Investigation of the Wet Extraction of Hydrocarbons from a Heterotrophic Microalga, *Aurantiochytrium* sp. 18W-13a, by Cell Disruption Using a High-pressure Homogenizer

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*Aurantiochytrium*, a heterotrophic microalga, produces hydrocarbons inside its cells and has a higher growth rate than other photosynthetic microalgae. To investigate a possible method of extracting hydrocarbons from wet *Aurantiochytrium* cells, cell cultures of *Aurantiochytrium* were treated with a high-pressure homogenizer. The influence of cell disruption and extraction time on the cell morphology, hydrocarbon yield, and energy balance of hydrocarbon extraction was evaluated. The hydrocarbon yield increased with an increase in extraction time regardless of cell disruption. After one round of the homogenizing treatment in which cells were fragmented (one-pass treatment), a second round (two-pass treatment) did not cause any additional morphological change. For the 40-min extraction, the yield of hydrocarbon from disrupted samples was found to be over ten times higher than that of undisrupted ones. In contrast, for the 180-min extraction, the yield of undisrupted samples was almost the same as that of disrupted ones because the cells of *Aurantiochytrium* were disrupted by extended contact with n-hexane over the longer extraction time. Furthermore, the input energy of the 40-min extraction with cell disruption was 78% lower than that of the 180-min extraction without cell disruption to obtain almost the same hydrocarbon yield.

Key Words

*Aurantiochytrium*, High-pressure homogenizer, Cell disruption, Hydrocarbon extraction, Solvent extraction

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1. Introduction

Microalgae are valuable sources of biofuel because the demand for microalgae does not compete with food production, and they produce higher yields of hydrocarbons and lipids per cultivation area than do energy crops such as beans and corn. Biofuel from microalgae is typically produced via cultivation, harvesting, drying, extraction, and purification processes. However, in such a production system, because the input energy for fuel production is higher than the energy of the fuel itself, this system does not utilize energy efficiently. Lardon et al. reported that omitting the drying process from the production system can lower its energy consumption by 60%. Therefore, to optimize a practical system for biofuel extraction from microalgae, a more extensive investigation of the effectiveness of a production system without inclusion of the drying process is necessary.

Microalgae are cultivated at low concentrations and accumulate hydrocarbons and/or lipids inside their cells. Hence, the extraction of sufficient amounts of hydrocarbons or lipids from wet microalgae without any appropriate pretreatment is difficult unless a lipid extraction is performed over a long time period while using n-hexane, which is widely used as an extractant. In order to release intracellular substances (e.g., lipids) from the cells, previous studies have used several different pretreatment methods, including lysis of cell walls and/or membranes using acids, bases, or enzymes, application of supercritical/subcritical water or other fluids to liquefy the biomass, or cell disruption by applying thermal explosions or mechanical forces. While chemical methods have relatively low energy consumption and higher selectivity for releasing target components compared to mechanical methods, the chemical reagents they use must be completely removed from the extraction products, which again raises production costs. In addition, supercritical/subcritical fluids require high temperature and pressure to maintain the reactions, which would not be feasible for mass production of products like biofuels.

In contrast, mechanical cell disruption methods are less likely to cause contamination of the product, can be scaled up to process large volumes, need no further costs for pretreatment (unlike chemical pretreatments), are readily controlled, and require less energy than processes requiring drying or extraction using supercritical fluids. High-pressure homogenizers, bead mills, sonicators, and microwave ovens are mainly used as cell disruption devices in mechanical methods. Previous studies reported that these methods enhanced the extraction yields of hydrocarbons or lipids. Lee et al. performed cell disruption for various kinds of microalgae using several disruption methods, and reported that the efficacy of the disruption process differs depending on the microalgal species used. Therefore, it is necessary to identify the most effective method for use on any given target microalgae.

Aurantiochytrium sp. 18W-13a is a heterotrophic microalga that produces and stores the hydrocarbon squalene. Unlike autotrophic microalgae, which perform photosynthesis, Aurantiochytrium requires organic compounds (e.g., glucose) as carbon sources. Furthermore, because the doubling time of Aurantiochytrium is two hours, which is shorter than that of other cultured microalgae such as Nannochloropsis oculata and Botryococcus braunii, the prospects for mass production of biofuel from Aurantiochytrium are promising. As Aurantiochytrium stores hydrocarbon inside its cells similarly to microalgae in general, cell disruption should allow for wet hydrocarbon extraction from this microalga using organic solvents. Byreddy et al. disrupted cells of Schizochytrium sp. using a bead mill and investigated the influence of cell disruption conditions on lipid extraction yields. They showed that yields of lipids extracted from Schizochytrium sp. increased with increased rotational speed of the bead mill and processing time. However, they did not evaluate the degree of cell disruption quantitatively, which is crucial for determining the optimal operational conditions because the extent of cell disruption is related to extraction yield. To examine the disruption phenomenon and the disruption conditions that enable extraction of sufficient amounts of intracellular hydrocarbons or lipids, the influence of changes in cell size and shape on hydrocarbon yields from disruption needs to be investigated. Although increased contact time between algal samples and the organic solvent used for extraction improves extracted lipid yields, a sufficient amount of lipid extraction often requires a problematically long extraction time. Conversely, the effects of pretreatments such as cell disruption prior to lipid extraction would be quite small if extraction time is long. Hence, the effects of the length of extraction time on the energetic feasibility of cell disruption must be evaluated.

In the present study, wet Aurantiochytrium cells in culture were disrupted using a high-pressure homogenizer, and hydrocarbons were then extracted from the samples using n-hexane over different extraction time periods. The high-pressure homogenizer has considerable cell disruption ability and is effective at cell disruption of many different kinds of microalgae. To investigate the influence of cell disruption on cell size and shape, cells...
before and after disruption were observed using optical and fluorescence microscopy, and the particle size and shape distributions of cells were measured. In addition, energy analysis of cell disruption and hydrocarbon extraction was performed to evaluate the energetic feasibility of cell disruption and extraction conditions.

2. Materials and methods

2.1 Heterotrophic microalga

* Aurantiochytrium* sp. 18W-13a (3.2-5.0 g dry cell weight/L) was used as the algal sample. The GTY medium was used for cell cultivation; it contains 50% seawater, 2% glucose, 1% tryptone, and 0.5% yeast extract. Culture bottles were placed in a temperature-controlled reciprocal shaker, and cells were cultivated for 5 days. The cultivation temperature was set at 25°C, and the shaking speed was maintained at 100 rpm. The glucose concentration of the culture medium was measured using a phenol-sulfuric acid method every 24 h, and 40% (w/v) glucose solution was added for maintaining this glucose concentration. Dry cell weight was measured by gravimetric analysis, while considering moisture content within the samples. The detailed procedure was as follows: 30 mL of the cell culture was centrifuged at 3400 × g for 15 min to eliminate the influence of components of culture media on the gravitational analysis, and the supernatant was removed. Note the algal suspension was cultured using seven 500 mL Erlenmeyer flasks containing 300 mL of GTY liquid medium. They were cultured at the same time and then mixed together. After the cell culture was prefrozen at -28°C, the culture was lyophilized at -45°C for 5 days using a lyophilization device (FDU-1200; Tokyo Rikakikai Co., Ltd., Tokyo, Japan). After lyophilization, the moisture content of the lyophilized sample was measured using a moisture meter (MOC63u; Shimadzu Co., Japan).

2.2 Cell Disruption

The cell disruption of *Aurantiochytrium* was performed using a high-pressure homogenizer (LAB 2000; APV, Delavan, WI, USA), which was previously used by Tsutsumi et al. Approximately 1 L of cell culture was introduced into the device and mechanically disrupted, and then the treated culture fluid was used as samples for subsequent extraction procedures. A high-pressure homogenizer is a flow disruption/dispersion device that applies external forces to cells, such as shear force, static pressure, and impulsive force caused by cavitation. In the present study, the processing pressure of the homogenizer was set at 30 MPa, and the number of passes was one or two. The cell suspension was fed in at the inlet of the device and disrupted, and then the disrupted sample was collected at the outlet of the device. This sequence of the process was defined as the one-pass treatment. Two such processes repeated on the same sample were defined as the two-pass treatment.

2.3 Evaluation of cell morphology

Optical and fluorescence microscopes connected to a digital camera (IX73PI-33FL/DIC-TR + DP73-SET-C-2: Olympus, Japan) were used for observation of treated and untreated cells. To stain the intracellular hydrocarbons and lipids, 15 µL of Nile red (WAKO Pure Chemical Industries, Japan) solution (0.5 mg/mL in acetone) was added to 3 mL of the samples. The samples were observed after the mixture was left in a dark room for 10 min. Particle size distributions of cells before and after cell disruption were measured using a fully automated dynamic flow particle imaging instrument (Sysmex FPIA-3000; Malvern Instrument, UK). Particle size and shape were evaluated by measuring the circle-equivalent diameter (defined by Eq. (1)) and roundness (defined by Eq. (2)) as shown below:

\[
\text{(Circle-equivalent diameter)} = \sqrt{\frac{4S}{\pi}} \tag{1}
\]

\[
\text{(Roundness)} = \frac{4\pi S}{L^2} \tag{2}
\]

Where \(L\) is the circumference of the particle, and \(S\) is the projective area of the particle. The number of target particles was 5000, and measurements were performed in triplicate.

2.4 Hydrocarbon extraction and quantification

To extract hydrocarbon from samples before and after cell disruption, 20 mL of sample and 50 mL of n-hexane were mixed in a separatory funnel and shaken with an AW-2 shaker (AS ONE, Japan) at 318 rpm for the extraction times listed in Table 1. The water fraction was discarded, and all the solvent fractions were recovered. The amount of extracted hydrocarbon was quantified using a gas chromatography-flame ionization detector (GC-FID) (GC 2014; Shimadzu Co.) according to the protocol described by Tsutsumi et al. *n*-triacontane (C30, purity: 99%; GL Sciences Inc., Japan) was used as the internal standard.

| Sample conditions | Extraction time [min] |
|-------------------|-----------------------|
| Samples with cell disruption | 3, 20, 40, or 180 |
| Samples without cell disruption in wet condition | 40 or 180 |
| Samples without cell disruption in dried condition | 40 or 180 |
standard. Since the dominant component of the extracted hydrocarbons and lipids was squalene (33-69 wt% of total lipid content), total extracted hydrocarbon was quantified in terms of this compound only. To define maximum hydrocarbon yields, hydrocarbon extraction from dried *Aurantiochytrium* was performed. To extract hydrocarbon from the lyophilized samples, the samples were soaked in 50 mL of *n*-hexane and shaken for 40 min or 180 min. The maximum amount of extractable hydrocarbons from *Aurantiochytrium* was defined as the yield of hydrocarbons extracted from lyophilized samples (originally 30 mL of cell culture) for 180 min. After 180 min of extraction, the sample was then further subjected to extraction for 24 h. The amount of hydrocarbons extracted from the sample was 1.67-1.82 wt% of dry cell weight. As the yields of hydrocarbons from the re-extracted samples were 0.07 wt% of dry cell weight, it is reasonable to define the yields of hydrocarbon from the sample lyophilized for 180 min as the maximum amount of the extractable hydrocarbon. The extracted hydrocarbon yields were calculated using the following equation:

\[
\text{Hydrocarbon yields} = \frac{\text{Extracted hydrocarbon weight}}{\text{Extracted hydrocarbon weight in dried sample}} \times 100
\]

The extraction was performed in triplicate for each extraction condition, and the averaged values of all three replicates were defined as the overall hydrocarbon yields.

2.5 Energy analysis

The influence of cell disruption on input energy was evaluated by comparing the energy costs of the following three conditions: the first one was the hydrocarbon extraction method with cell disruption (Condition A), the second was that without cell disruption included (Condition B), and the third was the drying method (Condition C). The one-pass cell disruption with a 40 min extraction was defined as Condition A, while the 180 min extraction without mechanical disruption was defined as Condition B. The input energy for the homogenizer was measured with an electrical energy meter (Power Analyzer 3390, HIOKI E. E Co., Japan). Conversely, the input energy for the extraction was assumed to equal the energy used in shaking the separation funnels. Since the apparent power of the shaker was 200 W, the product of the power and shaking time was defined as the input energy for the extraction. The input energies for the extraction per weight of treated sample were evaluated. Furthermore, the drying of algal suspension was defined as Condition C. In the drying method, we assumed that the algal suspension was dried by 7% of the moisture content and its oil was completely extracted in 180 min. Note that the latent heat of water at temperature of 20 °C is 2.45 kJ/g. The assumed amount of the extracted sample was estimated as follows: 10 funnels (the limitation number of funnels that could be attached to the shaker) were fixed to the shaker and 20 mL of the cell suspension was poured per funnel, similarly to the extraction experiment. The density of the cell suspension was assumed as 1 kg/L. Furthermore, the output energy of the hydrocarbon fuel (squalene) was calculated using the following Dulong's formula:

\[
Q [\text{MJ/kg}] = 33.83C + 144.2H - 18.03O
\]

where \(Q\) is the higher heating value of the hydrocarbon, and \(C\), \(H\), and \(O\) are the mass fraction in the fuel of carbon, hydrogen, and oxygen, respectively. The calculated higher heating value of squalene was 47.3 MJ/kg. To simplify this energy analysis, the dry cell weight of algal suspension and the oil content in the alga were assumed to be 5 g/L and 2 wt% of dry cell weight, respectively. The data for the actual experimental hydrocarbon yield in each condition were used in this calculation.

3. Results and discussion

3.1 Influence of cell disruption on cell morphology

Fig. 1 shows the optical and fluorescence micrographs (the cell concentration was 3.2-5.0 g dry cell weight/L).
Cells were observed in the untreated sample (Fig. 1 (a)), and, as the cells were stained using the Nile red solution, oil droplets could be observed inside the cells before disruption. In the disrupted sample shown in Fig. 1 (b) and (c), whole cells were not observed, whereas the particles stained by Nile red solution were present in the sample. The stained particles were cell fragments left after disruption by the high-pressure homogenizer, which contained hydrocarbons or lipids. Comparing cells with the one-pass treatment to those with the two-pass treatment revealed that the extent of cell disruption in both samples was not recognizably different, suggesting that when cells were disrupted by the homogenizer, they were completely fractionated even after a one-pass treatment.

The circle-equivalent diameter and roundness of cells before and after cell disruption were analyzed to evaluate the influence of cell disruption on cell size and shape. Figs. 2 and 3 show the particle size and roundness distributions. Cells were approximately 10 µm in diameter in the untreated sample. In the disrupted sample, particles with 10 µm in diameter were not present, and the number of particles under 2.5 µm in diameter increased. These data showed that almost all the cells were fractionated by cell disruption. Moreover, the particle size distributions did not change between the one-pass and two-pass treatments. Lander et al. showed that the gap of the flow channel of a homogenizer that had a similar disruption mechanism to the one used in the present study was related to treatment pressure. According to their study, when treatment pressure was 30 MPa, the gap of the flow channel was approximately 10 µm. Thus, at a treatment pressure of 30 MPa, the particle size after a one-pass treatment was 1/10 of the gap of the flow channel in the device. Therefore, the interaction between the wall of flow channel and particles (e.g., shear force between the wall and particles) was small in the second treatment. The roundness distribution of the untreated samples showed that the cells had a spherical shape. After cell disruption, the number of particles with roundness values of 0.7-0.9 increased. Similarly to the particle size distributions, the roundness distributions of the two-pass treatment were almost the same as those of the one-pass treatment, indicating that the second cell disruption did not affect cell morphology at all.

![Fig. 2](image1.png)  ![Fig. 3](image2.png)

Fig. 2  Particle size distributions of samples: (a) before cell disruption; (b) disrupted by the high-pressure homogenizer after the one-pass treatment; (c) disrupted by the high-pressure homogenizer after the two-pass treatment. Error bars represent the standard error (n = 3)

Fig. 3  Roundness distributions: (a) before cell disruption; (b) disrupted by the high-pressure homogenizer after the one-pass treatment; (c) disrupted after the two-pass treatment. Error bars represent the standard error (n = 3)
3.2 Hydrocarbon yields

The influence of cell disruption using the high-pressure homogenizer on hydrocarbon yields was evaluated. Fig. 4 shows the yields of extracted hydrocarbons for each sample at various extraction times and/or number of treatment passes. Hydrocarbon yields increased with longer extraction time, suggesting that hydrocarbon extraction from the alga required sufficient time for diffusion of the extractant into cell or cell debris interiors. Regardless of the number of passes, the hydrocarbon yields of disrupted samples were almost the same in the 3, 20, and 40 min extractions. Thus, the second treatment with the homogenizer had no influence on the hydrocarbon yield. The small sizes of the cell fragments and oil droplets compared to the gap of the flow channel of the homogenizer, caused by the first disruption pass, led to limited interaction between the wall of the flow channel and these small particles in the second treatment pass.

For the 40 min extraction, the hydrocarbon yield of the disrupted samples was significantly higher than that of the untreated ones, suggesting that the intracellular hydrocarbons were released outside of the cells, and \( n \)-hexane and the hydrocarbons came into contact well. Compared to the hydrocarbon yield of the dried sample, the yield of the disrupted sample was larger. This was because the diffusion of \( n \)-hexane into the dried sample required a long time. The hydrocarbon yield of the disrupted sample, extracted for only 40 min, was 47.4% of the hydrocarbons contained in the algal cell. Small cell fragments (with sizes under 2.5 µm) containing hydrocarbons were present in the disrupted sample, as shown in Fig. 1. Thus, to extract more hydrocarbon, disruption of those fragments or extraction time longer than 40 min is necessary. For the sample subjected to extraction for 180 min, no difference in hydrocarbon yields between the untreated and disrupted sample was observed. To investigate why large amounts of hydrocarbons were obtained from untreated samples, the cell morphology of the untreated sample after shaking with or without \( n \)-hexane for 180 min was evaluated. Figs. 5 and 6 show the micrographs and particle size and shape distributions of those samples. Cells were not observed in the untreated sample after shaking with \( n \)-hexane for 180 min. Moreover, as the particle size and shape distributions of the untreated sample were similar to those of the disrupted sample, the cells in the sample extracted for 180 min were disrupted similarly to the cells in the homogenized sample. In contrast, for the untreated sample without \( n \)-hexane shaken for 180 min, while the cells changed a little in shape, cell disruption was not observed as long as observing the micrographs. Furthermore, Fig. 6 shows that the roundness of particles after shaking was lower than that before shaking, whereas the particle size of the shaken samples was similar to that of the untreated samples without shaking. Thus, the above facts suggest that the cells were not disrupted by the shaking only, but rather by shaking in the presence of \( n \)-hexane for a long time. In previous studies, extractants such as \( n \)-hexane and ethanol have been shown to damage the cell wall. Hence, the walls of algal cells were damaged by prolonged contact with \( n \)-hexane as \( n \)-hexane diffused inside the cells, causing the cells to swell, eventually disrupting (i.e. rupturing) them.

The hydrocarbon yield of the disrupted sample, extracted for 180 min, was only 72.7% of the hydrocarbons
3.3 Energy analysis of cell disruption and extraction

The results of the present study showed that, for the purposes of extracting hydrocarbons from microalgae, the cell disruption step in this process could be omitted for Aurantiochytrium 18W-13a. However, since such results do not always reflect the actual energetic feasibility of alternative methods, energy analysis was performed. Fig. 7 shows the input and output energies of the three scenarios compared for this analysis. Conditions A (one-pass cell disruption and 40 min extraction), B (180 min extraction with no disruption), and C (drying and 180 min extraction). The input energies of all the conditions were far larger...
than their output energies. However, the input energy of Conditions A and B was significantly less than that of Condition C, suggesting that extraction with or without mechanical cell disruption was the more reasonable process than the drying method in terms of energy balance. In addition, the input energy of Condition A was markedly (78%) less than that of Condition B, suggesting that mechanical cell disruption of this alga was the more reasonable method in terms of energy balance. This occurred because the cell disruption accelerated the mass transfer of algal hydrocarbon into the extractant. In summary, when the extraction time was set at 180 min, cell disruption using mechanical disruption devices such as a high-pressure homogenizer was unnecessary for *Aurantiochytrium 18W-13a* in terms of obtaining sufficient hydrocarbon quantity. However, in terms of energy balance, the cell disruption is better for achieving economically feasible algal biofuel production.

4. Conclusion

In the present study, mechanical cell disruption was investigated as a pretreatment option to effectively extract hydrocarbons from *Aurantiochytrium sp. 18W-13a* without drying. A high-pressure homogenizer was used, and the influence of mechanical cell disruption and hydrocarbon extraction time on cell size, cell shape, hydrocarbon yields of samples, and energy balance of hydrocarbon extraction were investigated. In the one-pass treatment, cells of *Aurantiochytrium* were disrupted, and the hydrocarbon yield increased compared to the yield of the untreated sample in the 3, 20, and 40 min extractions. In the two-pass treatment, the cell size, cell shape, and hydrocarbon yields of the disrupted samples did not change compared to those in the one-pass treatment. This was caused by the small size of the cell fragments (with sizes under 2.5 µm) compared with the gap of the flow channel of the homogenizer after the first cell disruption, meaning that resultant small particles have limited interaction with the wall of the flow channel. With increased extraction time, hydrocarbon yields of extractions also increased, and when the extraction time was 180 min, the yield of the sample without cell disruption was almost equivalent to that of the disrupted sample. This is because the cells were damaged and disrupted by contact with n-hexane in the untreated sample for a long time. In the energy analysis of the cell disruption and hydrocarbon extraction performed, the input energy of the extraction with the cell disruption was reduced by 78% compared to that of the longer extraction only. These facts suggested that when the extraction time was set at 180 min, cell disruption using mechanical disruption devices such as a high-pressure homogenizer are unnecessary for this alga to obtain hydrocarbons. However, in terms of energy balance, the cell disruption would be more economically feasible algal biofuel production.

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