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Growth characteristics and lipid content of three Korean isolates of *Botryococcus braunii* (Trebouxiophyceae)

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Abstract

Three strains of the green microalga *Botryococcus braunii* (JJS, KCM, and KJD) were isolated from different water bodies in Korea and grown as batch cultures in the laboratory. The effects of different growth media and temperatures on the growth rate were investigated, as well as the effect of temperature on the total lipid content and lipid profile. All three strains had the highest growth rates in BG-11 medium and at 25°C. Maximal lipid production (g L^{-1}) was at 30°C in the JJS strain and at 25°C in the KCM and KJD strains. However, all the three strains produced the greatest percent dry weight of total lipids at 15°C and had the lowest percent dry weight of total lipids at 25°C. In general, oleic acid, linolenic acid, and behenic acid were the most common fatty acids in all three strains. However, the three strains varied considerably in their fatty acid profiles at different culture temperatures.

Key words: *Botryococcus braunii*, growth rate, lipid content, media, temperatures

INTRODUCTION

There has been increasing interest in the use of microalgae as sources of renewable energy and other economically important products (Sushchik et al. 2003, Chisti 2007, Mata et al. 2010, Abou-Shanab et al. 2011). *Botryococcus braunii* Kützing is a colonial green alga that lives in diverse habitats, such as reservoirs, ponds, and lakes that have fresh or brackish water (Wake and Hillen 1980, Hillen et al. 1982). *Botryococcus braunii* accumulates abundant oils in extracellular spaces (Largeau et al. 1980, Hillen et al. 1982, Chisti 2007), and lipids can account for 25-75% of the dry weight (Kalacheva et al. 2002, Metzger and Largeau 2005). Thus, this species seems well-suited as a source for renewable biofuel.

Numerous studies have attempted to optimize the conditions for growth and lipid production in *B. braunii* (Casadevall et al. 1985, Inoue et al. 1994, Li and Qin 2005, Dayananda et al. 2007, Yeesang and Cheirsilp 2011). Other studies examined the effect of variables such as growth medium, temperature, pH, light intensity, and CO_2 concentration on lipid production and growth of *B. braunii* (Lupi et al. 1991, Kalacheva et al. 2002, Ranga Rao et al. 2007). In Korea, two physiological studies of *B. braunii* used foreign strains of this species (Oh et al. 2009, Choi et al. 2011).

Previous experiments indicated significant physiological differences among isolates of microalgae of the same species, possibly due to adaptations to local environments (Gallagher 1986, Lee and Kim 2007, Kim et al. 2009). Gallagher (1982) and Soudek and Robinson (1983) reported genetic, biochemical, and physiological differences of algae isolated during different seasons and from different geographical regions. The growth characteristics

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of a *B. braunii* isolate also depend on its geographic origin, and lipid content can range from 15-76% of dry weight among local species (Kalacheva et al. 2002, Metzger and Largeau 2005). Thus, it is necessary to study the physiological characteristics of Korean isolates of *B. braunii* to determine their suitability as sources for domestic biofuel production.

The present research examined isolates of *B. braunii* from three different regions in Korea and compared their growth characteristics and lipid production at different temperatures and in different growth media.

MATERIALS AND METHODS

Isolation and culture conditions

The Korean strains of *B. braunii* used in this study were each isolated by pipetting a single colony from materials collected at water bodies in Jeju Island (JJS), Kyungpook (KCM), and Kangwon province (KJD) from April to September in 2010 (Fig. 1 and Table 1). Species identification was conducted using a Zeiss Axioskop2 (Carl Zeiss, Jena, Germany) equipped with differential interference contrast (DIC) optics. Micrographs were taken with an AxioCam HRC camera (Carl Zeiss).

Uni-algal stock cultures were maintained at 24°C under about 80 μmol m^{-2} s^{-1} of cool white fluorescent light on a 16 h light:8 h dark cycle in DY-III medium (Lehman 1976) with 100 mL min^{-1} aeration. DY-III medium contained the following components (g L^{-1}): 0.02, $CaCl_2 \cdot 2H_2O$; 0.074, $MgSO_4 \cdot 7H_2O$; 0.01, Na_2 glycerophosphate$\cdot 5H_20$; 0.02, $NaNO_3$; 0.015, $NaSiO_3 \cdot 9H_2O$; 0.01, NH_4NO_3; 0.01, KCl; 0.0002, Thiamine; 0.0000005, Biotin; 0.0000005, Vitamin B_{12}; and Trace metals solution. For growth medium experiments, the three strains were grown in DY-III, BG-11 (Stanier et al. 1971), and Chu-13 media (Largeau et al. 1980) at 24°C under about 80 μmol m^{-2} s^{-1} of cool white fluorescent light on a 16 h light:8 h dark cycle with 100 mL min^{-1} aeration. BG-11 medium consisted of (g L^{-1}): 1.5, $NaNO_3$; 0.04, K_2HPO_4; 0.075, $MgSO_4 \cdot 7H_2O$; 0.036, $CaCl_2 \cdot 2H_2O$; 0.006, Citric acid; 0.0006, Ammonium ferric citrate; 0.001, EDTA-Na_2; 0.02, Na_2CO_3; and Trace metals solution and Chu-13 medium contained the following components (g L^{-1}): 0.4, KNO_3; 0.08, K_2HPO_4; 0.2, $MgSO_4 \cdot 7H_2O$; 0.107, $CaCl_2 \cdot 2H_2O$;

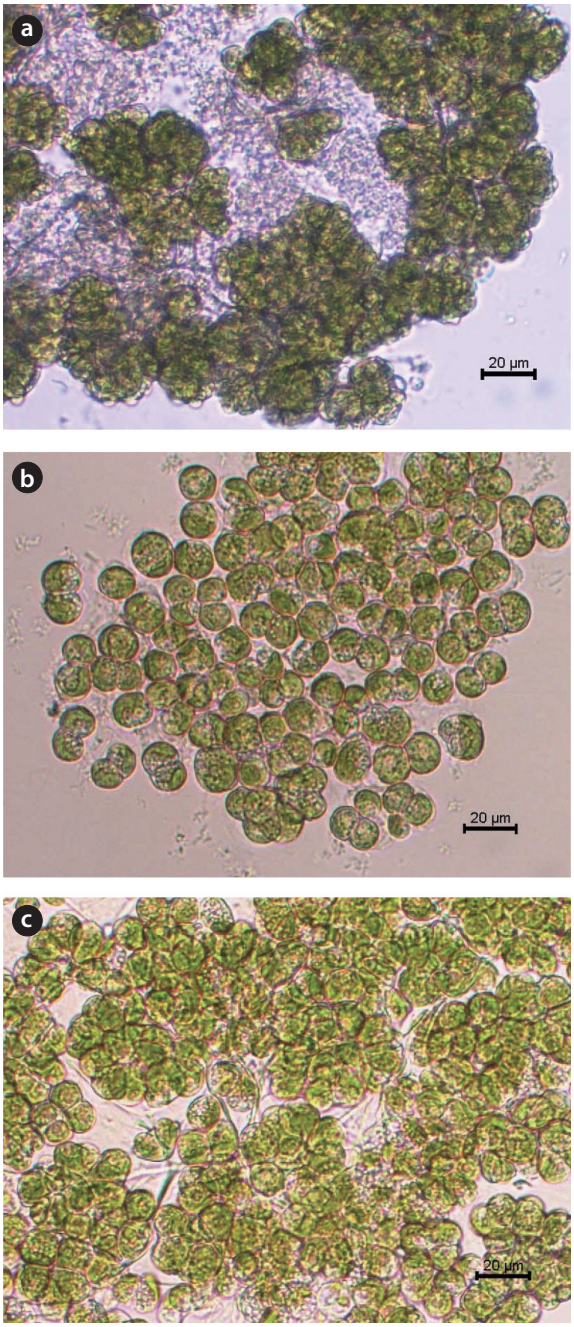

Fig. 1. Light micrographs of the JJS (a), KCM (b), and KJD (c) strains of *Botryococcus braunii*. Scale bars, 20 μm.

Table 1. Geographic location, collection date, and collection site altitude of the 3 Korean strains of *Botryococcus braunii* examined in the present study

Strain*	Location	Collection date	Altitude (m)
JJS	33°21′54″ N 126°27′03″ E	Sep. 14, 2010	980
KCM	36°22′14″ N 129°04′23″ E	Apr. 25, 2010	250
KJD	37°11′20″ N 128°48′39″ E	Apr. 24, 2010	1,136

*The three strains were collected at water bodies in the following sites: JJS, Jeju Island; KCM, Kyungpook province; and KJD, Kangwon province.

0.1, Citric acid; 0.02, Ferric citrate; and Trace metals solution. For temperature experiments, the three strains were cultured in BG-11 medium at 15°C, 20°C, 25°C, and 30°C under about 80 μmol m^{-2} s^{-1} of cool white fluorescent light on a 16 h light:8 h dark cycle with 100 mL min^{-1} aeration. All experiments were conducted in triplicate in 500-mL glass bottles after adaptation for two weeks in the selected growth medium and temperature.

Measurement of biomass and growth rate

For measurement of dry cell weight (DCW), 0.5 mL of each sample was filtered onto a weighed glass microfiber filter (25 mm GF/C; Whatman, Buckinghamshire, UK) every three days. These samples were washed with distilled water, dried at 70°C for 24 h, and weighed with a XM 1000P microbalance (Sartorius, Goettingen, Germany). The growth rate (g day^{-1}) is expressed as $\mu = \ln(N_2/N_1)/(t_2 - t_1)$, where N_2 and N_1 are the DCW during the period of exponential growth at times t_2 and t_1, respectively (Levasseur et al. 1993).

Extraction and analysis of total lipids

The total lipids produced at each temperature were extracted with a modification of the method described by Bligh and Dyer (1959), using a chloroform:methanol ratio of 1:2. The chloroform layer was separated and evaporated in the clean bench, and the remaining extract was weighed with a XM 1000P microbalance (Sartorius).

The lipid components were measured by gas chromatography (GC-2010; Shimadzu, Kyoto, Japan). First, 50 mg of each sample was saponified by addition of 1 mL of the saturated KOH-CH_3OH solution at 75°C for 10 min, and then submitted to methanolysis with 5% HCl in methanol at 75°C for 10 min. Then, the fatty acid layer was separated by adding 2 mL of distilled water. The peaks of the nine lipid components were identified by comparison with standards (Xu et al. 2001).

RESULTS

Growth in different media

After 21 days of growth, in the JJS strain, the DCW and the growth rate were greatest in BG-11 medium (0.430 g L^{-1} and 0.078 g day-1) and lowest in DY-III medium (0.222 g L^{-1} and 0.047 g day-1) (Figs. 2a and 3). In the KCM strain, the DCW and the growth rate were greatest in BG-11 me-

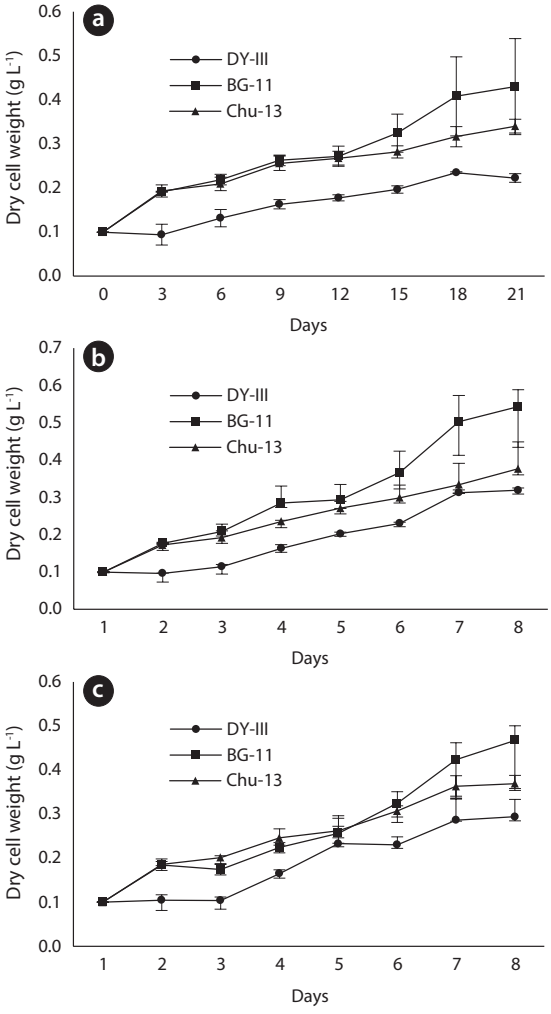

Fig. 2. Accumulation of dry cell weight (g L^{-1}) in the JJS (a), KCM (b), and KJD (c) strains of *Botryococcus braunii* that were cultured in different growth media. Error bars represent standard deviation.

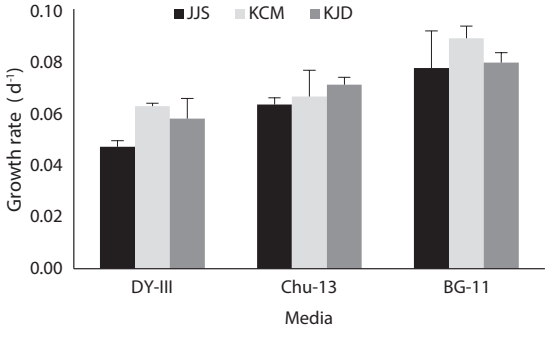

Fig. 3. Growth rate (g day^{-1}) of the three strains (JJS, KCM, and KJD) of *Botryococcus braunii* that were cultured in different growth media. Error bars represent standard deviation.

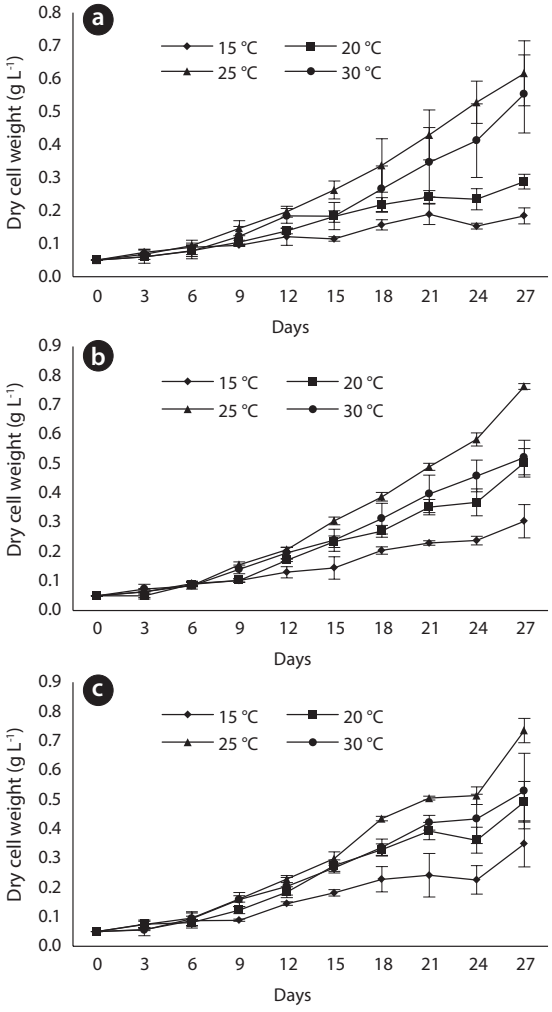

Fig. 4. Accumulation of dry cell weight of the JJS (a), KCM (b), and KJD (c) strains of *Botryococcus braunii* that were cultured at different temperatures in BG-11 medium. Error bars represent standard deviation.

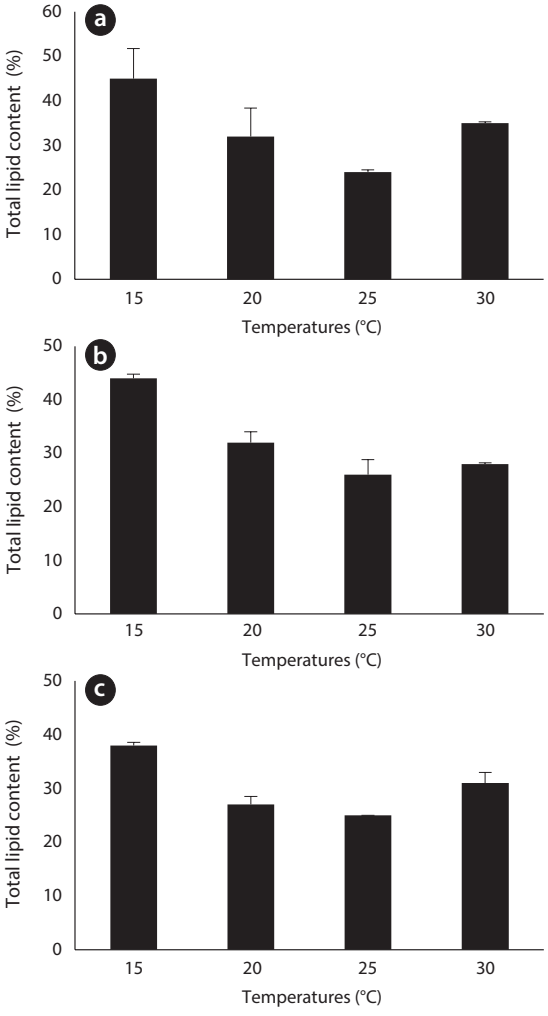

Fig. 6. Percent dry weight of lipids in the JJS (a), KCM (b), and KJD (c) strains of *Botryococcus braunii* that were cultured at different temperatures in BG-11 medium. Error bars represent standard deviation.

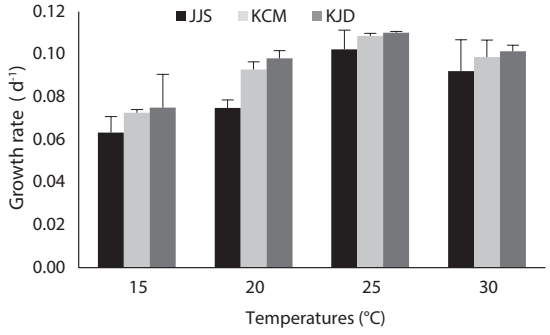

Fig. 5. Growth rate (g day^{-1}) of the three strains of *Botryococcus braunii* that were cultured at different temperatures in BG-11 medium. Error bars represent standard deviation.

dium (0.543 g L^{-1} and 0.090 g day-1) and lowest in DY-III medium (0.319 g L^{-1} and 0.063 g day^{-1}) (Figs. 2b and 3). In the KJD strain, the DCW and the growth rate were greatest in BG-11 medium (0.457 g L^{-1} and 0.080 g day^{-1}) and lowest in DY-III medium (0.294 g L^{-1} and 0.058 g day^{-1}) (Figs. 2c and 3).

Growth at different temperatures

After 27 days of growth, in the JJS strain, the DCW and the growth rate were greatest at 25°C (0.616 g L^{-1} and 0.102 g day-1) and lowest at 15°C (0.184 g L^{-1} and 0.063 g day-1) (Fig. 4a and 5). In the KCM strain, the DCW and the growth rate were greatest at 25°C (0.763 g L^{-1} and 0.109 g day-1) and lowest at 15°C (0.304 g L^{-1} and 0.073 g day-1) (Figs. 4b

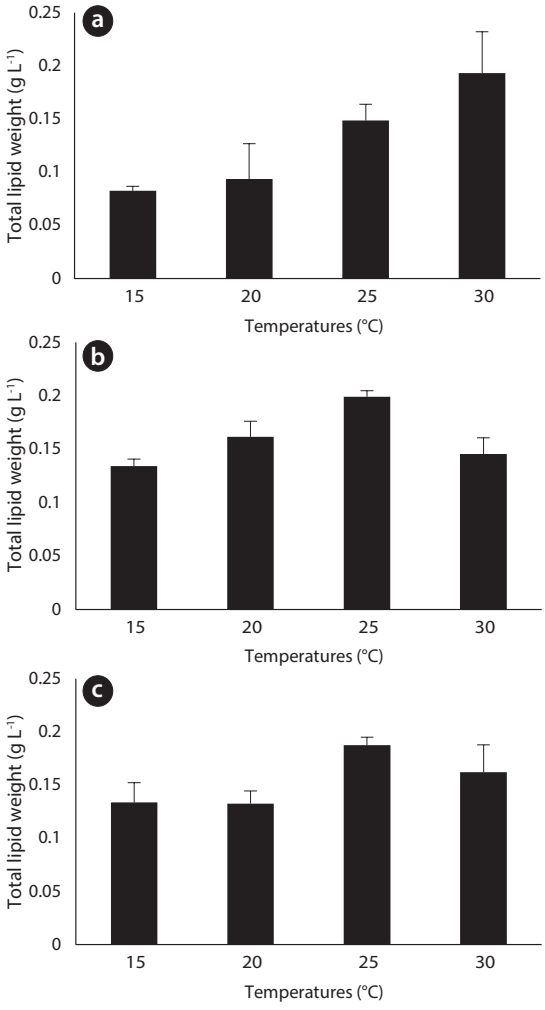

Fig. 7. Total lipid yield (g L^{-1}) of the JJS (a), KCM (b), and KJD (c) strains of *Botryococcus braunii* that were cultured at different temperatures in BG-11 medium. Error bars represent standard deviation.

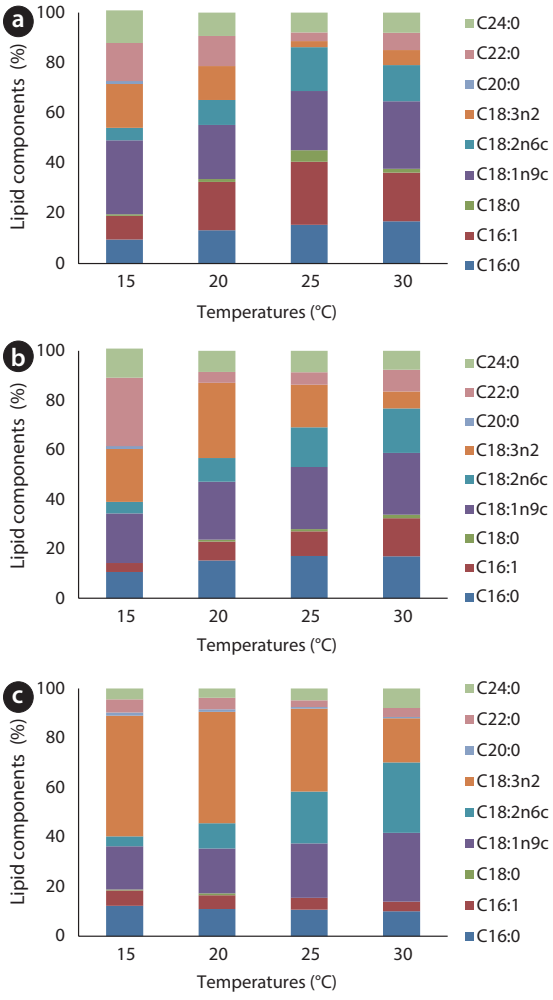

Fig. 8. Lipid profiles of the JJS (a), KCM (b), and KJD (c) strains of *Botryococcus braunii* that were cultured at different temperatures in BG-11 medium.

and 5). In the KJD strain, the DCW and the growth rate were greatest at 25°C (0.735 g L^{-1} and 0.110 g day^{-1}) and lowest at 15°C (0.349 g L^{-1} and 0.075 g day^{-1}) (Figs. 4c and 5).

Total lipid content at different growth temperatures

After 27 days of growth, in the JJS strain, it had the greatest percent dry weight of lipids at 15°C (45%) and the lowest at 25°C (24%) (Fig. 6a); the total lipid yield was greatest at 30°C (0.193 g L^{-1}) and lowest at 15°C (0.083 g L^{-1}) (Fig. 7a). In the KCM strain, it had the greatest percent dry weight of lipids at 15°C (44%) and the lowest at 25°C (26%) (Fig. 6b); the total lipid yield was greatest at 25°C (0.199 g L^{-1}) and lowest at 15°C (0.134 g L^{-1}) (Fig. 7b). In the KJD strain, it also had the greatest percent dry weight of lipids at 15°C (38%) and the lowest at 25°C (25%) (Fig. 6c); the total lipid yields were similar at temperatures below 20°C (0.132-0.134 g L^{-1}) and were maximal at 25° C (0.187 g L^{-1}) (Fig. 7c).

Lipid profiles at different growth temperatures

For all tested temperatures except 25°C, the amount of oleic acid (C18:1) was greatest in the JJS strain; at 25°C, the amount of palmitoleic acid (C16:1) was greatest, and oleic acid was second greatest (Fig. 8a). In the KCM strain, the amount of oleic acid was greatest at relatively high temperatures (25-30°C); linolenic acid (C18:3) was greatest at

15°C, behenic acid (C22:0) was greatest at 20°C, and oleic acid was second greatest at these two temperatures (Fig. 8b). The KJD strain produced the greatest amount of oleic acid at 30°C; at temperatures below 25°C, the amount of linolenic acid was greatest, and the proportion of oleic acid was second greatest (Fig. 8c).

DISCUSSION

Previous studies used the Chu-13 medium (Sawayama et al. 1994, Dayananda et al. 2005, Ranga Rao et al 2007, Ashokkumar and Rengasamy 2012) or the BG-11 medium (Dayananda et al. 2007, Ge et al. 2011) for culturing of *Botryococcus braunii*. Thus, the present study tested the effect of the growth medium on the three Korean strains of *B. braunii* by incubation in BG-11, Chu-13, and DY-III media. All three strains grew best in BG-11 medium and worst in DY-III medium. Previous papers reported a critical effect of nitrate on the growth of *B. braunii* (Xu et al. 2001, Zhila et al. 2005a, 2005b, Choi et al. 2011, Kim et al. 2012). In fact, the concentration of nitrate was highest in BG-11, intermediate in Chu-13, and lowest in DY-III, in a ratio of 49:11:1. Further studies of effects of nitrate and other nutrients on growth of *B. braunii* are required to determine whether the nitrate concentration is growth-limiting.

Lupi et al. (1991) reported that *B. braunii* cannot grow at temperatures higher than 32°C, and no previous research has examined the effect of growth below 18°C. Thus, we examined the effect of temperature on growth in the range of 15-30°C. All three of the studied strains grew best at 25°C and worst at 15°C, as previously reported for the UC58 strain (Lupi et al. 1991). In contrast, the LB807/1 strain (Kalacheva et al. 2002) and the CHN strain (Li and Qin 2005) grew best at 32°C and 20°C, respectively. However, the three strains of *B. braunii* examined in the present study had 63-68% of the maximum growth rate at 15°C. Thus, compared with other studied strains of *B. braunii* (Kalacheva et al. 2002, Li and Qin 2005), these three Korean strains are relatively tolerant to a wide range of temperatures.

The effect of temperature on the lipid content and profile were also analyzed to determine the optimal culture conditions for biodiesel production. All strains had the greatest amount of lipids per g DCW at 15°C and the lowest amount at 25°C. Kalacheva et al. (2002) reported that environmental conditions (e.g., temperature or light intensity) which favor a high growth rate in *B. braunii* lead to less storage of high energy products, in agreement with the results of the present study. However, the total lipid yield (g L^{-1}) is different from the amount of lipids per g DCW. In particular at 15°C, the total lipid yields of all strains were relatively low due to the low growth rate at this temperature, even though the lipids per g DCW were the highest at 15°C. The KCM and KJD strains had the highest lipid yields at 25°C, and their growth rates were also highest at this temperature. Interestingly, the JJS strain had the highest lipid yield at 30°C, but the highest growth rate at 25°C. Therefore, the optimal temperature for oil production in the JJS strain was 30°C, but the optimal temperature for the KCM and KJD strains was 25°C, similar to that reported in previous studies of non-Korean isolates (Kalacheva et al. 2002, Li and Qin 2005). Intriguingly, these two strains had 65-70% of the maximum yield at all tested temperatures. This may be an advantage, because it means reduced expenditures for maintenance of temperature in regions with variable climate and low-temperature regions.

The lipids extracted from microalgae consist of various fatty acids (Zhila et al. 2005a, 2005b). Oleic acid (C18:1) is easily converted into biodiesel due to its low melting point (-20°C) and strong stability (Knothe 2008). The present study examined the 9 lipid components, including oleic acid, palmitoleic acid (C16:1), and linolenic acid (C18:3), that were produced by three Korean strains of *B. braunii* at different temperatures. Oleic acid was most common lipid in the JJS strain at all temperatures except 25°C, in the KCM strain at 25°C and 30°C, and in the KJD strain at 30°C. At other temperatures, oleic acid was the second most common lipid in the KCM and KJD strains. Thus, all three of these Korean strains appear to be suitable as sources of biodiesel due to their high proportions of oleic acid.

Taken together, the results of this study indicate that these three Korean strains of *B. braunii* should be considered for the production of biodiesel. Additional studies of the effects of nutrients, light intensity, and of other isolates are still needed.

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