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Culture of the hydrocarbon producing microalga *Botryococcus braunii* strain Showa: Optimal CO$_2$, salinity, temperature, and irradiance conditions

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Abstract

Specific growth rates and hydrocarbon contents of *Botryococcus braunii* strain Showa were measured under a wide range of CO\(_2\), salinity, temperature, and irradiance conditions. The bubbling CO\(_2\) concentration of 0.2–5% and no addition of salinity were favorable conditions for growth. The strain cannot grow at 5°C and above 35°C under any irradiance levels. Maximum specific growth rate of 0.5 day\(^{-1}\) (doubling time of 1.4 days), the highest value reported for *B. braunii* in the past studies, was observed at 30°C and 850 µmol photons m\(^{-2}\) s\(^{-1}\). Since hydrocarbon productivity, shown as the product of hydrocarbon content and specific growth rate, increased with the increasing specific growth rate, we conclude that more efficient hydrocarbon production by the mass culture of strain Showa can be achieved by maintaining higher specific growth rate based on the culture conditions presented in this study.

**Keywords:**

*Botryococcus braunii*, Temperature, Irradiance, CO\(_2\), Salinity
1. Introduction

Much attention has focused on microalgae for their potential to produce energy through photosynthetic CO$_2$ fixation. To reduce CO$_2$ emissions to the atmosphere, a shift from fossil to renewable energy sources is clearly required. Biomass utilization is considered to be one of the answers to this serious issue. Because of its higher potential for lipids production than other biomass sources, microalgae are considered to be a significant source for conversion of CO$_2$ and sunlight into usable energies (Service, 2011). Among microalgal species, Botryococcus braunii has a very high lipid content, and much of the lipid is composed of hydrocarbon (Banerjee et al., 2002). The alga is classified into three chemical races A, B, and L according to the type of hydrocarbons accumulated. Furthermore, the hydrocarbons are produced under the active growth phase unlike other high lipid content species that accumulate lipids under nitrogen limited conditions (Casadevall et al., 1985). Hydrocarbon is easily transformed into fuels, so effective utilization of the potential of B. braunii would lead to a method of biofuel production (Yoo et al., 2010) and thus enable reduction of CO$_2$ emissions and a more sustainable society. Mass culture of B. braunii has a significant potential for biofuel production.

Microalgae have species and strain specific optimum growth conditions and different tolerance to environmental factors. The factors which have to be elucidated should include temperature, irradiance, CO$_2$, and salinity. A mass culture system for B. braunii also should be established and controlled based on the responses of the growth to environmental variations. In this context, many strains of B. braunii have been studied for their physiology and ecology in previous studies, reviewed by Banerjee et al. (2002). For example, change in the growth of B. braunii has been reported for a range of temperatures for strain UC 58 (Lupi et al., 1991), irradiance for race B strain BOT-22 (Sakamoto et al., 2012), CO$_2$ for strain 765 (Ge et al., 2011) and for race A strain LB-572 (Ranga Rao et al., 2007b), and salinity for strain LB-572 (Ranga Rao et al., 2007a). Recent studies have examined effects of multiple environmental factors on optimal growth conditions for the strains CHN 357 (Qin and Li, 2006), UK807-2 and NIES-836 (Li and Qin, 2005), and KMITL 2 (Ruangsomboon, 2012). On the other hand, combined effects of multiple factors that include, for example, quite basic parameters of temperature and irradiance remain to be clarified. In particular, irradiance is shown as a major
parameter in determining the system yield of *B. braunii* in an outdoor pilot-scale reactor under uncontrolled conditions (Bazaes et al., 2012). Comparable data sets are required for more strains to select a suitable strain for a given environmental condition of a culturing system. In addition to determining the optimal environmental conditions, improvement of the commonly used Chu 13 medium by supplementing potentially growth limiting micronutrients of vitamins (Croft et al., 2005) and selenium (Araie and Shiraiwa, 2009) which are lacking in the Chu 13 medium is necessary. Detailed data on the growth response of *B. braunii* to the medium improvement and the basic environmental conditions are required prior to constructing a mass culture.

Among the three races, B race which produces methylated triterpenes, known as botryococcenes, appears to be suitable for biofuel production because (1) hydrocarbon content is relatively high and the hydrocarbon is readily and quantitatively separated from the biomass (Eroglu et al., 2011); (2) produced hydrocarbon have a high unsaturation degree and thus suitable for fuels (Hillen et al., 1982); (3) derivatives originated from the B race indeed are found in a crude oil (Moldowan and Seifert, 1980). Within B race, the strain Showa has been used in many studies and findings on hydrocarbons and related substances produced have been accumulated. Recently three genes related to triterpene production were identified from this strain (Niehaus et al., 2011). Moreover, EST analyses and genome sequencing were carried out at the Joint Genome Institute (http://genome.jgi.doe.gov/genome-projects/pages/projects.jsf?searchText=Showa. Accessed 17 January 2013) by using strain Showa as a model of the B race. These data will be released to the public and this will accelerate the utilization of *B. braunii* for commercial biofuel production. Thus, detailed features of strain Showa on basic but important environmental factors should initially be clarified.

In the present study, we first examined the possibility of the improvement of the modified Chu 13 medium. Then the growth responses of the strain Showa to different CO₂ concentrations, salinities, temperatures, and irradiances were studied. Hydrocarbon content and elemental composition of carbon (C), nitrogen (N), and phosphorus (P) also were measured. The presented results enable the determination of optimal conditions for a culture of strain Showa.
2. Materials and methods

2.1. Algal strain and stock culture conditions

An isolate of nonaxenic *B. braunii* strain Showa belonging to the B race (Nonomura, 1988), also known as strain Berkeley, was examined in the present study. Stock culture of strain Showa was grown in a modified Chu 13 medium as shown in Okada et al. (1997) in a 45 mL borosilicate test tube (25 mm in diameter, Pyrex) containing 30 mL medium. The culture was maintained at 25°C under a photosynthetically active radiation (PAR) of 160 µmol m\(^{-2}\) s\(^{-1}\) using a cool white fluorescent lamp (FLR40S·W/M, Mitsubishi) with a 14 h:10 h light/dark cycle. Filter sterilized (0.20 µm) air enriched with 1% CO\(_2\) was bubbled directly into the medium at a rate of 10 mL min\(^{-1}\) throughout the culture period. The stock culture was inoculated periodically (ca. every two weeks) to new medium to maintain the exponential growth phase.

2.2. Estimation of specific growth rates

As *B. braunii* forms colonies, it is difficult to estimate its growth rate by counting the number of individual cells. On the other hand, estimation of the biomass from its dry weight takes a long time because the alga grows slowly. For quicker and easier estimation of the growth rate of *B. braunii*, the growth of strain Showa was monitored by directly measuring in vivo Chlorophyll-\(a\) (Chl-\(a\)) fluorescence of the culturing test tube using a fluorometer (model 10-AU, Turner Designs) with an in vivo chlorophyll optical kit (#10-096R). Specific growth rates were calculated from the linear regression of the natural log of in vivo Chl-\(a\) fluorescence vs. time during the exponential growth phase of acclimated cells. The specific growth rate can be translated to a doubling time by dividing \(\ln(2) = 0.693\) by the value of specific growth rate. To confirm the relationship between in vivo Chl-\(a\) fluorescence and biomass, the strain Showa was cultured in polycarbonate bottles with 2 L medium under the same conditions of the stock culture but with 186 µmol photons m\(^{-2}\) s\(^{-1}\). During the incubation, a portion of the culture was periodically sampled, and in vivo Chl-\(a\) fluorescence
and cellular dry weight were measured concurrently. For measuring the dry weight, the culture was filtered through a pre-washed and pre-weighed glass fiber filter (GF/F with pore size of 0.7 µm, Whatman). Then, the filter samples were weighed after freeze-drying. Nitrate and phosphate concentrations in the medium were determined colorimetrically with a segmented continuous flow analyzer (QuAAtro, Bran+Luebbe).

2.3. Medium improvement experiment

To optimize the medium for growth, vitamins and selenium were supplemented into the modified Chu 13 medium. Vitamins including thiamine hydrochloride (vitamin B<sub>1</sub>), biotin (vitamin B<sub>7</sub>), and cyanocobalamine (vitamin B<sub>12</sub>) were enriched with the same concentrations of f/2 medium (Guillard, 1975) which is a typical medium for marine phytoplankton. Selenium was enriched as disodium selenite (Na<sub>2</sub>O<sub>3</sub>Se) with the same molar concentration with copper in the modified Chu 13 medium (0.32 µmol L<sup>-1</sup> in final concentration). Four treatments of the modified Chu 13 medium as the control, the control + vitamins (+V), the control + selenium (+Se), and the control + vitamins + selenium (+V+Se) were examined to compare the growth under the same conditions of the stock culture but with 169 µmol m<sup>–2</sup> s<sup>–1</sup>. Acclimation to the new medium and PAR condition was achieved by two transfers of the culture to the new medium (approximately 10 generations). After the acclimation, the experiments were run in parallel by serial transfer of exponentially growing culture in each treatment. In this study we did not examine the effect of macronutrients (nitrate and phosphate) on the algal growth because plenty of macronutrients remained in the medium under culture conditions examined throughout the present study as shown in Fig. 1.

2.4. Effect of CO<sub>2</sub> concentrations on the algal growth

The growth of strain Showa under a wide range of CO<sub>2</sub> concentrations was measured. We investigated the growth under bubbling of ambient air (0.04% CO<sub>2</sub>) and air containing 0.1, 0.2, 0.5, 1, 3, 5, 10, 20, 30, and 50% CO<sub>2</sub>. The air-CO<sub>2</sub> mixtures (0.1–20% CO<sub>2</sub>) were purchased from a commercial gas supply company (Nissan-Tanaka Co., Japan). For 30 and
50% CO₂, ambient air and 100% CO₂ were mixed based on the flow rate using a flow controller (RK1200, Kofloc). Air-CO₂ mixture was bubbled directly into the 30 mL medium at a rate of 10 mL min⁻¹ throughout the culture period. The experiments were conducted under 25°C and 266 µmol m⁻² s⁻¹. After the acclimation to the new environment, the culture was inoculated into two test tubes for each CO₂ treatment and the specific growth rates were measured. pH of the media were measured using a glass electrode (744, Metrohm) after bubbling the air-CO₂ mixture into the media for two days without inoculating the alga and thus under a steady state condition between the bubbling gas CO₂ concentration and partial pressure of CO₂ in the medium.

2.5. Effect of salinity on the algal growth

The growth of strain Showa under a wide range of salinities was measured. Ultrapure water (Milli-Q Advantage, Millipore) and nutrient depleted (less than 0.3 µg L⁻¹ of PO₄) subtropical Pacific surface water with salinity of 36 were mixed to obtain the control and four different salinity treatments, and then modified Chu 13 nutrients were added to the control and treatments. As a result of the salinity measurements using a model EC300 (YSI), the salinity was 0.4 in the control, and 0.7, 2.1, 4.0, and 18.1 in the four salinity treatments. The experiments were conducted under 25°C and 255 µmol m⁻² s⁻¹. Air enriched with 1% CO₂ was bubbled directly into the medium at a rate of 10 mL min⁻¹ throughout the culture period. After the acclimation to the new environment, the culture was inoculated into two test tubes for each salinity treatment and the specific growth rates were measured.

2.6. Effect of various combinations of temperature and PAR on the algal growth

The growth of strain Showa under a wide range of temperature and PAR was measured. The experiments were conducted at nine temperatures levels (5, 15, 20, 25, 27.5, 30, 35, 38, and 45°C) and six to ten PAR levels in 0–2000 µmol m⁻² s⁻¹ for each temperature. A cool white fluorescent lamp (FL10W, Panasonic; http://www.taroto.jp/site/pdf/lump/03/hi-light.pdf. Accessed 17 January 2013) coupled with
an air circulating incubation chamber was used for the relatively low PAR range, whereas a halogen (EA864C-139,ESCO; http://ds.esco-net.com/out/DispDetail.do?volumeName=00003&itemID=t000100025091. Accessed 17 January 2013) or xenon lamp (SOLAX XC-100A, Seric; http://www.seric.co.jp/english/100wlightingproducts.htm. Accessed 17 January 2013) coupled with a temperature controlled water tank was used for the relatively high PAR range to prevent medium temperature increase by the heat of a lamp. The fluorescent lamp was used for all PAR levels at 5, 15, 35, and 45°C, for 10–450 µmol m⁻² s⁻¹ treatments at 25°C, and for 20–650 µmol m⁻² s⁻¹ treatments at 30°C. The halogen lamp was used for 250–2000 µmol m⁻² s⁻¹ treatments in 25°C and for 550–1800 µmol m⁻² s⁻¹ treatments in 30°C. The xenon lamp was used for all PAR levels at 20 and 27.5°C. Note that the light irradiated from the three types of lamps would have different light spectra. Even for identical PAR levels, different light sources would have different impacts on algal growth because the photosynthetic action spectra may differ from the light spectrum of a given light source. However, since wavelength does not influence the light-saturated region of photosynthesis (Pickett and Myers, 1966), we did not consider the difference in the type of lamp in discussing maximum specific growth rate at a certain temperature or among the temperatures examined. Air enriched with 1% CO₂ was bubbled directly into the medium at a rate of 10 mL min⁻¹ throughout the culture period. After the acclimation to the new environment, the culture was inoculated for two test tubes in each treatment and the specific growth rates were measured continuously in the serial transfer of the culture of each treatment.

2.7. Cellular hydrocarbon and C, N, and P contents

Strain Showa was cultured in polycarbonate bottles with 2 L medium. To determine the response to different temperatures and PAR, the cultures were conducted at 15°C (189 µmol m⁻² s⁻¹), 25°C (208 and 394 µmol m⁻² s⁻¹), and 30°C (91 and 311 µmol m⁻² s⁻¹) with bubbling of a constant 1% CO₂ enriched air. To determine the response to different CO₂ conditions, the cultures were incubated with bubbling of air containing 0.04, 1, 5, and 10% CO₂ at a constant 25°C and 274 µmol m⁻² s⁻¹ condition. During each culture, a portion of the culture was periodically sampled, and in vivo Chl-a fluorescence was measured to estimate
the specific growth rate. When the fluorescence reached around 100 (no dimension) under maintaining exponential growth phase, the incubation was ended. A portion of the culture was filtered onto a pre-combusted (450°C for 4 h) GF/F filter to analyze cellular C, N, and P. The C, N, and P contents on the filter samples were measured as described in Yoshimura et al. (2009) using a C/N elemental analyzer and colorimetric method for P. The rest of the culture was filtered through a 20 µm mesh plankton net, and the cells were analyzed for hydrocarbon content. Following freeze-drying of the harvested cells, the lipid fractions were extracted using acetone and then chloroform/methanol mixture (2:1, v/v). The extracts were combined and concentrated under reduced pressure. The residual oil was dissolved in n-hexane and subjected to silica gel column chromatography on Wakogel C-300 (Wako Pure Chemical Industries, Ltd., Japan) with n-hexane as the mobile phase. All eluates before a yellow band of carotenes were collected, evaporated to remove the solvent, dried under vacuum, and then weighed to determine the amounts of triterpene hydrocarbons.

3. Results and discussion

3.1. Estimation of algal specific growth rate by in vivo Chl-a fluorescence

The relationship between in vivo Chl-a fluorescence and cellular dry weight was demonstrated during the whole culture period of 54 days (Fig. 1). Initially, in vivo Chl-a fluorescence increased linearly with dry weight until ca. 100 (no dimension) of fluorescence, corresponding to a dry weight of 0.2 g L⁻¹. The fluorescence reached a maximum of ca. 160 at a dry weight of around 0.8 g L⁻¹, and then decreased to ca. 50 for all algal concentrations over the dry weight of 3 g L⁻¹. We considered that self-shading of light by algal cells growing in the culturing test tubes played an important role in the relationship. Furthermore, changes in the contents and profiles of chlorophyll pigments could occur in the aged and dense culture. Therefore the estimation of specific growth rate by in vivo Chl-a fluorescence may not be used for too dense or aged cultures. In this context, Tanoi et al. (2011) also estimated specific growth rates of *B. braunii* cultured in multi-well plates by in vivo Chl-a fluorescence with the initial concentrations of 15 to 20 mg L⁻¹ which were much lower than those used in this study. In our experiments, linearity between the in vivo Chl-a fluorescence and algal dry biomass
was found even at much higher concentrations, up to 0.2 g L\(^{-1}\). Therefore we concluded that measurements of in vivo Chl-\(\alpha\) fluorescence were useful to monitor the increase in the biomass of strain Showa as far as the cell density was under a certain level or the growth phase was at an early stage of a culture period.

The measurement of in vivo Chl-\(\alpha\) fluorescence provided a time and space saving method to monitor the growth of strain Showa. This was a quicker, simpler, and more sensitive method to measure the biomass rather than other conventional methods such as the dry weight method, because the test tube culturing algae itself was used as the measuring cuvette for the Turner fluorometer. Furthermore, the use of incubation room can be minimized by replacing the culture vessel from a large container to a test tube. This enables us to test many experimental treatments and replicates concurrently in an incubator. We estimated the specific growth rate successfully using the in vivo Chl-\(\alpha\) fluorescence throughout the present study. The technique can be applied to other \textit{B. braunii} strains and other algal species regardless of cell size including colony-forming species as examined in Brand et al. (1981).

Additionally, our data showed that the maximum yield of strain Showa was 3.3 g L\(^{-1}\) in the modified Chu 13 medium (Fig. 1). The culture started at 0.001 g L\(^{-1}\) and took 54 days to reach the maximum yield of 3.3 g L\(^{-1}\) at 25°C and 186 µmol m\(^{-2}\) s\(^{-1}\). At the end of the culture, nitrate was depleted in the medium (Fig. 1), so this is the maximum yield for strain Showa in the modified Chu 13 medium. Time course of the nutrient data showed that nitrate and phosphate were consumed at a molar ratio of 45, which was slightly higher than the optimal supply ratio of 35 for strain IPE 001 proposed in Xu et al. (2012). As a result, the phosphate concentration remained more than 1 mg L\(^{-1}\) at the final phase of the culture, because the initial nitrate to phosphate ratio was 17.4 in the modified Chu 13 medium. Thus, nitrate regulated the maximum yield of strain Showa in the medium.

### 3.2. Medium improvement experiment

Micronutrient additions can stabilize the growth of strain Showa (Table 1). Although the mean specific growth rates measured in the four treatments did not show any statistically significant difference, the growth in each treatment showed different ranges of variation
during the serial cultures. The specific growth rate dropped to less than 0.2 day$^{-1}$ in some cases in the control, +V, and +Se treatments. Coefficient of variance in the specific growth rates was minimized in the +V+Se treatment and was 35% lower than the +V and +Se treatments and less than half of the control. We conclude that the +V+Se medium enhanced the steady growth of strain Showa. To obtain reproducible result for the growth under various conditions, we cultured strain Showa in the modified Chu 13 medium supplemented with vitamins and selenium throughout the present study.

Vitamins and selenium are reported as a potential limiting factor for the growth of microalgae. Many microalgae have been found to be unable to synthesize vitamins themselves (Croft et al., 2005), thus they require exogenous vitamins for growth in culture. Selenium also is required for the growth in many microalgae for synthesizing selenoproteins (Araie and Shiraiwa, 2009). Vitamins can be provided through bacteria (Croft et al., 2005), if they exist in the culture, but selenium must be taken up from the medium. Our results showed that vitamins and selenium can influence the growth of strain Showa when a medium lacking these micronutrients was used. Effects of these micronutrients to other strains of *B. braunii* have not yet been clarified. The micronutrients should be considered as a potential limiting factor or as a factor enabling stable growth in the future mass culture of *B. braunii*.

3.3. Effect of CO$_2$ concentrations on the algal growth

Specific growth rate of strain Showa varied significantly with the CO$_2$ concentration (Fig. 2). The specific growth rates increased from 0.24 day$^{-1}$ in ambient air to 0.35 day$^{-1}$ in 0.2% CO$_2$, indicating that the supply of CO$_2$-enriched air is necessary to maintain a certain level of growth rate. This is qualitatively consistent with Wolf et al. (1985), in which the specific growth rate of strain Showa increased from 0.12 day$^{-1}$ in air to 0.42 day$^{-1}$ in 0.3% CO$_2$ under a 23–25°C and continuous illumination of 250 µmol m$^{-2}$s$^{-1}$ condition. Our data showed that the growth did not change significantly in the range of 0.2–5% CO$_2$, thus these were the optimal CO$_2$ conditions for strain Showa. The growth decreased over the 5% CO$_2$ and stopped at 50% CO$_2$. Because CO$_2$ concentration in the bubbling air affects the pH of the medium, the pH declined with increasing CO$_2$ concentrations (Fig. 2). The decline of pH by
addition of CO\textsubscript{2} may be mitigated by the use of a medium that contains a buffer such as Tris or Bicin. In our medium condition, optimal growth was observed in pH 5.44–6.65, and the growth fell to zero in pH 4.54. We considered that 1\% CO\textsubscript{2} should be bubbled into the medium to obtain optimal growth of strain Showa.

Optimal CO\textsubscript{2} conditions differ among strains. Strain Showa has been cultured in 0.3–2\% CO\textsubscript{2} in previous studies (Okada et al., 1995; Wolf et al., 1985), confirmed as an appropriate CO\textsubscript{2} conditions by the present result. Ranga Rao et al. (2007b) reported that strain LB-572 (A race) showed optimal growth and hydrocarbon content in 2\% among 0–2\% CO\textsubscript{2} enriched air, while strain 765 showed uniform growth among the 2–20\% CO\textsubscript{2} range examined (Ge et al., 2011). These results indicated that optimal CO\textsubscript{2} concentrations for the growth and tolerance to high levels of CO\textsubscript{2} are strain specific, so the optimal CO\textsubscript{2} condition should be determined depending on the utilizing strain’s characteristics.

3.4. Effect of salinity on the algal growth

We showed that salinity inhibited the growth of strain Showa. The specific growth rate decreased with increasing salinity although the specific growth rate in salinity 0.7 did not significantly decrease compared with the control (Fig. 3). The strain did not grow in salinity 18.1. Change in salinity from 0.4 to 18.1 corresponded to the change in pH from 5.97 to 6.68 probably due to changes in carbonate chemistry, but the change in pH did not explain the decrease in the specific growth rate because significant growth was observed in a similar pH range in the CO\textsubscript{2} experiment shown in Fig. 2. Salinity tolerance of strain Showa was relatively low compared with previous studies; for example, strain LB-572 showed optimal growth at 1 and 2‰ in the range of 1–5‰ of NaCl (Ranga Rao et al., 2007a), or Li and Qin (2005) reported reduced but significant growth of strains CHN 357, UK 807-2, and NIES-836 at 0.5 M (ca. 29‰) of NaCl. On the other hand, a similar tolerance to strain Showa was seen for strain KMITL 2 (Ruangsomboon, 2012). Thus all Botryococcus strains do not always have tolerance to salinity. The low tolerance of strain Showa to salinity is a disadvantage to culture it in open pond systems in areas where transport of sea-salt particles occur or by using seawater as a part of the medium to reduce freshwater utilization. We conclude that no salinity
should be added to the medium to obtain optimal growth, and thus we used freshwater medium throughout the present study.

3.5. Effect of various combinations of temperature and PAR on the algal growth

Strain Showa showed a wide range of specific growth rates depending on temperature and PAR conditions. This is the first detailed report of combined effect of temperature and PAR on the growth of *B. braunii*. The strain did not grow at all at 5 and 45°C (Table 2). At 35 and 38°C, the biomass, measured by in vivo Chl-a fluorescence, increased just after the inoculation from the stock culture but then decreased, indicating eventual extinction in these temperatures. On the other hand, persistent and stable growth was observed at 15–30°C. In this range, specific growth rate at a temperature increased with an increase in PAR with some inhibitions under higher PAR range (Fig. 4). Maximum specific growth rate at a certain temperature increased from 0.1 day\(^{-1}\) at 15°C and 70 µmol photons m\(^{-2}\) s\(^{-1}\) to 0.5 day\(^{-1}\) at 30°C and 850 µmol photons m\(^{-2}\) s\(^{-1}\). The PAR where a maximum specific growth rate at a certain temperature was observed became larger together with the rise of temperature. Among the temperatures and PAR examined, the maximum specific growth rate of 0.50 day\(^{-1}\) was observed at 30°C and 850 µmol m\(^{-2}\) s\(^{-1}\) (Fig. 4b). The specific growth rates at 30°C slightly decreased over the optimal PAR but were maintained at 95% of the maximum even at 1770 µmol m\(^{-2}\) s\(^{-1}\). The maximum specific growth rate revealed in this study is, to our best knowledge, the highest specific growth rate for *B. braunii* reported in the previous studies (Table 3).

3.6. Hydrocarbon contents of algae grown under different conditions

Strain Showa produced hydrocarbon at proportions with relatively small but significant variability over a wide range of culture conditions (Fig. 5a). In the range of temperature from 15 to 30°C and PAR from 91 to 394 µmol m\(^{-2}\) s\(^{-1}\), hydrocarbon contents were 30.3–39.2% under a constant CO\(_2\) concentration of 1%. In a range of CO\(_2\) concentration from 0.04 to 10%, hydrocarbon contents were 28.5–38.3% at 25°C and 274 µmol m\(^{-2}\) s\(^{-1}\).
The hydrocarbon contents were significantly reduced when the specific growth rate fell to a lower range rather than environmental conditions themselves, and thus the hydrocarbon contents showed a significant ($p = 0.003$) positive linear relationship with specific growth rate (Fig. 5a). The contents, however, represent small variability with $34.7 \pm 3.3\%$ (mean $\pm$ standard deviation) regardless of being under different temperature, irradiance, and CO$_2$ conditions. This is a contrasting result to Li and Qin (2005) that showed significantly different total lipid contents in strains CHN 357 (2–15%), UK 807-2 (20–80%), and NIES-836 (5–60%) varied due to the change in temperature, irradiance, and salinity though their hydrocarbon contents were not determined (approximate values were estimated from the figures). The value obtained in this study is consistent with previously reported hydrocarbon contents for strain Showa (Okada et al., 1995; Wolf et al., 1985) (Table 3). Among the strains of *B. braunii*, strain Showa showed intermediate hydrocarbon content whereas the content is much lower than strain IPE 001UK (Xu et al., 2012) (Table 3). Our data showed that the hydrocarbon production occurred during exponential growth phase, consistent with the previous report (Casadevall et al., 1985), contrasting to some another species producing high lipid content only under nutrient depleted and growth-arrested conditions such as *Chlorella* (Converti et al., 2009). This advantage should be utilized in the operational conditions of future mass culture system.

Hydrocarbon productivity should be considered to discuss optimal culture conditions for efficient hydrocarbon production. Hydrocarbon productivity can be shown as a specific hydrocarbon production rate, a product of specific growth rate and hydrocarbon content. We found that hydrocarbon productivity showed a clear positive correlation with the specific growth rate, indicating that hydrocarbon productivity increased with increasing specific growth rate. We conclude that more efficient hydrocarbon production can be achieved under the culture conditions enabling higher specific growth rate.

### 3.7. C, N, and P compositions

Cellular elemental composition tended to vary with the specific growth rate. Cellular C/N ratios had a range of 23–35 (molar) with a mean $\pm$ standard deviation of 27 $\pm$ 3 and
showed a significant ($p = 0.010$) negative linear relationship with the specific growth rate (Fig. 5b). Although the contents of hydrocarbon, made of only carbon and hydrogen, was lower in cells with a reduced specific growth rate, C/N ratio was higher in reduced specific growth rates. This suggests that cellular N content increased with specific growth rate more than C content. Increase in CO$_2$ concentration is expected to elevate C/N of microalgae (Urabe et al., 2003), but we did not observe the effect on strain Showa in the CO$_2$ range examined. On the other hand, N/P ratios have a range of 11–25 ($18 \pm 4$) and showed a significant ($p = 0.013$) negative linear relationship with the specific growth rate (Fig. 5c). This is explained by elevated cellular content of RNA in fast-growing cells (Dortch et al., 1983). Thus, similar to the hydrocarbon contents, cellular elemental composition varied with the growth conditions.

Cellular elemental composition of strain Showa can contrast with the Redfield ratio with C/N of 6.6 and N/P of 16, proposed for a typical elemental composition of microalgae (Redfield et al., 1963). Higher C/N of strain Showa than the Redfield ratio (27 vs. 6.6) must result from the unique high hydrocarbon content. This may be the reason why strain Showa needs supplementation of CO$_2$ for their optimal growth (Fig. 2). The N/P ratio of strain Showa was revealed to correspond to the typical Redfield ratio, indicating similar composition of N- and P-containing components compared with other standard microalgae. The cellular N/P of 18 differed from the nitrate to phosphate consumption ratio of 45, as mentioned in Section 3.1. A plausible explanation is the release of organic materials richer in N than in P as dissolved exudates during the exponential growth phase. Unfortunately, we found no studies for examining the characteristic of exudates from *B. braunii*. This should be further examined because nutrient uptake and assimilation provide evidence to determine the ratio of nutrient addition to the medium and the amount of nutrient addition is directly linked to the running costs of mass culture.

4. Conclusion

This study demonstrated that the growth of strain Showa was maximized under conditions of 0.2–5% CO$_2$, no salinity addition, 30°C, and 850 µmol photons m$^{-2}$ s$^{-1}$. Hydrocarbon productivity increased with increasing specific growth rate, so optimal growth
condition should be maintained to achieve more efficient hydrocarbon production by the mass culture of strain Showa. A good strategy for efficient hydrocarbon production described here should be combined with efficient hydrocarbon recovery from wet biomass of strain Showa by thermal pretreatment (Kita et al., 2010) or using dimethyl ether as an extraction solvent (Kanda et al., 2013).

Contributors

All authors have approved the article for publication. Individual author roles are as follows: Yoshimura – research design and management, laboratory operations, data analysis and interpretation, manuscript drafting; Okada – research design and management, laboratory operations, data analysis and interpretation, manuscript drafting; Honda – research design and management, data analysis and interpretation.

Disclosure Statement

The authors declare no actual or potential conflict of interest.

Role of the funding source

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References


Kanda, H., Li, P., Yoshimura, T., Okada, S., 2013. Wet extraction of hydrocarbons from Botryococcus braunii by dimethyl ether as compared with dry extraction by hexane. Fuel 105, 535–539.


Redfield, A.C., Ketchum, B.H., Richards, F.A., 1963. The influence of organisms on the


Figure Captions

Fig. 1. Relationship between in vivo chlorophyll-α fluorescence and cellular dry weight with nitrate concentrations in the medium during the time course of a culture in 2 L medium under 25°C and 186 µmol photons m⁻² s⁻¹. Enlarged view of the initial part of the culture is shown in the inset.

Fig. 2. Variations of specific growth rates and pH under different CO₂ concentrations bubbled into the medium. The specific growth rates in the shaded region did not differ significantly (p > 0.05).

Fig. 3. Specific growth rates in the medium with different salinities. Significant differences among the treatments were observed between the treatments shown with different letters (e.g. “a” and “b”) near points, while “ab” indicates data that was not significantly different from “a” and “b” (Tukey-Kramer’s test, p < 0.05).

Fig. 4. Plots showing the specific growth rate vs. photosynthetically active radiation (PAR) in (a) 15, 20, and 25°C, (b) 27.5 and 30°C. Error bars represent ± one standard deviation of replicate cultures.

Fig. 5. (a) Hydrocarbon content, (b) hydrocarbon productivity shown as a product of specific growth rate and hydrocarbon content, and cellular elemental compositions as (c) carbon to nitrogen and (d) nitrogen to phosphorus molar ratios plotted against the specific growth rate examined under different temperature, irradiance, and CO₂ conditions.
Table 1. Specific growth rates of strain Showa in the modified Chu 13 medium (the control) and additions of vitamins (V) and selenium (Se), either alone or in combination.

<table>
<thead>
<tr>
<th>Culture replicates</th>
<th>Mean (day⁻¹)</th>
<th>Minimum (day⁻¹)</th>
<th>Maximum (day⁻¹)</th>
<th>Standard deviation (day⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>0.22</td>
<td>0.16</td>
<td>0.29</td>
</tr>
<tr>
<td>Control + V</td>
<td>7</td>
<td>0.27</td>
<td>0.18</td>
<td>0.31</td>
</tr>
<tr>
<td>Control + Se</td>
<td>9</td>
<td>0.26</td>
<td>0.16</td>
<td>0.29</td>
</tr>
<tr>
<td>Control + V + Se</td>
<td>9</td>
<td>0.26</td>
<td>0.21</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Table 2. Maximum specific growth rates and minimum doubling times among the range of photosynthetically active radiation examined at each temperature.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Maximum specific growth rate (day⁻¹)</th>
<th>Doubling time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>15.0</td>
<td>0.095</td>
<td>7.30</td>
</tr>
<tr>
<td>20.0</td>
<td>0.207</td>
<td>3.34</td>
</tr>
<tr>
<td>25.0</td>
<td>0.392</td>
<td>1.77</td>
</tr>
<tr>
<td>27.5</td>
<td>0.431</td>
<td>1.61</td>
</tr>
<tr>
<td>30.0</td>
<td>0.496</td>
<td>1.40</td>
</tr>
<tr>
<td>35.0</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>38.0</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>45.0</td>
<td>0</td>
<td>–</td>
</tr>
</tbody>
</table>
Table 3. Culture conditions, specific growth rates, and hydrocarbon contents for some strains of *Botryococcus braunii* in past studies.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Race</th>
<th>Temp. (°C)</th>
<th>PAR and photoperiod in a day (µmol m⁻² s⁻¹ or as shown)</th>
<th>CO₂ enrichment of air (%)</th>
<th>Specific growth rate (day⁻¹)</th>
<th>Doubling time (days)</th>
<th>Hydro content</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Showa B</td>
<td>30</td>
<td>850 (14 h)</td>
<td>1</td>
<td>0.50</td>
<td>1.4</td>
<td>No data</td>
<td>No data</td>
<td>30–39</td>
</tr>
<tr>
<td>Showa B</td>
<td>25, 30</td>
<td>85–398 (14 h)</td>
<td>1–10</td>
<td>0.19–0.44</td>
<td>1.6–3.6</td>
<td>30–39</td>
<td>No data</td>
<td>37.9</td>
</tr>
<tr>
<td>Showa B</td>
<td>23–25</td>
<td>250 (24 h)</td>
<td>0</td>
<td>0.12</td>
<td>6</td>
<td>29–39</td>
<td>No data</td>
<td>30–39</td>
</tr>
<tr>
<td>Showa B</td>
<td>23–25</td>
<td>250 (24 h)</td>
<td>0.3</td>
<td>0.42</td>
<td>1.7</td>
<td>24–29</td>
<td>No data</td>
<td>37.9</td>
</tr>
<tr>
<td>Showa B</td>
<td>25</td>
<td>240 (12 h)</td>
<td>2</td>
<td>No data</td>
<td>No data</td>
<td>37.9</td>
<td>No data</td>
<td>37.9</td>
</tr>
<tr>
<td>B70 B</td>
<td>25</td>
<td>85 (12 h)</td>
<td>Mixotrophy</td>
<td>0.17</td>
<td>4.1</td>
<td>No data</td>
<td>No data</td>
<td>37.9</td>
</tr>
<tr>
<td>IPE 001 B</td>
<td>25</td>
<td>35 (16 h)</td>
<td>1</td>
<td>0.15#</td>
<td>4.5#</td>
<td>64.3</td>
<td>No data</td>
<td>30–39</td>
</tr>
<tr>
<td>Yayoi B</td>
<td>25</td>
<td>240 (12 h)</td>
<td>2</td>
<td>0.20</td>
<td>3.5</td>
<td>40.5</td>
<td>No data</td>
<td>37.9</td>
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<tr>
<td>Göttlingen 807/1</td>
<td>A</td>
<td>25</td>
<td>26 W m⁻² (14 h)</td>
<td>1</td>
<td>0.30</td>
<td>2.3</td>
<td>44</td>
<td>No data</td>
</tr>
<tr>
<td>LB-572 A</td>
<td>26</td>
<td>1.2 klux (16 h)</td>
<td>2</td>
<td>0.07#</td>
<td>10.6#</td>
<td>28</td>
<td>No data</td>
<td>37.9</td>
</tr>
<tr>
<td>765 No data</td>
<td>25</td>
<td>150 (24 h)</td>
<td>20</td>
<td>0.13#</td>
<td>5.5#</td>
<td>24</td>
<td>No data</td>
<td>37.9</td>
</tr>
<tr>
<td>CHN 357 No data</td>
<td>25</td>
<td>60 W m⁻² (12 h)</td>
<td>0</td>
<td>0.2#</td>
<td>3.5#</td>
<td>10#*</td>
<td>No data</td>
<td>37.9</td>
</tr>
<tr>
<td>KMITL 2 No data</td>
<td>25</td>
<td>200 (24 h)</td>
<td>0</td>
<td>0.10#</td>
<td>7.0#</td>
<td>55</td>
<td>No data</td>
<td>37.9</td>
</tr>
<tr>
<td>NIES-836 No data</td>
<td>25</td>
<td>60 W m⁻² (12 h)</td>
<td>0</td>
<td>0.09#</td>
<td>7.7#</td>
<td>35#*</td>
<td>No data</td>
<td>37.9</td>
</tr>
<tr>
<td>UC 58 No data</td>
<td>25</td>
<td>250 (24 h)</td>
<td>1</td>
<td>0.42#</td>
<td>1.7#</td>
<td>No data</td>
<td>No data</td>
<td>37.9</td>
</tr>
<tr>
<td>UK 807-2 No data</td>
<td>25</td>
<td>60 W m⁻² (12 h)</td>
<td>0</td>
<td>0.18#</td>
<td>3.8#</td>
<td>65#*</td>
<td>No data</td>
<td>37.9</td>
</tr>
</tbody>
</table>

# As the specific values were not described in the papers, their approximate values were estimated from the figures.

* Contents of total lipids not hydrocarbons determined by fluorescence after Nile Red staining.
Highlights

Optimal growth in the medium with 0.2–5% CO₂ and in freshwater conditions

Maximum specific growth rate of 0.5 day⁻¹ at 30°C and 850 μmol photons m⁻² s⁻¹

The highest specific growth rate for *B. braunii* ever reported

Positive correlation of hydrocarbon contents (29–39%) with specific growth rate

Hydrocarbon productivity increased with increased specific growth rate