Growth rate assessment of high lipid producing microalga *Botryococcus braunii* in different culture media

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Abstract

The green colonial microalga, *Botryococcus braunii* is well known for its high lipid content and has already been proposed as a renewable energy source for various aquaculture and biotechnological applications. However, due to its slow growth rate compared with other microalgae, *B. braunii* has not yet been used in mass culture to produce more biomass. Therefore, in this study we tested different culture media to enhance the growth rate of *B. braunii*. *B. braunii* samples were collected from natural habitat, isolated and purified by repeated streaking on agar plate. The purified samples were cultured in six treatments containing different culture media with incubation conditions of pH 7.5, temperature 25±1°C under 1.2±0.2 klux light intensity with 12 hr photoperiod to observe their growth rate and morphology. The results of this study showed the highest growth rate (μ=0.20 day^{-1}) in the autoclaved lake water with 427 colonies/ml. Bold's basal medium (BBM) and modified Chu No. 10 medium showed moderate growth after 24 days of incubation. The growth rates amongst all media were significantly different (p<0.05) except between un-autoclaved lake water and autoclaved water medium added with half concentration of BBM. Morphological observation showed that *B. braunii* varied in different culture media. This study illustrated that autoclaved lake water has the highest growth rate compared to formulated media such as BBM and modified Chu No. 10 medium. This indicates that the autoclaved lake water medium had high concentration of nutrients and can be used for the mass production of *B. braunii* at lower cost.

Keywords: *Botryococcus braunii*, Chlorophyceae, Specific growth rate, Microalgae, Culture medium

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Introduction

Microalgae are a group of tiny photosynthetic organisms, which have attracted much attention during the past decades due to their potential applications in aquaculture, biofuel, nutraceuticals, pharmaceuticals, cosmetics and food industries (Chisti, 2007; Mata *et al*., 2010; Sostaric *et al*., 2012). Microalgae play an important role as food for aquaculture animals and also for improvement of water quality in aquaculture systems (Khatoon *et al*., 2007). Several studies have demonstrated that many species of microalgae such as *B. braunii*, *Dunaliella primolecta*, *Chlorella* sp., *Crypthecodinium cohnii*, and *Nannochloropsis* sp., synthesize large amounts of hydrocarbons and lipids. Among these, *B. braunii* is one of the species capable of synthesizing high quantity of hydrocarbons relative to its biomass, as well as synthesizing other compounds such as polysaccharides and carotenoids with aquaculture benefits (Chisti, 2007; Hu *et al*., 2008; Niehaus *et al*., 2011).

B. braunii is a freshwater green colonial microalga (Chlorophyceae), commonly found in freshwater and brackish water lakes, reservoirs, ponds, or even ephemeral lakes situated in continental, temperate, alpine, or tropical zones (Huang *et al*., 1999; Yusoff *et al*., 2002; Volova *et al*., 2003). *B. braunii* is able to produce high level hydrocarbons in the range of 15-80% of the dry cell weight that is much higher than the other microalgae (Metzger *et al*., 1985; Sawayama *et al*., 1994; Banerjee *et al*., 2002; Powell and Hill, 2009). It is a notable microalga species that secretes hydrocarbons under different conditions (Metzger and Largeau, 2005), which is more similar to fossil oil to be converted into oxygen-free fuels (Banerjee *et al*., 2002). A few recent studies have reported that *B. braunii* has the capacity to bio-mitigate atmospheric CO_2 of up to 200-1300 mg/L/day (Chiu *et al*., 2008; Sydney *et al*., 2010; Rosenberg *et al*., 2011; Zhu *et al*., 2013).

According to aquatic species program (ASP) report (Sheehan *et al*., 1998), *B. braunii* is a relatively slow growing microalga compared to other species; doubling only each 72 hours (divide once per three days). Due to this factor, *B. braunii* has not yet been commercially used in mass cultures to produce biomass (Cepak and Lukavsky, 1994; Banerjee *et al*., 2002; Metzger and Largeau, 2005). There was a limited research conducted to enhance the growth rate of *B. braunii* by using different culture media, culture conditions and nutrients concentrations (Yin *et al*., 2008; Zhang *et al*., 2011; Rao *et al*., 2012; Yoshimura *et al*., 2013). The growth rate and hydrocarbon production in *B. braunii* is influenced by nitrogen limitation, NaF and bacterial populations (Wang and Xie, 1996; Zhila *et al*., 2005). Yonezawa *et al*. (2012) observed the enhancement in the growth rate and hydrocarbon production of *B. braunii* strain BOT-22 with the application of 1% and 2% soybean curd wastewater to the AF-6 medium. However, in this investigation we examined the growth rate of indigenous *B. braunii* strain by using different growth media to attain economically feasible media for its mass production.

Materials and methods

Chemicals and culture media

All chemicals used were purchased from Sigma-Aldrich and Merck laboratories. Six types of media were used in this experiment

including 1. BBM, 2. Modified Chu No.10 medium, 3.Soil-water medium, 4. Autoclaved lake water medium, 5. Un-autoclaved lake water medium, 6. Autoclaved lake water added with half concentration of BBM and 7. lake water sediment medium. The Lake water was bubbled for 10 days to remove COD and filtered through 0.45 µ glass fibre filter (GFF) before using as culture media. Soil-water medium was adopted from the method proposed by Pringsheim (1946). Lake water sediment medium was the interstitial water extracted from the Putrajaya Lake sediment (Yusoff *et al*., 2001).

Sampling, isolation and purification

The samples of *B. braunii* were collected from Putrajaya Lake, Malaysia (2.9419° N, 101.6891° E), using a plankton net with mesh size of 35 µm (Fig. 1). Collected *B. braunii* were initially left to float free in bottles filled with lake water assisted with aeration to keep them suspended in the laboratory. The presence of *B. braunii* colonies in the water samples were observed under microscope, picked up with pasture pipette and washed five times with sterile modified Chu No. 10 medium. The isolated colonies were purified by repeated streaking on agar plates (Ashokkumar and Rengasamy, 2012; Boonma *et al*., 2014). The purity of the cultures was ensured by regular observation under microscope.

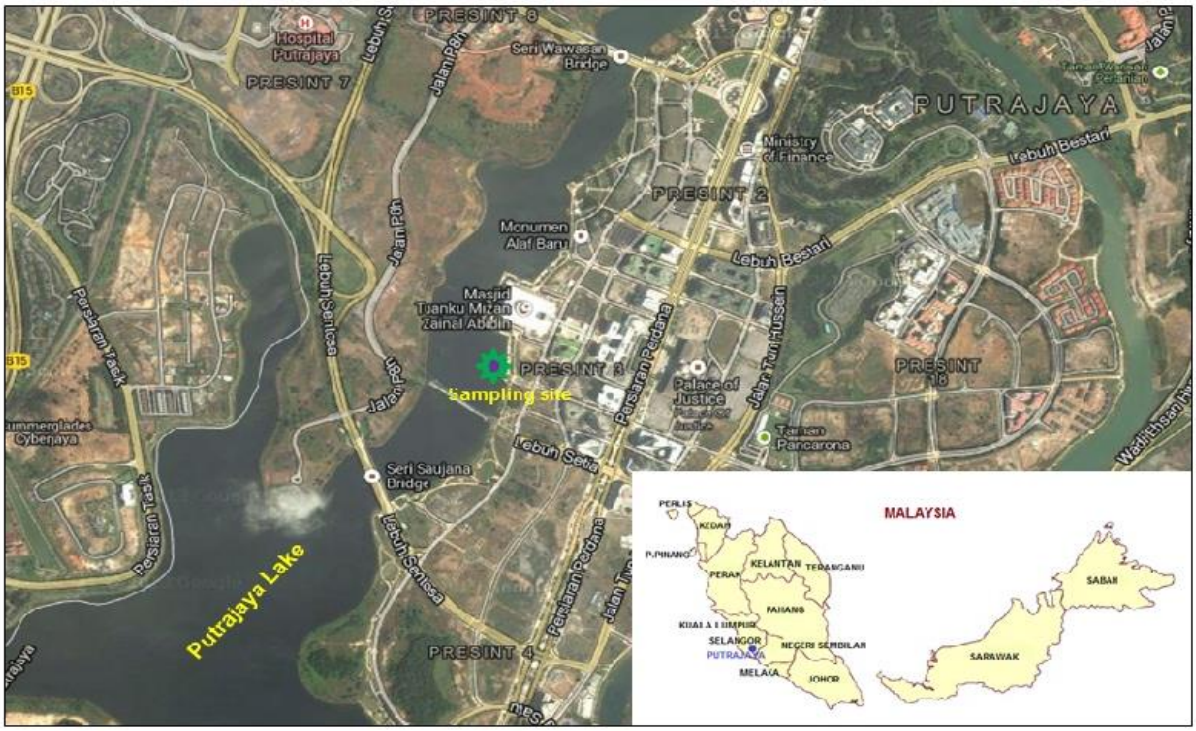

Figure 1: Location of sampling site at Putrajaya Lake (2.9419° N, 101.6891° E), Putrajaya, Malaysia.

Assessment of growth rate on different media

For the growth experiments, 400 ml of each media was inoculated with 10 colonies of *B. braunii* and incubated in orbital shaker at 25±1°C under 1.2±0.2 klux light intensity with 12:12h light and dark cycle. The growth and morphology of the cultures were monitored everyday under the microscope for 24 days. All experiments

were done in triplicates. Colony counting was done every day under microscope by pipetting 10 ml of culture media into the petri dish and pictures were recorded. Cell counting in *B. braunii* was difficult due to its complex colonial organization. Therefore, the growth rate assessment was performed by measuring the optical density at 680 nm. The specific growth rate was determined using the following formula (Guillard, 1973):

$$\mu\ (d^{-1}) = \frac{\ln (F_1 / F_0)}{t_1 - t_0}$$

where, F_1 = Biomass at time of harvest (t_1) and F_0 = Biomass at times zero (t_0).

Statistical analysis

The data for all the experiments were analyzed by univariate of general linear model and one-way ANOVA in SPSS. Multiple pair wise comparisons were carried out by using Tukey HSD test to observe statistical differences between different culture media. Graphical results were constructed using Microsoft Excel.

Results

Isolation, purification and morphology

B.braunii blooms were quite common in Putrajaya Lake during most of the year. Water samples containing *B. braunii* blooms were collected from presint-3 of Putrajaya Lake. Microscopic observation of collected water samples showed varied microbial diversity containing majority of *B. braunii*. Observations under dissecting and compound microscope showed the colonies of purified *B. braunii* as large colonial complex (104.79 - 237.73 × 79.50 – 176.15 µm). Each large colony is formed by the interconnection of small colonies (43 –61.75×30–45µm) with hyaline mucilaginous like structure. Each colony contains about 100 individual pear shaped cells (10 – 30 × 5 – 10 µm) organized into the smaller clusters and embedded in a hydrocarbon made extra cellular matrix (h-ECM). Colonies appeared in green and yellow brown lumps depending on the medium and age of the cells (Fig. 2).

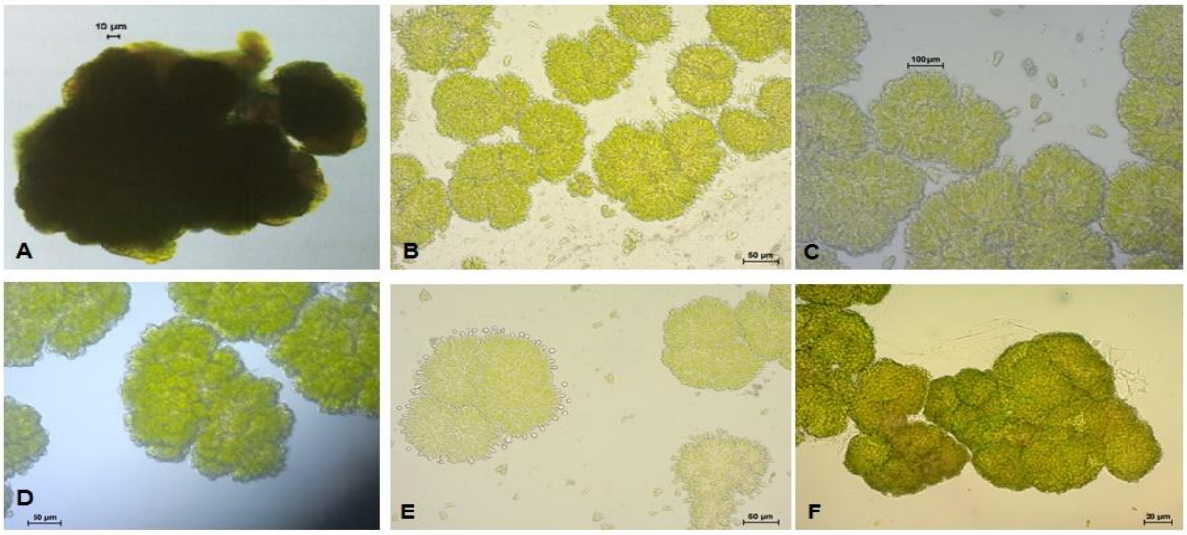

Figure 2: Morphological appearance of *Botryococcus braunii* colonies grown in different culture medium: A. Bold's basal medium (BBM), B. Modified Chu No. 10 medium, C. Soil-water medium, D. Autoclaved lake water medium, E. Un-autoclaved Lake water medium and F. Lake water sediment medium.

Growth assessment in different culture media

Among the six types of culture media used, autoclaved lake water medium showed stable and enhanced growth rate (μ=0.20 day^{-1}) throughout the period of observation. The growth rate of *B. braunii* in un-autoclaved lake water medium showed progressive increment with 0.24 μ day^{-1} from the second day onwards until the 20th day and a decrease in the number of colonies was observed afterwards (Fig. 3). BBM showed the positive progression of the growth (μ= 0.24 d^{-1}) from the 8th day of culture until the 24th day and the stationary period was observed between the 12th and 18th day of culture. In modified Chu No. 10 medium, the growth started within 12 days and gradually increased on the following days. There was no significant lag phase observed. Autoclaved lake water added with half concentration of BBM did not show proper growth. In this medium growth was detected on 8th day and the highest growth was observed on 12th day with 133 colonies.

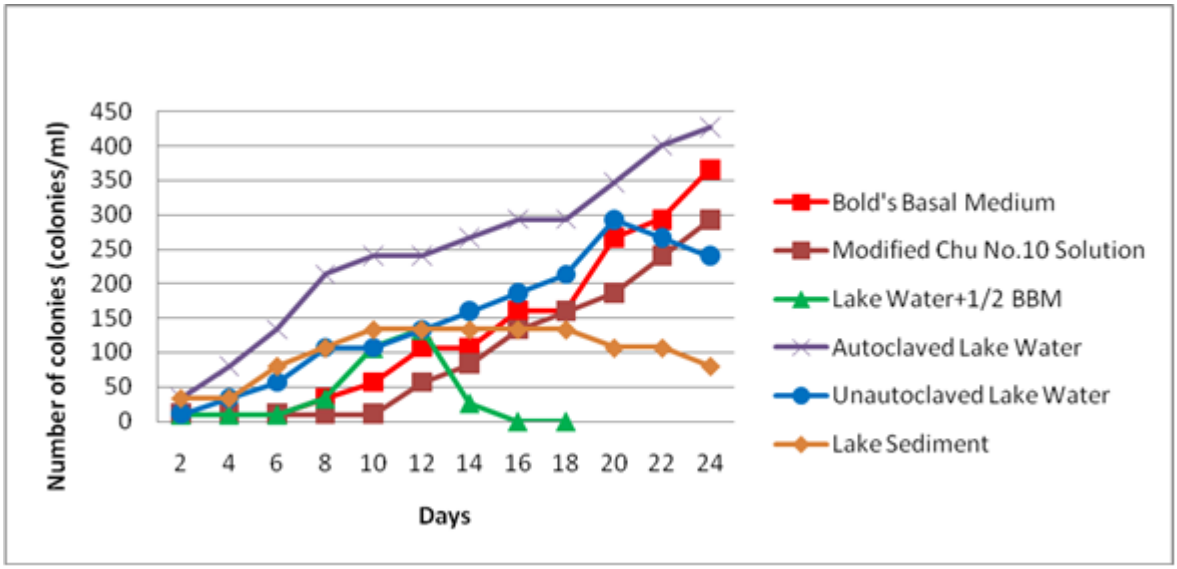

Figure 3: Comparison of the growth of *Botryococcus braunii* in six different culture media.

Mean growth differences were significant (p<0.05) between all media except between BBM and CHU, BBM and lake sediment and also between BBM and un-autoclaved lake water media. CHU No. 10 medium also showed insignificant difference with lake water sediment media (p>0.05). From post hoc test, it was demonstrated that *B. braunii* exhibited significantly higher growth rate in autoclaved lake water than the lake water mixed with BBM at half concentration. The latter media showed an early crash on day 12, most probably due to the competition between *B. braunii* with other microorganisms that might have grown in the unsterilized lake water after several days of incubation, whereas the autoclaved lake water showed continuous growth. In terms of specific growth rate significant difference (p<0.05) was observed between each media except between un-autoclaved lake water media and autoclaved lake water added with half concentration of BBM (Fig. 4).

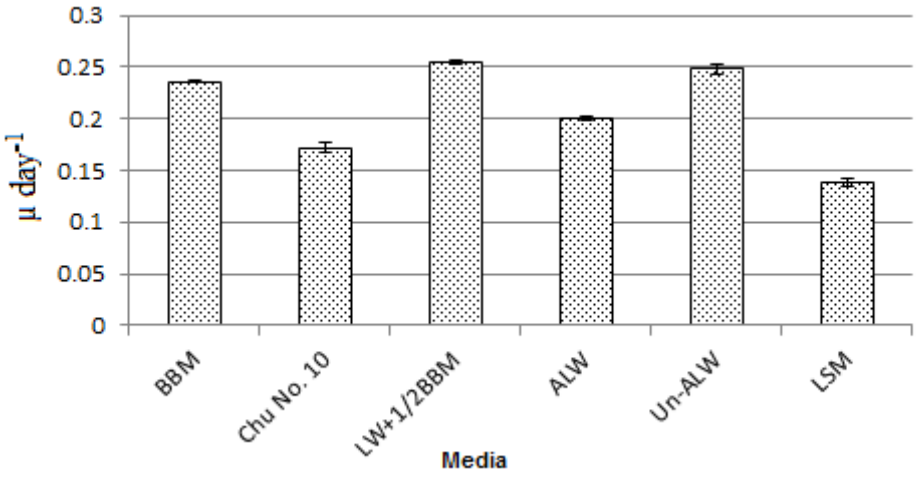

Figure 4: Specific growth rate of *Botryococcus braunii* in different culture media. Data are reported as mean ± standard error of triplicates from one-way ANOVA.

Discussion

Putrajaya Lake is a manmade lake of about 650 ha and the main purpose for the development of this lake is for natural cooling of the city and also for fishing, water sports and recreation. The pH of the lake is 6.99 with a mean temperature of 28.32°C at the surface; and slightly lowers pH (6.22) and temperature (28.23°C) in the bottom layer. In this work, *B. braunii* was cultivated for 24 days for growth assessment experiment. Similarly Dayananda *et al*. (2005), Velichkova *et al*. (2012) and Dayananda *et al*. (2007) studied the growth of *B. braunii* in different autotrophic media and concluded that the BG11 as the best medium for the growth and hydrocarbon production. In a recent study, Tanoi *et al*. (2011) studied the effect of different carbon source on growth and morphology of *B. braunii*. The growth of *B. braunii* is also influenced by a number of biotic and abiotic factors such as interactions with bacterial biofilms (Rivas *et al*., 2010), salinity, temperature, CO_2 levels and irradiation conditions (Rao *et al*., 2007; Yoshimura *et al*., 2013). Baba *et al*. (2012) studied the effect of monochromatic light on growth, photosynthesis, and hydrocarbon production of *B. braunii* Bot – 144 (Race B) and determined that the growth was higher in order of red, blue, and green light.

In the present work, the colonies of *B. braunii* appeared in different color and morphology (Fig. 2). This variation mainly depends on the culture media and age of the cells (Prescott, 1978; Metzger and Largeau, 2005). Individual cells could scarcely be seen and size of the cells ranged between 10 µm to 30 µm. Komarek and Marvan (1992) classified the genus *Botryococcus* into 13 species based on morphological differences. As shown in Fig. 3 and 4, autoclaved lake water was the best media for the growth of *B. braunii* with specific growth rate of 0.20±0.002 μd^{-1}. The growth of *B. braunii* in water sediment media showed the growth resembling the standard growth curve of most microalgae (Fig. 3). Similar studies were conducted on the growth rate of *B. braunii* under different temperatures, salinity levels and light intensities (Li and Qin, 2005; Qin and Li, 2006). There were

also a few recent studies that showed enhanced growth rate and hydrocarbon production in *B. braunii* strain under different cultivation methods and culture conditions (Zhang *et al*., 2011; Ashokkumar and Rengasamy, 2012; Rao *et al*., 2012). Autoclaving the lake water actually might cause some chemical changes that alters the chemical constituents of the lake water. These chemical alterations may enhance the growth of *B. braunii*. Based on our observations, autoclaved lake water medium was the best medium for *B. braunii* and showed the highest number of colonies compared to other media. Therefore, autoclaved lake water medium can be used for the mass production of *B. braunii* for various aquacultural and biotechnological purposes.

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