1 **The surface structure of *Botryococcus braunii* colony prevents the entry of**
2 **extraction solvents into the colony interior**

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16 Abbreviations¹

¹energy profit ratio, EPR; pre-heating, PH; culture in brackish medium, CB; no treatment, NT

17 **Abstract**

18 The green colonial microalga *Botryococcus braunii* (race B) has attracted considerable
19 attention because it is known to produce many hydrocarbons. The hydrocarbons are
20 present in the extracellular matrix that connects the cells located in the interior of a *B.*
21 *braunii* colony; however, the surface of each colony is covered with a retaining wall and
22 a fibrillar colony sheath. This unique colony structure is thought to be associated with
23 hydrocarbon storage and known to affect the extractability of hydrocarbons by using an
24 organic solvent. In this study, we investigated the relation between hydrocarbon
25 extractability and changes in the colony surface structure after two distinct treatments:
26 culture of the algae in brackish medium or thermal pretreatment of the algae before
27 solvent extraction. Both the treatments improved hydrocarbon extractability when
28 non-polar solvents such as *n*-hexane were used. Thermal pretreatment inhibited the
29 formation of fibrils on the surface of algal colonies, as revealed by rapid-freezing and
30 freeze-substitution electron microscopy. However, when the alga was cultured in
31 brackish medium, the fibrils on the surface shortened and became less dense. Thus, the
32 fibrillar colony sheath that mainly consisted of saccharic components prevented the
33 entry of organic solvents into the colony interior, and thus hydrocarbon extraction.

34

35

36 **Keywords**

37 *Botryococcus braunii*; Colony sheath; Hydrocarbon; Extraction; Electron microscopy.

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39

40 **1. Introduction**

41 Microalgae have attracted considerable attention because some of them are used as
42 functional food and for extracting pigments [1,2]. They produce not only
43 high-value-added products but also biofuel and commodity chemicals via
44 photosynthesis [3]. The freshwater green microalga *Botryococcus braunii* is considered
45 as a source of biofuel because of its unique features that other algae lack. Generally,
46 oil-accumulating microalgae produce fatty acids or triglycerides under environmental
47 stresses such as depletion of nutrients [4]. *B. braunii* produces hydrocarbons at a high
48 rate per unit dry cell weight during the growth phase [5–7]. It is classified into three
49 races according to the types of hydrocarbons produced [8]. The A race produces
50 *n*-alkadienes and *n*-alkatrienes [9]; B race, mainly C₃₀ to C₃₇ triterpenoids [9,10]; and L
51 race, tetraterpenoids known as lycopadiene [11]. In particular, the B race stores
52 hydrocarbons at a relatively high rate, and these hydrocarbons can be easily
53 decomposed to gasoline via the hydrocracking reaction [12].

54 Another characteristic feature of *B. braunii* is that it stores the hydrocarbons in the
55 oleophilic extracellular biopolymer that connects cells [7,13,14]. In other algae, the cell
56 wall needs to be degraded to recover the fatty acids or triglycerides stored in the cell
57 interiors [15]. Hydrocarbon recovery from *B. braunii* was thought to be easier by using

58 non-polar solvents such as *n*-hexane without requiring cell disruption. These solvents do
59 not mix with the water phase, and their recovery and reuse is easier than those of other
60 solvents. Oil extraction with *n*-hexane is a popular and industrially applicable method
61 for the extraction of food oil. However, hydrocarbons could not be effectively extracted
62 from wet *B. braunii* only by adding non-polar solvents [16]. Although the algal biomass
63 could be dried before extraction, a large amount of thermal energy was required for this
64 process [17]. The extraction rate of hydrocarbons could be improved without requiring
65 the drying of microalgae by pre-heating wet *B. braunii* or by culturing it in brackish
66 medium [18–20].

67 The colony structure of *B. braunii* hinders the extraction of hydrocarbons despite their
68 presence in the extracellular biopolymer. In the colony interior of race B, three to six
69 thin layers containing hydrocarbons cover the basolateral side of each cell surface and
70 connect the cells [7,21]. On the colony surface, these cells are entirely enclosed by a
71 retaining wall covered with fibrillar structures, i.e., the colony sheath [21,22]. Largeau
72 et al. identified these fibrils on the cell apex and showed that they continuously
73 surrounded a part of the colony by using ruthenium red treatment [13]. Subsequently,
74 these fibrils were found to cover not only the apical region of the cells but also the
75 entire colony surface [22]. Moreover, Uno et al. used rapid-freezing and

76 freeze-substitution electron microscopy and showed that fibrils on the cell apex were
77 generated in a different manner from those on the extracellular biopolymer [21]. Fibrils
78 on the cell apex were replaced during every cell division, whereas those on the
79 extracellular biopolymer were never replaced. Electron microscopy analysis revealed
80 that these fibrils showed positive signals after silver hexamine treatment [21], a kind of
81 periodic-acid Schiff's reaction; hence, they were thought to contain saccharic
82 components. Therefore, these fibrils might act as a hydrophilic fibrillar layer and
83 prevent the entry of non-polar solvents such as *n*-hexane in the colony interior during
84 hydrocarbon extraction.

85 In this study, two treatments, i.e., pre-heating and culture in brackish medium, which
86 have been shown to improve hydrocarbon extractability from *B. braunii*, were used to
87 investigate whether the formation of colony sheath and retaining wall on the colony
88 surface was associated with hydrocarbon extractability. Transmission electron
89 microscopy was used to examine the structural changes in the colony after the two
90 treatments.

91

92 2. Materials and methods

93 2.1. Microalgae cultivation

94 In this study, *B. braunii* race B (Showa strain) was cultured in 1.2-L glass flasks
95 aerated with 1% CO₂ at 25°C under illumination of 100 μmol·m⁻²·s⁻¹ with a 12-h
96 light-dark cycle for 18 days. Two types of culture media were used. One was modified
97 Chu 13 medium as freshwater medium [20], and the other was a brackish medium with
98 a salinity of 7 g·L⁻¹ (0.7% SM) that was prepared by diluting commercial artificial
99 seawater (Daigo's Artificial Seawater SP for Marine Microalgae Medium; Wako Pure
100 Chemical Industries; total salinity, 36 g·L⁻¹). Nutrients and trace metals, KNO₃, K₂HPO₄
101 ·3H₂O and FeNaEDTA, were already included in modified Chu 13 medium, but not in
102 the artificial seawater; hence, they were added to the artificial seawater at the same final
103 concentrations as those present in Chu 13 medium.

104

105 2.2. Pre-heating and the amount of sugars recovered by hot water extraction

106 The alga was pre-heated as follows. The alga was harvested with vacuum filtration by
107 using a 20-μm-mesh nylon plankton net, and the obtained algal pellets were rinsed with
108 deionized water. The moisture content of the algal pellets was measured using a
109 moisture analyzer (MX-50; A&D) and adjusted to 95 wt% by adding deionized water.
110 This algal slurry was heated at 90°C for 20 min by using a hot stirrer. Next, the heated
111 sample was centrifuged (2,320 g, 10 min), and the water phase was recovered to

112 measure the amount of sugars released by hot water extraction by using the
113 phenol-sulfuric acid method with D-glucose (Wako Pure Chemical Industries) as the
114 standard [23]. Into 10 mL test tube, 400 μ L of the sample of fluid and 400 μ L of 5%
115 (w/w) phenol aqueous solution were transferred. After 2 mL of 98% (w/w) concentrated
116 sulfuric acid was added into the mixture rapidly, the sample was cooled in water bus at
117 30°C for 1 h and measured with a UV-visible spectrophotometer (UV 1800; Shimadzu)
118 at 485 nm.

119

120 2.3. Evaluation of hydrocarbon extractability

121 Hydrocarbon recovery rate was used as an index to evaluate hydrocarbon extractability
122 from the alga. The algal density of each sample was adjusted to 4.1 $\text{g}\cdot\text{L}^{-1}$ in terms of dry
123 weight to perform extraction under the same solid concentration after the sample was
124 harvested with vacuum filtration through a 20- μ m nylon mesh.

125 Hydrocarbon content—denominator of the hydrocarbon recovery rate—was measured
126 using the following method. Algal slurry was freeze-dried, and the dried sample was
127 soaked in *n*-hexane with sonication. Some carotenoids in the form of secondary
128 metabolites, which were present in the colony interior, together with the hydrocarbons
129 turned the extraction solvent to yellow. The yellow colored *n*-hexane was separated by

130 centrifugation at 2,320 *g* for 10 min, and fresh solvent was added to the extraction
131 residue. This process was repeated until the extract became colorless. All the extracts
132 were combined and concentrated using a rotary evaporator. The obtained yellow oily
133 material was subjected to silica gel column chromatography (Wakogel C-300; Wako
134 Pure Chemical Industries) by using *n*-hexane as a mobile phase to remove components
135 other than hydrocarbons. Pure hydrocarbons were obtained by collecting fractions until
136 yellow carotenoids were eluted. The amounts of hydrocarbons were determined
137 gravimetrically after the solvent was removed using a rotary evaporator, followed by
138 drying in a vacuum desiccator for 24 h.

139 The amounts of hydrocarbons recovered from wet algae were measured using the
140 following method. This value was the numerator of the hydrocarbon recovery rate. In a
141 16-mL glass test tube, 5 mL *n*-heptane and 5 mL algal slurry were placed and shaken
142 using a shaker (MMV-1000W; Tokyo Rikakikai) at 300 rpm at 70°C for 1 min. The
143 recovered upper *n*-heptane phase was subjected to gas chromatography (GC) with flame
144 ionization detection (GC-2014; Rtx-1 capillary column; Shimadzu). The temperature
145 program was set at 50°C for 1 min, from 50°C to 220°C at 10°C·min⁻¹, 220°C for 3 min,
146 from 220°C to 260°C at 2°C·min⁻¹, and 260°C for 3 min. The amount of hydrocarbons
147 extracted by *n*-heptane were calculated as the sum of peak areas of hydrocarbons

148 compared to those for known amounts of standard hydrocarbons extracted and purified
149 from freeze-dried algal samples [24,25].

150

151 2.4. Fluorescence and electron microscopy

152 For microscopy observation, the algal colony was first concentrated. Colonies in Chu
153 13 medium were naturally concentrated; they came up to the surface of the standing
154 medium and were collected. The colonies in brackish medium or those subjected to
155 pre-heating culture were filtered using a 10- μ m nylon mesh. The colonies on the filter
156 were used for the observations.

157 For fluorescence microscopy, 1:200 volume of 1 mM Nile Red solution dissolved in
158 dimethyl sulfoxide was added to the concentrated algal slurry. The algal colony was
159 observed under BX51 fluorescence microscope by using an FITC filter set (Olympus)
160 after the colony was stained for 10 min.

161 For electron microscopy, the concentrated algal slurry was attached to a Formvar film
162 mounted on copper wire loops (diameter, 8 mm) and rapidly frozen in liquid propane.
163 Frozen samples were soaked in acetone containing 3% osmium tetroxide and 0.2%
164 uranyl acetate at -85°C for over 72 h. Subsequently, the temperature of the frozen
165 sample was increased to 4°C. The sample was treated with fresh acetone containing

166 osmium tetroxide and uranyl acetate for 1 h at room temperature and washed with fresh
167 acetone. The acetone in the sample was gradually substituted with Spurr resin from 1%
168 to 100%, and samples in 100% resin were polymerized at 60°C. Sections were stained
169 with lead citrate for 10 min at room temperature and observed under JEM-1230
170 transmission electron microscope (JEOL).

171

172 3. Results and discussion

173 3.1. Changes in hydrocarbon extractability with treatments

174 The hydrocarbon contents of the Showa strain cultured in freshwater medium (Chu 13
175 medium) and brackish medium (0.7% SM) were 34.3% and 35.5%, respectively. The
176 difference in culture medium did not significantly affect the hydrocarbon content as was
177 found in our previous study [20]. Hydrocarbon extractabilities after the two
178 treatments—pre-heating (PH) and culture in brackish medium (CB)—were compared
179 with that of no treatments (NT), that is, direct extraction from wet alga cultured in Chu
180 13. Frenz et al. reported that the water content of algal slurry affected hydrocarbon
181 extractability [16]. In this study, algal density was adjusted to $4.1 \text{ g} \cdot \text{L}^{-1}$ in terms of dry
182 weight before extraction in order to exclude the effect of the difference in the ratio of
183 solvent and algal density (Table 1). Even after shaking for only 1 min, more than 90%

184 of hydrocarbons were recovered after the two treatments. Compared with no treatment,
185 the two treatments had considerable positive effects on hydrocarbon extractability.

186 Saga et al. compared the energy profit ratio (EPR) of the wet process with pre-heating
187 to that of the dry process for extracting hydrocarbons from the same strain of *B. braunii*
188 [17]. Wet process with pre-heating required extra latent heat for water and solvent
189 evaporation from the extraction residue. Nevertheless, the EPR of the wet process was
190 approximately twice that of the dry process. Culture in brackish medium might have
191 overcome the need for sensible heat for pre-heating.

192

193 3.2. Colony organization under fluorescence and electron microscopy

194 The three colony samples of *B. braunii*, i.e., NT, CB, and PH, were observed under
195 fluorescence and electron microscopes. The samples used for hydrocarbon extraction
196 and colony observation were obtained from the same lot. For fluorescence microscopy
197 observation, samples were mounted on a slide glass and slightly pressed with a
198 coverslip to clearly observe each cell while maintaining the colony organization like
199 that in a flower bouquet in which the rounded cell apex is directed toward the outer
200 surface of the colony (Fig. 1) [7]. However, the colonies of CB and PH could not be
201 pressed well because the former was excessively large and the latter became rigid by the

202 heat treatment. Nile red staining showed that all the samples had oils in the cell
203 connections of extracellular matrices (Fig. 1-A1, B1, C1). Colonies of CB were
204 considerably larger than those of NT and PH. Unlike in NT and PH, the cells of CB had
205 markedly larger oil bodies internally.

206 The colony organization of the three samples observed under an electron microscope is
207 shown in Figure 1-A2, B2, and C2. In NT, a fibrillar layer of colony sheath of about less
208 than 5 μm (indicated by double-ended arrows) covered the entire colony surface, as
209 reported previously [21]. The fibrillar layer of CB or PH could not be clearly observed
210 unlike than in NT under this low magnification.

211

212 3.3. Changes in the colony sheath after treatments for improving hydrocarbon 213 extractability

214 The three samples were observed under high magnification to investigate the detailed
215 ultrastructural changes in the colony sheath of CB and PH. The vertical section of NT
216 from the surface of colony sheath to the center of cells is shown in Figure 2-A.
217 Branched fibrils not only extended from the retaining wall on the cell apex (c-RW) but
218 were also found on the extracellular biopolymers (h-RW), which were thin layers of
219 polymerized extracellular hydrocarbons and contained liquid hydrocarbons. These

220 fibrils covered the entire colony surface without any space, and an electron-dense thin
221 layer was observed on the outer surface of the fibril layer (Fig. 2-A2, arrowhead), which
222 was responsible for the unique colony characteristics of *B. braunii* (Fig. 1-A2). Two
223 contacting colonies are shown in Figure 2-B. The border (black and white arrow) had
224 high electron density reflecting two electron-dense layers on the outer surface of the
225 colony sheath. The cross-section parallel to the surface of the colony is shown in Figure
226 2-C. The fibrils formed tiny mesh structures of about 50 to 500 nm. Electron-dense
227 materials (double arrowheads) were found in some spaces among the fibrils.

228 Conversely, the sample of CB showed different fibrillar structure in the colony sheath
229 (Fig. 3). Fibrils of CB were also branched, but their densities were lower than those of
230 NT (Fig. 2-A). The thin layer at the surface of the fibril layer had disappeared, and
231 electron-dense materials among fibrils were not clear compared with those in the fibril
232 layer of NT. The magnified view of CB colony to finely observe the cell surface is
233 shown in Figure 3-B. The high electron density layer indicated by arrows (c-RW and
234 h-RW) was the retaining wall that enclosed the colony surface under the fibrillar layer
235 and was first reported by Weiss et al. [22]. The retaining wall was intact in contrast to
236 the fibrils on both cell wall (c-RW) and extracellular biopolymers (h-RW) suggesting
237 that culturing in brackish media did not affect the colony structure.

238 The colony organization of sample CB is shown in Figure 4-A, B, C, and D. A part of
239 the colony surface had complete fibrils and the remaining parts had less dense fibrils
240 (Fig. 4-A). The less-dense fibrils were shorter and fatter than those observed in Figure
241 4-A (Fig. 4-B). Almost no fibrils were observed in some parts (Fig. 4-C, D). Colony
242 sheath in the state of Fig 4-C was observed most frequently in this observation..

243 The colony of *B. braunii* is organized like a flower bouquet in which the rounded cell
244 apex is directed toward the outer surface of the colony (Fig. 1-A) [7], and its surface is
245 increased by the addition of new daughter cells and layers of extracellular biopolymers
246 newly formed after cell division. On the increased area of colony surface, new retaining
247 wall is produced, and then the fibrils of the colony sheath elongate from the retaining
248 wall [21]. Fibrils on c-RW are replaced, and those on h-RW increase in number with cell
249 divisions. If the production of new fibrils was inhibited from the retaining wall, both
250 fibrils on c-RW and h-RW would be shorter or missing with continuous cell divisions.
251 Eukaryotic algae require some processes and time to completely acclimate to salinity
252 conditions [26]. The growth of a part of the colony was suggested to become very slow
253 or even stop by rapid salinity changes compared with that in the other parts and in the
254 remaining fibrils (Fig. 4-A). The colony shown in Figure 4-B might not have
255 completely acclimated and showed incomplete inhibition. In Figure 4, not one but four

256 fibril conditions are noted, indicating that culture in brackish medium did not break or
257 remove the fibrillar layer of colony sheath because of some physical or chemical
258 reaction, but inhibited the production of new fibrils from the retaining walls both on the
259 cell wall (c-RW) and extracellular biopolymers (h-RW). Fibrils showed regression in
260 the order from Figure 4 A to D with continuous growth in brackish medium.

261 Photographs of PH are shown in Figure 5. No fibrils were found on both the cell apex
262 and extracellular biopolymer. Saccharic components consisting of fibrils might have
263 become dissolved in the hot water. However, the retaining wall of PH was intact on the
264 colony surface as was noted in the sample of CB (Fig. 5-B1, arrowhead). Fibril foots
265 observed in NT (Fig. 2-A2) were not noted in CB on the retaining wall, but granules
266 were embedded in the retaining wall (Fig. 5-B2, arrowhead).

267 These findings suggest that the CB or PH samples that showed improved hydrocarbon
268 extractability led to a decrease or inhibition of fibril formation on the colony sheath,
269 respectively. Further, the retaining wall was intact after both the treatments and was not
270 associated with the improvement of hydrocarbon extractability. Weiss et al. corrected
271 the apical parts of the thin layers with fibrils that had separated from the cell apex
272 during culture [22]. They named these parts “shells,” which consisted of fibrils,
273 protuberant tubular elements, and the retaining wall. Moreover, Uno et al. used silver

274 hexamine treatment combined with electron microscopy and revealed that only fibrils
275 on c-RW and h-RW had sugar components except on the retaining wall and protuberant
276 tubular elements that were present on the foot of fibrils on the retaining wall [21]. The
277 retaining wall was thought to be mainly composed of polymerized hydrocarbons like
278 the extracellular biopolymers. This was the reason why the retaining wall was not
279 associated with an improvement in hydrocarbon extractability from the samples of CB
280 and PH. However, fibril foots were removed by pre-heating unlike the retaining wall
281 that did not show positive signals for silver hexamine reaction. Instead of fibril foots,
282 granules were observed in the c-RW of PH (Fig. 5-B). These granules of high-electron
283 density might have been the traces of fibril foots that were decomposed by pre-heating,
284 and the composition of fibril foots might have been different from that of the retaining
285 wall because of the difference in appearances. Thus, fibril foots might be the basis for
286 the formation of fibrils of the colony sheath and might be composed of saccharic
287 components produced during enzymatic synthesis of fibrils.

288

289 3.4. Decrease in the number of fibrils of colony sheath leading to the improvement of
290 hydrocarbon extractability

291 None of the colonies of PH had fibrils in the colony sheath (Fig. 5). Fibrils were

292 thought to have dissolved into hot water at 90°C. Atobe et al. reported that the contents
293 of arabinose and galactose, which were the constituents of saccharic components,
294 increased when the colony was extracted with water at 90°C compared with that when
295 the extraction was performed at 60°C [27]. After pre-heating at lower than 60°C, protein
296 was not denatured, and the amount of hydrocarbon extracted was less. The saccharic
297 components extracted by hot water at 90°C had amphiphilic characteristics and
298 prevented hydrocarbon extraction by emulsifying the water-organic solvent systems
299 [28]. Weiss et al, reported that 81% of “shells” containing fibrils were composed of
300 arabinose and galactose [22]. These findings suggested that saccharic components
301 extracted by pre-heating were mainly the components of shells, namely, fibrils.

302 The sample of CB that showed low fibrillar density was heated at 90°C, and the fibrils
303 were extracted in order to measure the decreased amounts of sugars in the fibrils. The
304 amounts of sugars extracted from the fibrils between NT and CB by using the
305 phenol-sulfuric acid method are shown in Table 2. The amounts of sugars extracted
306 from CB were 64% lesser than those extracted from NT. This difference quantitatively
307 explained the low density of fibrils on algal colonies cultured in brackish medium
308 observed by electron microscopy.

309 CB reduced the density and number of fibrils. PH inhibited fibril formation because of

310 the dissolution into the aqueous phase. These decreases or disappearance of fibrils led to
311 the improved hydrocarbon extractability despite the retention of the retaining wall. *B.*
312 *braunii* prevents the entry of organic solvents during extraction into the colony interior
313 by the fibrils of the colony sheath that cover the colony surface.

314 If a strain that had no fibrils or a low fibrilliform density such as the algae cultured in
315 brackish medium in this study could be produced on a large scale in a pond,
316 hydrocarbon extractability from such a strain would be improved, and the production
317 costs and input energy would be greatly reduced because of the elimination of the need
318 of pretreatment processes that consume large amounts of heat energy for efficient
319 hydrocarbon extraction. In solvent extraction process, Saga et al. reported that the input
320 energy of pre-heating was $1.6 \text{ MJ} \cdot \text{kg-algae}^{-1}$ compared to total input energy (4.1
321 $\text{MJ} \cdot \text{kg-algae}^{-1}$) [17]. Moreover, the input energy of drying was over five times that of
322 pre-heating. Producing the strain which has no fibrils or a low fibrilliform density can
323 reduce the input energy by 39% compared to solvent extraction process with pre-heating.

324 Moreover, milking process which is a way to continuously extracting hydrocarbons from
325 *B. braunii* without destruction of the cells is proposed, because *B. braunii* stores the
326 most of the hydrocarbons in the extracellular biopolymer that connects the cells [29-31].
327 This milking process has a possibility of reduction in input energy by 70% compared to

328 conventional process [32]. However, long contact time with organic solvent results in
329 significant reduction in the photochemical efficiency of *B. braunii* indicating the cells
330 are damaged [30]. Our research might lead to efficient milking process to shorten the
331 contact time with organic solvent without killing the cells.

332

333 4. Conclusions

334 *B. braunii* prevented solvents from entering the colony internal because of the
335 presence of fibrils of colony sheath that covered the colony surface. Hydrocarbon
336 extractability of *B. braunii* was improved by the pretreatment of the culture with
337 brackish medium or pre-heating at 90°C; these pretreatments decreased or dissolved the
338 amphiphilic fibrils of colony sheath, respectively. When the alga was cultured in
339 brackish medium, the fibrillar layer was not broken or removed because of some sort of
340 physical or chemical reaction, but the production of fibrils from both cell wall (c-RW)
341 and extracellular biopolymers (h-RW) was inhibited. However, the retaining wall was
342 intact after both pre-heating and culturing in brackish medium. The retaining wall was
343 relatively resistant to external pretreatments and did not affect the hydrocarbon
344 extractability because it lacked saccharic components.

345

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349

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460

461 **Table 1.** Hydrocarbon recovery rate from the three samples: pre-heating (PH), culture in
 462 brackish medium (CB), and no treatments (NT)

463

Sample	Hydrocarbon content (%)	Hydrocarbon recovery rate (%)
NT	34.3	<0.1
CB	35.5	93.8 (± 2.0)
PH	42.9	95.3 (± 0.8)

464 ($\pm$) indicates the standard deviation ($n = 3$).

465

466 **Table 2.** The amounts of sugars extracted by hot water from the two samples: no
467 treatments (NT) and culture in brackish medium (CB) measured using the
468 phenol-sulfuric acid method

469

Sample	sugars (mg·g ⁻¹)
NT	97.5 (±0.3)
CB	62.4 (±0.8)

470 (±) indicates the standard deviation (n = 3).

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480 **Figure legends**

481

482 **Fig. 1.** Fluorescent and electron microscopy images of *Botryococcus braunii* colony. A:
483 no treatment (NT). B: culture in brackish medium (CB). C: pre-heating (PH). 1. Nile red,
484 fluorescent microscopy. Oil bodies in the cytoplasm and extracellular matrix are stained
485 with Nile red as yellow. Chloroplast is stained red. Colonies in CB became very large. 2.
486 Electron microscopy. Colony sheath observed around each colony in NT (double-ended
487 in A2) are not clearly visible in CB and PH (see Figs. 3–5 for details). Small-sized
488 colonies were selected for B2 to view several central sections of the colonies in a
489 limited space of the photograph. Size bars, 10 μm in panel 1 and 5 μm in panel 2.

490

491 **Fig. 2.** Electron microscopy images of colony sheath of no treatment (NT). A: The
492 vertical section cut from the colony sheath to the center of the colony. A2 is the
493 magnified photograph of a part of A1 indicated by a box and shows electron-dense fibril
494 foots on the retaining wall. B: A part of the contacting site (black and white arrows) of
495 two colonies. B2 is the magnified photograph of a part of B1 indicated by a box. C: The
496 cross-section parallel to the surface of the colony. C2 is the magnified photograph of a
497 part of C1 indicated by a box. The networks of fibrils contain electron-dense materials

498 (double arrowheads). ch, chloroplast; c-RW, retaining wall on the cell wall; G, Golgi
499 body; h-RW, retaining wall on the layers of biopolymer containing hydrocarbons;
500 m-CW, mother cell wall; n-CW, new daughter cell wall; PM, plasma membrane;
501 arrowhead, colony surface; *, extracellular biopolymers containing hydrocarbons;
502 arrowhead, surface layer of the colony sheath. Size bars, 2 μm in panel B1; 0.5 μm in
503 panels A, B2, C1; and 0.1 μm in insert A of panel C2.

504

505 **Fig. 3.** Electron microscopy images of colony sheath of culture in brackish medium
506 (CB). A: The vertical section cut from the colony sheath to the center of the colony. A2
507 is the magnified photograph of a part of A1 indicated by a box. B: The magnified
508 photograph to observe the colony sheath. A retaining wall covering cells and the
509 extracellular matrix is clearly noted, but electron-dense materials in fibrillar networks
510 are not clear (compared to that in Fig. 2C). ch, chloroplast; M, mitochondria, N,
511 nucleus; c-RW, retaining wall on the cell wall; h-RW, retaining wall on the layers of
512 biopolymer containing hydrocarbons; *, extracellular biopolymers containing
513 hydrocarbons; **, oil body. Size bars, 5 μm in panel A1, 1 μm in panel A2, and 0.5 μm
514 in panel B.

515

516 Figure 4. Regression of the colony sheath in the order from A to D by the treatment of
517 culture in brackish medium (CB). A. A part of colony sheath remains complete. B. A
518 surface layer of colony sheath is not visible, and fibrils composing colony sheath are
519 shorter and fatter than those in the complete form (arrow; compares to A and Fig. 2-A1).
520 C, D. Fibril layers of colony sheaths are not visible. *, extracellular biopolymers
521 containing hydrocarbons. Size bars, 0.5 μ m.

522

523 Figure 5. Electron microscopy images of colony sheath of pre-heating (PH) treatment.
524 A: A part of the colony. B: Remaining retaining wall on the cell apex and extracellular
525 matrix. B2 is the magnified photograph of a part of B1 indicated by a box. Fibril foots
526 on the retaining wall are not visible, but granules embedded in the retaining wall
527 (arrowhead) are visible. c-RW, retaining wall on the cell wall; h-RW, retaining wall on
528 the extracellular biopolymers containing hydrocarbons; *, extracellular biopolymers
529 containing hydrocarbons; **, oil body. Size bars, 1 μ m in panels A and B, and 0.25 μ m
530 in the insert of B.

531

532

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5