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Wavelength specificity of growth, photosynthesis, and hydrocarbon production in the oil-producing green alga *Botryococcus braunii*

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Abstract

The effect of monochromatic light on growth, photosynthesis, and hydrocarbon production was tested in *Botryococcus braunii* Bot-144 (race B), which produces triterpenoid hydrocarbons. The growth was higher in order of red, blue, and green light. The color of red light-grown cells became more orange-yellow and their shape dominantly changed to grape-like with long branches. Photosynthetic carbon fixation activity was higher in order of blue, red, and green light-grown cells, but photosystem activities showed no difference. In the pulse-chase experiments with $^{14}\text{CO}_2$, no major difference was observed in the production of lipids, hydrocarbons, polysaccharides, or proteins among the three kinds of cells, although hydrocarbon production was slightly lower in green light-grown cells. These results indicate that blue and red light were more effective for growth, photosynthetic CO_2 fixation, and hydrocarbon production than green light, and that red light is the most efficient light source when calculated based on photoenergy supplied.

Keywords: *Botryococcus braunii*; Growth; Hydrocarbon production;
Monochromatic light; Photosynthesis; Wavelength effect

1. Introduction

Current human activities are strongly dependent on petroleum as an energy source and as an industrial material; thus, we are facing serious global environmental problems such as the greenhouse effect and air pollution. An oil-producing green alga *Botryococcus braunii* is a promising renewable source of petroleum substitute, as it produces hydrocarbons by fixing atmospheric CO₂ photosynthetically (Banerjee et al., 2002; Casadevall et al., 1985; Largesu et al., 1980; Metzger and Largeau, 2005; Wolf et al., 1985).

The hydrocarbons extracted from cells as a hexane-soluble component (Yamaguchi et al., 1987) can be converted to liquid fuels such as gasoline (Hillen et al., 1982) and jet fuels after catalytic cracking.

The control and optimization of light quality and quantity is one of the most important parameters for the mass culture of such a photosynthetic microorganism (Ugwu et al., 2008). The rate of extracellular polysaccharide production in race B of *B. braunii* decreases under low light intensity (Zhang and Kojima, 1998), and growth is also affected by changes in light intensity in the range of 3–10 klx but hydrocarbon production is not (Zhang and Kojima, 1999). However, these studies did not consider the outer wall, a biopolymer derived from very long chain fatty acids (Banerjee et al., 2002).

No information is available on the wavelength specificity for growth and the photosynthetic characteristics of *B. braunii* but there are many reports on the topic in a variety of other photosynthetic organisms (Goodwin and Britton, 1988; Larkum, 2003; Moore et al., 1995). Therefore, we examined the effect of light wavelength on growth, colony color, colony shape, photosystem activity, photosynthetic CO₂ fixation, and metabolite production in *B. braunii* Bot-144 using tiles of aligned blue, green, and red light emitting diodes for illumination. Our results clearly showed that blue and red light were more effective for growth, photosynthetic CO₂ fixation, and hydrocarbon production than green light, and that red light was the most efficient light for hydrocarbon production by *B. braunii*.

2. Materials and Methods

2.1. Strain and culture conditions

B. braunii Bot-144 was isolated by Dr. M. Kawachi, National Institute for Environmental Studies, Japan, from a pond in Nong Kra Ting public park, Thailand. Cells were cultured in a flat-oblong glass vessel containing 500 mL modified AF-6 medium (Watanabe et al., 2000) in darkness or under continuous illumination at a photon flux density (PFD) of 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for monochromatic blue ($\lambda_{\text{max}} = 470 \text{ nm}$), green (525 nm), and red light (660 nm)

for 6 months. The half-band widths are 22, 33, and 21 nm for blue, green and red light, respectively, according to manufacturer's instruction (CCS Inc., Kyoto, Japan). Temperature was maintained at 25°C. Cultures were bubbled with sterilized-filtered air at a flow rate of 100 mL min⁻¹.

2.2. Chlorophyll determination and analysis of the cellular extract absorption spectrum

After harvesting, the cells were transferred to 90% methanol (final concentration) and then disrupted by sonic disintegration. Cell debris was removed by centrifugation at 17,000 × *g*. After centrifugation, the chlorophyll concentration of the supernatant was determined by the method of MacKinney (1941). The absorption spectrum of the extracts obtained in the supernatant was determined with a spectrophotometer (Shimadzu, Kyoto, Japan).

2.3. Photosystem parameters

Photosystems status was observed using an Open FluorCam 701MF imaging fluorometer (Photon Systems Instruments, Brno, Czech Republic). Minimum fluorescence (F_o) was determined after dark adaptation for at least 30 min. Maximum fluorescence (F_m) was obtained by applying a 0.8 s saturating light pulse (3,000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). The intensity of the

actinic light was $60 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, and the duration of illumination was 15 min. The maximum quantum yield of PSII (F_v/F_m) was calculated as $(F_m - F_o) F_m^{-1}$. The steady state quantum yield of PSII (F_v'/F_m') was calculated as $(F_m' - F_o') F_m'^{-1}$. The effective efficiency of photosynthetic electron transport (ϕII) was calculated as $(F_m' - F_s) F_m'^{-1}$. Non-photochemical quenching (NPQ) was calculated as $(F_m - F_m') F_m'^{-1}$. Photochemical quenching (qP) was calculated as $(F_s - F_o') (F_m' - F_o')^{-1}$.

2.4. *Fluorescent microscopy*

A bright field microscope and a fluorescence microscope with a WU filter (excitation, 330–385 nm; emission, >420 nm) were used for microscopic observations. Cells were incubated in medium with a 1/1000 volume of Nile Red stock solution (Sigma-Aldrich, St. Louis, MO) prepared in DMSO (1 mg ml^{-1}) to observe neutral lipids such as hydrocarbons. After 5 min, Nile red fluorescence was observed under a microscope with a NIBA filter (excitation, 470–490 nm; emission, 515–550 nm).

2.5. *Photosynthetic radioisotope labeling of metabolites*

Pre-grown cells were suspended in fresh medium containing 10 mM NaHCO_3 (final concentration) at $\text{OD}_{750} < 0.3$ for short-term experiments. The cells were incubated with gentle stirring in the monochromatic blue, green,

and red light at a PFD of $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 25°C for 10 min. ^{14}C -labeling was initiated by adding $25 \mu\text{L NaH}^{14}\text{CO}_3$ (136 kBq ml^{-1} , 2 GBq mmol^{-1} ; final concentration, 0.2 mM) to a 5 ml cell suspension. An aliquot of cell suspension was taken from the culture at intervals, and metabolites were extracted with methanol ($80\% [\text{v/v}]$, final concentration) at 70°C .

For pulse-chase experiments, ^{14}C -labelling was first initiated by adding $\text{NaH}^{14}\text{CO}_3$ (136 kBq ml^{-1} , 2 GBq mmol^{-1} ; final concentration, 0.01 mM) into the reaction medium in Erlenmeyer flasks (0 h). A very low concentration of $\text{NaH}^{14}\text{CO}_3$ was added so that it would be depleted during the short term for pulse ^{14}C -labeling of photosynthetic metabolites. After injecting the $\text{NaH}^{14}\text{CO}_3$, air was bubbled for 1 min to mix, and the cells were incubated in the light without bubbling for 2 h . During this period $\text{NaH}^{14}\text{CO}_3$ was nearly depleted and most metabolites were well labeled with ^{14}C (pulse labeling period). Then the suspension was bubbled with air to purge the ^{14}C -dissolved inorganic carbon (DIC) remaining in the medium and to further proceed with the experiment (chase period). Aliquots of cell suspension (10 ml each) were harvested for metabolite analysis at intervals. To determine the ^{14}C -DIC remaining in the medium, collected samples were immediately mixed with 0.1 N NaOH (final concentration) to prevent the release of unfixed ^{14}C -DIC from the medium into the air. Samples were passed through a filter to remove cells and cell debris before determining radioactivity in the medium.

All radioactive data were calculated by assuming that 10,000 dpm of ^{14}C -radioactive substrate was added to 1 ml of reaction medium, although the exact ^{14}C -radioactivity injected was slightly different among experiments (see Fig. 3b).

2.6. Fractionation of ^{14}C -labeled metabolites

Cells in 80% methanol (final concentration) were disrupted by sonication. The homogenates (300 μl) were centrifuged at $17,000 \times g$, and the pellet was washed with a small amount of 80% methanol. The resulting supernatants and precipitates were used as methanol soluble and insoluble fractions, respectively, for further analysis. After bringing up the methanol soluble fraction to 500 μl with DW, 300 μl chloroform was added. After centrifugation ($17,000 \times g$), low molecular compounds (LMC) and lipids were obtained in the upper and lower layers, respectively. The methanol insoluble fraction was treated with 5% [v/v] trichloroacetate (TCA) at 60°C for 15 min and then centrifuged to obtain TCA-soluble and -insoluble fractions that contained polysaccharide plus poly-nucleotides and a protein fraction, respectively. To avoid protein contamination, the pH of the extracts was maintained at neutral but not alkaline conditions. The radioactivity of the fractions was determined using a liquid scintillation counter (LS5000TD, Beckman, Fullerton, CA, USA).

2.7. Quantification of ^{14}C -hydrocarbons by thin-layer chromatography

For thin-layer chromatography (TLC), lipid fractions were concentrated by evaporation and then dissolved in a small volume of 80% [v/v] ethanol. The sample was applied to a Silica gel 70 TLC plate (Wako, Osaka, Japan) and developed with *n*-hexane. After drying, the TLC plate was placed into contact with an imaging plate (Fuji Film, Tokyo, Japan). Radioactive spots were quantified by BAS 1800 and Image Gauge software (Fuji Film, Tokyo, Japan). Hydrocarbon spots were identified by referring to previous results (Kalacheva et al., 2002; Okada et al., 2000).

3. Results and Discussion

3.1. Growth, pigment composition, photosystem activity, and colony shapes

After pre-cultivating the cells under fluorescent light, they were incubated in the dark for 1 week to reduce storage compounds and avoid the influence of light on pre-culture. Then, cells were transferred to continuous illumination with monochromatic light of $60 \mu\text{mol m}^{-2} \text{s}^{-1}$. Under such conditions, cell density (OD_{750}) remained constant in the dark and then started to increase in the light. Algal growth rates (μ , d^{-1}) were higher in the

order of red, blue, and green light-grown cells with values of 0.19, 0.15, and 0.09, respectively.

The color of red light-grown cells changed to yellow during the 6 months that the cells were maintained under monochromatic illumination (data not shown). This change seemed to be due to an increase in the ratio of carotenes/chlorophyll content, based on the absorption spectrum of the 90% methanol extracts, as previously reported in the cyanobacteria *Synechococcus* and *Prochlorococcus* (Moore et al., 1995) and *B. braunii* during light-acclimating processes (Wolf et al., 1985). In contrast, the photosystem activity parameters such as Fv/Fm, qP, ϕ II, and NPQ showed no difference among the blue, green, and red light-grown cells (Fig. 1). These results indicate that pigment composition, but not the photosystem machinery, was affected when light quality was changed during growth.

Interestingly, colony shapes were obviously specific to each wavelength during the 6-month growth period (data not shown). Microscopic images revealed that colonies of red light-grown cells had highly branched grape-like shapes, whereas blue light-grown colonies showed more branchless grape-like shapes. Green light-grown cells were in-between the blue and red light-grown cells. There was no visible difference in the status of Nile red stained neutral lipids including hydrocarbons. Differences in light quality may change colony morphogenesis, as the outer wall of *B. braunii* has been

suggested to be comprised of a biopolymer derived from a very long chain fatty acid (Banerjee et al., 2002).

3.2. Photosynthetic $^{14}\text{CO}_2$ fixation by blue, green, and red light-grown cells

The rate of photosynthetic $^{14}\text{CO}_2$ fixation calculated per chlorophyll molecule was highest in blue light-grown cells and decreased in the order of red and then green light-grown cells when determined under the respective monochromatic light (Fig. 2). The rate was 1.8-fold higher in the blue than in the red light-grown cells, although the reason was not clear. This trend was different than that of growth (see Fig. 3a), suggesting that photosynthesis and growth are not directly correlated.

3.3. Effect of wavelength on carbon metabolism in long-term pulse-chase experiments

We conducted long-term pulse-chase experiments in blue, green, and red light-grown cells to study the effect of wavelength on carbon metabolism. During the experiment for 8 days, growth rates calculated from changes in cell density (μ, d^{-1}) in blue-, green-, and red-light-grown cells were 0.16, 0.15, and 0.15, respectively (Fig. 3a).

During photosynthetic $^{14}\text{CO}_2$ fixation, about 58 ± 14 , 72 ± 2 , and 84 ± 2 % of $\text{NaH}^{14}\text{CO}_3$ added to the medium was used after 2, 3, and 5 h,

respectively (pulse-labeling period) (Fig. 3b, c). After 5 h, air-bubbling was started to purge the remaining ^{14}C -DIC, supply CO_2 for maintaining active photosynthesis, and decrease the specific activity of ^{14}C by dilution (change to chase period). Radioactivity in the medium was maintained at a steady value after about 5 h, showing almost no further incorporation of $^{14}\text{CO}_2$ from the medium into cells. Under such conditions, the route of ^{14}C -transfer among metabolites can be chased clearly (chase period).

The time course of ^{14}C -transfer during the chase period clearly indicated that lipids, particularly hydrocarbons, were the final products of photosynthetic $^{14}\text{CO}_2$ fixation in *B. braunii* Bot-144, as ^{14}C -radioactivity increased only in lipids, decreasing in other products (Fig. 4a, b, c). The lipid fraction occupied about 70% of ^{14}C -labeled compounds and had a high hydrocarbon content (50% in blue and red light-grown cells, 40% in green light-grown cells; Fig. 4a–d). These results clearly indicate that the major photosynthetic products were hydrocarbons. ^{14}C that was incorporated into LMC, proteins, and polysaccharides + nucleic acids changed from 30 to 20%, 17 to 10%, and 8 to 2%, respectively. Hydrocarbon production was slightly lower in green light-grown cells than in blue and red light-grown cells at 8 d (Fig. 4d). Carbon metabolism is regulated by a particular wavelength of light in some organisms such as *Chlorella* and land plants (Kami et al., 2010; Kamiya and Saitoh, 2002). However, our results suggest that the metabolism

of *B. braunii* Bot-144 is not regulated by a specific wavelength of monochromatic light.

4. Conclusions

Growth, pigment composition, colony shape, and the rate of photosynthetic CO₂ fixation in *B. braunii* Bot-144 were regulated by changes in light quality, whereas the photosystem machinery and metabolic pathways for hydrocarbon production were not. This suggests that the stimulation of the activity of carbon input reactions is more important for increasing the production of biofuels such as hydrocarbons and lipids. According to the calculation of photoenergy utilization efficiency, red light was the most effective and useful light source for the production of hydrocarbons as a renewable energy source.

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Figure Captions

Fig. 1. Comparison of the photosystem activity parameters determined by FluorCam. F_v/F_m , the maximum quantum yield; qP, photochemical quenching; ϕ_{II} , effective efficiency of photosynthetic electron transport; NPQ, non-photochemical quenching.

Fig. 2. Time courses of photosynthetic $^{14}\text{CO}_2$ fixation determined for monochromatic blue, green, and red light-grown cells of *Botryococcus braunii* Bot-144. The photon flux density for photosynthesis was $60 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Fig. 3. Changes in cell density and ^{14}C fixed by blue, green, and red light-grown cells of *Botryococcus braunii* Bot-144 during the ^{14}C -pulse-chase experiments. Cells which had been maintained under blue, green, and red lights for 6 months were used for the ^{14}C -labelling experiment under respective light. (a) Change in cell density. The specific growth rates (μ, d^{-1}) were 0.16, 0.15, and 0.15 in blue, green, and red light-grown cells, respectively; (b) ^{14}C -radioactivity that was fixed by cells (^{14}C -fixed) and remained in the medium (^{14}C -medium). Broken line, ^{14}C -radioactivity added to the medium; (c) ^{14}C in the cells. Photon flux density was set at $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ during both growth and ^{14}C -labelling experiments.

Fig. 4. Percent distribution of ^{14}C -radioactivity in the lipid, low molecular weight compound (LMC), protein, and polysaccharide plus nucleic acids fractions during long-term ^{14}C pulse-chase experiments in blue, green, and red light-grown cells. (a) blue light-grown cells; (b) green light-grown cells;

(c) red light-grown cells; (d) ^{14}C incorporation into hydrocarbons and other lipids in cells at 8th day.

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