Kawamura et al., J Ecosys Ecograph 2020, 10:3

Journal of Ecosystem and Ecography

Review Article Open Access

The Oil-Producing Microalga *Botryococcus Braunii*: A Method for Isolation from the Natural Environment and Perspectives on the Role of Ecological Studies in Algal Biofuel Production

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Received: July 21, 2020; **Accepted:** August 04, 2020; **Published:** August 10, 2020

Abstract

The oil-producing microalgae *Botryococcus braunii* accumulates petroleum-like hydrocarbons in large quantities that can be processed into renewable biofuels such as biodiesel. Algal biofuel is expected to become a next-generation, carbon-neutral bioenergy source, as microalgal oils are made from carbon dioxide fixed by photosynthesis, but industrial-scale algal biofuel production has not yet been achieved. Algal biofuel research tends to be laboratory-based, such as for bioreactor optimization and the genetic engineering of algae, but ecological research conducted in the field, such as investigating wild algae ecology in natural environments, promises breakthrough potential in algal biofuel studies. This paper aims to introduce a simple method for isolating *B. braunii* from natural environments and our recent work on a DNA-based method for the detection of *B. braunii* in natural environments, and to discuss how ecological studies may contribute to the development of the algal biofuel production.

Keywords: *Botryococcus braunii*; Biofuel; Phytoplankton; Microalgae; Renewable energy

Introduction

Oil-producing microalgae accumulate oil compounds in large quantities that can be processed into renewable biofuels such as biodiesel [1]. Algal biofuel is expected to become a next-generation, carbon-neutral bioenergy source, as microalgal oils are made from carbon dioxide fixed by photosynthesis. Algal-biofuel research started in the 1970's, provoked by the shock of the oil crisis, and was reactivated in the early 2000s in response to global warming [2]. Large, government-financed research projects, such as the Aquatic Species Program (ASP) in the United States [3] and the New Sunshine project in Japan [4], aimed to develop renewable biofuels from algae, but industrial-scale algal biofuel production has not yet been achieved.

Botryococcus braunii has a few outstanding characteristics compared to the other oil-producing microalgae. This algae accumulates petroleum-like hydrocarbons up to 70% of its dry weight [5]. This hydrocarbon, called *botryococcene*, has higher energetic values than the neutral lipids constituting the major oil components of most microalgae [6]. *Botryococcene* can be converted into gasoline and diesel by a simple catalytic hydrocracking [7]. Geological research suggests that B. braunii is one of the organisms that produced fossil fuels such as petroleum and oil shales [8-10]. Furthermore, *B. braunii* accumulates its hydrocarbon oil in the intra-colony matrix outside the cell wall, which may enable 'milking' the hydrocarbon without killing the algae [11]. Despite these remarkable characteristics, *B. braunii* has a weak point of slow growth (typical doubling time is 3-4 days), which makes outdoor mass-cultivation of this algae difficult to control, due to contamination by faster-growing species [12].

Algal biofuel research tends to be laboratory-based, such as for bioreactor optimization and the genetic engineering of algae, but ecological research conducted in the field, such as investigating wild algae ecology in natural environments, promises breakthrough potential in algal biofuel studies. In the study of *B. braunii*, field-based studies on (i) wild genetic resources, (ii) population ecology, and (iii) life history of the species may be expected to contribute to the realization of algal biofuel production as follows:

(i) As noted earlier, *B. braunii* has a weak point of slow growth. The fastest growth rate in the species was a 1.4 day doubling time recorded for a strain called Showa [13]. The Showa strain is a wild strain collected in U.S. at 1980s [14] and was not selected by screening nor modified genetically. Therefore, there is a high possibility of finding novel wild strains faster than Showa, but few studies have investigated the wild genetic resources of this species. Although the wild population of *B. braunii* can be found in a range of freshwater environments, the population density is generally low, and exploiting wild genetic resources is not simple.

(ii) In contrast, there are several reports on natural blooms of *B. braunii* [15-17]. The ecological mechanisms of how this slow-growing microalgae comes to dominate are completely unknown. There might be some bacterium coexisting with *B. braunii* that helps them to dominate in natural environments [18]; or *B.braunii* might produce some chemicals that inhibit the growth of other microalgae. If one can elucidate the ecological mechanisms of natural *B. braunii* blooms, that knowledge will be very helpful for developing outdoor mass-cultivation of this algae [19].

(iii) To the best of our knowledge, there has been no report on sexual reproduction nor dormancy of *B. braunii*, but many microalgae are known to change their morphology and to perform sexual reproduction and/or become dormant in response to environmental stimuli such as nutrient deficiency and darkness [20]. We have recently identified a set of meiosis-specific genes in the genome of the Showa strain [21], implying that *B. braunii* is capable of sexual reproduction, but have failed to induce sexual reproduction in the laboratory. Monitoring and observing wild populations may offer a chance to detect sexual reproduction and dormancy in natural environments. If we can induce sexual reproduction of *B. braunii*, cross breeding, as has been done in crop plants, should dramatically improve productivity.

This paper aims to introduce a simple method for isolating *B. braunii* from natural environments and our recent work [22] on a DNA-based method for the detection of *B. braunii* in natural environments, and to discuss how these methods contribute to tackle the above-mentioned research goals (i)–(iii).

Materials and Methods

Isolation of wild strains

Botryococcus braunii inhabits a range of water environments from brackish to fresh lakes and rivers, but their population density is normally low. Therefore, sampling must effectively concentrate *B. braunii* in the water. We developed a 8-step isolation protocol (Figure 1).

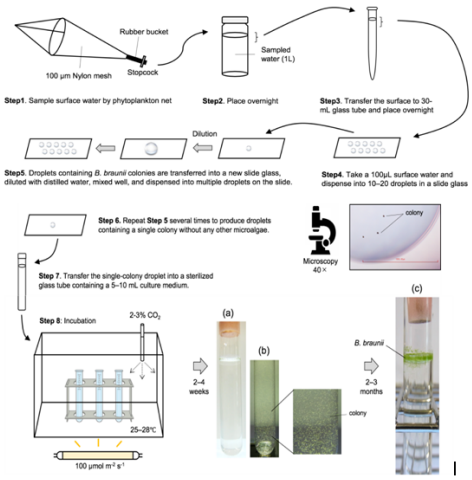

Figure 1: Original protocol for isolation of *Botryococcus braunii* from natural environments. (a) A picture of glass tube one month after isolation. Color of media did not change, and colonies are almost invisible. (b) The same glass tube illuminated from beneath by a LED of smart phone: Green colonies are turned to be visible. (c) After a few months, aggregated colonies make a green band in the surface of glass tube.

Step 1: Due to their colony-forming characteristics and high hydrocarbon content, *B. braunii* is expected to float up to the water surface with a 100–300 μm colony size. We therefore sample surface water using a phytoplankton net with 100 μm mesh size. A relatively large mesh size is effective for concentrating *B. braunii* colonies by passing the abundant single-cell phytoplankton.

Step 2: The water sample of 0.5–1 L, gathered by phytoplankton net, is left in a bottle overnight so that the *B. braunii* colonies can float up and aggregate in a surface layer.

Step 3: The surface water of the bottle is transferred to a 30 mL glass tube and left overnight, which makes *B. braunii* concentrate further still due to the narrow surface area within the glass tube.

Step 4: A 50–100 μL sample is taken by micropipette from the surface of the water in the glass tube, dispensed into 10–20 droplets in a slide glass, and observed by microscopy with a 4X objective lens (total magnification 40X) without a cover glass. *B. braunii* colonies can be seen as black pieces. Once a *B. braunii* colony is found, it can be isolated by further dilution.

Step 5: Droplets containing *B. braunii* colonies are transferred into a new slide glass, diluted with distilled water, mixed well, and dispensed into multiple droplets on the slide.

Step 6: Each droplet including a target colony is transferred to a new slide glass, and Step 5 is repeated several times to produce droplets containing a single colony without any other microalgae or zooplankton.

Step 7: Each single-colony droplet is transferred into a sterilized glass tube containing a 5–10 mL culture medium. We use AF-6 medium [23], but in some cases, pond water (filter-sterilized) is better than artificial medium for growth after isolation.

Step 8: The glass tube containing an isolated *B. braunii* colony is incubated at 25–28℃ with 12 h illumination per day at 100 μmol m^{-2} s^{-1}, and 2%–3% CO_2 (which, if available, will be much better for microalgal growth than normal air). The glass tube is capped loosely to permit air exchange. After 2–4 weeks, multiplied colonies can be seen by illuminating from beneath. After 2–3 months, aggregated *B. braunii* colonies can form a green layer, visible to the naked eye, on the top of the culture medium.

Sampling locations

We sampled pond water by the above-mentioned method in order to isolate wild *B. braunii*.

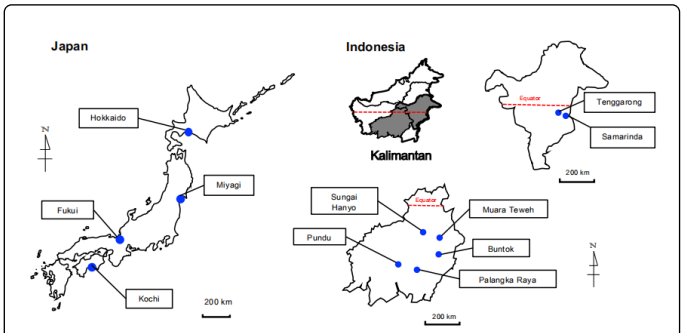

Figure 2: Samping location in Japan and Indonesia for isolation of wild *Botryococcus braunii*.

A total 57 ponds in lowland area in Japan (temperate climate) and in Indonesia (tropics) were sampled from 2016 to 2018 (Figure 2). pH and electric conductivity (EC) were measured at the time of sampling, and the abundance of *B. braunii* was scored by the number of colonies (N) found in a 100 μL sample at Step 4: (Abundance score,

Criterion)=(***, N ≥ 10), (**, 1 ≤ N<10), (*, 0<N<1), (/, No colonies found in total 500 μL).

No.	Location	Sampling Date	pH	EC (mS/m)	*Botryococcus braunii* †	
					Abundance score	Isolation
No.6	Miyagi, Japan	Summer (July-August) 2016	7	No data	/	
No.7			8		/	
No.8			7.4		/	
No.9			8.7		***	8
No.10			7.7		/	
No.12	Fukui, Japan		7		/	
No.13			6.4		/	
No.14			6.8		/	
No.15			6.9		/	
No.16			6.7		***	8
No.34	Kochi, Japan	Spring (March) 2017	8		*	5
No.35			7.1		/	
No.36			6.8		/	
No.37			7.1		/	
No.57	Hokkaido, Japan	Spring (March) 2018	8.2	21.5	/	
No.58			7.6	8.5	/	
No.59			7.4	8.7	/	
No.60			6.9	0.6	/	
No.61			7.4	42.2	/	
No.62			7.3	44.8	/	
No.63			7.4	63	/	
No.64			7.3	39.6	/	
No.65			7.5	27.2	/	
No.17	Palangka Raya, Indonesia	Rainy Season (February-March) 2017	4	4.3	/	
No.18			4.1	5.9	/	
No.22			5	3	***	32
No.23			4.1	3.8	/	
No.24			6.1	0.8	/	
No.26			4.8	2.6	/	
No.27			7.2	2.2	/	
No.28	Pundu, Indonesia		7.3	3.5	**	12
No.29			6.9	3.2	***	18
No.30			6.6	3.1	/	

No.31	Bunto, Indonesia		6.5	3.8	*	4
No.32			5.8	4.5	/	
No.38	Muara Teweh, Indonesia	Dry season (August-September) 2017	6.6	3.3	/	
No.39			6.7	2.9	/	
No.40			6.2	8.5	/	
No.41			6.7	7.2	/	
No.42			6.5	3.2	*	16
No.43			6.5	16	/	
No.44			6.4	62.6	/	
No.45	Plangka Raya, Indonesia		6	4.9	**	2
No.46			5.9	3.6	/	
No.49	Sungai Hanyo, Indonesia		5.9	0.7	/	
No.50			6	0.5	/	
No.51			5.8	1.6	/	
No.72	Tenggarong, Indonesia	Dry season (September) 2018	7.6	32	/	
No.73			7.7	0.5	/	
No.74			7.7	14.5	***	18
No.75			7.5	17.4	**	8
No.76	Samarinda, Indonesia		9.4	26	/	
No.77			8.5	19.3	/	
No.78			8.1	18.5	/	
No.79			9.9	20	/	
No.80			8.9	9.8	/	
No.81			5.2	6.5	/	
Key :†, Abundance score (See text for definition) and number of isolated strains were shown.						

Table 1: List of sampling ponds and resuIts of identification of *Botryococcus braunii* populations.

Results and Discussion

In temperate parts of Japan, we sampled water in a total of 23 ponds in Shikoku, Honshū, and Hokkaidō, finding *B. braunii* population in three ponds (Table 1). All these ponds are artificial water reserves, in which the water looks green, indicating eutrophication (Figure 3).

Both in summer and early spring, we found *B. braunii*, but seasonal changes in *B. braunii* abundance in natural environments have not been studied yet. In tropical Indonesia, we sampled water in 34 ponds in central and East Kalimantan and found 8 populations (Table 1).

The pH of the ponds inhabited by *B. braunii* were above 5 and tended to be green. We did not find any *B. braunii* in brown-coloured ponds in peat swamps, which show high acidity (pH <5) probably due to large amounts of humic acids. The EC of the *B. braunii*-inhabited ponds ranged from 3–17.4 mS/m. We did not find any *B. braunii* in oligotrophic ponds with low EC levels (<3 mS/m) nor in salty marshes with high EC (>20 mS/m).

We have hypothesized that *B. braunii* populations can be more easily found in the dry season than in the rainy season because of their concentration due to lesser precipitation. Contrary to the hypothesis, *B. braunii* was found in 33% of ponds (4 ponds per 12) in the rainy season, but in only 18% (4 ponds per 22) in the dry season, although the studied sites were not the same between seasons.

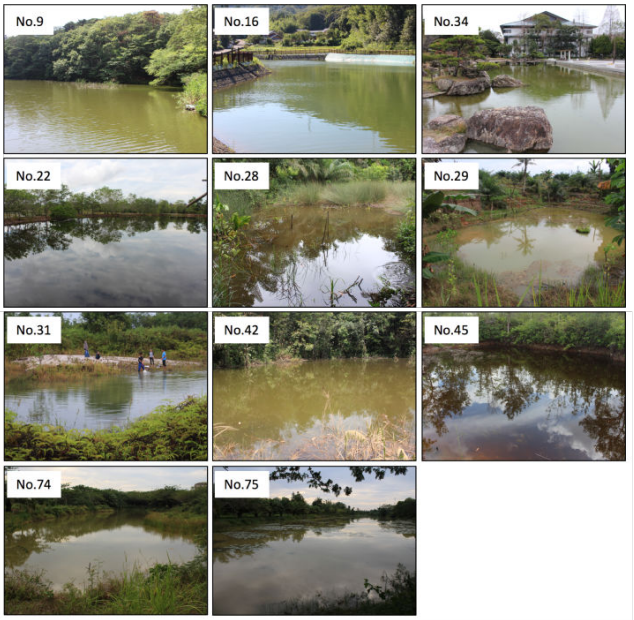

Figure 3: Natural habitats of the oil-producing microalga *Botryococcus braunii*. Wild *B. braunii* populations were found in these ponds; No.9: Miyagi, Japan (pH 8.7, Jul. 2016); No.16: Fukui, Japan (pH 6.7, Aug. 2016); No.34: Kochi, Japan (pH 8, Mar. 2017); No.22: Palangka Raya, Indonesia (pH 5, Feb. 2017); No.28: Pundu, Indonesia (pH 7.3, Feb. 2017); No.29: Pundu, Indonesia (pH 6.9, Mar.2017); No.31: Bunto, Indonesia (pH 6.5, Mar. 2017); No.42: MuaraTeweh, Indonesia (pH 6.5, Aug. 2017); No. 45: Palangka Raya, Indonesia (pH 6.0, Aug. 2017); No.74: Tenggarong, Indonesia (pH7.7, Sep. 2018); No.75: Tenggarong, Indonesia (pH7.5, Sep. 2018).

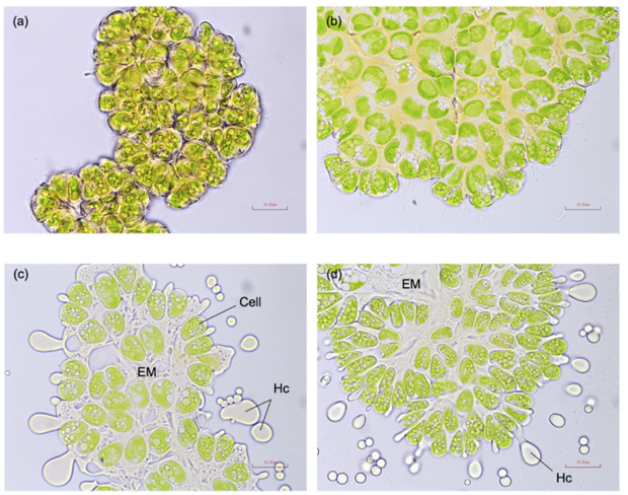

Figure 4: Isolated wild strains of *Botryococcus braunii*. (a) OIT-623, isolated from pond No. 9 (SY), Miyagi, Japan. (b) OIT-777, isolated from pond No. 16 (TK), Fukui, Japan. (c) OIT-678, isolated from pond No. 28 (IF), Pundu, Indonesia. (d) OIT-685, isolated from pond No. 22 (IE), Plangka Raya, Indonesia. EM: Extracellular Matrix, Hc: Hydrocarbon. A molecular phylogenetic analysis of the 18S ribosomal RNA gene [22] indicated that OIT-623 and OIT-777 belong to the clade S and L, respectively, while OIT-678 and -685 belong to the clade B. Strains belong to the different clades are expected to produce hydrocarbons with different chemical structures.

The total number of isolated strains were 21 strains from 3 ponds in Japan and 110 strains from 8 ponds in Indonesia (Table 1).

A molecular phylogenetic analysis of the 18S ribosomal RNA gene [22] indicated that they belong to the clade of *B. braunii* and include all four sub-clades corresponding to different chemical races based on hydrocarbon structures (Figure 4).

We hence demonstrated that wild *B. braunii* population can be found and isolated from natural environments in both temperate and tropical regions. Based on our 8-step method, we expected to find *B. braunii* in approximately 1 out of every 5 ponds in Kalimantan, Honshū and Shikoku. But, the method does not permit quantification, i.e., it does not measure the absolute density of *B. braunii* in natural ponds, and moreover requires a large amount of labour for sampling. The method targets the colony-forming individuals floating in surface water so cannot find the target organism in other forms such as single cells, gametes, or zygotes,nor less buoyant cells distributed below the surface or in bottom sediments.

In order to tackle the above-mentioned subjects (genetic resource, population ecology, life cycle), we have developed a DNA-based, quantitative detection method of wild *B. braunii* in natural environments [22]. The method detects and quantifies *B. braunii* by PCR amplification of a species-specific, hydrocarbon-biosynthesis gene.

The PCR method is highly sensitive and can detect as little as 1 colony of *B. braunii* in a water sample. As the method is a DNA-based detection method, it can find *B. braunii* regardless of morphology. Furthermore, the new method is high-throughput, processing 100 samples in a few days. We could apply this PCR-based detection method of *B. braunii* to resolve research goals (i)–(iii) as follows:

(i) Genetic resources: There may be wild genetic resources to improve the greatest fault of the species, i.e. slow growth. We have proposed a strategy for exploring fast-growing strains [22]: By quantifying the density of *B. braunii* in multiple ponds with the PCR-method and identifying a high-density pond, the effort of isolation can be concentrated on the high-density ponds. This strategy may increase the chance of finding fast-growing strains, because high growth rates contribute to higher population density. However, the population densities of microalgae in natural environments are affected not only by their intrinsic growth rates, but also by the densities of competitors and enemies, water quality, disturbance frequency, etc.

(ii) Population ecology: In order to clarify the factors controlling the population density of *B. braunii* in natural environments, the water samples are simultaneously analysed for microbiome and physicochemical parameters of water quality. Landsat spectral data of ponds and their surrounding areas at the time of sampling and their temporal changes over different time scales (months, years, decades) will also provide useful information about water quality, irradiance levels, and disturbance frequency of the ponds and may hence inform prediction of *B. braunii* population density in natural environments. Artificial Intelligence (AI) will help processing of these multiple, big

data sets. The knowledge will be very useful for mass-cultivating *B. braunii* in outdoor environments [22].

(iii) Life cycle: As noted earlier, the DNA-based method can detect *B. braunii* regardless of morphology. Investigating seasonal changes in *B. braunii* population density in different layers of a pond may catch a sign of sexual reproduction or dormancy and lead to the elucidation of unknown life-cycle phases of the species in nature.

Conclusion

Algal biofuel is one possible solution to the problems of sustainable energy supply and global warming. The history of cultivating and breeding microalgae is much shorter than for crop plants and is at a primitive stage, open to improvements. In such an early stage of cultivation, field-based ecological studies of the target organism usefully complement laboratory-based biotechnology development and may contribute to breakthroughs that influence the potential for future growth. This paper describes an isolation method for extracting the oil-producing microalga *Botryococcus braunii* from natural environments and a recently-developed, DNA-based detection method for the species, and discusses how ecological studies may contribute to the development of the algal biofuel production.

Acknowledgment

This study was partially supported by a fund from JSPS KAKENHI (No. 26660179, 20K06215). We appreciate the members of Laboratory of Bio-environmental Sciences of OIT, Hendrik Segah, Sulmin Gumiri, Dontes R Siburian Zakiah, Noorkumala Sari Nisa, Andri Cornelius of UPR, and Widha Prahastika of UNMUL for their helpful support with the fieldwork.

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