Iron and glucose effects on the morphology of *Botryococcus braunii* with assumption on the colony formation variability

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Abstract The axenic strain BOT-22 (race B) of *Botryoco ccus braunii* was investigated for the influence of iron and glucose on its morphology to reveal the relationship with growth and oil production. The average size of the iron-rich cells was larger than that of iron-limited cells. The shapes of iron-rich cells were elliptical and that of iron-limited cells were conical. Several cells were tightly connected to form large colonies in the iron-rich culture; however, only a few cells were sparsely arranged to form the small colonies in the iron-limited culture. Glucose addition to the iron-rich culture further increased the size of the cells and colonies. The growth increased in the following order: iron-limited, iron-rich, and iron-rich with glucose cultures. The same was observed for the volume of hydrocarbons in iron-rich cultures. It was also speculated that the shapes of cells and the amount of extracellular oil are most likely related to colony size and form.

Keywords *Botryococcus braunii* · Chlorophyta · Colony size · Iron-rich · Iron-limited · Glucose

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Introduction

The colony-forming green alga *Botryococcus braunii* Kützing produces a high amount of oil, representing a potential alternative to fossil fuel resources. Several transmission and scanning electron micrographs and confocal laser scanning micrographs revealed that *B. braunii* has complicated cell and colony structures (Hirose and Ogasawara 1977; Wolf and Cox 1981; Largeau et al. 1980; Berkaloff et al. 1984; Plain et al. 1993; Beakes and Cleary 1999). This alga produces liquid hydrocarbons in its cells and accumulates them into the intracolonial extracellular matrix space filled with a cross-linked hydrocarbon network (Weiss et al. 2012).

A cell of *B. braunii* is covered with an outer wall and a polysaccharide internal layer (Berkaloff et al. 1984; Casadevall et al. 1985; Allard and Casadevall 1990; Metzger et al. 1990; Simpson et al. 2003). The outer cell wall is mainly composed of algaenans consisting of two components: (1) a thin and transparent cell cap that covers the apical part of the cell and extrudes from the colony (Wolf and Cox 1981; Weiss et al. 2012) and (2) a thick basal envelope that is embedded in a colony filled with hydrocarbons (Plain et al. 1993).

Although many studies have been carried out on the effects of environmental factors and nutrients on growth and hydrocarbon contents of *B. braunii*, little is known about their effects on its morphology. The morphological variation in *Botryococcus* colonies is extensive (Komarek and Marvan 1992). Samples collected in nature generally comprise bulky colonies containing many cells. In contrast, liquid laboratory cultures frequently produce small colonies

(Berkaloff et al. 1984). Zhang and Kojima (1998) found that both cell and colony size increased significantly with light intensity, inferring that colony size was related to the effect of light intensity on the concentration of polysaccharides, which aid in colony formation, or to the dynamic balance between the mechanical strength of colonies and hydrodynamic stress from turbulence. In our previous study (Tanoi et al. 2011), it was revealed that, in the presence of glucose and light conditions, not only the growth of axenic strain B70 of *B. braunii* was greatly enhanced, but also its cell and colony size and the intracellular granules containing oil were markedly larger than those cultured without glucose. Because this was true even in static cultures, we considered that turbulence was not a major factor but large cells that form colonies or extracellular secretions that function to hold cells together to form colonies (e.g., algaenan) were responsible for increasing colony size. However, the relationship among colony morphology, growth, and oil production still remains unclear.

In this study, we found that iron and glucose not only affect the growth but also the morphology and most possibly oil production of *B. braunii*. Here, we show that cells, colony sizes and shapes, growth, and oil production are affected by different concentrations of iron and also by glucose addition. Based on the results, we can speculate about colony-forming processes that takes place beginning with a single cell.

Materials and methods

Strain BOT-22 of *B. braunii* was isolated from a dam in Ishigaki Island, Okinawa Prefecture in Japan. This strain was sterilized with sodium hypochlorite containing 0.01 % available chlorine, and its axenic state was observed and confirmed using a microscope and by agar plate check with bacterial contamination test medium containing yeast extract (4 g L^{-1}), malt extract (10 g L^{-1}), glucose (4 g L^{-1}), and agar (20 g L^{-1}) (adjusted to pH 7.3). This strain mainly produces $C_{34}H_{58}$ triterpenoid (Prof. Kunimitsu Kaya of the University of Tsukuba, personal communication).

Modified Chu medium was used for subculturing the strain and for the present experiments. The medium contained KNO_3 (0.2 g L^{-1}), K_2HPO_4 (0.04 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.1 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.054 g L^{-1}), iron(III) citrate hydrate (0.1 mg L^{-1}), and citric acid monohydrate (100 mg L^{-1}). In addition, the medium also contained microelements: $MnCl_2 \cdot 4H_2O$ (18.1 µg L^{-1}), $ZnSO_4 \cdot 7H_2O$ (2.22 µg L^{-1}), $Na_2MoO_4 \cdot 2H_2O$ (0.5 µg L^{-1}), and $CoCl_2 \cdot 6H_2O$ (0.8 µg L^{-1}). The pH was adjusted to 7.0 with 0.1 N NaOH, and the medium was autoclaved. During preliminary experiments, we confirmed that citrate alone had no effect on the growth of *B. braunii* (data not shown).
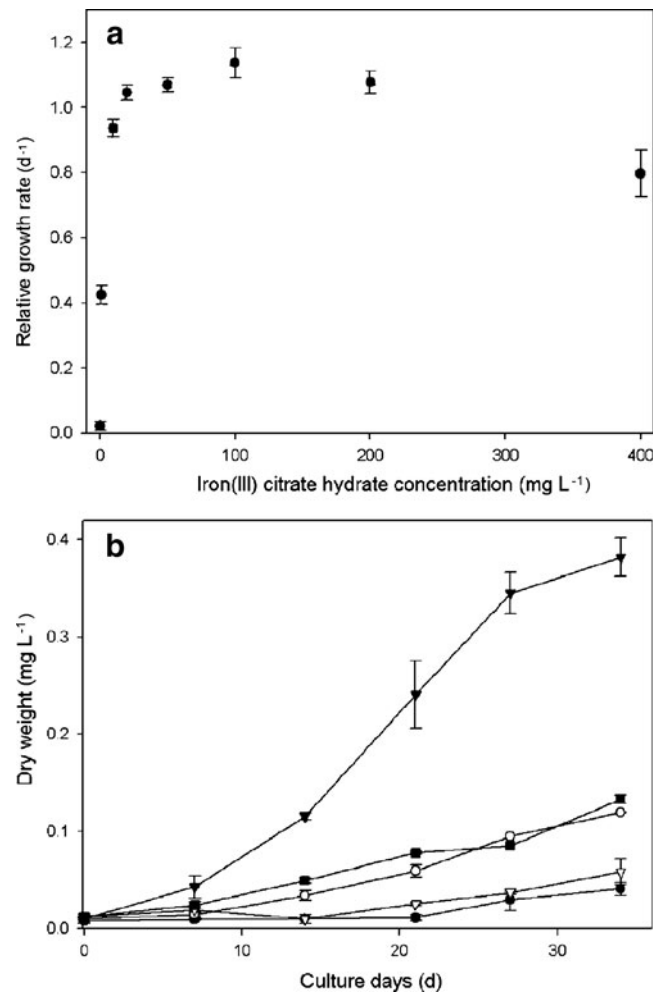

Fig. 1 a Effects of iron(III) citrate hydrate on the growth rate of *B. braunii* strain BOT-22 in microplate culture. **b** The growth curve of *B. braunii* strain BOT-22 at different combinations of iron(III) citrate hydrate concentrations from the precultures (0.1 mg L^{-1} in *open triangle* and *filled square*; 20 mg L^{-1} in *filled circle*, *open circle*, and *filled triangle*) to the experimental cultures (0.1 mg L^{-1} in *open triangle* and *filled circle*; 20 mg L^{-1} in *filled square*, *open circle*, and *filled triangle*); 1 g L^{-1} of glucose was added in experimental culture of *filled triangle*

The effect of Fe citrate hydrate on the growth is therefore due to the iron and not due to the citrate component. To determine the appropriate iron concentrations for this experiment, the subcultured algae cells were inoculated into a 96-well microplate with the media containing 0.1, 1, 10, 50, 100, 200, and 400 mg L^{-1} of iron(III) citrate hydrate and the relative growth rates were measured using the in vivo fluorescence method (Tanoi et al. 2011) from day 1 to day 23 after inoculation when the logarithmic growth phase continued.

In order to investigate the effects of iron and glucose on the morphology of strain BOT-22, cells were cultured at 25 °C in a 500-mL flask with 200 mL of modified Chu medium containing 0.1 mg L^{-1} of iron(III) citrate hydrate

Table 1 Growth rates and size of cells cultured under different conditions. The growth rates were calculated from sequential daily dry weight up to 21 days; (*n*) are the replicated numbers for measurements and (±) the standard deviation

Concentration of iron(III) citrate hydrate		Growth rate (day^{-1}) (*n*=4)	Cell area (µm^2) (*n*=40)	Cell length (µm) (*n*=40)	Cell width of lower 1/3 ordinate (µm) (*n*=40)	Cell width of upper 1/3 ordinate (µm) (*n*=40)
Preculture	Culture					
0.1 mg L^{-1}	0.1 mg L^{-1}	0.037±0.006	71.2±11.5	12.1±1.16	4.73±0.47	6.17±0.66
	20 mg L^{-1}	0.091±0.006	74.2±9.6	12.1±1.38	5.81±0.70	6.10±0.83
20 mg L^{-1}	0.1 mg L^{-1}	0.039±0.007	74.4±9.5	12.8±0.94	4.98±0.59	6.46±0.64
	20 mg L^{-1}	0.093±0.018	83.6±11.4	12.5±1.43	6.03±0.67	6.93±0.82
	20 mg L^{-1}+glucose 1 g L^{-1}	0.153±0.012	119.1±15.0	14.5±1.37	7.74±1.00	8.68±0.93

for 1 month under a 12 L:12 D cycles with cool white fluorescent illumination (100 µmol photons m^{-2} s^{-1}).

The cultured cells were inoculated into 500-mL flasks with 200 mL of two kinds of modified Chu medium containing 0.1 mg L^{-1} of iron(III) citrate hydrate or 20 mg L^{-1} of iron(III) citrate hydrate and then precultured under the same above-mentioned conditions. One month after inoculation, the precultured cells were filtered with a sterilized 5 µm pore polycarbonate membrane (Nuclepore, Whatman) and rinsed with sterilized water. Next, approximately 0.01 mg L^{-1} (dry weight) of cells was transferred to each 500 mL experimental culture flask with 200 mL modified Chu medium supplemented with (A) 0.1 mg L^{-1} of iron(III) citrate hydrate, (B) 20 mg L^{-1} of iron(III) citrate hydrate, or (C) 20 mg L^{-1} of iron(III) citrate hydrate and 1 g L^{-1} of glucose. Each culture experiment was carried out in duplicate.

For the dry weight measurement, cells were firstly filtered on a dried glass fiber filter (GF/C 25 mm diameter, Whatman), then rinsed with distilled water and finally dried at 70 °C for 24 h. Loss of water-soluble exocellular biomass was ignored. The specific growth rates were calculated from the 0 to 21 days sequential values of dry biomass.

Measurement of cells and particle size

To measure the cell area, length, and diameter, digital photos were taken by mounting cells on a microscope slide with a cover glass. The cover glass was supported by two other cover glasses so as to not apply pressure on cells. Forty cells were measured from each culture conditions.

Maximum Feret diameter is the greatest distance between any two points along the boundary of a region of interest. Maximum Feret diameters of 300 particles, as indicated by a two-dimensional display, were measured with a FlowCAM (Fluid Imaging Technologies). The instrument conditions used to measure colony size were as follows: gain=4, threshold=100, cell size range=5 to 300 µm, and minimum distance to nearest neighbor=25.

Observation by fluorescence microscope

For comparing the relative abundance of oil content, Nile red solution in ethanol (0.1 mg mL^{-1}) was added to the algal suspension at a final concentration of 1 µg mL^{-1}. Fluorescence of Nile red was observed using a fluorescence mirror unit U-MWB2 (Olympus) that contained a 460- to 490-nm excitation filter and a 510-nm absorption filter. Chloroplasts were observed using a fluorescence mirror unit U-MWU2 (OLYMPUS) that contained a 330- to 385-nm excitation filter and a 420-nm absorption filter.

Observation by scanning electron microscope

Algal cultures were fixed in mixture of 2.5 % glutaraldehyde and 1 % OsO$_4$ in 0.1 M cacodylate buffer with 0.1 M

Fig. 2 Light micrographs of single cells at different concentrations combinations of iron(III) citrate hydrate from the precultures (**a** and **b** at 0.1 mg L^{-1}; **c**–**e** at 20 mg L^{-1}) to the experimental culture conditions (**a** and **c** at 0.1 mg L^{-1}; **b** and **d** at 20 mg L^{-1}; **e** at 20 mg L^{-1} with 1 g L^{-1} of glucose). Same magnification in **a**–**e**, the scale bar=10 µm
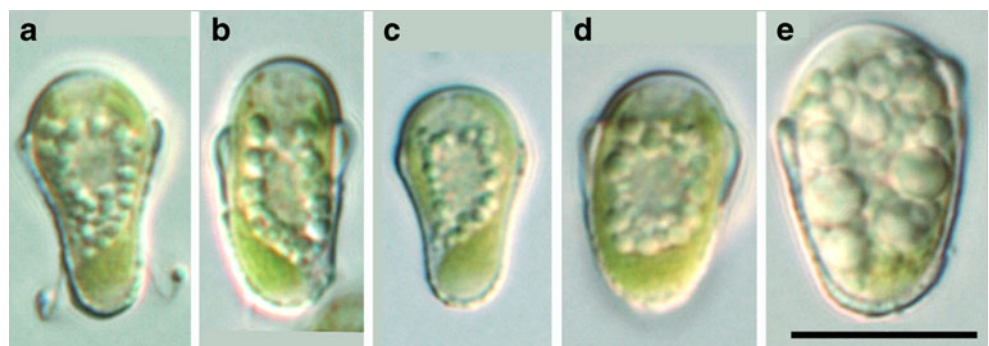
sucrose (pH 7.2) for 12 hours at 5 °C. The samples were centrifuged then washed with 0.1 M cacodylate buffer twice. Supernatants were removed and the fixed materials were put on glass plates coated with poly-L-lysine and dehydrated with a graded ethanol series. Ethanol was then replaced with *t*-butyl alcohol. The materials were frozen at 4 °C and vacuum dried. Dried samples were coated with OsO_4 by osmium coater (Vacuum Device Inc., HPC-1S) and then examined with a scanning electron microscope (Hitachi, S-4800).

Results and discussion

The influence of iron and glucose on cell and colony size

Because iron-limited and maximum relative growth rates were obtained at 0.1 to 1.0 mg L^{-1} and 10 to 200 mg L^{-1} of iron(III) citrate hydrate concentrations, respectively (Fig. 1a), hence, 0.1 and 20 mg L^{-1} of iron(III) citrate hydrates were chosen for the study on the influence of iron on morphology, each of which hereafter is designated as "iron-limited" or "iron-rich,"
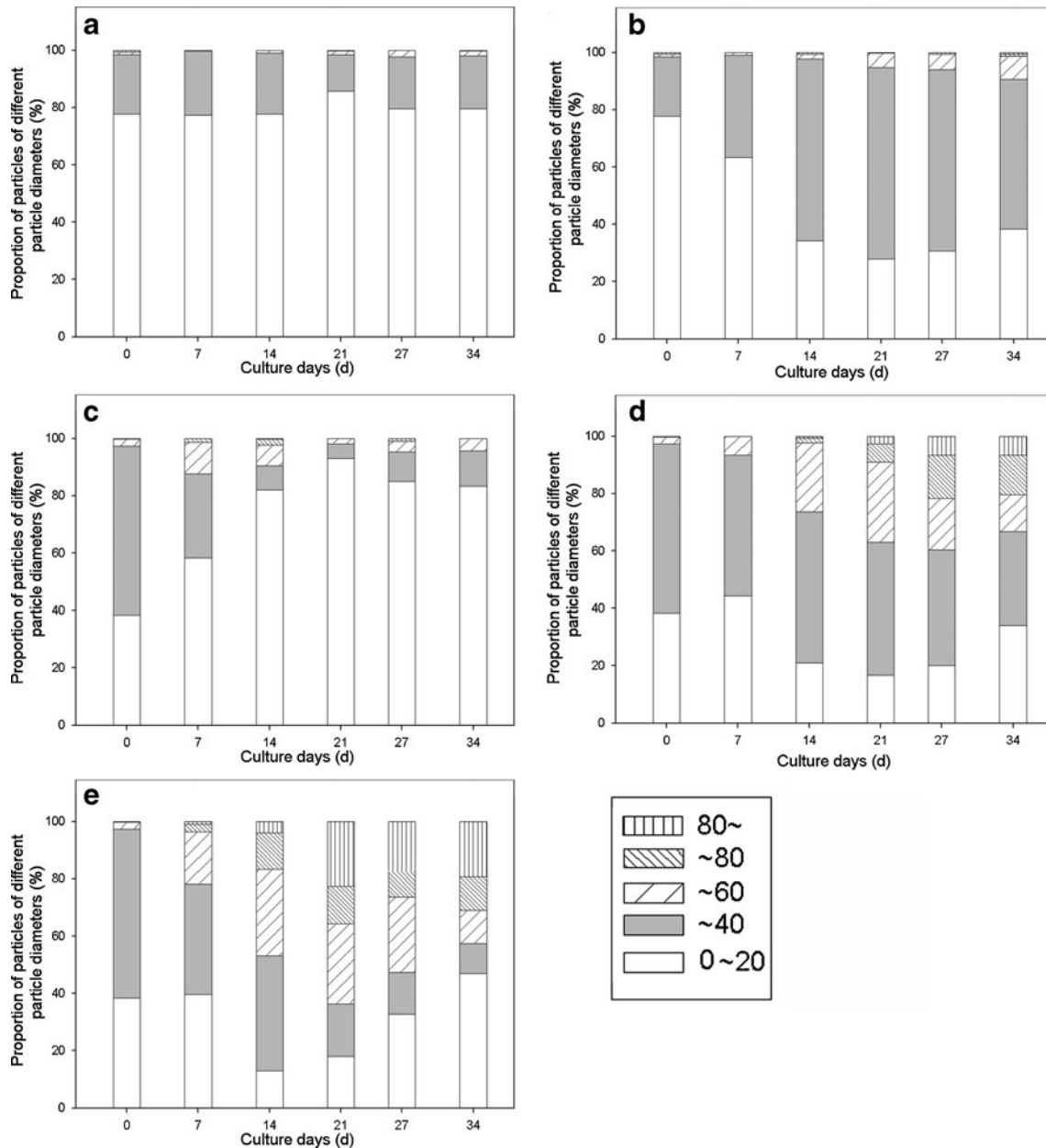

Fig. 3 Proportions of particles of different diameters (less than 20, 20–40, 40–60, 60–80 μm, and more than 80 μm), including cells and colonies at different combinations of iron(III) citrate hydrate concentrations from the precultures (**a** and **b** at 0.1 mg L^{-1}; **c–e** at 20 mg L^{-1}) to the experimental cultures conditions (**a** and **c** at 0.1 mg L^{-1}; **b** and **d** at 20 mg L^{-1}; **e** at 20 mg L^{-1} with 1 g L^{-1} of glucose)

Fig. 4 Light and fluorescence micrographs of colonies stained with Nile Red. The colonies were cultured at different combinations of iron(III) citrate hydrate concentrations from the precultures (**a–c** at 0.1 mg L^{-1}; **d–i** at 20 mg L^{-1}) to the experimental culture conditions (**a–c** at 0.1 mg L^{-1}; **d–f** at 20 mg L^{-1}; **g–i** at 20 mg L^{-1} with 1 g L^{-1} of glucose). All scale bar = 10 μm
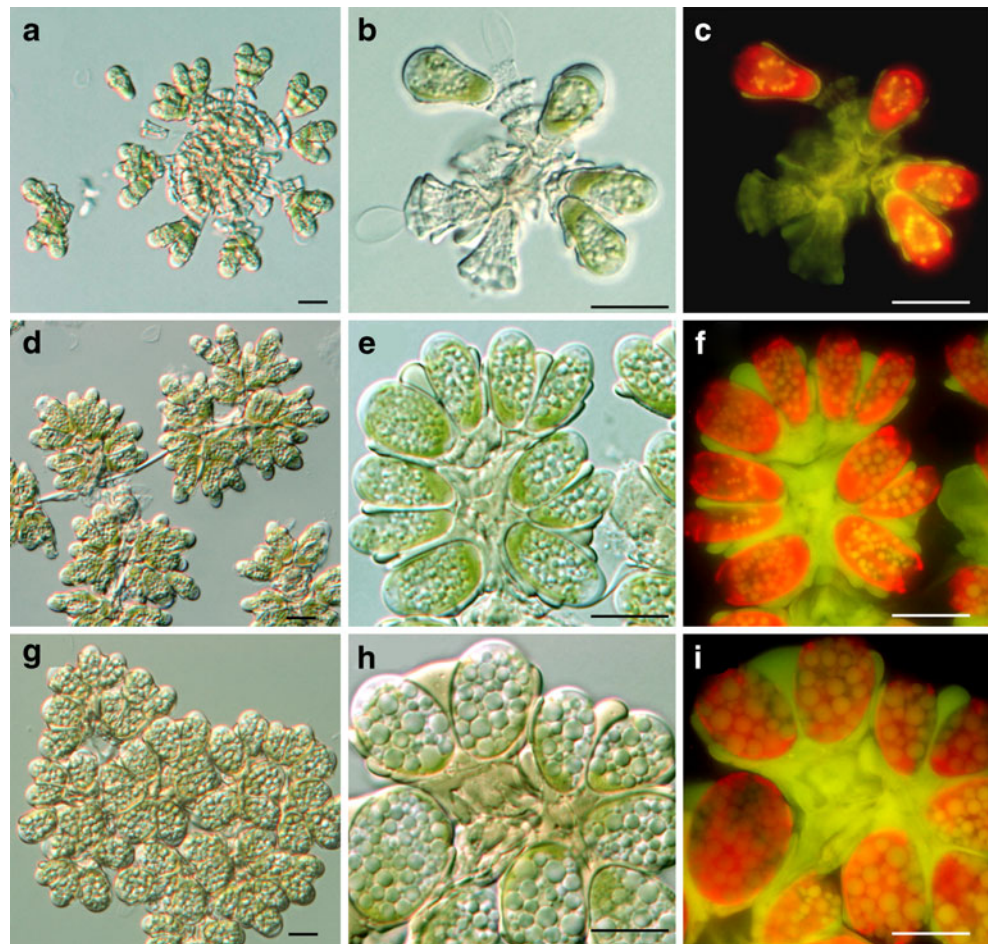
respectively. The growth rates were not affected by iron concentration in the preculture (Fig. 1b and Table 1). The average diameter of the lower part of the cells in iron-rich cultures was larger than that in iron-limited cultures, although the cell lengths were approximately equal to each other (Figs. 2a–d and 5b, c and Table 1). The shapes of iron-rich cultured cells were elliptical (Fig. 2b and d), but those of iron-limited cultured cells were conical (Fig. 2a, c). The maximum cell length and width and the maximum growth rate (Fig. 1b and Table 1) were obtained in the presence of iron and glucose (Figs. 2e and 5d).

The proportion of particles containing cells and colonies was constant when cells were precultured with 0.1 mg/L^{-1} of iron(III) citrate hydrate (Fig. 3a). Approximately 80 % of the particles consistently maintained sizes less than 20 μm, and thus, there were many single cells in the culture. This suggests that large colonies were minutely formed in the iron-limited culture. After the iron-limited precultured cells were transferred to the iron-rich medium, the number of particles with sizes less than 20 μm decreased and the number of particles with sizes 20 to 80 μm increased (Fig. 3b). The large particles occupied more than 60 % of total particles after 14 days culture. On the other hand, when the cells were transferred from an iron-rich medium to an iron-limited medium, the growth was very slow compared to that of the iron-rich culture (Fig. 1b) and the particle size decreased and particles with a size of less than 20 μm accounted for more than 80 % of total particles after 14 days culture (Fig. 3c). In this case, the proportion after culturing for 14 days approximated that of the iron-limited preculture particles (Fig. 3a). This suggests that single cells and small colonies were released from large colonies and the growth shifted to that in iron-limited cultures. When iron-rich precultured cells were cultured in iron-rich medium again, the ratio of large particles not only increased but also there was a significant increase in particles with a size of more than 60 μm (Fig. 3d). In the presence of sufficient iron and glucose, the particles became larger than those without glucose (Fig. 3e). Particles with more than 80 μm in diameter occupied 20 % of the total particles after culturing for 21 days. However, the ratio of particles less than 20 μm increased after 21 days in both iron-rich culture and iron-rich culture with glucose (Fig. 3b, d, e), indicating that cells and small colonies were released from the large colonies
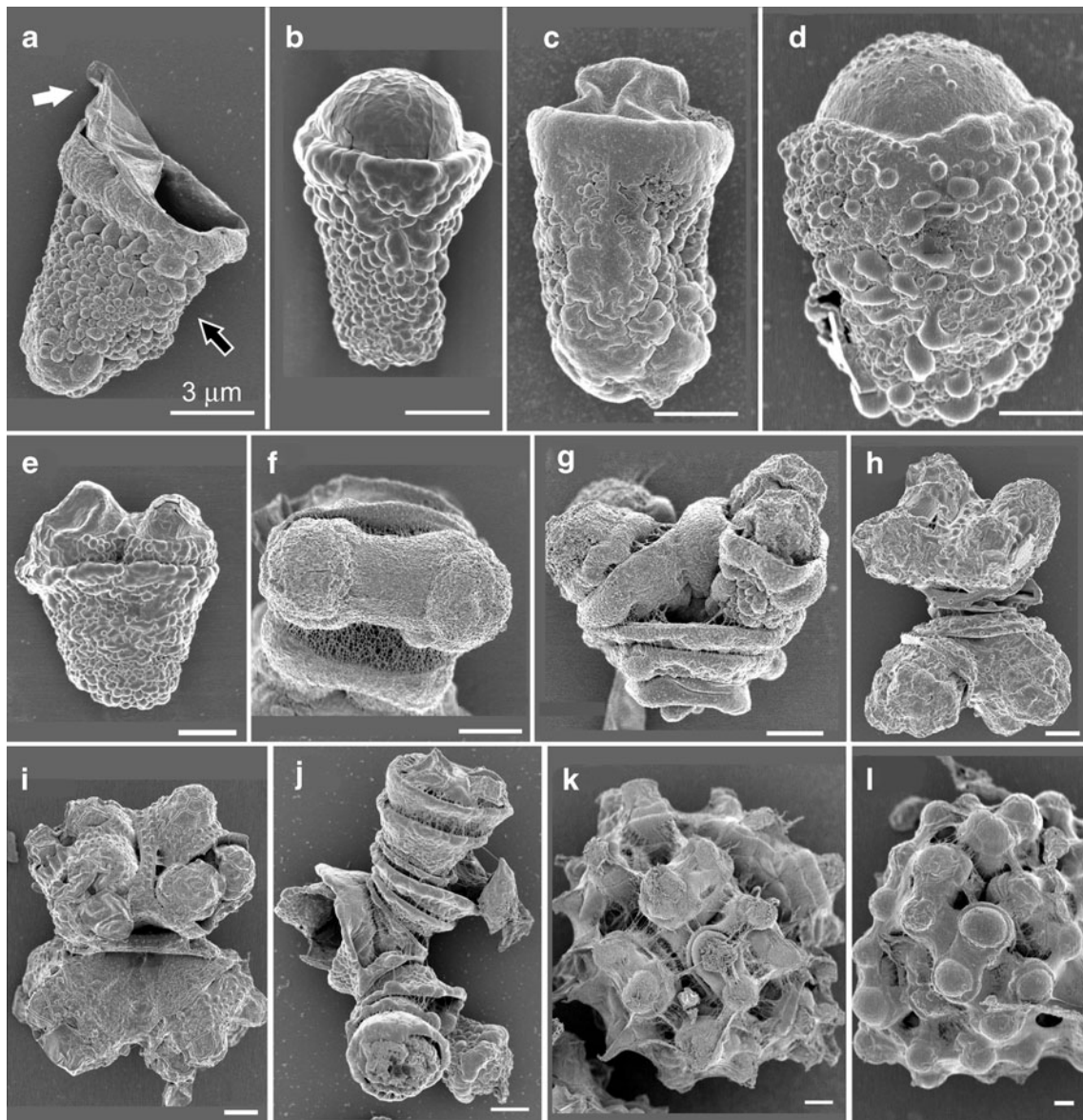
Fig. 5 SEM micrographs of different stages of *B. braunii* strain BOT-22. **a** A single cell wall showing cell cap (*white arrow*) and basal envelope (*black arrow*). **b, e–j** cells cultured with 0.1 mg L^{-1} of iron(III) citrate hydrate including a single cell (**b**), 2-cell stage (**e, f**), 4-cell stage (**g**), 8-cell stage (**h**), and 16-cell stage (**i**). **c, k** A single cell and colony cultured with 20 mg L^{-1} of iron(III) citrate hydrate. **d, l** A single cell and colony cultured with 20 mg L^{-1} of iron(III) citrate hydrate and 1 g L^{-1} of glucose. All scale bar = 3 μm

according as culture ages. It is speculated that there are two reasons for the release of cells from colonies: (1) cells were easy to drop out of the mother's basal envelope because their shape changed from elliptical to conical (Fig. 2a, c), and (2) as described later, the decrease in the growth rate under iron-limited conditions causes the reduction of materials such as oil and algaenan, which connect cells to each other (Fig. 4b, c).

The typical shape of the colony also changed because of different iron concentrations and also by glucose addition. Cells were sparsely arranged in colonies when cultured under the iron-limited condition (Fig. 4a). On the other hand, cells were tightly connected in colonies under the iron-rich condition (Fig. 4d, g). The largest colonies were composed of units of small sub-colonies that were connected by stalk-like structures containing oil (Fig. 4d). The sizes of each unit cultured without glucose (Fig. 4d) were smaller than those cultured with glucose (Fig. 4g). We did not perform quantitative analysis of lipids produced by BOT-22 cultured with iron; however, it is probable that iron increased the oil contents in the colony. We make this presumption because yellow fluorescence of Nile Red revealed that the volume of hydrocarbons surrounding cells was greater in highly dense

colonies seen in iron-rich cultures (Fig. 4e, f, h, i) than in sparse colonies seen in iron-limited cultures (Fig. 4b, c). This consideration can be supported by the recent report of Yeesang and Cheirsilp (2010) that iron increased oil accumulation in four strains of *Botryococcus*. In addition, a colony cultured with glucose had larger oil bodies in cells and larger amount of oils between cells than those cultured in iron-rich without glucose (Fig. 4f, i). Thus, supplements of sufficient iron or iron with glucose activated a higher production of secondary metabolites such as oils that strongly bonded between cells or between cells and mother's basal envelope, as shown in Fig. 4e, f, h, i, resulting in development into large colonies. Dayananda et al. (2005) reported that ferric citrate tended to have negative effect on the biomass yield of *B. braunii* A race, especially in the presence of magnesium. Such contradiction with our results might come from the race bias or the potential effect of iron–manganese concentrations levels used in the media. Specific experiments are needed to answer this disparity.

It should be noted that glucose addition significantly increased cell and colony size, further enhancing growth and possibly oil accumulation (Figs. 1b, 2e, 3e, and 4h, i). The same was suggested in strain B70 of *B. braunii* (Tanoi et al. 2011). Glucose is considered to be an effective organic carbon source for promoting growth and oil accumulation and increasing cell and colony size in *B. braunii*; however, glucose addition caused the release of colonies less than 20 μm at a culture age earlier than that of the additive-free, iron-rich culture (Fig. 3b, d, e), and at times, bleached the cells. Further investigation is needed to evaluate the effects of glucose.

Wake and Hillen (1980) reported a "bloom" of *B. braunii* in the surface water of Darwin River Reservoir, Australia. Iron concentration was low at the surface water of reservoir during the bloom but supply of iron from deeper waters was speculated. Indeed, deeper waters were reported to show discoloration and an iron concentration reaching 0.5 mg L^{-1} at 16 m depth was measured, together with seasonal water column vertical mixing. In nature, organic compounds like glucose would be immediately degraded by bacteria, but iron would affect significantly the growth of *B. braunii*.

The process of forming colonies

The cell division processes were observed under light and scanning electron microscopes. A part of the apical cell cap often attached to the basal envelope after the cell was released (Fig. 5a, white arrow). Thickening of the wall of the basal envelope was observed at the upper side around the joint of the cell cap. The cell division occurred at the basal part of the envelope so that the daughter cells were kept inside the mother's basal envelope (Fig. 5e, f). Old basal envelopes were accumulated below the cells which anchored the cells as a colony with alternate generations (Fig. 5g–i). In the iron-limited culture, basal envelopes sometimes developed into stalk-like structures (Fig. 5j). Much more extracellular matrix was observed among cells in colonies cultured with glucose (Fig. 5l) than those cultured without glucose (Fig. 5k).

From our observations on colony features in iron-limited, iron-rich and glucose-additive cultures, we propose the process of cell division and colony formation of *B. braunii* as shown in Fig. 6. During cell division, subsequent to the formation of a new cell wall, a mother's cell cap was detached from the basal envelope by the pressure of growing daughter cells while the basal envelope remained in the matrix. The basal envelopes of former
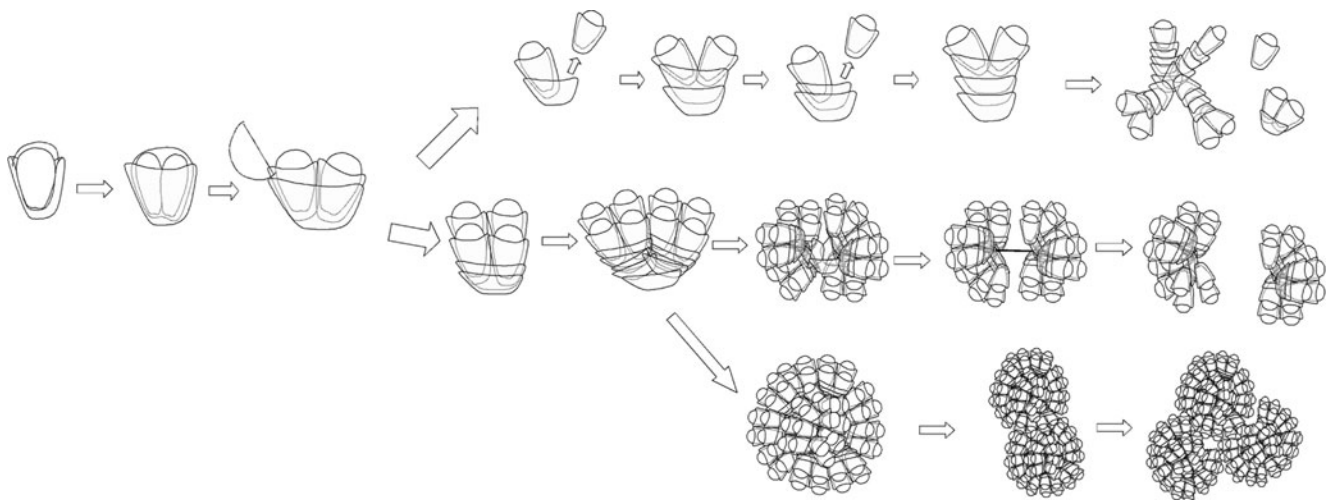

Fig. 6 Illustration of the cell division and colony formation of *B. braunii*. *Upper*, formation process of sparsely arranged colonies. *Middle*, process of colony division. *Bottom*, formation of larger size of colony

generations were accumulated below new cells and anchored the cells into a colony. This structure may play a fundamental role in the formation of colonies and as a reservoir for hydrocarbons secreted from cells. In cultures without iron, the conical shape of the daughter cells made it difficult for them to stick into the mother's basal envelope and basal envelopes did not bind them strongly into a colony because extracellular space was not filled with oils (Fig. 4c); therefore, small or sparsely arranged colonies with stalk-like structures made of cell walls were produced (Fig. 6, upper scheme). Zhang and Kojima (1998) reported that the average diameter of colonies of *B. braunii* decreased in low light. This suggests that low light may have similar physiological effects on forming colonies of *B. braunii* under iron-limited conditions.

On the other hand, in the presence of an abundance of iron, large and high-density colonies developed because the elliptical shape of daughter cells made it easier for them to remain in their mother's basal envelopes after division and adjacent daughter cells could be arranged tightly by increasing secretions of metabolites such as oils (Fig. 4g); however, the pressure between neighboring cells associated with colony development would cause the division of the colony (Fig. 6, middle scheme). Colony sizes may be decided by the angle between cells and the amount of secretions that hold the colony together. Glucose addition caused an increase in both cell size and the amount of secretions. Consequently, the colony size would be the largest (Fig. 6, bottom scheme). It should be noted that not only the iron and glucose but also other factors influencing the growth may cause similar morphological changes in *Botryococcus*. Namely, if the condition is good for growth, the shape is preferentially elliptical, while, if the condition is not optimal for growth, the shape may become conical. As the results of such morphological changes in cells, the colony size and shape gradually change. In this work, we focused on the iron and glucose factors, keeping the other factors constant. Iron and glucose critically enhanced the growth and resulted in clear morphological changes.

We conclude that iron concentration strongly influences the size, shape, growth, and oil production of a cell and colony. Glucose addition further enhances the effects of iron. It is a major finding that cell and colony sizes are closely related to growth and oil production. The appropriate additions of iron and glucose are highly effective in increasing the biomass and oil production of *B. braunii*.

References

Allard B, Casadevall E (1990) Carbohydrate composition and characterization of sugars from the green microalga *Botryococcus braunii*. Phytochemistry 29:1875–1878

Beakes GW, Cleary AL (1999) Visualization of plastids and lipophilic components in living colonies of a wild strain of the hydrocarbon-forming, green alga *Botryococcus* by laser scanning confocal microscopy. J Appl Phycol 10:435–466

Berkaloff C, Rousseau B, Couté A, Casadevall E, Metzger P, Chirac C (1984) Variability of cell wall structure and hydrocarbon type in different strains of *Botryococcus braunii*. J Phycol 20:377–389

Casadevall E, Dif D, Largeau C, Gudin C, Chaumont D, Desanti O (1985) Studies on batch and continuous culture of *Botryococcus braunii*: hydrocarbon production in relation to physiological state, cell ultrastructure, and phosphate nutrition. Biotechnol Bioeng 27:286–295

Dayananda C, Sarada R, Bhattacharya S, Ravishankar GA (2005) Effect of media and culture conditions on growth and hydrocarbon production by *Botryococcus braunii*. Process Biochem 40: 3125–3131

Hirose H, Ogasawara N (1977) Fine structural evidence for the systematic position of *Botryococcus braunii* Kutzing as a member of Chlorophyceae. Bull Jpn Soc Phycol 25:61–69

Komarek J, Marvan P (1992) Morphological differences in natural populations of the genus *Botryococcus* (Chlorophyceae). Arch Protistenkd 141:65–100

Largeau C, Casadeval E, Berkaloff C, Dhamelincourt P (1980) Sites of accumulation and composition of hydrocarbons in *Botryococcus braunii*. Phytochemistry 19:1043–1051

Metzger P, Allard B, Casadevall E, Berkaloff C, Couté A (1990) Structure and chemistry of a new chemical race of *Botryococcus braunii* (Chlorophyceae) that produces lycopadiene, a tetraterpenoid hydrocarbon. J Phycol 26:258–266

Plain N, Largeau C, Derenne S, Coutè A (1993) Variabilité morphologique de *Botryococcus braunii* (Chlorococcales, Chlorophyta): corrélations avec les conditions de croissance et la teneur en lipides. Phycologia 32:259–265

Simpson AJ, Zang X, Kramer R, Hatcher PG (2003) New insights on the structure of algaenan from *Botryococcus braunii* race A and its hexane insoluble botryals based on multidimensional NMR spectroscopy and electrospray–mass spectrometry techniques. Phytochemistry 62:783–796

Tanoi T, Kawachi M, Watanabe MM (2011) Effects of carbon source on growth and morphology of *Botryococcus braunii*. J Appl Phycol 23:25–33

Wake LV, Hillen LW (1980) Study of a "Bloom" of the oil-rich alga *Botryococcus braunii* in the Darwin River Reservoir. Biotech Bioeng 12:1637–1656

Weiss TL, Roth R, Goodson C, Vitha S, Black I, Azadi P, Rusch J, Holzenburg A, Devarenne TP, Goodenough U (2012) Colony organization in the green alga *Botryococcus braunii* (Race B) is specified by a complex extracellular matrix. Eukaryot Cell 11:1424–1440

Wolf F, Cox ER (1981) Ultrastructure of active and resting colonies of *Botryococcus braunii* (Chlorophyceae). J Phycol 17:395–405

Yeesang C, Cheirslip B (2010) Effect of nitrogen, salt, and iron content in the growth medium and light intensity on lipid production by microalgae isolated from freshwater sources in Thailand. Biores Technol 102:3034–3040

Zhang K, Kojima E (1998) Effect of light intensity on colony size of microalga *Botryococcus braunii* in bubble column photobio reactors. J Ferment Bioeng 86:575–576