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Isolation of herbicide-resistant mutants of *Botryococcus braunii*

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ABSTRACT

Aiming at herbicide-assisted cultivation of *Botryococcus braunii* for prevention of algal contamination, herbicide-tolerant mutant lines of *B. braunii* were established for two widely used herbicides, methyl viologen and glufosinate. Some established mutant lines exhibited vigorous oil production and growth in herbicide-containing media. Because the two herbicides were effective in controlling the growth of the algal competitors of *B. braunii*, these mutants can be directly used in industrial attempts for cost-effective oil production in herbicide-assisted non-axenic systems. This is the first report of mutagenesis of *B. braunii*.

1. Introduction

Botryococcus braunii is a colonial green alga found in fresh and brackish water (Wake and Hillen, 1980, 1981; Aaronson et al., 1983; Huszar and Reynolds, 1997; Huang et al., 1999; Metzger and Largeau, 2005; Volova et al., 2003). This green alga is unique in that it biosynthesizes large amounts of hydrocarbon oils from photosynthetic products, excrete the oils outside the cells, and accumulate the oils in the extracellular matrices. The hydrocarbons biosynthesized by *B. braunii*, such as botryococcenes, botryoxanthins, and various high molecular weight phenoxy ether lipids, chemically resemble petroleum (Huang and Murray, 1995; Okada et al., 1996; Metzger and Pouet, 1995). These features render *B. braunii* one of the most useful renewable source of fossil-fuel substitutes.

Aiming at practical industrialization of this organism for oil production, reduction of the production cost is demanded. The most cost-effective cultivation system for large-scale production probably is to simply grow *B. braunii* in an outdoor open-air puddles or ponds. However, trials to cultivate *B. braunii* in non-axenic systems often fail due to thriving of other green algal and cyanobacterial species because of the relatively slow growth of *B. braunii*. Therefore, methodological or technological breakthrough to control the growth of these contaminating species is awaited to minimize the oil production cost using *B. braunii*.

For crop cultivation, utilization of herbicides for weeding is commonly practiced (ISAAA, 2009). To enable herbicidal control of *B. braunii* cultivation, mutants exhibiting resistance to methyl viologen and glufosinate were isolated from mutagenized cells of a Race A strain of *B. braunii* (BOT88-2). These mutants exhibited herbicide tolerance at concentrations that inhibit the growth of the wild-type BOT88-2 and competing algal species. These results indicate that the newly isolated mutants of *B. braunii* can be used for cultivation in herbicide-containing media to prevent algal contaminations.

2. Methods

2.1. Algal materials and growth

The original cultures of *B. braunii* (strain BOT88-2) and its algal competitors *Chlorella vulgaris* [NIES-227], *Chlamydomonas reinhardtii* [NIES-2235], *Scenedesmus dimorphus* [NIES-119], *Pseudokirchneriella subcapitata* [NIES-35] and *Merismopedia tenuissima* [NIES-230] were provided from Masanobu Kawachi (National Institute for Environmental Studies, Japan). Each species was cultured in its optimized medium under continuous light (90 μmol/m^2/s) at 25 °C. *B. braunii* was cultured in the BOT medium (0.2 g/L KNO_3, 0.04 g/L K_2HPO_4, 0.1 g/L $MgSO_4{\cdot}7H_2O$, 0.054 g/L $CaCl_2{\cdot}2H_2O$, 10 mg/L Fe citrate·nH_2O, 100 mg/L citric acid monohydrate, 18.1 ng/L $MnCl_2{\cdot}4H_2O$, 2.22 ng/L $ZnSO_4{\cdot}7H_2O$, 0.5 ng/L $Na_2MoO_4{\cdot}2H_2O$, 0.8 ng/L $CoCl_2{\cdot}6H_2O$, pH 7) developed by Takako Tanoi and Masanobu Kawachi (personal communication) for optimal growth of *B. braunii*. All the other species were grown in the C medium (150 mg/L $Ca(NO_3)_2{\cdot}4H_2O$, 100 mg/L KNO_3, 50 mg/L β-Na_2

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glycerophosphate·5H_2O, 40 mg/L $MgSO_4·7H_2O$, 0.1 mg/L vitamin B_{12}, 0.1 mg/L biotin, 10 mg/L thiamine HCl, 33 mg/L $Na_2EDTA·2H_2O$, 6.5 mg/L $FeCl_3·6H_2O$, 1.2 mg/L $MnCl_2·4H_2O$, 0.73 mg/L $ZnSO_4·7H_2O$, 0.13 mg/L $CoCl_2·6H_2O$, 0.083 mg/L $Na_2MoO_4·2H_2O$, 500 mg/L Tris(hydroxymethyl)aminomethane, pH 7.5) as described by Ichimura (1971).

The algal growth was monitored by measuring the absorbance at 660 nm (A_{660}) using a spectrophotometer. The specific growth rate (μ, day^{-1}) was calculated using the following formula: $\mu = \ln(A_{t_2}/A_{t_1})/(t_2 - t_1)$, where A_{t1} and A_{t2} was the A_{660} at times t_1 and t_2, respectively.

2.2. Herbicide treatment

The liquid medium was inoculated with a 1/10 volume of a log-phase pre-culture. They were grown in capped test tubes with 5 mL culture medium under condition described above. Either methyl viologen (Nacalai Tesque, Kyoto, Japan; also known as Paraquat) or glufosinate (Basta; Bayer CropScience, Tokyo, Japan) was added to the culture at standard concentrations (50–100 μM for methyl viologen and 50–100 mg/ml for glufosinate).

2.3. EMS mutagenesis

Cells were harvested from 40-ml culture of the BOT88-2 strain in the log phase by centrifugation (3000 rpm 10 min). Subsequently, the cells were suspended in culture medium containing 1% ethyl methanesulfonate (EMS, Nacalai Tesque, Kyoto, Japan) for 1–24 h. After the EMS treatment, cells were washed twice with 40 ml of the culture medium and cultured for a week under continuous light. For the calculation of survival rate, EMS treated cells were placed on a slide glass and the colonies were crushed by pressing the cover glass. Damaged cells were identified by the blue-green autofluorescence and absence of the red chlorophyll fluorescence via epifluorescence microscopy under UV excitation. The photographs were taken under a BH-2 microscope with a DP12 digital camera (Olympus, Melville, NY, USA) and the red, green, and blue intensity levels were adjusted using the Photoshop 6.0 software (Adobe Systems Inc., San Jose, CA, USA).

2.4. Mutant screening

Mutagenized cells were propagated on herbicide-containing solid media contained 100 μM methyl viologen or 100 mg/L glufosinate. They were incubated for 2 months. In order to confirm herbicide tolerance, green colonies were spread on another herbicide-containing solid media with platinum loop, then mutant lines which grew on the loop trace were confirmed as herbicide resistant mutants.

2.5. Oil measurement by Nile Red fluorescence quantification

The oil content was quantified via the Nile Red method according to Lee et al. (1998) using a spectrofluorometer (FP-6500; JASCO Corp., Tokyo, Japan) set at 490 nm (excitation) and 585 nm (emission). A mixture of isooctane, cetane, benzene at the volume ratio of 37.5%, 37.5%, and 25.0% (OCB standard solution) was used to calibrate the system. The oil content per biomass was determined by dividing the values by absorption at 560 nm (Casadevall et al., 1985) for each mutant line and the wild-type BOT88-2 strain.

3. Results and discussion

3.1. Effects of commercially available herbicides on growth of algal competitors of B. braunii

Controlling the growth of algal competitors seemed to be a key to successful cultivation of *B. braunii* in non-axenic systems, which are significantly more cost-effective than axenic systems (Sheehan et al., 1998). The idea here was to inhibit the thriving of algal competitors by addition of herbicides. Because information about effectiveness of herbicides on aquatic microorganisms was limited, the effectiveness of two widely used herbicides, methyl viologen and glufosinate, were first examined using algal species typically found in similar environments to *B. braunii*. Four green algal species (*C. vulgaris*, *C. reinhardtii*, *S. dimorphus*, and *P. subcapitata*) and one cyanobacterial species (*M. tenuissima*) were selected and obtained from Microbial Culture Collection at National Institute for Environmental Studies (http://mcc.nies.go.jp/localeAction.do;jsessionid=EE75D310D881EE65136DD4032D67747B?lang=en). These photosynthetic organisms are found in freshwater and exhibit optimal growth temperatures of 20–25 °C.

The herbicide was added to the algal culture in the early log phase at standard concentrations (50 μM for methyl viologen and 50–100 mg/ml for glufosinate). The growth of all algae tested was inhibited by methyl viologen (Fig. 1a). Glufosinate was less effective than methyl viologen for *C. vulgaris*, *S. dimorphus*, and *M. tenuissima* but effectively inhibited the growth of *C. reinhardtii* and *P. subcapitata* (Fig. 1b).

Such results indicated that methyl viologen is effective in removing a wider range of competing species than glufosinate (Fig. 1). Use of methyl viologen, a blocker of electron transport systems, should also be advantageous in the sense that it can be used to control the growth of contaminating bacterial species as well. Downside of methyl viologen is its high toxicity to a wide range of organisms including human (Franco et al., 2010). Compared with methyl viologen, glufosinate is less toxic to animals (Bayer CropScience, 2007). When choosing herbicides to use for large-scale cultivation, it will be important to consider the side effects on other organisms and the environment besides the effects on target organisms.

3.2. Effects of commercially available herbicides on growth of wild-type BOT88-2 strain of B. braunii

Subsequently, the effects of the two herbicides on the growth of *B. braunii* were examined. The growth of the wild-type BOT88-2 cells was abolished by the herbicide treatments at standard concentrations (50 μM for methyl viologen and 50 mg/ml for glufosinate) (Fig. 2). The herbicide-treated cells became yellow around 2 weeks after the herbicide application, then turned white 3–4 weeks after the herbicide application. These results indicated that herbicides can be used to inhibit the growth of contaminating algae, but the growth of *B. braunii* would also be retarded. Acquisition of herbicide-tolerant mutants of *B. braunii* seemed to be one of the most effective tactics to realize the utilization of herbicides to prevent the thriving algal competitors.

3.3. Mutagenesis of B. braunii (strain BOT88-2)

Because there was no preceding report on mutagenesis of *B. braunii*, preliminary experiments were carried out to optimize the conditions for mutagenesis. The BOT88-2 cells were treated with a 1% ethyl methanesulfonate (EMS) solution for varying durations of time (1–24 h). The survival rate of *B. braunii* cells after the EMS treatment decreased with increasing duration of the treatment. Four-hour EMS treatment gave the 50% lethal dose,