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Improvement of Hydrocarbon Recovery by Two-Stage Cell-Recycle Extraction in the Cultivation of *Botryococcus braunii*

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Abstract *In situ* extraction by organic solvent was studied in order to improve the recovery yield of hydrocarbon from the culture of *Botryococcus braunii*, a green colonial microalga. When the solvent mixture of octanol as an extractive solvent and *n*-octane as a biocompatible solvent was added to a two-phase column, the algal growth was seriously inhibited, even at a low concentration of polar octanol. Therefore, a two-stage cell-recycle extraction process was proposed to improve the contact area between the organic phase and the aqueous phase. The hydrocarbon recovery with *in situ* cell-recycle extraction showed a three-fold increase (57% of cell) in yield over that with two-phase extraction. In addition, over 60% of the hydrocarbon could be recovered without serious cell damage by downstream separation when this process was applied to the culture broth after batch fermentation.

Key words: *Botryococcus braunii*, hydrocarbon, two-stage cell-recycle extraction, *in situ* extraction

The green alga, *Botryococcus braunii*, has an unusually high hydrocarbon content, ranging from 15 to 75% of dry weight, as a long-chain unsaturated hydrocarbon. It could have potential as a renewable source of chemical feedstock or fuel [1, 2, 3, 8, 9, 11, 12, 16].

Hydrocarbons have been separated from harvested wet algal cells of *B. braunii* by extraction with organic solvent after drying, filtering, or freeze drying on laboratory scale. However, these separation processes would not be suitable for large-scale cultivation due to their complexity and high cost [4, 7]. Mechanical and magnetic stirring of cell broth and hydrophobic solvent leads to the formation of clumps which shield a fraction of the cell from exposure to solvent. These clumps result in phase-separation difficulties and a decrease in hydrocarbon recovery and cell viability.

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In situ two-phase extraction by effective contact between cell broth and organic solvent may be an alternative way of overcoming these problems. Moreover, it is possible to harvest and re-cultivate biomass after extraction without serious damage because a drying process is not required.

In situ extraction using inert hydrophobic chemicals has been widely used in biotechnology for the recovery of water-insoluble products from cell suspension [15]. The key factor is the selection of a suitable solvent as the secondary phase, which must have a low inhibition to cell viability, be immiscible with culture broth with favorable partitioning of the compound of interest, and have a suitable density for phase separation in the extraction culture.

In the two-phase extraction culture of *B. braunii*, the polarity of the solvent is also an important property affecting hydrocarbon recovery efficiency. The presence of water surrounding the cells acts as a barrier to the recovery of hydrocarbons. The inaccessibility of weakly polar solvents, such as alkanes, for extraction to the cell surface, where the hydrocarbons are located, appears to protect the cell from toxic effects, but also prevents efficient recovery of the hydrocarbons. On the other hand, the good accessibility of a polar solvent, such as octanol or butoxyethoxyethane, to the cell surface leads to high efficiency of recovery, but also results in rapid release of other cellular contents and loss of cell viability [5].

The hydrocarbons produced by *B. braunii* are insoluble in the aqueous medium surrounding the cells, and are stored in the outer walls that build up the matrix of *B. braunii* colonies. Therefore, the hydrocarbons do not readily pass through the medium [6]. For this reason, poor recovery (18–32%) was obtained by two-phase extraction with various solvents in previous works [5, 6]. In order to enhance the recovery yield of hydrocarbons, optimal selection of solvents is required. An alternative approach would be to enhance the contact between the cell broth and the organic solvent.

In this study, the solvent mixture of extractive/biocompatible solvents was tested in a two-phase column and two-stage cell-recycle extraction.

A two-phase separator was proposed in order to obtain h gh recovery of hydrocarbon. We also investigated the possibility of *in situ* extraction, periodic extraction, downstream separation of the hydrocarbons using a two-stage cell-recycle extraction system, and the re-cultivation of biomass a'ter downstream separation, respectively.

MATERIALS AND METHODS

Algal Strain

Botryococcus braunii UTEX 572 was grown on a modified Chu13 medium [17]. Pre-cultures were carried out in a 250-ml Erlenmeyer flask with shaking at 100 rpm; at 25°C; light flux density 40 μE/m/s; light-dark cycle 14 h/10 h; inoculation at 10 % (v/v).

Bubble Column Culture Conditions

A bubble column, 30 mm diameter and 600 mm height, was operated at 25°C, illuminated continuously at 100 μE/m/s. Sterile-air containing 1% (v/v) CO_2 by filtering using glass fiber was aerated into the column through an air sparger at the bottom of the column at a rate of 100 ml/min.

Two-Stage Cell-Recycle Extraction

The schematic diagram of the two-stage cell-recycle extraction is shown in Fig. 1. The two-stage cell-recycle extraction process consisted of the bubble column (500 ml) for the algal growth and the two-phase separator (500 ml) for hydrocarbon extraction. The algal broth was recycled between the bubble column and the two-phase separator by peristaltic pumps (Tandem™ 1081, SciLog Inc, U.S.A.). The culture conditions for *in situ* extraction were the same as for bubble column extraction, except for the sparging of 1% (v/v) CO_2 air at the bottom of the two-phase separator at a rate of 50 ml/min.

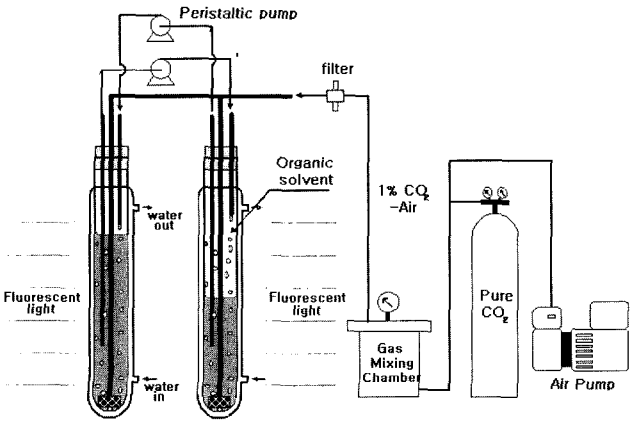

Fig. 1. Schematic diagram of two-stage cell-recycle extraction.

Algal Growth and Chlorophyll Analysis

Dry biomass weight was measured by filtration of aliquots with a pre-weighted Whatman filter (GF/C). The filtrated algae were rinsed with 10 ml distilled water twice and dried at 80°C for 24 h. Growth was determined spectrophotometrically at 680 nm. There was a direct correlation between optical density and dry biomass up to 2.5 g/l: dry biomass (g/l)= $0.515 \times OD_{680}$.

The pellet, after centrifugation of the cell broth (10 ml), was resuspended in methanol and a and b chlorophyll were extracted by sonication (VC 100, Materials & Sonics Inc., U.S.A.) for 10 min. Cell debris was removed by centrifugation (2,000 ×*g*, 5 min) and the chlorophyll in the cell debris was re-extracted by sonication for 10 min with a further volume of methanol. The absorbance of the green supernatant was measured at 665 nm and 650 nm using a spectrophotometer (U 2,000, Hitachi, Japan). The a and b chlorophyll concentrations were calculated according to the following formula [10]: Chl(chlorophyll) a (mg/l)= $16.5\ A_{665} - 8.3\ A_{650}$, Chl b (mg/l)=$33.8\ A_{650} - 12.5\ A_{665}$, and Total Chl (mg/l)=$25.8\ A_{650} + 4.0\ A_{665}$.

Hydrocarbon Analysis

The algal sample dried at 80°C for 24 h was extracted by sonication for 30 min with 10 ml acetone to which a known amount of squalene was added as an internal standard, and stirred for 3 h. After evaporation of the acetone in a rotary evaporator, the residue was dissolved in *n*-hexane and subjected to column chromatography (7× 2 cm) on silica gel [13]. The hydrocarbons were completely eluted with *n*-hexane before a yellow band. They were analyzed by a gas chromatography (HP-5890, Hewlett Packard, U.S.A.) with an FID detector.

RESULTS AND DISCUSSION

In Situ Two-Phase Extraction Culture with Solvent Mixture

In previous works [5, 6, 14], poor recovery (18-32%) of hydrocarbon was obtained by two-phase extraction culture with organic solvent. This was the result of insufficient mixing between the aqueous phase and the organic phase. In addition, hydrocarbon was entrapped tightly in the cell colonial matrix, which made it difficult to obtain a high recovery of hydrocarbon [14].

Polar solvents such as butoxyethoxyethane or octanol are most effective in hydrocarbon extraction. However, they showed very high toxicity [5]. Octane was most biocompatible in their work. Since octanol was very toxic to the cell,

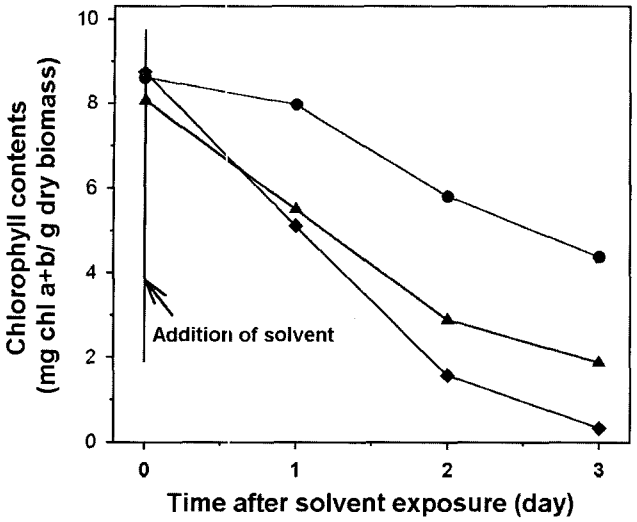

Fig. 2. Effects of mixed solvents on the photosynthetic activity estimated by chlorophyll contents.
(●) Control (without solvent); (▲) 1% octanol-octane; (◆) 3% octanol-octane.

several solvent mixtures were tested, such as 1% octanol-octane or 3% octanol-octane in the two-phase column, for *in situ* extraction.

The effects of solvent composition on the photosynthetic activity were estimated by chlorophyll contents (Fig. 2). Eighty ml solvent mixture of extractive solvent and biocompatible solvent was added to 400 ml cell broth in the two-phase column for *in situ* extraction. The cell was seriously inhibited even at low concentration of octanol. In the case of 3% octanol-octane, especially, algal cell was thoroughly bleached out and chlorophyll contents became nearly zero on the third day after 80 ml solvent had been added to 400 ml cell broth. Because cellular contents (chlorophyll) as well as hydrocarbon were recovered from the solvents layer by low concentration of polar octanol, cell viability could not be maintained in the two-phase column. Therefore, two-phase extraction with a solvent mixture of polar solvent and weakly polar solvent was not suitable for *in situ* extraction.

In Situ Two-Stage Cell-Recycle Extraction Culture

It was difficult to maintain cell viability when mixed solvent was used in the two-phase column for *in situ* extraction. Therefore, two-stage cell-recycle extraction was developed in order to obtain high recovery by improving contact between the cell broth and the organic solvent. The two-stage cell-recycle extraction system consisted of the bubble column for the algal growth and the two-phase separator for the hydrocarbon extraction. The algal broth was recycled between the bubble column and the two-phase separator by a peristaltic pump. When the algal broth dropped to the organic solvent in the two-phase separator, hydrocarbon attached on the alga colonial matrix was transferred to the organic solvent in the two-phase separator. As the algal broth was dropped in the form of small droplets into the solvent layer, the surface area and contact time between the cell broth and the organic solvent increased, which resulted in an improvement in hydrocarbon recovery.

In previous work [14], two-phase extraction with weakly polar dihexyl ether was able to obtain higher hydrocarbon recovery (32%) than with nonpolar alkanes such as *n*-octane, *n*-dodecane, *n*-tetradecane, and *n*-hexadecane (18–21%). In spite of growth inhibition, weakly polar dihexyl ether was selected as the optimum solvent for two-phase extraction because this solvent showed higher recovery yield than other solvents, although there was cell growth inhibition in the two-phase extraction. However, dihexyl ether has several shortcomings; it is expensive and requires more separation stages for purification of hydrocarbon due to its higher boiling point (228°C). Improving the contact area and time between two phases leads to a high recovery of hydrocarbon, but causes an increase in toxicity to the cell. Therefore, a two-stage cell-recycle extraction system requires a higher biocompatible solvent for successful *in situ* extraction.

Figure 3 shows the chlorophyll contents after solvent exposure in the cases of two-phase extraction with dihexyl ether and *n*-octane. The photosynthetic activities estimated by the chlorophyll contents in the cases of dihexyl ether and *n*-octane were 62% and 90% of the control culture, respectively. In the two-stage cell-recycle extraction culture, therefore, *n*-octane was selected as the optimum solvent due to its lower cost, lower boiling point (120°C), and lower toxicity to the cell than those of dihexyl ether.

Figure 4 shows the hydrocarbon concentration recovered in the solvent layer in two-stage cell-recycle extraction and

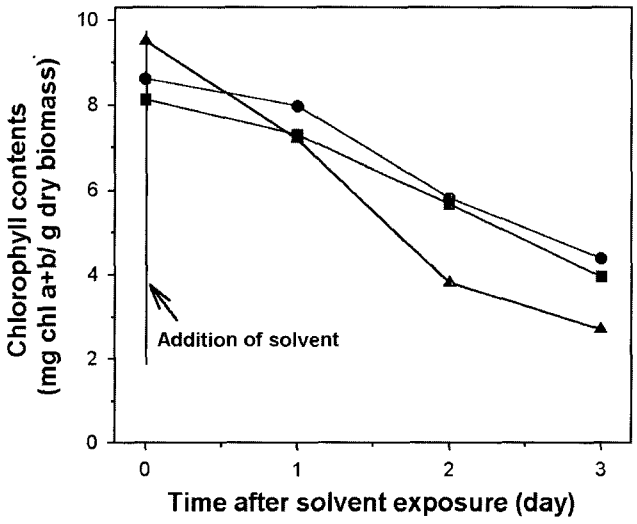

Fig. 3. Effects of *n*-octane and dihexyl ether on the photosynthetic activity estimated by chlorophyll contents.
(●) Control (without solvent); (■) *n*-octane; (▲) dihexyl ether.

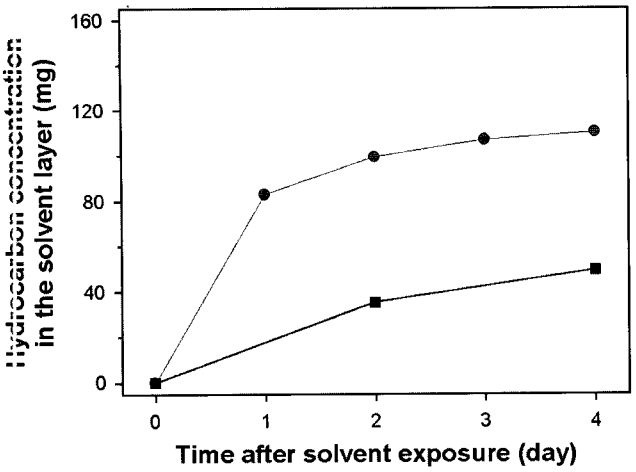

Fig. 4. Time courses of recovered hydrocarbon in the solvent layer (*n*-octane) by different extraction processes.
(◗) Two-stage cell-recycle extraction; (■) two-phase extraction.

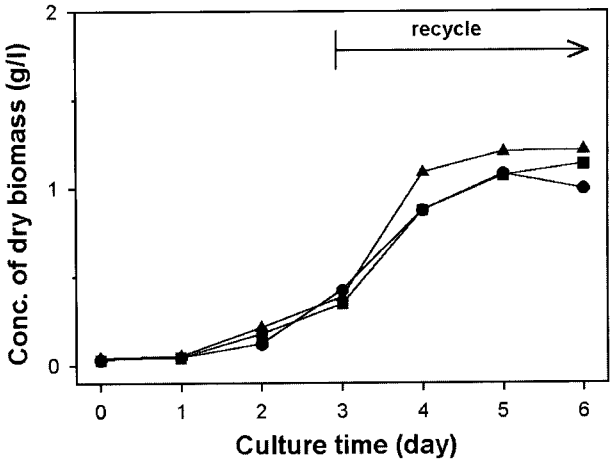

Fig. 5. Effects of recycle flow rate on the algal growth in the two-stage cell-recycle extraction culture.
(●) 12 ml/min; (■) 6 ml/min; (▲) 3 ml/min.

two-phase extraction with *n*-octane, respectively. After 3-day cultivation, 80 ml *n*-octane was added to the bubble column for two-phase extraction and the cell broth was recycled at a flow rate of 12 ml/min between the bubble column and the two-phase separator containing 150 ml *n*-octane. The hydrocarbon recoveries by two-phase extraction and two-stage cell-recycle extraction with *n*-octane were 20% and 57% after 4-day *in situ* extraction, respectively. The hydrocarbon recovery showed a 2.9-fold increase over two-phase extraction. On the other hand, the algal growth became more retarded (μ=0.012 h^{-1}) than in two-phase extraction (μ=0.029 h^{-1}). It seems that the more frequent solvent-to-culture-medium-contact resulted in lower cell growth rate and higher recovery yield in two culture modes. The hydrocarbon recovery and the growth rate under different extraction modes with various solvents are summarized in Table 1.

Effects of the Recycle Flow Rate on Hydrocarbon Recovery and Algal Growth

To investigate the effects of recycle flow rate on hydrocarbon recovery and algal growth, the cell broth was recycled at a flow rate of 3 ml/min, 6 ml/min, and 12 ml/min, respectively, between the bubble column and the two-phase separator after 3-day growth. Figures 5 and 6 show the algal growth in the bubble column and recovered hydrocarbon concentration in the two-phase separator. The hydrocarbon recoveries were 26%, 39%, and 52% for 3-day *in situ* extraction at a flow rate of 3 ml/min, 6 ml/min, and 12 ml/min, respectively. The specific growth rates were 0.017 h^{-1}, 0.016 h^{-1}, and 0.012 h^{-1} at a flow rate of 3 ml/min, 6 ml/min, and 12 ml/min, respectively. The hydrocarbon recovery increased and the algal growth retarded with increasing recycle flow rate.

Downstream Separation by Two-Stage Cell-Recycle Extraction and Recultivation of *B. braunii* after the Separation

The algal growth retarded considerably even at a low recycle flow rate between the bubble column and the two-phase separator. Therefore, two-stage cell-recycle extraction was applied to the downstream separation for hydrocarbon recovery after batch culture. Also, *B. braunii* inhibited by the solvent exposure was re-cultivated in order to investigate the possibility of biomass reuse after the downstream separation.

Figure 7 shows the recovered hydrocarbon concentration in the two-phase separator for 6 h at a flow rate of 100 ml/min, 150 ml/min, and 200 ml/min between the bubble column and the two-phase separator. The dry biomass and hydrocarbon concentration of the algal broth after batch culture were 1.6 g dry biomass/l and 194 mg hydrocarbon/l, respectively. The hydrocarbon recoveries were 45%, 62%, and 53% at a flow rate of 100 ml/min, 150 ml/min, and 200 ml/min, respectively. Because sudden contact at high recycle flow rate brought about the formation of large

Table 1. Specific growth rate and recovery yield of hydrocarbon under two-phase extraction and two-stage cell-recycle extraction culture.

Culture type	Solvent	Specific growth rate (h^{-1})	Hydrocarbon recovery (%)
Two-phase extraction culture	Dihexyl ether	0.019	32
	n-Octane	0.029	20
Two-stage cell-recycle extraction culture	*n*-Octane	0.012	57

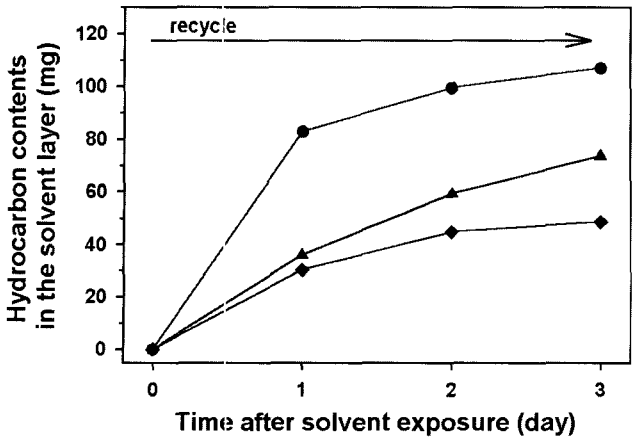

Fig. 6. Time courses of recovered hydrocarbon in the two-phase separator at various recycle flow rates by the two-stage cell-recycle extraction.
(●) 12 ml/min; (◆) 6 ml/min; (▲) 3 ml/min.

clumps, this phenomenon was minimized by a gradual increase in the recycle flow rate. As shown in Fig. 7, hydrocarbon recovery increased sharply after 3 h extraction. It seems that wetting cells with organic solvents may make it easier for the solvents to access the hydrocarbon located on the cell surface. At a flow rate of 200 ml/min, large clumps were formed and they floated upward toward the solvent layer. This resulted in lower hydrocarbon recovery than that at a flow rate of 150 ml/min.

To investigate the possibility of biomass reuse after downstream separation, 40 ml of the inhibited algae from the above experiments (downstream separation for 6 h) was inoculated in a 500-ml bubble column. Figure 8 shows the algal growth in the case of the re-cultivation after downstream separation by two-stage cell-recycle extraction.

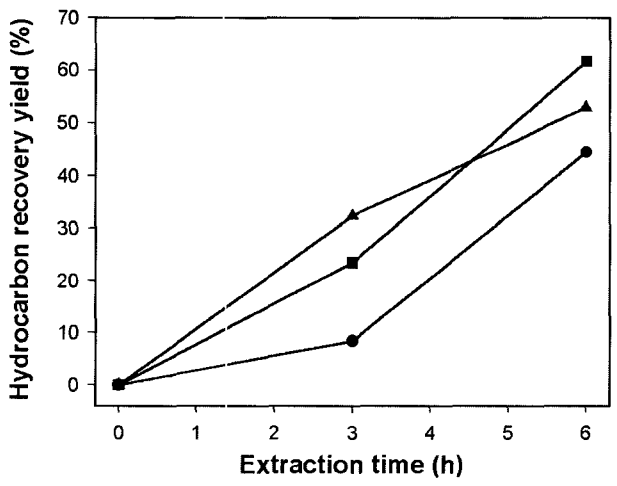

Fig. 7. Effects of recycle flow rate on the hydrocarbon recovery by downstream separation using two-stage cell-recycle extraction.
(●) 100 ml/min; (■) 150 ml/min; (▲) 200 ml/min.

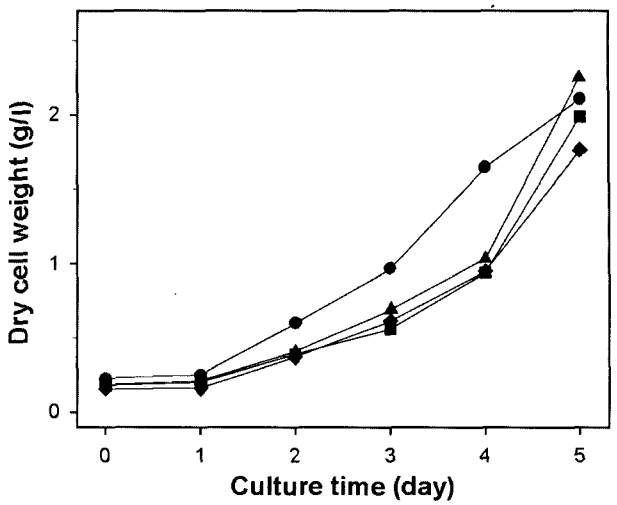

Fig. 8. Growth curve of *B. braunii* in the case of recultivation after downstream separation for 6 h by the two-stage cell-recycle extraction.
(●) Control; (■) 100 ml/min; (▲) 150 ml/min; (◆) 200 ml/min.

B. braunii was grown at a reduced rate of 0.021 h^{-1}–0.022 h^{-1} for 4 days. After a period of adaptation, however, *B. braunii* was grown at a high rate of 0.031 h^{-1}–0.032 h^{-1} and the concentration of dry biomass was similar to that of the control culture at day 5.

In this study, we showed that two-stage extraction could be successfully employed for high recovery yield of hydrocarbon. A water removal process, such as centrifugation, drying, and filtration, which is not suitable for a large cultivation, was not required in two-stage cell-recycle extraction. The efficient contact between the cell broth and organic solvent by two-stage cell-recycle extraction solved the problem of poor recovery in a two-phase extraction. The hydrocarbon recovery in a two-stage cell-recycle extraction culture for *in situ* extraction was two times higher than in two-phase extraction culture. Also, through downstream separation in this way, over 60% of hydrocarbon could be recovered without serious cell damage. Therefore, we anticipate that two-stage cell-recycle extraction may contribute to the design of the continuous and periodic removal process of a useful product from microalgae. The periodic extraction process in a large cultivation using piggery wastewater is now under development on the basis of these results.

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