Bioresource Technology 133 (2013) 232–239

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Culture of the hydrocarbon producing microalga *Botryococcus braunii* strain Showa: Optimal CO_2, salinity, temperature, and irradiance conditions

Takeshi Yoshimura [a,*], Shigeru Okada [b], Masaki Honda [a]

[a] Central Research Institute of Electric Power Industry, 1646 Abiko, Abiko, Chiba 270-1194, Japan
[b] Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

HIGHLIGHTS

- Optimal growth in the medium with 0.2–5% CO_2 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.

ARTICLE INFO

Article history:
Received 13 July 2012
Received in revised form 17 January 2013
Accepted 20 January 2013
Available online 29 January 2013

Keywords:
Botryococcus braunii
Temperature
Irradiance
CO_2
Salinity

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.

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

* Corresponding author. Tel.: +81 4 7182 1181; fax: +81 4 7183 2966.
E-mail address: ytakeshi@criepi.denken.or.jp (T. Yoshimura).

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http://dx.doi.org/10.1016/j.biortech.2013.01.095

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 CO_2 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. 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 2 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_1), biotin (vitamin B_7), and cyanocobalamine (vitamin B_{12}) 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_2O_3Se) with the same molar concentration with copper in the modified Chu 13 medium (0.32 μmol L^{-1} 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^{-2} s^{-1}. 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_2 concentrations on the algal growth

The growth of strain Showa under a wide range of CO_2 concentrations was measured. We investigated the growth under bubbling of ambient air (0.04% CO_2) and air containing 0.1%, 0.2%, 0.5%, 1%, 3%, 5%, 10%, 20%, 30%, and 50% CO_2. The air–CO_2 mixtures (0.1–20% CO_2) were purchased from a commercial gas supply company (Nissan-Tanaka Co., Japan). For 30% and 50% CO_2, ambient air