Extraction of carotenoids and lipids from algae by supercritical CO₂ and subcritical dimethyl ether

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Abstract

Algae contain lipids and functional compounds such as carotenoids. Especially, microalgae are recently focused as a source of biofuel. To extract these components, organic solvent or supercritical carbon dioxide have been used. We have been developing wet extraction process using liquefied (subcritical) dimethyl ether (DME) as solvent at around 0.59 MPa. The extraction process usually requires energy consuming drying and grinding process as in the case of supercritical CO₂. We have applied liquefied DME for the extraction of lipids and functional compounds from various kinds

of algae. Since the liquefied DME extraction process can eliminate the process for drying, cell disruption, and solvent evaporation, it can realize simpler and low energy consumption system. In this paper, our studies on the extraction from algae by using supercritical CO₂ or subcritical DME are reviewed.

Keywords: Algae, lipid, carotenoid, extraction, carbon dioxide, dimethyl ether

1. Introduction

Algae are a diverse group of eukaryotic organisms, ranging from unicellular to multicellular forms, and various species contain valuable organic compounds. Algae are known to possess several functional properties such as antioxidative, anticancer, antiviral, and antiobesity activities. They contain lipids, sulfated cell-wall polysaccharides such as fucoidans, and several functional compounds. Recently, microalgae have become the focus of several research topics owing to their utility as sources of biofuel. A well-known species is the brown alga *Undaria pinnatifida*, commonly known as “wakame” in Japan. Wakame is an edible seaweed that is widely consumed and is a part of the diet in several countries, especially Korea and Japan. The microalga *Haematococcus pluvialis* is the richest known natural source of the ketocarotenoid astaxanthin. Astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione) is vibrantly red pigmented; therefore, it is used as a source of pigments in poultry and aquaculture industries. Furthermore, astaxanthin also has several key biological functions in poultry animals; it serves as a precursor of vitamin A and is associated with cell reproduction, embryo development, as well as protection against oxidative damage. Astaxanthin has been gaining widespread popularity as a human dietary supplement owing to its superior antioxidant properties compared to those of β -carotene, α -carotene, lutein, lycopene, canthaxanthin, and vitamin E [1-4]. Several astaxanthin-based products are now commercially available and are being promoted as anticancer, anti-inflammatory, and immunostimulating agents. Another important microalgal species is *Chlorella vulgaris*, which is known to contain very high concentrations of chlorophyll and a large amount of carotenoids like astaxanthin and canthaxanthin and minor amounts of β -carotene and lutein. Carotenoids derived from *C. vulgaris* have already been

successfully tested sources of pigments for poultry products, along with their ability to impart functional activity (e.g., antioxidant activity) [5-8]. Lutein is one of the most abundant carotenoids acquired through diet. Lutein is a desirable carotenoid since it is inexpensive and possesses antioxidant and anticarcinogenic properties. It can be obtained from animal sources such as egg yolk and plant sources such as marigold. However, the production of lutein from these sources is limited by a number of reasons. For example, homogeneous biomass cannot be produced at a constant rate at all times and under various weather conditions because marigold is a plant.

In this study, subcritical dimethyl ether (DME; critical temperature, 126.85 °C; critical pressure, 5.37 MPa) [9-12] and supercritical carbon dioxide (CO₂) were used [12-15] to extract lipids and the functional compounds (carotenoids) from various algae. Supercritical fluids are widely accepted for extraction, purification, recrystallization, and fractionation operations in many industries and are known to be more efficient extraction fluids than traditional liquid solvents. Supercritical fluids act as selectively dissolving liquid solvents when pressure and temperature are adjusted. Supercritical CO₂ is by far the most common supercritical fluid used in extraction of natural compounds and in food processing. Since CO₂ can be readily separated by process depressurization, the extracts obtained after using supercritical CO₂ are highly concentrated. This leaves no trace of harsh organic chemicals or residues in the product. Furthermore, since the CO₂ gas stream can be recycled, supercritical CO₂ extraction can be regarded as an environmentally friendly process. Liquefied (subcritical) DME has also been used as an extractant in place of CO₂ to extract fucoxanthin from *Undaria pinnatifida* [12, 16]. DME is the simplest form of ether, possessing the following characteristics: (i) DME has a low normal boiling point (−24.8 °C); therefore, it is not present in the final products at

normal temperatures [17]; (ii) Relative permittivity of DME is 1.08 and 5.34 at 30.5 °C, in gaseous and liquid states, respectively. Liquefied DME has high affinity for oily substances [18] and partial miscibility with water [19]; (iii) DME has been approved as a safe extraction solvent for the production of foodstuffs and food ingredients by the European Food Safety Authority (EFSA) [20], by the Food Standards Australia New Zealand, and by the United States [21]. The EFSA panels also consider the intended use of dimethyl ether as an extraction solvent to remove fat from animal protein raw materials provided: (a) the defatted animal protein is submitted to vacuum, which assures that most of the volatile dimethyl ether is eliminated from the final animal protein products; (b) the maximum residual limit of dimethyl ether is 9 μgkg^{-1} of extracted animal proteins and; (c) the extracted proteins are used at a level of up to 2% in the final foodproduct, at which the Panel considers that there is no safety concern; (iv) DME exhibits resistance to autoxidation, unlike other alkyl ethers [22].

Based on the characteristics of DME mentioned above, Kanda reported that liquefied DME can be used as a solvent to extract water from high-water content solids [23, 24], including low rank coals [23, 25-27], plants, foods [24, 28], sewage sludge [29], and polymers [30]. Similarly, Catchpole et al. reported the extraction of pungent components from low-water content chili, black pepper, and ginger by liquefied DME [31]. Sato and Matsumura reported extraction of phenol in water phase by liquefied DME [32]. Thereafter, Kanda [33] and Catchpole et al. [34] applied the water extraction method for the extraction of oily substances from high-water content solids. Kanda et al. reported extraction of odorous substances from sewage sludge [29, 35, 36], extraction of heavy oil from oil-polluted soil [37], extraction of polychlorinated biphenyls from river sediment [38, 39], extraction of caffeine and catechins from green tea leaves [40], and

extraction of vegetal oil from plants [28]. Catchpole et al. further reported the use of DME as an effective antisolvent for proteins in an aqueous solution and for water-soluble coating compounds. Lipids were extracted from aqueous protein/lipid mixtures, and this method was demonstrated on fresh and reconstituted egg yolks and on selected dairy by-product streams [41, 42]. Catchpole et al. have also reported the extraction of lipids from yeast and soil fungi [43]. Kanda et al. and Billakanti et al. have also reported on the extraction of carotenoids and lipids from the macroalga *U. pinnatifida* [12, 44] and microalgae *Botryococcus braunii*, *Oscillatoria agardhii*, *Microcystis aeruginosa*, and several more by using liquefied DME [8-12].

2. Materials and Methods

2.1 Materials

All algae used in this study as starting materials were commercially available in Japan. Samples were stored at 0°C in tightly sealed aluminum bags until use to prevent degradation of labile compounds. Standard lutein (70% purity), β -carotene (80% purity), chlorophyll a (90% purity), and chlorophyll b (80% purity) and HPLC grade ethanol, acetone, methanol, THF, and chloroform used for analysis were purchased from Wako Pure Chemical Industries, Ltd., Japan. CO₂ and DME were obtained from Uchimura Co. Japan, Sogo Kariya Sanso, Inc. Japan and Tamiya, Inc. Japan, respectively.

2.2. Analytical Methods

The major carotenoids were determined by using HPLC (high-performance

liquid chromatography). The organic components in algal extracts were recovered with 2 mL of analytical solvent. All solutions were filtered using a disposable 0.45- μ m filter prior to HPLC analysis. Calibration curves (5 points) were obtained by using pure carotenoid standards (fucoxanthin, astaxanthin, lutein, chlorophyll a, chlorophyll b, and β -carotene). The HPLC instrument was equipped with an ultraviolet–visible spectroscopy detector (UV-970; JASCO, Tokyo, Japan) and a diode array detector SPD-M10A (Shimadzu, Japan), equipped with an ODS-3 column (5 μ m; 250 mm $\times$ 4.6 mm; GL Sciences, Tokyo, Japan), STR ODS-II column (5 μ m; 4.6 $\times$ 250 mm; Shinwa Chemical Industries, Ltd., Japan), and 5C18-MS Waters column (5 μ m; 4.6 $\times$ 150 mm; Nacalai Tesque, Inc., Japan). The analytical methods used have been previously described in detail [12–14]. All extracts were analyzed in duplicate.

2.3. Experimental Apparatus

Figure 1 shows a schematic diagram of the supercritical CO₂ extraction apparatus. This apparatus includes a high-pressure pump for CO₂ (PU-2086; Jasco, Hachioji, Japan), a heating chamber (WFO-400; EYELA, Tokyo, Japan), a 25-mL extraction cell (Thar Technologies, Inc., Pittsburgh, PA, USA), and a backpressure regulator (AKICO, Tokyo, Japan). In this study, the extraction of carotenoids by supercritical CO₂ was conducted at temperatures of 40–80 °C and pressures of 20–40 MPa by using a semi-continuous flow-type system under various CO₂ flow rates. In each experiment, 3–7 g of dry algae sample was loaded into the extraction vessel, filled with glass beads at the bottom and top. The extraction vessel was placed in the heating chamber to maintain the operating temperature. Extracts were collected every 30–60 min for 5 h and were weighed and analyzed immediately after

collection. Bottles used for extract collection were wrapped in aluminum foil, and in all experiments, including DME extraction, products were stored at $-60\text{ }^{\circ}\text{C}$ without further treatment. Extracts were maintained under the conditions mentioned above until further analysis. Co-solvent effect of ethanol on supercritical CO_2 extraction was also examined. Ethanol flow rates supplied by using high-pressure pump (PU-2080; Jasco, Hachioji, Japan) were between 0.05 and 0.5 mL min^{-1} .

For liquefied DME extraction, a semi-continuous flow-type apparatus was used. Figure 2 depicts the scheme of apparatus used to evaluate the extraction efficiency of the process. The major components of this apparatus were an extractor (HPG-10-5; Taiatsu Techno Corp., Saitama, Japan; volume, 10 cm^3), a needle valve to control the DME flow rate, and an extract storage tank (HPG-96-3; Taiatsu Techno Corp.; volume, 96 cm^3). These components were connected in series. The extractor and DME storage tank were made of pressure-resistant glass coated with polycarbonate. Raw algae (water content: 93.2%) was loaded into the lower half of the extractor, and the upper half was loaded with colorless glass beads. DME flow rate was maintained at $10 \pm 2\text{ cm}^3\text{ min}^{-1}$. All experiments were carried out in a temperature-regulated room, and the column temperature was adjusted to approximately $25 \pm 1\text{ }^{\circ}\text{C}$ and measured by an infrared thermometer. The pressure was approximately $0.59 \pm 0.02\text{ MPa}$, which is the saturated vapor pressure at $25 \pm 1\text{ }^{\circ}\text{C}$. Liquefied DME was passed through the extractor at different time intervals after which it was evaporated by opening the reducing valve of the storage vessel. Following this, liquefied DME was passed through the lower half of the extractor whereby the color of the glass beads in the upper half changed to olive-green. Since the glass beads and liquefied DME were colorless, a change of the glass bead color to olive-green indicated probable presence of extract. The extraction

test was completed when the color of the glass beads was restored to colorless. The total extraction time was less than 43 min.

3. Results and Discussion

3.1 Supercritical CO₂ extraction

Figures 3a and 3b show the fucoxanthin extraction curves from *U. pinnatifida* at different extraction times and under varying pressure (10–40 MPa; 60 °C) (Fig. 3a) and varying temperature (40–70 °C; 40 MPa) (Fig. 3b). The physical appearance of the extracts was oily and their amount increased with increase in pressure at constant temperature. This tendency is attributed to a direct increase in density and hence the solvating power of supercritical CO₂. The yield of fucoxanthin increased significantly up to an extraction time of 150 min. For example, the yield was about 47 µg g⁻¹ dry weight of *U. pinnatifida* after extraction for 30 min at 40 MPa and 60 °C. This increased gradually to 58 µg g⁻¹ dry weight at an extraction time of 150 min. Beyond an extraction time of 150 min, the yield of fucoxanthin did not increase significantly. At the same extraction temperature, a similar trend was observed at 10, 20, and 30 MPa. As previously mentioned, the effect of pressure can be attributed to the increase in solvent power due to the strengthening of intermolecular physical interactions. An increase in pressure also affected the solubility of fucoxanthin in supercritical CO₂. These results are in agreement with the trends reported in other studies.

At constant pressure (40 MPa), the yield of fucoxanthin increased with increasing extraction temperature. The yield increased rapidly until 150 min of extraction and then remained constant after 200 min at each extraction temperature. The extraction rate of fucoxanthin was highest when the extraction temperature was 60 °C

(Fig.3b). This indicated the dependence of fucoxanthin extraction on solute vapor pressure which increased with an increase in temperature. Increasing the temperature contributed to the decomposition of cell walls, and as a result the amount of fucoxanthin and other extractable compounds increased. Figure 3b also showed that an increase in temperature likely had a stronger effect on the solubility than an increase in pressure. At 70 °C, the yield of fucoxanthin is lower than at 60 °C. This might be possible due to the degradation of fucoxanthin at higher temperature, considering that carotenoids are heat-sensitive.

Figure 4 shows the amounts of astaxanthin extracted from *Haematococcus pluvialis* by supercritical CO₂ extraction with and without ethanol as an entrainer, under various pressure conditions at 40 °C, when CO₂ flow rate is maintained at 3 mL min⁻¹. In this work, the effect of addition of the entrainer was studied mainly at low temperature, since the rate of extraction under this condition was low. Experiments were conducted at up to 40 MPa, since the maximum capacity of the entrainer pump was 50 MPa. In this work, the use of ethanol at a concentration of 1.67% was found to enhance the amount of extracted astaxanthin more than 2-fold. A significant increase in extraction efficiency was due to ethanol that enhanced the solubility of specific compounds in supercritical CO₂. Ethanol functioned as polarity modifier and led to swelling of the matrix [45-49], thus increasing the internal volume and the surface area for the contact with supercritical CO₂. The addition of ethanol in supercritical CO₂ also resulted in decomposition of the cell wall [49-51], resulting in increased astaxanthin available for extraction. Under 40 MPa pressure and at 40 °C, the amount of extracted astaxanthin also increased with increasing ethanol concentration (up to 5% (v/v) ethanol). At this condition, more than 80% of astaxanthin could be extracted.

However, at a higher entrainer concentration of 7.5% (v/v), lower amount of astaxanthin was extracted. This was probably due to the lowering of the density of the supercritical fluid.

Table 1 shows the main components extracted from *C. vulgaris* by supercritical CO₂ using entrainer (based on HPLC chromatogram). The yield of each pigment in the extract, obtained by supercritical CO₂ with ethanol as an entrainer, was higher than that obtained with and without acetone as a co-solvent. This is because the solubility of pigments, especially lutein and chlorophyll, is higher in ethanol than in acetone. Pigments are soluble in alcohol but have low solubility in ketones. In addition, ethanol present in supercritical CO₂ led to modification of the characteristics of supercritical CO₂, and resulted in enhancement of its solvent properties, thus facilitating the extraction of pigment components. In this case, the undesirable components were not extracted together with the target component. Furthermore, also when food safety is considered, ethanol is more appropriate as an entrainer than acetone owing to its low toxicity compared with acetone.

3.2 Liquefied (Subcritical) DME Extraction

Figure 5 shows the yield of fucoxanthin extracted from *U. pinnatifida* with liquefied DME. Increasing amount of DME led to an increase in the amount of extracted fucoxanthin. When 286 g DME was used (at the last time point of extraction process), the residue of the *U. pinnatifida* was almost completely dehydrated and almost resembled a dried paper. At this point, the color of *U. pinnatifida* residue was light green and the amount of fucoxanthin was approximately 390 µg g⁻¹ dry weight of *U. pinnatifida*. It is well known that liquefied DME can dissolve a wide range of polar and non-polar

substances. They are also good solvents for many hydrogen-bonded substances. To dissolve hydrogen-bonded substances, high solvation energy was needed. In this case, DME has the ability to form hydrogen bonds with hydrogen-bonding solutes. Therefore, liquefied DME can penetrate into *U.pinnatifida* cells and extract components like fucoxanthin into the medium. The cumulative fucoxanthin extracted seemed significantly enhanced with increasing DME consumption. Like previously mentioned, despite possessing a high dissolving ability, liquefied DME could also generate a less viscous matrix, and therefore allowed better diffusion of the solute from the solid phase to the solvent. Consequently, the extraction of fucoxanthin in *U.pinnatifida* was achieved easily.

Lipids can be extracted from various types of algae by liquefied DME owing to its characteristic properties. A basic scheme of the process of extraction of lipids is depicted in Fig. 6. The operational temperature shown in Fig. 6 can be set arbitrarily close to the ordinary room temperature, and 30 °C, which is depicted in the figure, is a representative example. The operational pressure is the saturated vapor pressure of DME. After partial dewatering, microalgae are collected and packed in the extraction column. Water and lipids are further extracted using liquefied DME and are discharged from the extraction column in the form of a mixture. In case the lipid concentration is not sufficiently high, the mixture is returned to the extraction column. The DME with saturated extracted oil is heated and evaporated using hot water. The residual waste heat from a high-grade heat source can be used for this purpose. However, sun-warmed hot water or heat generated from small biomass electric generator with not much of electricity generation efficiency is desirable. 45 °C setting of sun-warmed water as depicted in the figure is representative, and it is advisable to increase the approach

temperature in order to reduce the size of the heat exchanger. The evaporated DME is in a gaseous high-pressure state at ordinary temperature that can be condensed by using seawater and reused as liquefied DME. In order to reduce the size of the heat exchanger, it is advisable that the temperature of the cold source is low. In this context, the actual temperature of circulating DME may be decided based on the temperature of the cold source and the approach temperature of the heat exchanger.

Figure 7 shows a comparison of the yield of lipid extraction from various algal sources using the liquefied DME and Bligh-Dyer method. Microalgae are initially partially dried by centrifugation and filled in a column of inner volume of 5cm³ in a state of 70%–90% water content. The quantity of oil obtained after passing sufficient amount of liquefied DME through the column is shown in Fig. 7. The proportion of lipids obtained by means of liquefied DME, based on dry weight of microalgae, is shown in white. Lipids obtained using the Bligh-Dyer method, wherein the microalgae are dried and cell disrupted, are shown in black. The ratio of the weight of the lipid obtained by DME to that extracted by Bligh-Dyer method is mentioned. The Bligh-Dyer method makes use of a methanol-chloroform mixture and is regarded effective for the extraction of lipids of various polarities. As seen in Figure 7, at least 97% lipid was obtained from the different algal sources by using liquefied DME by considering Bligh-Dyer method as a reference. Maximum amount of lipids was obtained from the blue-green microalgae collected from Hirosawalake in Kyoto city, Japan, and it accounted for 40.1% of dry weight in case of liquefied DME. In the Bligh-Dyer method, it was 40.2%. In this case, if the cell-rupture treatment was not used in the Bligh-Dyer method, the quantity of extracted oil was not more than 0.6%. Thus, it is clear that microalgal cell disruption, which is a necessary step in the Bligh-Dyer method to

increase the amount of extracted lipids, can be skipped when the liquefied DME method is used. Based on the analysis of lipid properties; such as molecular weight distribution determined by gel permeation chromatography, elemental composition and calorific value; it was concluded that the properties of lipids obtained by using liquefied DME were almost the same as that obtained by the Bligh-Dyer method. Furthermore, the extraction of hydrocarbons from *Botryococcus braunii* by using DME, without using any pretreatment like cell-drying or cell-disruption, could be confirmed.

3.3 Development of Extraction Technology

The process of extraction of bioactive compounds from plants has usually been accomplished by traditional methods such as solid-liquid extractions employing methanol, ethanol, acetone, or hexane and through steam distillation or evaporation processes for removal of solvents from extracts. Currently, the demand for natural bioactive compounds is on the rise due to their application in the functional food and pharmaceutical industry [52]. To cope with this demand, the development of an appropriate extraction technology that is able to provide high-quality extracts is needed. Development of an efficient extraction technology is also needed for the following reasons: (a) tightening government regulations on toxic-chemical solvent residues and pollution control, (b) consumer concerns over the use of toxic-chemical solvents during processing, and (c) increased demand for higher quality products that traditional processing techniques cannot meet [52]. In this study, the connection between supercritical CO₂ and liquefied DME as clean extraction processes for bioactive compounds from algae has been demonstrated. This process could be operated safely and is one of the major advantages associated with the development of new extraction

processes. As a non-polar substance, SCCO₂ mainly solubilizes low molecular weight, non-polar compounds and is a poor solvent for high molecular weight, polar compounds. This makes extractions using SCCO₂ very selective due to penetration of CO₂ into complex matrices. Further, the solubility of polar solutes in SCCO₂ could be significantly increased by adding a small amount of polar co-solvent. However, this process might be difficult to apply due to potential issues concerning separation of the co-solvent at a later time point. This problem was overcome by employing DME as an extractant. In summary, we developed a very simple, versatile, and feasible technique for extraction of bioactive compounds from microalgae.

4. Conclusions

This study provides an overview about the extraction of carotenoids and lipids from algae by using supercritical carbon dioxide. This process employing subcritical DME saved energy and was effective for extraction of bioactive compounds from wet algae. Since the water content of biomaterials is very high, thorough drying is necessary. However, drying of wet biomaterials is highly energy consuming. Subcritical (liquefied) DME is unique and suitable for extraction of water and oily substances from high water-content biomaterials and has potential to cut energy consumption of extraction procedures.

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Figure captions

Figure 1. Schematic diagram depicting supercritical CO₂ extraction

Figure 2. Schematic diagram depicting liquefied DME extraction

Figure 3a.Effect of pressure on fucoxanthin extraction as a function of time at an extraction temperature of 60 °C [12].

Figure 3b.Effect of temperature on fucoxanthin extraction as a function of time at an extraction pressure of 40 MPa[12].

Figure 4.Comparison of supercritical CO₂ extraction with and without 1.67% entrainer at various pressure conditions, at 40 °C and a flow rate of 3 mLmin⁻¹ [13].

Figure 5.Fucoxanthin yield in the extract obtained from wet *Undaria pinnatifida* by liquefied DME [12].

Figure 6.Lipid extraction from wet microalgae by using liquefied DME.

Figure 7.Lipid extraction ratio for several species of natural microalgae by using the DME method [10].

Table 1. Composition of *Chlorella vulgaris* extract at 60 °C, 30 MPa, and CO₂ flow rate of 2.5 mL min⁻¹, with 7.5% ethanol for 120 min [13].

Retention time [min]	Component	Peak area [%]
2.22	Unidentified	16.91
2.84	Lutein	19.00
3.63	Unidentified	6.32
5.10	Chlorophyll b	2.83
7.17	Chlorophyll a	37.50
7.89	Unidentified	9.70
15.41	Unidentified	4.71
19.23	β-carotene	2.93

Figure 1

Figure 2

Figure 3a

Figure 3b

Figure 4.

Figure 5

Figure 6

Figure 7

Graphical Abstract

Solvent cycle for lipid extraction process from wet microalgae by using subcritical dimethyl ether (DME).