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Isolation and characterization of *Botryococcus braunii* from a freshwater environment in Tenggarong, Kutai Kartanegara, Indonesia

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Abstract. Nugroho RA, Subagyono DJN, Arung ET. 2020. Isolation and characterization of *Botryococcus braunii* from a freshwater environment in Tenggarong, Kutai Kartanegara, Indonesia. *Biodiversitas* 21: 2331-2336. The use of microalgae as an alternative source of oil has gained much attention in recent years. The present study was conducted to isolate and characterize microalgae *Botryococcus braunii* from a freshwater environment, located in Tenggarong, Kutai Kartanegara, East Kalimantan, Indonesia. *B. braunii* were cultured and determined their growth in terms of biomass yield and the presence of lipid globules by red Nile staining. Dry biomass, phosphate, and protein content of cultured *B. braunii* were measured at 3 days of intervals for 24 days. Biomass yield, hydrocarbon, carotenoid, and chlorophyll content were determined at the pH range of 6, 6.5, 7, 7.5, and 8 and various CO₂ concentrations. The result showed that *B. braunii* collected from freshwater in Tenggarong can produce lipid globules. The highest growth of *B. braunii* was reached at day 15, while the optimal phosphate content was on the 12th day, and the protein was relatively stable during culture. The optimal weight of *B. braunii* biomass was reached at pH 7 and 4% CO₂ and the highest hydrocarbon content was obtained at pH 8 and 4% CO₂. The highest chlorophyll and carotenoid contents were obtained 4% CO₂. In conclusion, *B. braunii* collected from Tenggarong was successfully cultured and able to produce lipid globules. The optimal conditions for its growth and production of hydrocarbon, phosphate, protein, chlorophyll, and carotenoid were at pH of 7-8 and 4% CO₂.

Keywords: *Botryococcus braunii*, growth, hydrocarbon, lipid droplets

INTRODUCTION

The need for renewable energy resources, particularly biofuel, has become a significant concern in the last few decades. This demand has been primarily driven by population growth and industrialization, both of which depend on fossil fuels (Medipally et al. 2015; Shuba and Kifle 2018). However, fossil-based fuel sources are continually declining, so it becomes a big problem for most countries (Mercure et al. 2018). As an alternative energy source, the biomass of organisms such as microalgae, namely *Botryococcus braunii* is a potential renewable source for biofuel (Chaudry et al. 2018; Sadeghin et al. 2018; Tsutsumi et al. 2018).

Botryococcus braunii is a microalga with a pyriform shape and is green plankton, with a cell size of 80-139 nm. It can be found in freshwater environments, brackish lakes, reservoirs, and ponds (Tasić et al. 2016). It can produce lipids up to 65%, contain high amounts of hydrocarbons and other types of lipids (Banerjee et al. 2002; Metzger and Largeau 2005; Tasić et al. 2016). High lipid producing microalgae has the potential to substitute fossil-based energy sources by processing their biomass into biofuels (Nurhatika et al. 2018). Lipid content in microalgae can be detected using a simple and rapid method of Nile red dye staining. Nile red contains a dye that fluoresces mainly in non-polar environments, lipophilic and emits intense

fluorescence in hydrophobic organic solvents. Nile red stain can contact lipid bodies, therefore it has the potential for lipid staining and quantification (Kimura et al. 2004; Huang et al. 2009; Bertozzini et al. 2011; Storms et al. 2014).

The growth and production of lipids in *B. braunii* are affected by several factors, such as nutrition, temperature, salinity, pH, CO₂, DO and turbidity (Al-Hothaly et al. 2016; Jin et al. 2016; Concha et al. 2018; Cheng et al. 2019). Optimum growth conditions result in producing a high amount of biomass, hydrocarbons, chlorophyll, phosphates, proteins carotenoids (Cepák and Přibyl 2018; Uchida et al. 2018; Wan et al. 2019). Previous research has determined that 2% (v/v) CO₂ increased the growth performance of *B. braunii* (Ruangsomboon et al. 2017), enhanced chlorophyll content (Uquiche et al. 2016), produced more hydrocarbon and led to a two-fold increase of carotenoid content (Ranga Rao et al. 2007). According to Jin et al. (2016), pH is an important factor for biomass and hydrocarbon production in *B. braunii* cultures. The optimal pH for hydrocarbons production in *B. braunii* is 6.5 while pH 6.0 affected biomass production.

Our preliminary observation conducted by author also showed that the freshwater environment in Tenggarong, Kutai Kartanegara, East Kalimantan supported the growth of *B. braunii*. However, the isolation and characterization of *B. braunii* have not been done yet. The present research aimed to isolate and characterize the growth of *B. braunii*

from the freshwater environment at Tenggarong, Kutai Kartanegara, East Kalimantan, Indonesia.

MATERIALS AND METHODS

Sampling site of *Botryococcus braunii* sampling

Microalga was collected and isolated from the freshwater environment in Tenggarong, Kutai Kartanegara District, East Kalimantan Province, Indonesia (0°25'38.35" S; 117°00'23.21" E) (Figure 1). Samples were collected using a 100 μm mesh size plankton net. Individual colonies of *Botryococcus braunii* from the freshwater sample were subjected to purification by serial dilution. *B. braunii* OIT 413 strain from the Osaka Institute of Technology, Osaka, Japan was used as a reference. Individual colonies of *B. braunii* collected from freshwater in Tenggarong were isolated and inoculated into AF 6 liquid medium (Watanabe et al. 2000; Watanabe 2005). *B. braunii* inoculum was incubated under 12-hrs light: 12 hrs dark conditions (12L: 12D) photoperiod and 1.2 ± 0.2 klux light intensity at 25-27°C. Pure colonies/culture of *B. braunii* were obtained from repeated inoculation and regularly observed under the light microscope.

Morphological profile and Nile red assay

The obtained colonies of *B. braunii* wild type were observed for their morphological profile using a light microscope (Primo Star, Carl Zeiss Microscopy GmbH, Germany). The potential oil production of *B. braunii* was detected by a simple staining method (Nile red). Nile red dye (9-(diethylamino) benzo[a]phenoxazin-5(5H)-one) was obtained from Sigma-Aldrich Pty. Ltd. (Singapore). A few

drops of *B. braunii* pure culture were added to a drop of Nile red solution. The mixture was allowed to stand for one minute at room temperature. The development of fluorescence of the stained mixture was observed using a fluorescence microscope (Nikon Corporation, Japan).

Growth of *Botryococcus braunii*

Botryococcus braunii collected from freshwater was cultured in 150 mL Erlenmeyer flasks in triplicate. Forty mL of AF 6 medium was inoculated with *B. braunii* at 20% (v/v), then incubated with 12L: 12D photoperiod and light intensity of 1.2 ± 0.2 klux at 25-27°C. Cultures were harvested at 3 days of intervals for 24 days, and dry biomass was calculated until day 24th. The dry biomass weight was determined as follows: the harvested of *B. braunii* culture was centrifuged at 5000 rpm, and then pellets were washed using distilled water and freeze-dried. The dried pellets were weighed and evaluated gravimetrically. The growth of *B. braunii* was expressed in terms of dry weight. Besides dry biomass, phosphate and protein content were also determined.

Estimation of phosphate and protein content

Phosphate content in the harvested *B. braunii* culture was measured as follows: The harvested *B. braunii* was centrifuge (Beckman Allegra X-22R, Beckman Coulter, USA) at a speed of 8000 rpm. The supernatant was collected, and the phosphate content was analyzed using Fiske-Subbarao's method (Fiske and Subbarow 1925). *B. braunii* protein content was determined with a Protein Assay Kit (Invitrogen, Q33211 from Thermo Fisher Scientific) and detected using a Qubit™ 4 fluorometer (Thermo Fisher Scientific, USA).

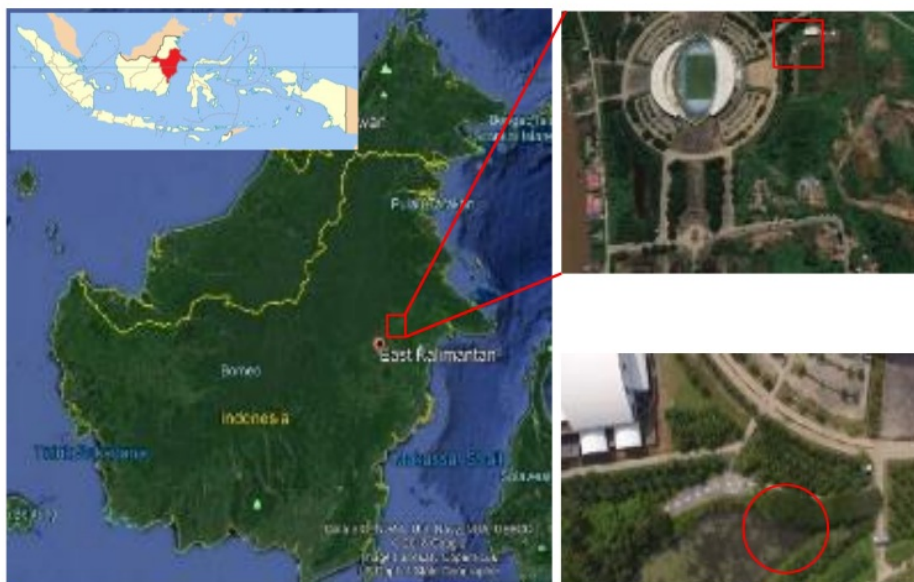


Figure 1. Sampling site (0°25'38.35" S; 117°00'23.21" E) of wild type *Botryococcus braunii* from the freshwater environment, Tenggarong, Kutai Kartanegara, East Kalimantan, Indonesia

The effect of pH on biomass and hydrocarbon content

The biomass and hydrocarbon content of *B. braunii* cultures were measured at the pH range of 6, 6.5, 7, 7.5, and 8. Forty mL of AF6 medium in the Erlenmeyer flask (150 mL) and the pH of culture medium was adjusted before sterilization. *B. braunii* was inoculated at 25% (v/v) and incubated for three weeks under a 12L: 12D photoperiod, 1.2 ± 0.2 klux light intensity, and 26-28 °C. After three weeks, the culture was harvested, and biomass and hydrocarbon content were analyzed

The effect of carbon dioxide on biomass weight, hydrocarbon, chlorophyll-a, and carotenoid content

Two-tier Erlenmeyer flasks were used for photoautotrophic growth experiments. Two-week-old *B. braunii* culture was inoculated at 25% (v/v) in the upper chamber, while the lower chamber was filled with 100 mL of carbonate (3M) and bicarbonate (3M) mixture solutions. To obtain 0.5, 1.0, 2, and 4 % (v/v) partial pressures of CO₂, the mixture of carbonate (3M) and bicarbonate (3M) solutions were established as per Tripathi et al. (2001). The Erlenmeyer flask was tightly sealed using a cotton plug and aluminum foil. The culture was incubated for three weeks under 12L: 12D photoperiods, 1.2 ± 0.2 klux light intensity, and 26-28 °C. The assays were performed in triplicate. At the end of the incubation period, the culture was harvested, and biomass, hydrocarbon, carotenoid, and chlorophyll yields were measured.

Chlorophyll content

Chlorophyll content was determined spectrophotometrically using a Jenway 6305 UV/VIS spectrophotometer at 750 nm, 664 nm, 647 nm, and 630 nm. Before analysis, the *B. braunii* culture was extracted using a centrifuge at 4000 rpm for 10 min. To obtain a turbidity-corrected value, the absorbance at 750 nm was subtracted from three different wavelengths, with 90% acetone being used as a blank. Chlorophyll a content was calculated following the equation provided by Johan et al. (2014).

$$\text{Chl-a } (\mu\text{g/L}) = (11.85 \times E_{664}) - (1.54 \times E_{647}) - (0.08 \times E_{630}) \quad (1)$$

$$\text{Concentration of Chl-a } (\text{mg/L}) = [\text{Chl-a} \times v] / V \times L \quad (2)$$

Where:

v : Volume of acetone 90% (L)

V : Volume of water sample (L)

L : Lightpath of cuvette (cm)

E₆₆₄ : Value of absorbance at wavelength 664 nm

E₆₄₇ : Value of absorbance at wavelength 647 nm

E₆₃₀ : Value of absorbance at wavelength 630 nm

Carotenoid content

Carotenoid content was determined according to the methods of Lichtenthaler (1987). Respectively, a known volume of *B. braunii* culture was centrifuged at 8000 rpm for 10 min. The resulting pellet was added to a known volume of methanol and kept in a water bath at 60°C for 30 min to extract the carotenoid, the absorbance of carotenoid was measured at 652 and 665 nm.

Hydrocarbon extraction and analysis

Dry biomass of *B. braunii* was extracted using hexane. The dry biomass in hexane was centrifuged at 5000 rpm for 10 min and the recovered supernatant was evaporated to dryness. Hydrocarbon content was measured gravimetrically and expressed as a dry weight percentage (Dayananda et al. 2005, 2006).

RESULTS AND DISCUSSION

Morphological profile

Isolation and characterization of potential renewable sources for sustainable energy production are very important to be carried out. For several years, there have been various attempts to find oil-producing microalga. Oil production from microalga is still far from economically feasible for the public market, however, its significant potential needs to be studied and developed. The result of this study showed that *B. braunii* collected from a freshwater environment in Tenggarong, Kutai Kartanegara, East Kalimantan, Indonesia showed the presence of oil lipid globules in *B. braunii* (Figure 2).

In Figure 2, the Nile red dye reacted with lipid globules, results in emitting either orange or yellow fluorescence, whereas the chlorophyll in *B. braunii* cells emits red autofluorescence. Nile red staining has been used in several previous studies to monitor lipid globules under different physiological conditions in a number of different microorganisms, such as oleaginous yeasts (Rostron et al. 2015), oleaginous fungus *Penicillium citrinum* (Bardhan et al. 2019) and various microalgal species belonging to Bacillariophyceae (Natunen et al. 2017) and Chlorophyceae classes (Kamalanathan et al. 2018).

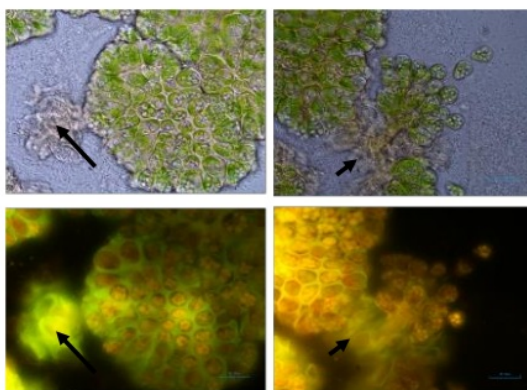


Figure 2. The colony of *Botryococcus braunii* before (upper) and after (lower) being stained with Nile red dye. Note: → : lipid globule

Nile red staining is a simple and rapid alternative method that has frequently been used to identify or quantify lipid bodies in various organisms, including microalgae. Nile red contains a dye that fluoresces preferentially in non-polar environments and is a lipophilic dye that emits intense fluorescence in hydrophobic organic solvents. This dye can contact lipid bodies, making a natural candidate for lipid staining and quantification (Kimura et al. 2004; Huang et al. 2009; Bertozzini et al. 2011; Storms et al. 2014).

Growth of *Botryococcus braunii*

The growth curve of *B. braunii* which was isolated from a freshwater environment in Tenggarong, Kutai Kartanegara, East Kalimantan, Indonesia was presented in Figure 3. A single colony of *B. braunii* was cultured in AF6 medium and sub-cultured every two weeks. Figure 3 showed that the growth of *B. braunii* showed an incremental increase up to day 2 under 12L: 12D photoperiods, 1.2 ± 0.2 klux light intensity, and 26-28 °C. Previous research also noted successful isolation of *B. braunii* from several freshwater environments (Szóke-Nagy et al. 2015; Prasertsin and Peerapornpisal 2018; Xu et al. 2018), and the optimal biomass dry weight of *B. braunii* was on day 15 using BG11 medium at an ambient temperature of 28 °C with a light intensity of $65 \mu\text{mol m}^{-2}\text{s}^{-1}$ (Xu et al. 2018).

Phosphate content in culture media decreased on days 12-15, indicating that phosphate utilization is required for *B. braunii* growth, whereas the level of protein slightly increased after day 15 (Figure 4). This finding is following the previous study that increasing protein content is due to cellular lysis (Casadevall et al. 1985). Phosphate was used at the beginning of the exponential growth of *B. braunii*, followed by an increase in the level of phosphate at the final stages of culture. *B. braunii* has a lower concentration of phosphorus compared to many other algae on an organic basis (Tsukahara and Sawayama 2005).

Effects of pH on growth and hydrocarbon content

Botryococcus braunii growth is affected by several factors, such as light, nutrients, CO₂, temperature, water, and pH (Ruangsomboon 2012; Onalo et al. 2014; Gani et al. 2015). *B. braunii* has a specific feature concerning its growth-related hydrocarbon requirements (Kojima and Zhang 1999). The results showed that pH 7 in culture media produced optimal biomass dry weight but lower hydrocarbon content. In contrast, the highest hydrocarbon content was at pH 8 but reduced biomass dry weight (Figure 5). These findings are consistent with the results of Jin et al. (2016) that optimal hydrocarbon content was reached at pH 6.5, whereas pH 6.0-8.0 had less effect on the biomass dry weight.

Botryococcus braunii is known as a long-chain hydrocarbon producer. There are several long-chain hydrocarbons produced by *B. braunii*, including C₂₇ diene, C₂₇ triene, C₃₀ botryococcene, squalene, tetramethyl squalene, and trs,rs-lycopadiene (Metzger and Largeau 2005). According to Largeau et al. (1980), *B. braunii* accumulates hydrocarbon in different locations, mainly in

cytoplasmic inclusions (internal) and outer walls or derived globules (external). Besides, hydrocarbons with shorter chains can be found in a higher degree in the internal pool. Almost 95% of hydrocarbons are also found in the external pool, whereas a normal level of about 0.75% is located within the cells.

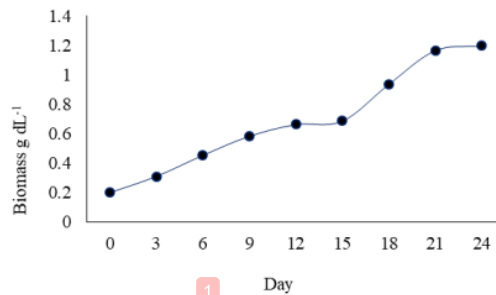


Figure 3. Growth curve of *Botryococcus braunii* isolated from the freshwater environment in Tenggarong, Kutai Kartanegara, East Kalimantan, Indonesia

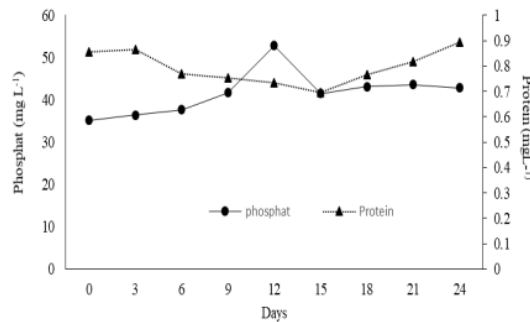


Figure 4. Phosphate concentration and protein content in *Botryococcus braunii* culture

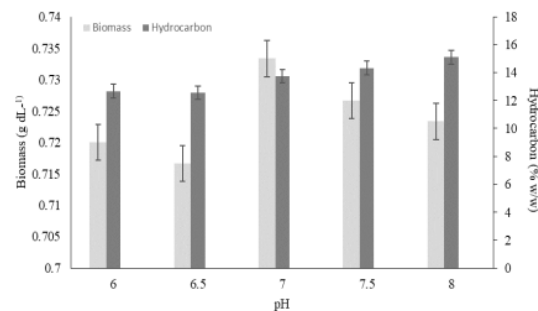


Figure 5. Effect of pH on biomass weight and hydrocarbon production of *Botryococcus braunii*

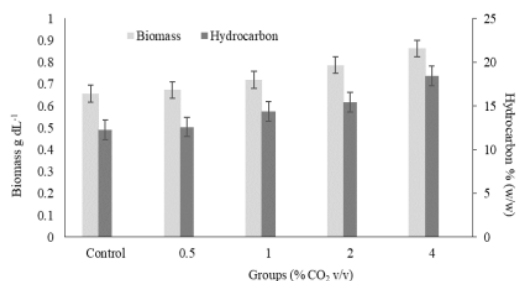


Figure 6. Effect of CO₂ on biomass yields and hydrocarbon production of *Botryococcus braunii*

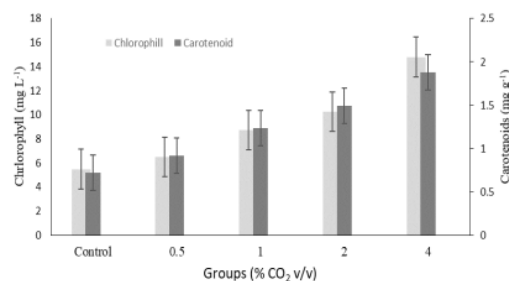


Figure 7. Effect of CO₂ on chlorophyll-a and carotenoid content of *Botryococcus braunii*

Besides pH, CO₂ concentration is also considered to be an important factor in *B. braunii* culture. Based on the current findings, a 4% CO₂ concentration is suitable for improving *B. braunii* biomass yield, hydrocarbon, chlorophyll-a, and carotenoid production (Figures 6 and 7). This finding is in agreement with previous research by Yoshimura et al. (2013), that CO₂ concentration of 0.2 - 5% without salinity was suitable conditions for *B. Braunii* growth. This condition is positively correlated with hydrocarbon production. Biomass dry weight of *B. braunii* was also correlated with chlorophyll-a production during cultivation; hence, chlorophyll-a is a useful parameter for monitoring *B. braunii* biomass growth. Hifney and Abdel-Basset (2014) revealed that the addition of 5 mM bicarbonate in combination with 6.4 mM nitrate increased the accumulation of carotenoid in *B. braunii*, which was positively affected by successive increases in sodium bicarbonate concentrations.

To conclude, *B. braunii* was successfully isolated from a freshwater environment in Tenggarong, Kutai Kartanegara, East Kalimantan, Indonesia, and cultured. *B. braunii* culture has optimum growth at pH 7-8 and 4% CO₂ to produce a high yield of dry biomass, hydrocarbon, chlorophyll, and carotenoid. *B. braunii* may also serve as a potential renewable raw material for oil or lipid production. Further research is required to evaluate the strain type and hydrocarbon composition of this particular *B. braunii* isolate.

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