FAMEs were identified by comparing their retention time against those of authentic standards.

2.5 Carbohydrate and protein estimation

The *B. braunii* cells which cultured in the two experiments were rinsed and re-suspended with de-ionized water and then freeze-drying. A small amount of dry algae powder was added for carbohydrate and protein assay. Carbohydrates were quantified by the phenol–sulfuric acid colorimetric method of Hodge and Hofreiter[27]. Protein content was analyzed by Bradford protein assay according to Zor and Selinger[28].

All the experiments were carried out in duplicate and average values were reported. Results were performed with Excel (Microsoft Office Enterprise, 2010) and SPSS 11.5 for Windows (SPSS Inc., 2007) and analysis of variance (ANOVA) was determined wherever applicable.

3 Results and discussion

3.1 Growth of *B. braunii* with attached and aqueous-suspension cultivation

In the present experiments, the growth of *B. braunii* in flat plate and attached photobioreactors was

investigated (Figure 2). All the experiments were cultured at identical conditions. The growth of microalgae was almost no difference in the two experiments for the first two days. Afterward, the *B. braunii* growth with attached cultivation showed a rapid growth. Even though they shared the same initial inoculum concentration (6.58 g/m^2), by the end of experiment, biomass was differences between the two cultivation methods (flat panel and attached reactors), correspondingly reaching 42.03 g/m^2 and 44.73 g/m^2. The final biomass productivity of *B. braunii* with attached cultivation was 4.78 g/(m^2·d), which was higher than that with aqua-suspended cultivation in flat panel reactors, correspondingly reaching 4.43 g/(m^2·d).

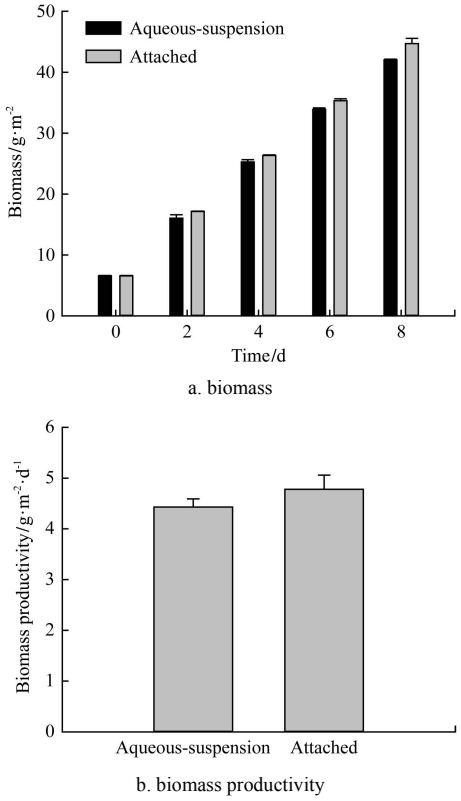

Figure 2 Growth of *B. brauni* with two cultivation methods

Most importantly, algae grew well in Chu 13 medium and remained immobilized during the experimental period without leakage of cells into the water. Microalgae self-adhere to the surface of the substrate layer and are effectively separated from the flow of medium by the microporous nature of the substrate layer. However, the biomass productivity of *B. braunii* with attached cultivation was less than that of an open pond and closed photobioreactor cultivating *Nannochloropsis*, respectively. The main reasons for the observed low areal productivity can be attributed to *B. braunii* being a notoriously slow grower with a doubling time ranging from 40 h to 6 d compared with *Nannochloropsis* sp. having a doubling time of about 29 h[29]. In addition, temperature is another critical parameter significantly affecting the biomass productivity of algae cultivating systems. The attached photobioreactors are more prone to cultivation temperature fluctuations as these systems contain significantly smaller quantities of water than aqua-suspended systems which help buffering the system temperature. Therefore, we used selective covers that are transparent in the attached reactors to keep temperature stable.

3.2 Hydrocarbon production and FAME content with two experiments

Figure 3 shows the effects of the two different cultivation methods on hydrocarbon production. The content of hydrocarbon of *B. braunii* with attached cultivation was 52.64%, which was lower than the values of algae cells in aqua-suspended systems, i.e., 53.5%. Accordingly, the hydrocarbon productivity of algae was reached 2.52 g/(m^2·d) and 2.37 g/(m^2·d), respectively. The different reactors resulted in the change of content of crude hydrocarbon and hydrocarbon productivity marginally. *B. braunii* is characterized by a conspicuous ability to synthesize and accumulate a variety of lipids. These lipid substances include numerous hydrocarbons, i.e., highly reduced compounds comprising only carbon and hydrogen as elements, which is more similar to fossil oil to be converted into oxygen-free fuels. However, hydrocarbon levels and distributions varied with algae origin. Although the effects of various cultivation parameters on hydrocarbon production is now well known, cultivation on a large scale of the best candidate species for renewable hydrocarbons have not yet proved financially worthwhile[8].

The compositional distribution of fatty acids extracted from the microalgae is shown in Table 1. In the present study, the FAME compositions of *B. braunii* cultured by the two reactors mainly consisted of C18:1 (oleic acid), C18:3 (octadecatrienoic acid methyl ester) and C16:0 (palmitic acid methyl ester). Obviously, the C18:1 of algae in attached and aqua-suspended cultivation

represented a major portion of fatty acid methyl esters compositions, accounting for a total of 52.25% and 47.28%, respectively. The portion of C18:1 in attached cultivation was higher than in flat panel reactors. However, the portion of C16:0 was lower than the values in aqua-suspended cultivation, which reached 11.32% and 17.28%, respectively. The portions of C18:0, C18:2, C18:3 of *B. braunii* in the two photobioreactors were similar with each other. The unsaturated fatty acid methyl esters were predominant in the FAME profile, while the counterpart in the two cultivation methods shared small amount of portion. Previous studies have reported that lipid content and fatty acid composition were greatly affected by culturing conditions, growth period and environmental situations, and thus it was not surprising to observe the little difference FAME profiles between the two experiments in this study[30,31].

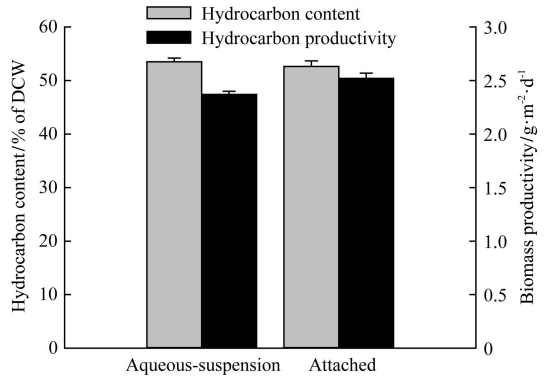

Figure 3 Hydrocarbon accumulation of *B. braunii* with the two cultivation methods

Table 1 Comparison of fatty acids composition of *B. braunii* SAG807-1 under attached and aqueous-suspension cultivation

Fatty acids composition	Relative percentage/%	
	Attached cultivation	Aqueous-suspension cultivation
16:0	11.32	17.28
16:1	2.73	2.68
16:2	0.32	0.38
16:3	2.20	2.58
18:0	2.15	3.36
18:1	52.25	47.28
18:2	6.69	6.57
18:3	15.81	14.41
19:0	2.78	2.22
20:1	0.54	0.92
20:5	2.72	1.85
21:5	0.49	0.48

Botryococcus braunii is a cosmopolitan green colonial microalga characterized by a considerable production of lipids, notably hydrocarbons. The use of different reactors resulted in the change of hydrocarbon production and FAME content with the two cultivation methods marginally. However, attached algae self-adhere to the surface of the substrate layer and were effectively separated from the flow of medium, which would reduce the cost of harvest.

3.3 Carbohydrate and protein production

The content of carbohydrate and protein on different days with flat panel and attached reactors are shown in Figure 4. The carbohydrate content of algae cells cultured by attached reactors was similar with those obtained in aqua-suspended systems, respectively reaching 13.5% and 12.8% (Figure 4a). Carbohydrates are major energy resources that could be directly consumed by the algae cells. The change style of carbohydrates can be served as an indicative of how the organism adapted to the environment. Casadevall et al.[32] reported that *B. braunii* of A race can produce considerable amounts of carbohydrate. However, the amount of carbohydrate production varies with the strains, the race, physiological conditions as well as culture conditions[9]. In this research, *B. braunii* SAG 807-1 (A race) produced carbohydrate content of biomass was little different between the two cultivation methods. It was worth noting that *B. braunii* is capable of synthesizing exopolysaccharides, which slowly dissolved in the culture medium and caused an increase in viscosity, as was first reported for the A race[32].

Also the protein content of *B.braunii* with attached cultivation as much as that of cultivating in flat panel reactor, where the values were 8.6% and 7.6%, respectively (Figure 4b). In general, the algae rich in protein have been used for various applications such as biofertilizer, soil conditioner, and as feed for animals on land and in aquaculture. In this research, the construction and performance of photobioreactor had little influence on biosynthesis of carbohydrate and protein with *B. braunii*.

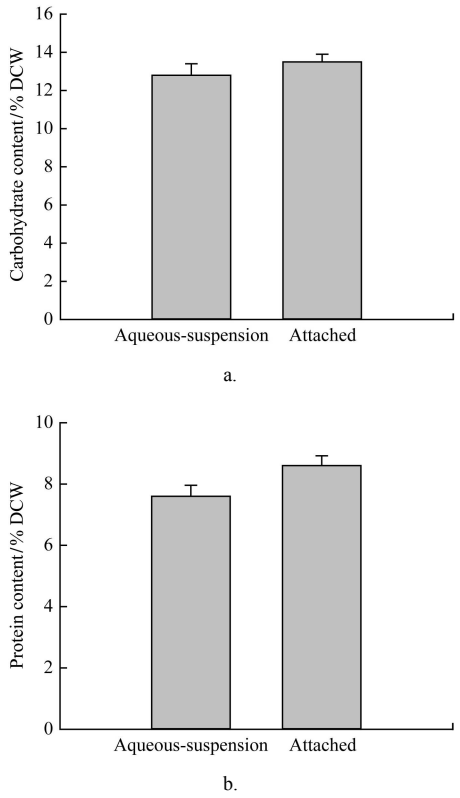

Figure 4 Carbohydrate and protein production of *B. braunii* with the two cultivation methods

3.4 Potential of attached cultivation of microalgae

The most common types of flat panel reactors could address some of the limitations which have been reported for raceway ponds, e.g. evaporation losses of water. However, flat panel reactors need use gas flow for mixing within the system to ensure high mass transfer and much cost to build and operate. The construction and performance of attached photobioreactor offers a significant reduction of the energy and water requirements of cultivation. Ji et al.[33] reported that biofilm method is the huge water saving potential due to the separation of aqua-medium with the algae biomass. In other words, the biomass concentration in these conventional open ponds and closed photobioreactors demand for and handling over 99% water with less than 1% solids coupled with low biomass productivity makes microalgae mass cultivation for biofuels neither economically viable nor sustainable from both energy and water supply standpoints[34,35]. Finally, the biofilm photobioreactor required 4.71 MJ of energy per kg dry biomass produced. This corresponds to a net energy ratio (NER) of 6.01. The energy requirements of raceway ponds and flat plates which are 9.18 and 16.96 MJ/kg, respectively, corresponding to a NER of 3.44 and 1.86 respectively, based on 24 h of daily pumping[4,14]. It should be noted that a biomass productivity of 0.7 g/(m^2·d) attained with the attached system, the net energy output is equal to that from an open pond with a productivity of 9.3 g/(m^2·d), indicating the importance of biomass harvest concentrations[36]. However, algae attached photobioreactors are not mature technologies but hold potential to be further developed for achieving energy and water efficient algae cultivation targeted for biofuel production and recovery of nutrients from wastewater.

4 Conclusions

This study compared the growth or metabolites of *Botryococcus braunii* between attached cultivation and aqueous-suspension cultivation. Due to the low productivity, high costs and poor efficient light transport for traditional algae aqua-suspended cultivation systems, *B. braunii* generally had many difficulties for cultivating and limited amount of substantive scale-up and productivity assessments, and the industrial biofuel production of microalgae was faltering and formidable. This research based on attached cultivation, proved the feasibility in biomass production for *B. braunii*. Biomass production and hydrocarbon productivity of algae with attached cultivation were higher than the values in aqueous-suspension cultivation systems. Microalgae with attached cultivation holds potential to be further developed for wastewater treatment and biofuel production.

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