



Simultaneous culture and hydrocarbon recovery from *Botryococcus braunii* with squalane as a solvent

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Received: 1 August 2025 / Revised: 22 September 2025 / Accepted: 26 September 2025 / Published online: 21 October 2025
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Abstract

This study aimed to examine a novel milking process for hydrocarbon recovery from the green microalga *Botryococcus braunii* with squalane as a solvent. Squalane is a hydrogenated derivative of squalene, one of the hydrocarbon components produced by *B. braunii* (Race B), and it is considered harmless to the alga. First, we conducted comparative experiments with previously reported organic solvents, followed by two-phase culture experiments to assess the feasibility of long-term culture and extraction. Solvent comparison revealed that squalane can effectively recover hydrocarbons while maintaining high cell viability, particularly in a modified medium supplemented with 0.5% sodium chloride. In a two-phase culture system with squalane as the upper phase, simultaneous hydrocarbon extraction and algal culture were achieved, providing a hydrocarbon recovery rate of 45.1% without compromising photosynthetic activity. Furthermore, the total hydrocarbon production increased by 19.5% compared to that under the control conditions. When unsaturated long-chain hydrocarbons are used as biofuels, hydrogenation followed by cracking is typically necessary. The proposed process, in which the hydrogenated hydrocarbon product itself is employed as the solvent, may reduce the energy input required by eliminating the need for fractional distillation between the solvent and the target product. Future research should aim to develop a practical process by verifying the reuse of botryococcane—the hydrogenated form of the main hydrocarbon produced by *B. braunii*—as a solvent.

Keywords Chlorophyceae · *Botryococcus braunii* · Hydrocarbon · Milking · Extraction · Organic solvent

Introduction

Biofuels derived from microalgae have attracted much attention in recent years (Wang et al. 2024). Among biomass resources, microalgae are characterized by their ability to use land without competing with food production (Sharma et al. 2023) and by their fast growth and high lipid productivity (Zhang et al. 2022). Traditionally, lipid recovery from microalgae involves a series of processes: culture, harvesting, concentration, drying, cell disruption, solvent extraction, and solvent separation. Among these processes, drying and cell disruption are highly energy- and cost-intensive processes (Acién Fernández et al. 2019; Branco-Vieira et al. 2020; Pôjo et al. 2021; Vázquez-Romero et al. 2022). Therefore, there is a need to eliminate energy-consuming

processes or increase their efficiency in relation to lipid recovery from microalgae.

Milking could solve the problems associated with conventional lipid recovery processes. Milking is a process in which target substances secreted outside the cells are recovered from microalgae nondestructively and which can be repeated several times by re-culturing the extraction residues (Hejazi and Wijffels 2004; Uchida et al. 2020). Milking can recover lipids from microalgae more efficiently because it eliminates energy-intensive processes such as harvesting, concentration, and heating (Kleinert and Griehl 2021b). *Botryococcus braunii* is a freshwater green alga that produces hydrocarbons, as are *Botryococcus alkenealis*; and *Botryococcus lycopadienior*, which were previously considered as the same species but were recently classified as closely related species (Metzger and Largeau 2004; Weiss et al. 2010; Tasić et al. 2016; Boland et al. 2024). Hydrocarbon milking is only applied to *B. braunii* and its close relatives (Moheimani et al. 2014). This occurs primarily because the hydrocarbons produced by these algae are secreted extracellularly and accumulate in the extracellular

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matrix (ECM), making them particularly suitable targets for nondestructive extraction via milking (Weiss et al. 2010). Considering factors such as hydrocarbon content, growth rate, biocompatibility with organic solvents, and nutrient consumption, *B. braunii* is identified as a particularly promising species for milking compared to related species (*B. alkenealis* or *B. lycopadienior*) (Kleinert and Griehl 2021a). Additionally, it has been reported that when *B. braunii* is recultured after hydrocarbon extraction, the depletion of hydrocarbons stimulates their production while potentially reducing the nitrogen requirement for hydrocarbon biosynthesis (Jackson et al. 2017).

A crucial factor in achieving hydrocarbon milking from *Botryococcus* involves increasing the contact between hydrocarbons and organic solvents and increasing the biocompatibility of the solvents. In terms of contactability, the challenge is that contact between organic solvents and hydrocarbons accumulated in the external cell matrix (ECM) is prevented by the polysaccharide layer covering the entire colony (Furuhashi et al. 2016a). For this reason, physical methods using a pulsed electric field (PEF) (Ahirwar et al. 2022), mechanical pressing (Moheimani et al. 2013), solvent extraction in a high-shear environment (Jackson et al. 2019) and cavitation (Tsutsumi et al. 2018) have been reported to remove the polysaccharide layer. Conversely, culture techniques that do not require energy input and increase the salinity of the medium to suppress the production of polysaccharide layers have also been reported (Furuhashi et al. 2016a, 2022). In particular, *B. braunii* cultured in the medium with salt concentrations of 0.3% or 0.5% showed dramatically improved extraction rates (Furuhashi et al. 2016b).

In terms of solvent biocompatibility, highly hydrophobic solvents are considered advantageous because they do not disrupt the cell membrane (Zhang et al. 2011). For example, short-chain hydrocarbons and polar solvents exhibit high extraction efficiency but often lead to a loss of cell viability (Jackson et al. 2017). In contrast, long-chain hydrocarbons, which are more hydrophobic than short-chain hydrocarbons, exhibit lower extraction efficiency but higher biocompatibility, making them more suitable for long-term, nondestructive extraction processes such as milking. However, a key drawback of long-chain hydrocarbons is that their boiling points are close to those of the hydrocarbons produced by *Botryococcus*, resulting in increased energy requirements for separation via fractional distillation.

In this study, we investigated a novel milking process for hydrocarbon recovery *B. braunii* with squalane as the extraction solvent. Squalane is a hydrogenated derivative of squalene—a minor hydrocarbon component naturally produced by *B. braunii*. Notably, the major hydrocarbon produced by *B. braunii*, i.e., botryococcene, is a structural isomer of squalene (Niehaus et al. 2011; Uchida et al. 2015).

We suggest that squalane is likely to exhibit excellent biocompatibility with *B. braunii*. Furthermore, when hydrocarbons derived from *B. braunii* are to be utilized as biofuels in internal combustion engines for automobiles or aircraft, hydrogenation followed by catalytic cracking is typically needed (Hillen et al. 1982). Within this context, the use of squalane as a solvent offers a unique advantage: squalane can be directly introduced into the downstream cracking process along with the extracted hydrocarbons, thereby eliminating the need for energy-intensive separation between the solvent and the product.

To evaluate the feasibility of this squalane-based milking process, we conducted two types of experiments. First, we compared the extraction efficiency and biocompatibility of squalane with those of short-chain hydrocarbon solvents to assess its suitability for milking. We subsequently developed a two-phase culture system with squalane as the upper phase, in which hydrocarbon extraction and algal culture were performed simultaneously.

Materials and methods

Materials

The Showa strain of *Botryococcus braunii* was grown in 1.5-L Roux culture flasks at a constant temperature of 25 °C. The Showa strain was originally isolated at UC Berkeley USA (Nonomura 1988) and was transferred to the University of Tokyo by the late Prof. Iwamoto at Meiji University. The culture bottles were supplied with 1.0% carbon dioxide-enriched air at a rate of 200 mL min⁻¹ through a high-efficiency particulate air (HEPA) filter. The photosynthetic photon flux density (PPFD) of 200 μmol photons m⁻² s⁻¹ on the surface of the bottle was provided by fluorescent lighting, with a 12-h light/dark cycle. Three media were employed: modified Chu13 medium (hereafter, referred to as Chu13 medium) (Dayananda et al. 2007), modified Chu13 medium enriched with 3 g L⁻¹ NaCl (referred to as the 0.3% medium), and modified Chu13 medium enriched with 5 g L⁻¹ NaCl (referred to as the 0.5% medium).

Solvent extraction test

The algae were pre-cultured for 11–13 days and were concentrated by filtration on a 20-μm nylon mesh to a concentration of 15 g L⁻¹. Although the culture days differed slightly for repeated tests, they were at the same growth stage. Six milliliter of the concentrated culture was placed in a 15-mL centrifuge tube, and 3 mL each of *n*-hexane, *n*-decane, or squalane was then added, followed by stirring at 40 rpm under inversion conditions at room temperature (ACR-100, AS ONE, Osaka, Japan). This extraction test was conducted

in the dark and in a sealed environment to prevent changes in hydrocarbon amounts in the tube. The extraction times were set to 10, 30, 60, 120, 240, 360, 720, and 1440 min. On the basis of preliminary experiments, sampling was omitted at time points when no significant changes were observed compared with the previous measurement. At each designated time point, the upper layer (solvent layer) and the lower layer (culture layer) containing algal cells were collected separately from the centrifuge tube, and the hydrocarbon recovery in the upper layer and photosynthetic activity of the algae in the lower layer were measured. The experiment was repeated three times.

Two-phase culture test

A schematic diagram of the two-phase culture experiment is shown in Fig. 1. Cultures were established in 1-L cylindrical glass bottles (inner diameter 92 mm). The temperature, light, and aeration conditions were the same as those described above. After 2 weeks of preculturing in the 0.5% medium, algal cultures were filtered within a cylinder with a wedge wire screen (a mesh size of 50 μm) until all the old culture media was discharged as filtrate. Fresh medium (0.5% medium) was added to achieve an initial algal density (1.2 g L^{-1}) and a total volume of 850 mL. Subsequently, 50 mL of squalane was added to each test section, a magnetic stirrer was added to agitate the culture layer, and the two-phase culture test was conducted for 23 days. Sampling was every 3–5 days by collecting 50 mL of the culture layer and 2 mL of the upper solvent layer to determine the amount of hydrocarbons contained by the algae, photosynthetic activity, nitrate concentration, the chlorophyll concentration in the culture layer, and amount of hydrocarbon recovery from the solvent layer. Fifty milliliter of new medium and 2 mL of new squalane were added at each sampling time. As a control, a culture system without squalane (control) was

established, and the experiment was repeated three times. In the control culture, the sampling volume of the culture layer and the amount of new medium added were equivalent to those in the two-phase culture.

Measurement of hydrocarbon recovery

Hydrocarbon quantification in the solvent was by gas chromatography (GC-2014 with an Rtx-1 capillary column, Shimadzu, Japan) as reported by Furuhashi et al. (2020). The column was maintained at 50 $^{\circ}\text{C}$ for 1 min, after which the temperature increased by 10 $^{\circ}\text{C min}^{-1}$ until it reached 220 $^{\circ}\text{C}$, maintained at 220 $^{\circ}\text{C}$ for 3 min, and then increased by 2 $^{\circ}\text{C min}^{-1}$ until it reached 260 $^{\circ}\text{C}$. The hydrocarbon mass was calculated from the sum of the peak areas corresponding to hydrocarbons with respect to the peak areas for known amounts of standard hydrocarbons extracted by *n*-hexane and purified from freeze-dried algal samples in each medium. The reference material (hydrocarbons) was changed because its composition varies slightly depending on the culture in the different media. Freeze-dried algal samples were soaked in *n*-hexane and the extraction was repeated until colors were lost by yellow pigments co-existing with hydrocarbons in the ECM. Subsequently, the extracts were combined and subjected to silica-gel column chromatography for purifying. The hydrocarbon recovery was defined as the ratio of the amount of extracellular hydrocarbons transferred into the solvent to the amount of extracellular hydrocarbons contained in the algae.

Photosynthetic activity (F_v/F_m) measurements

The biocompatibility of the solvent was evaluated by measuring the F_v/F_m ratio through pulse-amplitude modulated (PAM) fluorometry (JUNIOR-PAM (Heinz Walz GmbH, Germany)). After 5-min dark acclimation, *B. braunii* was concentrated on glass fiber filter paper (GF/B 47 mm, Cytiva, USA) by filtration until no water droplets were visible on the algal surface for measurement. Preliminary tests confirmed that extended dark time or changes in moisture content on filter caused by slight variations in filtration time did not affect F_v/F_m . The amount of algal slurry for filtration was 2 mL in the extraction test and 10 mL in the two-phase culture test.

Algal biomass and chlorophyll measurements

The dry weight of the algae was determined by filtration of the culture medium through glass fiber filter paper (GF/A 70 mm, Cytiva, USA), which had been previously dried at a temperature of 105 $^{\circ}\text{C}$ for 3 h to a constant weight, followed by washing with pure water to prevent any salts or dissolved polysaccharides in the culture medium from remaining on

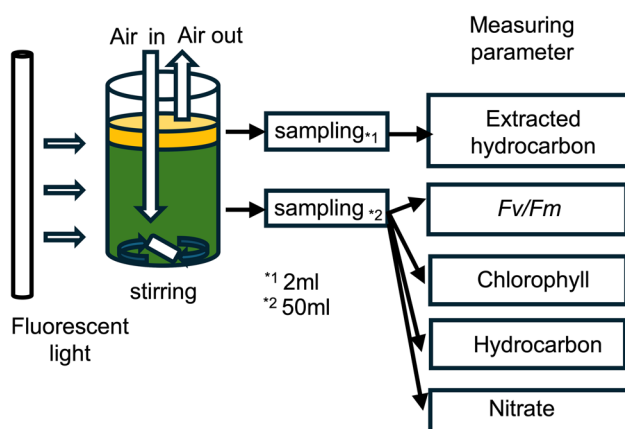


Fig. 1 Schematic diagram of the two-phase culture test

the filter paper. The weight of the filter paper was determined by measuring the weight of the filter paper dried at 105 °C for 24 h. The chlorophyll content in the culture medium was measured as described by Porra et al. (1989).

Measurement of nitrate concentration

The culture medium containing algae was filtered through glass fiber filter paper (GF/A; diameter: 110 mm; Cytiva, USA), and passed through a 0.45- μm filter, and the nitrate concentration in the filtrate was measured by suppressed ion chromatography (HIC-ESP/NS, Shimadzu, Japan). The analysis conditions included a data collection time of 30 min, a pump flow rate of 1.0 mL min⁻¹, a column oven at 40 °C, and rinse solution volume of 2500 μL . The analysis was performed using a Shim-pack IC-SA4 column (Shimadzu) for anion analysis. The eluent used for measuring anions was 12 mM sodium bicarbonate and 0.6 mM sodium carbonate. The calibration curve range for nitrate ions was set from 50 to 250 ppm.

Statistical analysis

Unpaired Student's *t* tests were employed for comparisons before and after solvent treatment and for comparisons between the two-phase culture and the control in the two-phase culture test using Microsoft Excel for Mac ver. 16.89.1 (Microsoft Japan, Japan). The results are presented as means $\pm$ standard deviations.

Results

Extraction tests with different solvents

Squalane and common short-chain hydrocarbons were compared as solvents, and the effects of the three solvents (*n*-hexane, *n*-decane, and squalane) on the hydrocarbon recovery rate and F_v/F_m ratio of the algae are shown in Fig. 2. Algae cultured in the three different media (Chu13, 0.3% medium, and 0.5% medium) were used.

When the Chu13 medium was used, *n*-hexane resulted in an increased recovery of hydrocarbons into the solvent phase, accompanied by a notable decrease in F_v/F_m . The hydrocarbon recovery with *n*-hexane was the greatest among the three solvents, with a value of $90.4 \pm 1.4\%$ at 1440 min (24 h).

The hydrocarbons recovery decreased with increasing carbon number of the solvent (*n*-hexane, *n*-decane, and squalane), while biocompatibility tended to improve. The salt concentration of the medium also contributed to the increase in the hydrocarbon recovery. In particular, squalane exhibited a minimal decline in F_v/F_m and simultaneously

achieved a high recovery rate of hydrocarbons from the alga cultured in 0.5% medium. Details regarding the changes in hydrocarbon recovery rate and biocompatibility for each medium are described below.

When the Chu13 medium was used, *n*-hexane achieved a hydrocarbon recovery of $90.4 \pm 1.4\%$ at 1440 min (24 h), although this was accompanied by a notable decrease in F_v/F_m . Conversely, the hydrocarbon recovery was below the detection limit of the GC-FID instrument ($< 1\%$ for all experimental conditions, 100 ppm) for *n*-decane and squalane even after the maximum contact time of 24 h. For *n*-decane, F_v/F_m decreased slightly after 24 h of contact (unpaired Student's *t* test, one-sided test, $p < 0.05$), but the decrease was limited to 2.1%. Squalane did not decrease F_v/F_m ($p > 0.05$).

Hydrocarbons were recovered from algae cultured in the 0.3% medium in all solvents. The hydrocarbon recovery was highest for *n*-hexane, ($92.6 \pm 0.5\%$ at 24 h). However, as hydrocarbon recovery progressed, F_v/F_m gradually decreased and reached 0 at 720 min (12 h) (hydrocarbon recovery, $61.9 \pm 1.0\%$). Hydrocarbons were extracted more slowly with *n*-decane, with a concurrent decrease in F_v/F_m . After 24 h of contact, the hydrocarbon recovery was $9.1 \pm 0.3\%$, and the F_v/F_m had decreased by 12.9%. For squalane, the hydrocarbon recovery at 24 h was $16.6 \pm 0.5\%$. Conversely, even after 24 h, there was no significant decrease in F_v/F_m compared with that before extraction ($p > 0.05$).

The hydrocarbon recovery of algae cultured in the 0.5% medium rapidly increased soon after the start of extraction in all the solvents. For *n*-decane and squalane in particular, hydrocarbon recovery increased dramatically compared to that in other media, reaching approximately 60% in 24 h. Furthermore, the decrease in F_v/F_m after 24 h was limited to 10.0% for squalane, even though the hydrocarbon recoveries were comparable.

Two-phase culture test

In the two-phase culture (upper solvent phase and lower algal suspension phase) test with squalane, hydrocarbons were successfully recovered in the solvent layer without negatively affecting photosynthetic activity. After 23 days of culture, 233.5 ± 51.3 mg of extracellular hydrocarbons were recovered from the solvent layer (Fig. 3). F_v/F_m in the two-phase culture test similar to that in the control at 23 days (unpaired Student's *t* test, one-sided test, $p > 0.05$). The total hydrocarbon production in the culture system, which is determined as the sum of hydrocarbons in the culture and those in the solvent layers, was 524.2 ± 27.7 mg at 23 days, which is 19.5% greater than in the control (438.5 ± 9.6 mg) ($p < 0.01$).

The chlorophyll content increased continuously in both the two-phase culture and the control culture (Fig. 3). In the

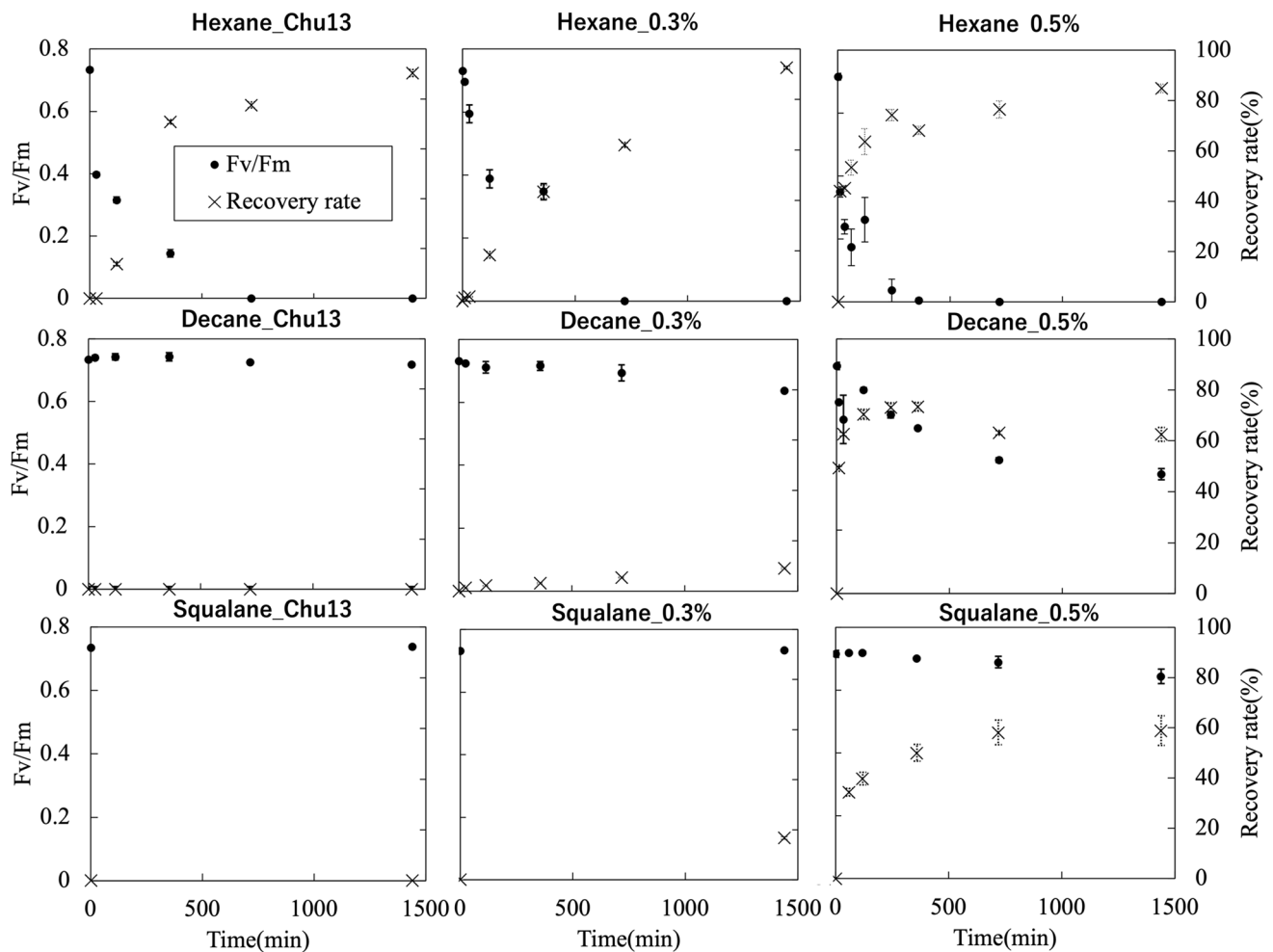


Fig. 2 Effects of the solvent (*n*-hexane; *n*-decane; squalane) and medium (Chu13; 0.3% medium; 0.5% medium) on the photosynthetic activity (F_v/F_m) and extracellular hydrocarbon recovery rate of algae over time ($n=3$, mean $\pm$ standard deviation (SD))

two-phase culture, the chlorophyll content was 39.8 ± 3.2 mg L^{-1} at 23 days, which was significantly (26.8%) greater than the value of 31.4 ± 1.6 mg L^{-1} in the control culture ($p < 0.01$). Nitrate was also continuously consumed and decreased in both the two-phase culture and the control culture. Nitrate was consumed at 45.7 ± 8.2 mg L^{-1} in the two-phase culture and at 47.6 ± 3.5 mg L^{-1} in the control culture throughout the entire culture period. There was no significant difference in nitrate concentrations between the two cultures on the last day of incubation ($p > 0.05$).

Discussion

Solvent evaluation

A major barrier for milking *B. braunii* is the difficulty of simultaneously achieving biocompatibility and high hydrocarbon recovery (Frenz et al. 1989). Therefore, to increase

the extraction efficiency as much as possible with a solvent that is less toxic to the cells, efforts are often made to increase the contact between the solvent and the algae during extraction via pretreatment with a low damage to the cells through cavitation (Tsutsumi et al. 2018), PEF (Ahirwar et al. 2022), or mechanical pressing (Moheimani et al. 2013). Furuhashi et al. (2016b, 2022) reported that *B. braunii* cultured in media with higher osmotic pressures than that of freshwater, such as diluted seawater media, did not develop hydrophilic colony sheaths, facilitating hydrocarbon recovery with hydrophobic solvents. The report also indicates that differences in salt concentration do not affect hydrocarbon content. In this study, we applied these results to experiments in which three media with various osmotic pressures (Chu13, 0.3%, and 0.5%) were tested. The results showed that increased osmotic pressure enhanced hydrocarbon recovery in all solvents (*n*-hexane, *n*-decane, and squalane). When *n*-decane and squalane were selected as solvents, hydrocarbons could

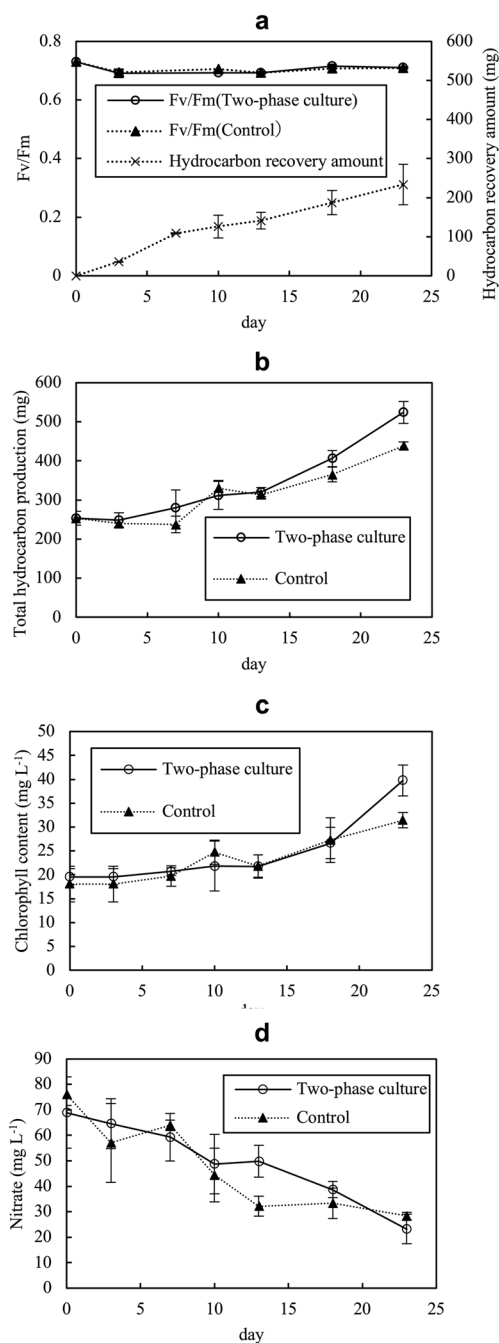


Fig. 3 Hydrocarbon recovery amount and F_v/F_m in the two-phase culture test (a), the total hydrocarbon production in the culture system (b), which is the sum of hydrocarbons in the culture and solvent layers, chlorophyll content (c) and nitrate concentration in the culture medium (d) ($n = 3$, mean $\pm$ SD)

not be extracted from the algae cultured in the Chu13 medium, but for those cultured in the 0.5% medium, the hydrocarbon recovery rate increased dramatically. The use of *n*-hexane also increased the recovery immediately after the start of extraction by increasing the osmotic pressure of the culture media.

In many species of microalgae, including *B. braunii*, solvent biocompatibility is highly correlated with the water–octanol coefficient $\log P_{oct}$, which indicates the hydrophobicity of the solvent; hydrophilic solvents with a low $\log P_{oct}$ typically disrupt the cell membrane structure (Sikkema et al. 1994; León et al. 2001; Zhang et al. 2011; Concha et al. 2018). In alkanes, shorter-chain hydrocarbons exhibit a lower $\log P_{oct}$ value, i.e., a lower biocompatibility (León et al. 2001). Therefore, *n*-hexane is not suitable for nondestructive extraction because it significantly reduces the photosynthetic activity of *B. braunii*, despite its high hydrocarbon recovery (Moheimani et al. 2013). In this study, squalane, a C₃₀ alkane and the longest-chain hydrocarbon ever used as a solvent, was the most biocompatible for algae. Conversely, the hydrocarbon recovery of squalane was inferior to that of *n*-hexane, but a hydrocarbon recovery of approximately 60% was obtained within 24 h from algae cultured in the 0.5% medium. As previously reported, *n*-decane is more biocompatible than *n*-hexane, but it exhibits a higher boiling point (Concha et al. 2018; Mehta et al. 2019). In wet extraction processes involving the mixing of *B. braunii* slurry with organic solvents, a portion of the solvent is absorbed into the ECM of *B. braunii* after extraction. During the recovery of hexane retained within the ECM, significant energy input is needed to cause simultaneous unintended water evaporation from the slurry (Saga et al. 2015). The energy input in solvent recovery is even greater for *n*-decane, which exhibits a boiling point closer to that of water than *n*-hexane does in the milking process. In addition, longer-chain hydrocarbons such as *n*-tetradecane, which were not included in this experiment, require more energy for solvent recovery because their boiling points are even higher (Mehta et al. 2019). While there are challenges regarding the availability and price of squalane as discussed later, these results indicate that the milking process with squalane is highly promising. When combined with culture in medium supplemented with 0.5% NaCl, it achieves both high biocompatibility and efficient hydrocarbon extraction. Furthermore, when biofuel production is the objective, squalane does not need to be separated from extracted hydrocarbons by fractional distillation, thus making the overall process more energy efficient and practical.

Milking via squalane

Approximately half of the total hydrocarbon production was recovered in the solvent layer (upper squalane phase) at the final day and F_v/F_m remained stable throughout the culture period in the two-phase culture experiment. To our knowledge, there are no examples of such high hydrocarbon recovery rates attained without lowering F_v/F_m . Moheimani et al. (2014) reported that in a continuous milking test with *n*-heptane, which has higher biocompatibility than *n*-hexane,

total hydrocarbon production increased. However, F_v/F_m gradually decreased over time. In our extraction test, F_v/F_m of the algae cultured in 0.5% medium decreased slightly by the extraction of squalane, whereas F_v/F_m did not decrease at all in the two-phase culture test. This may be due to the higher mixing ratio of the solvent to the culture medium. Solvent toxicity in organic solvent–water two-phase systems is caused not only by contact with the solvent dissolved in the water layer, but also through direct contact with the solvent layer, which prevents nutrient absorption (Bar 1988; Vermue et al. 1993). Concha et al. (2018) reported that even with biocompatible solvents in the A race of *B. braunii* (*B. alkenealis*), cell proliferation was reduced at higher solvent mixing ratios. In the extraction test, the mixing ratio of solvent to culture medium was greater than that in the two-phase culture test. In addition, the solvent and culture medium were separated into upper and lower layers via gentle agitation in the two-phase culture test, whereas the algae and the solvent were vigorously agitated through inverted agitation in the extraction test. Thus, the extraction test conditions easily inhibited nutrient uptake. In particular, squalane has significantly higher viscosity compared to conventional organic solvents. Technological development is required to simultaneously enhance extraction efficiency and suppress nutrient uptake reduction through methods such as micronization, which further improves extraction efficiency in two-phase culture systems.

The total hydrocarbon production and chlorophyll concentrations were greater in the two-phase culture than in the control culture. In this experiment, it was difficult to observe an increase in algal biomass density based on dry weight because squalane replaced hydrocarbons within the ECM. In this experiment, since F_v/F_m did not decrease, it is considered that there was no significant impact on intracellular chlorophyll between the control culture and the two-phase culture. Therefore, we consider that algal growth can be

tracked by changes in chlorophyll concentration, assuming the chlorophyll concentration within each cell is constant. Organic solvents have been shown to provide greater solubility of CO_2 and O_2 than water does; thus, moderate concentrations of solvents support gas exchange and can positively influence algal growth (Jia et al. 1997). Indeed, the addition of 10% (v/v) *n*-hexadecane to the culture medium increased the lipid production of *Nannochloropsis* sp. by 28.9% (Zhang et al. 2011). The addition of *n*-decane and limonene within a certain concentration range also promoted the growth of the A race of *B. braunii* (*B. alkenealis*) (Concha et al. 2018). The addition of squalane in the two-phase culture was also appropriate and may have caused increased CO_2 and O_2 levels in the culture medium, resulting in chlorophyll increases (algal growth) and total hydrocarbon production.

However, challenges remain in the milking process involving squalane. The recovery of extracellular hydrocarbons by squalane entails a displacement reaction that occurs through the colony surface layer. As extracellular hydrocarbons are recovered in the solvent, squalane simultaneously diffuses into the ECM. In this case, the net hydrocarbon recovery will not increase unless the production of hydrocarbons is high relative to the increase in the volume of the ECM (which holds the hydrocarbons) due to cell proliferation. In other words, a condition in which cell growth is inhibited and hydrocarbon production is promoted is desirable. However, in this study, the same amount of nitrogen was consumed as that in the control, the algae were growing, and the amount of hydrocarbons increased with their growth. This is also explained by the fact that no significant difference was observed in the ratio of total hydrocarbon production to chlorophyll concentration between the control culture (16.4 ± 0.9) and the two-phase culture (15.6 ± 1.3) on the final day. Therefore, certain innovations are necessary to increase the net recoverable hydrocarbons. For example, one possible approach

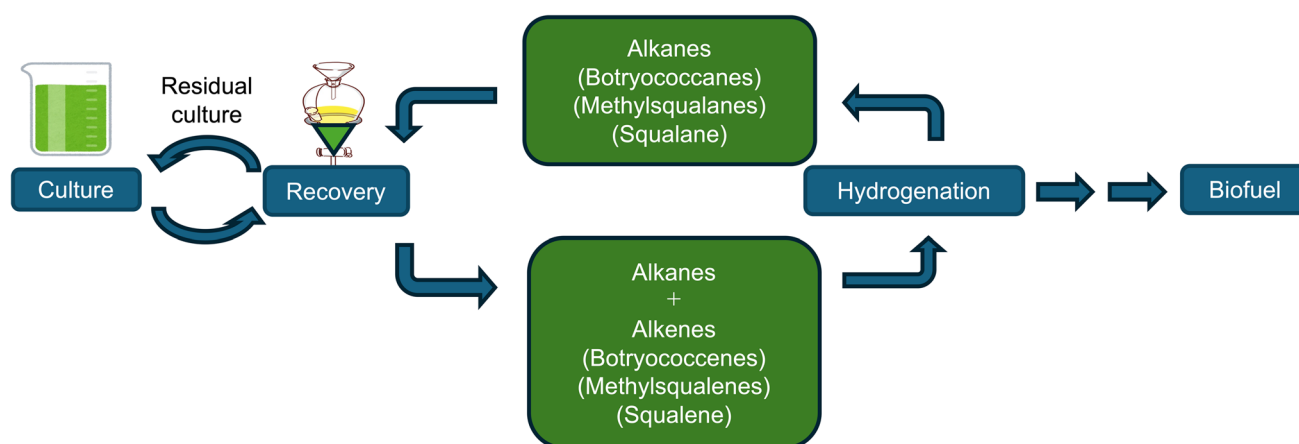


Fig. 4 Milking process using botryococcane as a solvent

is the use of the target of rapamycin (TOR) protein kinase, which regulates the switch between cell growth and hydrocarbon production (Loewith and Hall 2011; Imamura et al. 2015).

While milking involving squalane combined with saline culture conditions seems to be feasible over the long term due to its high biocompatibility, the availability and cost of squalane remain significant challenges. Squalane is traditionally purified from the liver oil of deep-sea sharks, although this process has raised concerns regarding environmental and ecological sustainability. Although plant- and microbially-derived squalane are also available, the plant-derived route suffers from low yield and high cost, while the microbial route faces challenges such as raw material costs and strain stability (Mendes et al. 2022; Shalu et al. 2024). To address this issue, a promising alternative is the use of botryococcane—the hydrogenated derivative of botryococcene, which is the main hydrocarbon produced by *B. braunii*—as an extraction solvent (Fig. 4). In this study, due to our capacity limitations, it was difficult to secure sufficient quantities of botryococcene to use as a solvent through large-scale culture. In this process, hydrocarbons are nondestructively extracted from *B. braunii*, and the culture medium is reused for the subsequent reculturing and hydrocarbon production. Moreover, the recovered botryococcene, together with the botryococcane used as the solvent, is subjected to hydrogenation. A portion of this mixture is not directed to downstream biofuel production (e.g., catalytic cracking) but is recycled instead as the solvent for the next extraction cycle (Hillen et al. 1982). Future studies including milking tests utilizing botryococcene are necessary to assess the feasibility of this process.

Conclusion

In this study we investigated the feasibility of using squalane as a solvent for hydrocarbon milking. Unlike conventional solvents, squalane may reduce energy input requirements by eliminating the need for fractional distillation between the solvent and the product. Compared with previously used solvents, squalane exhibited excellent biocompatibility, and efficient hydrocarbon extraction was achieved when *B. braunii* was cultured in a modified Chu13 medium supplemented with 0.5% NaCl. In a two-phase culture system with squalane, $45.1 \pm 7.6\%$ of the extracellular hydrocarbons was recovered without a decrease in F_v/F_m . To our knowledge, no previous study has indicated this level of hydrocarbon recovery without causing detectable damage to the cells. Moreover, an increase in hydrocarbon production was observed in

the process. These findings demonstrate that squalane, a hydrogenated derivative of the hydrocarbons produced by *B. braunii*, is an effective solvent for milking. This process represents a major advancement over traditional milking approaches that depend on energy-intensive distillation. However, for future practical applications, the supply and cost of squalane remain significant challenges. Therefore, replacing squalane with botryococcane, which is the hydrogenated form of botryococcene (the main hydrocarbon produced by *B. braunii*), is desirable, and the feasibility of this approach should be examined further.

Acknowledgements We express our sincere gratitude to Prof. T. Nishimura, Dr. T. Yamasaki, and Ms. N. Miyamoto for their technical support.

Author contributions Conceptualization: [T.M.], [K.F.], [K.I.]; Methodology: [T.M.], [K.F.], [S.O.]; Formal analysis and investigation: [T.M.], [K.F.]; Writing—original draft preparation: [T.M.]; Writing—review and editing: [K.F.], [S.O.], [Y.K.], [K.I.]; Funding acquisition: [K.F.], [S.O.], [K.I.]; Resources: [K.F.], [Y.K.]; Supervision: [K.F.]

Funding Open Access funding provided by The University of Tokyo. This work was supported by JSPS KAKENHI Grant Numbers JP21K19163, JP22K14975, JP25H00938.

Data availability The data during and/or analysed during the current study available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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