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Optimization of light for growth, photosynthesis, and hydrocarbon production by the colonial microalga *Botryococcus braunii* BOT-22

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Abstract

Optimization of the light conditions for biofuel production by the microalga *Botryococcus braunii* BOT-22 (Race B) was performed using monochromatic red light. The lipid and sugar contents were approximately 40 and 20-30% of the cell dry weight, respectively, and about half of the lipids were liquid hydrocarbons. The half-saturation intensities for the production rate of lipids, hydrocarbons, and sugars were 63, 49, and 44 μmol m^{-2} s^{-1}, respectively. Fluorescence microscopic images of Nile Red-stained cells showed an increased number of intracellular neutral lipid granules due to increased light intensity. After 16 days of incubation in the dark, lipid and sugar**, but not hydrocarbon** content decreased. Growth, metabolite production, and photosynthesis were saturated at 100, 200 and 1,000 μmol m^{-2} s^{-1}, respectively. These results indicate that photosynthetically captured energy is not used efficiently for metabolite production; thus, improvements in metabolic regulation may increase hydrocarbon production.

Keywords: Biofuel, Botryococcus braunii, Hydrocarbon production, Light intensity, Lipid production

1. Introduction

Microalgal biofuels are expected to be more ecologically friendly and economically efficient energy sources than biofuels produced from food resources which can significantly influence crop prices (Mitchell, 2008). Furthermore, land use efficiency is remarkably high for microalgal biofuel production compared with that for land plants typically used for biofuel production, including maize, jatropha, and palm (Chisti, 2007; Mata et al., 2011).

The colonial green alga *Botryococcus braunii* has been proposed as a promising renewable petroleum substitute because of its ability to fix atmospheric CO_2 photosynthetically and produce long-chain hydrocarbons (Banerjee et al., 2002; Casadevall et al., 1985; Largesu et al., 1980; Metzger and Largeau, 2005; Wolf et al., 1985). *Botryococcus braunii* produces hydrocarbons (Metzger and Largeau, 2005), which can easily be converted to liquid fuels such as gasoline and jet fuel after catalytic cracking (Hillen et al., 1982).

Optimization of light conditions is one of the most important factors in establishing cost- and energy-efficient mass cultivation of photosynthetic microorganisms (Ugwu et al., 2008)**; however,** conflicting reports have been published concerning the optimization of light intensity. **A** two-fold increase

in hydrocarbon productivity and **biomass were reported by** Brenckmann et al. (1985), **but not** Kojima and Zhang (1999). In the race B **strain,** *B. braunii* BOT-144, isolated in Thailand, Baba et al. (*in press*) reported that there was no significant difference in hydrocarbon production **when using** blue, green, and red light emission diode (LED) lights; however, they concluded that red light was the most efficient wavelength for hydrocarbon production when calculated on the basis of supplied energy.

In **the present** study, a representative of race B, *B. braunii* BOT-22 isolated at Okinawa prefecture, Japan, **was** examined **for** the effect of **intensity and duration** of light on growth, photosynthesis, and production of sugars and lipids, including hydrocarbons. This strain will frequently be used in future work since studies on the pathway of hydrocarbon biosynthesis and the expressed sequence tag are already completed (Ishimatsu et al., *in press*; Ioki et al., *in press*).

2. Materials and Methods

2.1. Strain and culture conditions

The original axenic culture of *B. braunii* strain BOT-22 collected from a reservoir of Ishigaki Island in Okinawa Prefecture, Japan, was provided by Dr. M. Kawachi (National Institute for Environmental Studies, Ibaraki,

Japan). The culture medium was modified Chu 13 medium (Largesu et al., 1980). Stock cultures were maintained in a 50-mL Erlenmeyer flask under light/dark conditions with a regimen of 16 h/8 h. The culture was shaken by hand once a day.

A previous study **had shown** that cell growth and hydrocarbon production proceeded stably under both blue and red light in *B. braunii* (Baba et al., *in press*), although blue and red light are generally known to alter the direction of metabolism toward amino acid and carbohydrate synthesis, respectively, in other microalgae such as the green alga *Chlorella* (Miyachi et al., 1978).

Cells (500 mL as suspension) were cultured in a flat-oblong glass vessel (38.5 cm length, 11 cm width and 3.5 cm thickness) with a bottleneck (10 cm length and 2.5 cm in internal diameter). A glass tube (4 mm in internal diameter) for aeration was inserted **into the vessel** to the bottom through a cotton plug.

The cultures were pre-cultured under continuous illumination by fluorescent light (photosynthetic photon flux density (PPFD): 40 μmol photons m^{-2} s^{-1}) and then transferred to darkness or continuous illumination by a red monochromatic LED (λ_{max} = 660 nm) at various intensities. The light intensities as PPFD were 20, 40, 100, and 200 μmol m^{-2} s^{-1}. The half-band width of the LED was 21 nm according to the manufacturer (CCS Inc., Kyoto, Japan). The cultures were aerated with **sterile**-filtered air at a flow rate of 100

mL min^{-1}. The stock and experimental cultures were maintained at 25 ºC. Changes in pH of the cell suspension were periodically monitored with a pH meter (HM-30S; TOA-TDK, Tokyo, Japan) after harvesting.

2.2. Chlorophyll determination

After harvesting by filtration through either GF/A or GF/C glass filter (2.5 cm in diameter, GE healthcare UK Ltd., Buckinghamshire, UK), cells were transferred to 90% methanol (final concentration) and large pellet was properly disrupted by ultrasonic disintegration (several times with output dial 3 at 10 sec-intervals, Ultrasonic Disruptor, TOMY SEIKO Co. Ltd., Tokyo, Japan). Then, the cell suspension was shaken by hand at 70 ºC for 5 min and thereafter kept at room temperature in the dark for 30 min. Cell debris was removed as a precipitate by centrifugation at 17,000×*g* for 5 min. **T**he chlorophyll concentration of the supernatant was determined by the method of MacKinney (1941) using a spectrophotometer (Shimadzu, Kyoto, Japan).

2.3. Determination of growth rate

Cell turbidity was determined by measuring the optical density at 750 nm (OD_{750}). The cell dry weight was determined by weighing **with** an electric balance (Mettler Toledo Co. Ltd., Greifensee, Switzerland) after

lyophilization of the cells from 20 mL of culture. The specific growth rate (μ) was determined for each growth parameter by calculating the growth speed at the logarithmic growth phase according to a standard method (Levasseur et al., 1993). The equation used for calculating growth rate (μ) was as follows: $(\ln T_b - \ln T_a)(t_b - t_a)^{-1}$, where T_x represents a parameter of cell mass at time x (t_x day).

2.4. Fluorescence microscopy

Observations were made by bright field and fluorescence (excitation, 330-385 nm; emission, >420 nm) microscopy. Cells were incubated in culture medium with a 1/1,000 volume of Nile Red stock solution (Sigma-Aldrich, St. Louis, MO) prepared in DMSO (1 mg mL^{-1}) to observe neutral lipids, including hydrocarbons. After 5 min, Nile Red fluorescence was observed under a microscope with a narrow band interference-blue (NIBA) filter (excitation, 470-490 nm; emission, 515-550 nm).

2.5. Determination of the rate of photosynthetic O_2 evolution

A Clark-type oxygen electrode (Rank Brothers, Bristol, UK) was used to examine photosynthetic O_2 evolution and dark respiration. The reaction vessel contained a 5-mL cell suspension in modified Chu 13 medium. $NaHCO_3$ (final concentration, 10 mM) was added as a carbon source just

before the measurements were made. The light source was a red LED lamp (HLV-24SR-3R; CCS Inc.) with the properties described above.

2.6 Quantification of hydrocarbons and total sugars

Total lipid was extracted by the method of Folch et al. (1957). Lyophilized cells of *B. braunii* were dissolved in 8 mL of chloroform:methanol (2:1 [v/v]) and mixed vigorously. **After storage at 4 ºC for one day, the suspension was disrupted by sonic disintegration for 1 min using the Ultrasonic Disruptor (output dial 3 at 10 sec-intervals, TOMY SEIKO)**. Next, 0.2 mL of 1 mg mL^{-1} *n*-triacontane ($C_{30}H_{62}$) as an internal standard and 1.6 mL of 0.8% NaCl were added and vigorously mixed. The suspension was centrifuged (2,300 ×*g* for 5 min) at 20 ºC and the bottom layer was obtained. After drying and weighing for the calculation of total lipids, the sample was dissolved in a small amount of chloroform, then applied to a silica gel column for chromatography. Both hexane and hexane:chloroform (9:1 [v/v]) extracts were stored at -20 ºC.

Quantification of hydrocarbons of BOT-22 was according to Yonezawa et al. (*in press*). An FID-equipped capillary gas chromatograph (GC-2014AFSC; Shimadzu) with an Rtx-5 capillary column (30 m, 0.32 mm I.D., 0.25 µm; Restek Corp., Bellefonte, PA) was used for quantification of the hydrocarbons. The carrier gas and split ratio were He (2.48 mL min^{-1}) and

50:1, respectively. The column temperature was set to 60 ºC for the first 3 min, heated at a speed of 20 ºC min^{-1} to 250 ºC, then taken at 4 ºC min^{-1} to 300 ºC. The temperature of the injection port and FID were 320 ºC. The highest hydrocarbon peak, at around 15.6 min, was quantified by comparing it to the peak from an *n*-triacontane standard.

Total sugars were quantified by the phenol-sulfuric acid colorimetric method (Hodge and Hofreiter, 1962). After mixing the cell suspension with the phenol reagent, sulfuric acid was injected and shaken vigorously. The absorbance at 490 nm was then determined using a spectrophotometer and calibration made with glucose as a standard.

3. Results and Discussion

Changes in color of colonies, cell growth, chlorophyll concentration, and the production of metabolites (lipids, hydrocarbons, and sugars) in *B. braunii* BOT-22 were determined in cells illuminated under red light at an intensity of 20, 40, 100 and 200 μmol m^{-2} s^{-1}. Actual values in each experiment **are** shown in Table S1-S3. The colonies changed color from green to yellowish when the light intensity was increase**d** from low to high (Fig. 1A-D). Spectroscopic pigment analysis showed that this change was due to an increase in the ratio of carotenoids/chlorophyll (Fig. 1E). This trend agreed

with a previous observation in *B. braunii* (Wolf et al., 1985; Baba et al., *in press*).

The algal growth parameters monitored by cell turbidity (OD_{750}), cell dry weight, and chlorophyll concentration were similarly saturated at 100 μmol m^{-2} s^{-1} (Fig. 2A-C), although the chlorophyll content was slightly **lower** at high light intensities (Fig. 2C). The decrease in chlorophyll content may indicate cellular acclimation to avoid excess light, although further analyses **are** required to demonstrate the possibility. Quantitative analyses showed that the production of lipids, hydrocarbons, and sugars increased along with an increase in light intensity (Fig. 2D-F). Fluorescence microscopic observations of Nile Red-stained colonies showed that the number of intracellular granules (namely, neutral lipids) within the egg-shaped cells increased at high light intensities (Fig. 2G)**, but** the amount of liquid (namely, hydrocarbons) that accumulated outside of the cells in the intercellular spaces of the colony was not remarkably different.

The results shown in Fig. 2 were confirmed by a second light-transient experiment in which the light intensity was suddenly increased to 200 μmol m^{-2} s^{-1} after growth at 40 μmol m^{-2} s^{-1} for eight days (Fig. 3). The turbidity and cell dry weight increased slightly, while the chlorophyll content decreased with increases in the light intensity (Fig. 3A-C). The lipid, hydrocarbon, and sugar contents increased about 1.5-, 1.5-, and 1.7-fold,

respectively (Fig. 3D-F), and the number of Nile Red-stained granules (namely, neutral lipid granules) also increased (Fig. 3G).

The lipid, hydrocarbon, and sugar contents were approximately 36±6%, 27±8%, and 23±2% of cell dry weight, respectively, throughout the experiments, which lasted for 20 days (data not shown), although the cell dry weight increased (Figs. 2B and 3B). These results indicate that the cellular composition was the same irrespective of light intensity. **Hydrocarbon production was maintained nearly constant during entire growth stage when it was calculated based on chlorophyll content (Fig. 2E)**.

No change in cell turbidity or cell dry mass was detected after 15 days of incubation in the dark (Fig. 4A, B); however, the chlorophyll content showed a slight gradual decrease (Fig. 4C). No change in the amount of hydrocarbons was observed by chemical analysis and microscopic observation (Fig. 4E, G); however, the amounts of lipids and sugars decreased slightly (Fig. 4D, F). The dark-incubated cells regained their previous growth rate when transferred **to light (data not shown), indicating that the cells had maintained cellular physiological activities to grow by the consumption of** storage molecules such as lipids and sugars. This assumption was confirmed by the decrease in **the** number of intracellular lipid granules observed during dark incubation (Fig. 4G). Tanoi et al. (2010) reported that *B. braunii* BOT-70 (race B), grown mixotrophically or heterotrophically, produced larger lipid

granules than when grown autotrophically. The neutral lipids may have been triacylglycerols (TAG), a group of ubiquitous non-membranous lipids (Voelker and Kinney, 2001). Detectable amounts of TAG are known to be contained in all race A strains of *B. braunii* (Metzger and Largeau, 1999); however, this has not been observed for race B strains to date. In any case, the hydrocarbons produced by *B. braunii* were not consumed by dark respiration even when cells were grown under a light/dark cycle.

The relationship between light intensity and the specific growth rate (μ), calculated from changes in cell turbidity, cell dry weight, and chlorophyll content at the logarithmic growth phase, is summarized in Fig. 5A. Similarly, changes in the lipid, hydrocarbon, and sugar contents as well as photosynthesis per chlorophyll unit are shown in Fig. 5B. The growth rate of *B. braunii* was saturated at around 50 μmol m^{-2} s^{-1} (Fig. 5A). A drastic decrease in chlorophyll content at over 50 μmol m^{-2} s^{-1} is considered to be due to cellular acclimation in order to avoid damage by excess light energy. The production of lipids, hydrocarbons, and sugars, however, was saturated at around 200 μmol m^{-2} s^{-1}; the half-saturating light intensities were 63, 44, and 49 μmol m^{-2} s^{-1} for the production of lipids, hydrocarbons and sugars, respectively (Fig. 5B). **These results indicate that physiological cellular activities such as growth, photosynthesis and metabolism differentially** regulated by light. In other words, both **biomass** and hydrocarbon production

were improved by optimization of the light conditions, as reported previously (Brenckmann et al., 1985). These metabolic characteristics were confirmed by microscopic observation of Nile Red-stained intracellular neutral lipid granules (Figs. 2-4).

In *B. braunii* BOT-22, photosynthetic O_2 evolution per unit of chlorophyll was nearly saturated (93%) at 600 μmol m^{-2} s^{-1} with a rate of 41.5 μmol O_2 mg Chl^{-1} h^{-1} under red LED illumination in cells grown at 40 μmol m^{-2} s^{-1} (Fig. 5C). The half-saturating light intensity for photosynthesis and the saturated light intensity for hydrocarbon production were the same (200 μmol m^{-2} s^{-1}). These data indicate that an increase in photosynthetic activity above 200 μmol m^{-2} s^{-1} does not stimulate hydrocarbon productivity per unit of chlorophyll in wild-type *B. braunii* BOT-22. Further analysis will be needed to elucidate which kind of metabolism is dominantly driven above 200 μmol m^{-2} s^{-1}.

4. Conclusions

Photosynthesis, growth, and metabolic productivity of *B. braunii* BOT-22 cells were saturated at approximately 1,000, 100, and 200 μmol m^{-2} s^{-1}, respectively, under monochromatic red light. The production of lipids and hydrocarbons was not directly supported by an increase in photosynthetic

activity. Metabolic manipulation, therefore, will be essential for further improvement of hydrocarbon production. In addition, the length of the dark period did not affect hydrocarbon production or the efficiency of light energy utilization for hydrocarbon production by *B. braunii*.

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Figure Captions

Fig. 1 Changes in color of cell suspension and spectroscopic patterns of pigments. A-D, Color of cell suspension under 20, 40, 100, and 200 μmol photons m^{-2} s^{-1}, respectively. E, Absorption spectra of methanol extracts of cells grown under a red LED light at intensities of 20 (♦), 40 (■), 100 (▲) and 200 (●) μmol photons m^{-2} s^{-1}, respectively.

Fig. 2 Changes in lipid, hydrocarbon, and sugar production under various light intensities during the growth of *Botryococcus braunii* BOT-22. A, cell turbidity (OD_{750}); B and C, cell dry weight and chlorophyll concentration, respectively, calculated on the basis of cell suspensions; D-F, total lipid, total hydrocarbon, and total sugar contents, respectively, calculated on the basis of the chlorophyll concentration. Cells were illuminated by a red LED at an intensity of 20 (♦), 40 (■), 100 (▲), or 200 (●) μmol photons m^{-2} s^{-1}. The values given are the averages of two separate experiments, each with triplicate samples. G, micrographic images of colonies from a 20-day-old culture grown under various light intensities. The cells were stained with Nile Red and observed by bright field (a) and fluorescence microscopy with UV light to show chlorophyll fluorescence (b) and NIBA filters to show neutral lipids (c).

Fig. 3 Changes in lipid, hydrocarbon, and sugar production with increased light intensity during the growth of *B. braunii* BOT-22. A-G, same as in Fig. 2. The red light intensity was changed on day 8 from 40 to 200 μmol photons m^{-2} s^{-1} (40→200) or remained 40 μmol photons m^{-2} s^{-1} (40→40) as a control. The values given are the average of three separate experiments with triplicate samples.

Fig. 4 Changes in lipid, hydrocarbon, and sugar production during dark incubation of *B. braunii* BOT-22. A-F, as described in Fig. 2. G, micrographic images of a colony from a 16-day-old culture incubated in the dark. The labels are the same as in Fig. 2. The cells were grown under fluorescent light at 40 μmol m^{-2} s^{-1} then transferred to the dark. The suspension was bubbled with air at 100 mL min^{-1} throughout the experiments. Each experiment was performed twice (Experiments 1 [♦] and 2 [■]) with triplicate samples.

Fig. 5 Changes in growth, metabolite production, and photosynthesis in response to change in light intensity in *B. braunii* BOT-22. A, changes in growth parameters such as cell turbidity (●), cell dry weight (×), and chlorophyll content (+). B, changes in the rates of hydrocarbon (▲), lipid (■),

and sugar (♦) production normalized by the chlorophyll content (Chl). C, changes in the rates of photosynthetic O_2 evolution (❇), cell growth based on dry weight (×) and lipid production (■). The maximum values were: A, 0.093 day^{-1} for cell turbidity (μ_1), 0.114 mg mL^{-1} day^{-1} for cell dry weight (μ_2) and 0.067 μg mL^{-1} day^{-1} for chlorophyll concentration (μ_3); B, 1.6 μg (μg Chl)$^{-1}$ day^{-1} for hydrocarbons, 2.64 μg (μg Chl)$^{-1}$ day^{-1} for lipids, 2.56 μg (μg Chl)$^{-1}$ day^{-1} for total sugars; C, 44.1 μmol (mg Chl)$^{-1}$ h^{-1} for the rate of photosynthetic O_2 evolution. Dry weight and lipid production are the same as in A and B, respectively.

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