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

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ABSTRACT

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

1. Introduction

Microalgae have attracted considerable attention because some of them are used as functional food and for extracting pigments [1,2]. They produce not only high-value-added products but also biofuel and commodity chemicals via photosynthesis [3]. The freshwater green microalga *Botryococcus braunii* is considered as a source of biofuel because of its unique features that other algae lack. Generally, oil-accumulating microalgae produce fatty acids or triglycerides under environmental stresses such as depletion of nutrients [4]. *B. braunii* produces hydrocarbons at a high rate per unit dry cell weight during the growth phase [5–7]. It is classified into three races according to the types of hydrocarbons produced [8]. The A race produces *n*-alkadienes and *n*-alkatrienes [9]; B race, mainly C_{30} to C_{37} triterpenoids [9,10]; and L race, tetraterpenoids known as lycopadiene [11]. In particular, the B race stores hydrocarbons at a relatively high rate, and these hydrocarbons can be easily decomposed to gasoline via the hydrocracking reaction [12].

Another characteristic feature of *B. braunii* is that it stores the hydrocarbons in the oleophilic extracellular biopolymer that connects cells [7,13,14]. In other algae, the cell wall needs to be degraded to recover the fatty acids or triglycerides stored in the cell interiors [15]. Hydrocarbon recovery from *B. braunii* was thought to be easier by using non-polar solvents such as *n*-hexane without requiring cell disruption. These solvents do not mix with the water phase, and their recovery and reuse is easier than those of other solvents. Oil extraction with *n*-hexane is a popular and industrially applicable method for the extraction of food oil. However, hydrocarbons could not be effectively extracted from wet *B. braunii* only by adding non-polar solvents [16]. Although the algal biomass could be dried before extraction, a large amount of thermal energy was required for this process [17]. The extraction rate of hydrocarbons could be improved without requiring the drying of microalgae by pre-heating wet *B. braunii* or by culturing it in brackish medium [18–20].

The colony structure of *B. braunii* hinders the extraction of hydrocarbons despite their presence in the extracellular biopolymer. In the colony interior of race B, three to six thin layers containing hydrocarbons cover the basolateral side of each cell surface and connect the cells [7,21]. On the colony surface, these cells are entirely enclosed by a retaining wall covered with fibrillar structures, i.e., the colony sheath [21,22]. Largeau et al. identified these fibrils on the cell apex and showed that they continuously surrounded a part of the colony by

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

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

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)

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

using ruthenium red treatment [13]. Subsequently, these fibrils were found to cover not only the apical region of the cells but also the entire colony surface [22]. Moreover, Uno et al. used rapid-freezing and freeze-substitution electron microscopy and showed that fibrils on the cell apex were generated in a different manner from those on the extracellular biopolymer [21]. Fibrils on the cell apex were replaced during every cell division, whereas those on the extracellular biopolymer were never replaced. Electron microscopy analysis revealed that these fibrils showed positive signals after silver hexamine treatment [21], a kind of periodic-acid Schiff's reaction; hence, they were thought to contain saccharic components. Therefore, these fibrils might act as a hydrophilic fibrillar layer and prevent the entry of non-polar solvents such as *n*-hexane in the colony interior during hydrocarbon extraction.

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

2. Materials and methods

2.1. Microalgae cultivation

In this study, *B. braunii* race B (Showa strain) was cultured in 1.2-L glass flasks aerated with 1% CO_2 at 25 °C under illumination of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a 12-h light–dark cycle for 18 days. Two types of culture media were used. One was modified Chu 13 medium as freshwater medium [20], and the other was a brackish medium with a salinity of 7 $g \cdot L^{-1}$ (0.7% SM) that was prepared by diluting commercial artificial seawater (Daigo's Artificial Seawater SP for Marine Microalgae Medium; Wako Pure Chemical Industries; total salinity, 36 $g \cdot L^{-1}$). Nutrients and trace metals, KNO_3, $K_2HPO_4 \cdot 3H_2O$ and FeNaEDTA, were already included in modified Chu 13 medium, but not in the artificial seawater; hence, they were added to the artificial

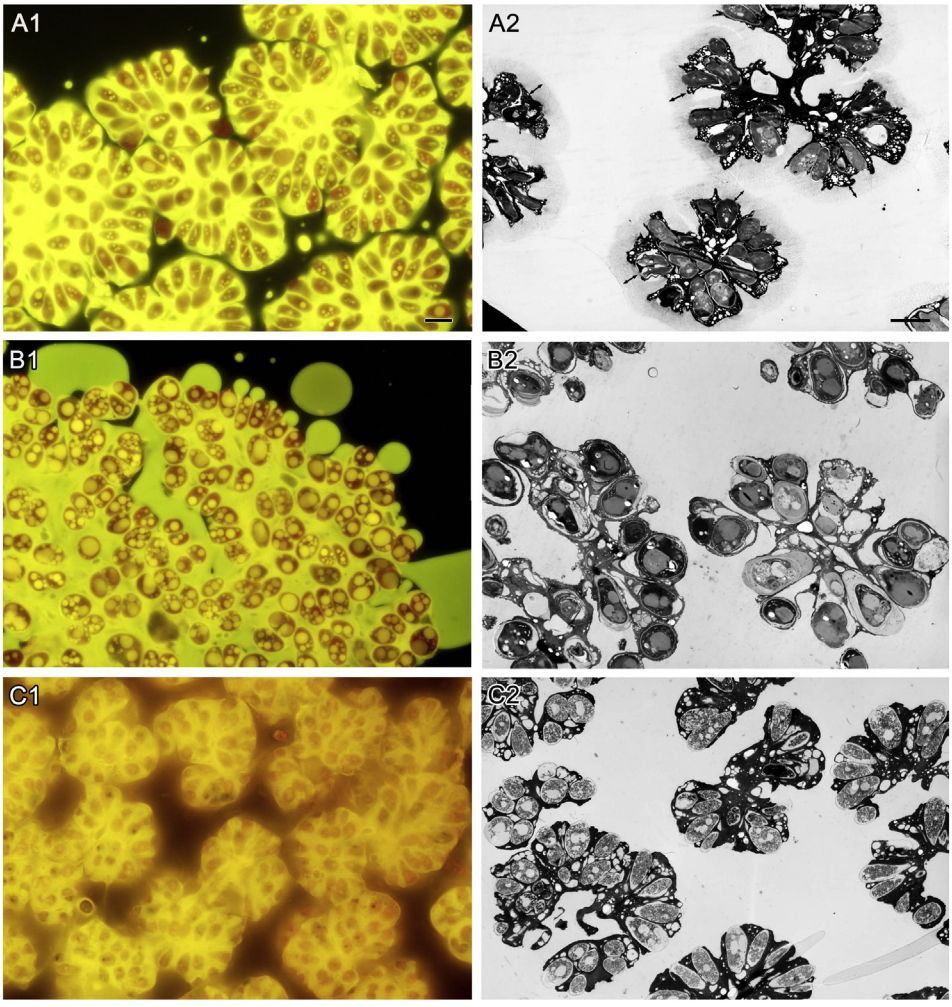

Fig. 1. Fluorescent and electron microscopy images of *Botryococcus braunii* colony. A: no treatment (NT). B: culture in brackish medium (CB). C: pre-heating (PH). 1. Nile red, fluorescent microscopy. Oil bodies in the cytoplasm and extracellular matrix are stained with Nile red as yellow. Chloroplast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