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Extraction of Astaxanthin from *Haematococcus pluvialis* Using Supercritical CO_2 and Ethanol as Entrainer

Siti Machmudah,[†] Artiwan Shotipruk,[‡] Motonobu Goto,*[,†] Mitsuru Sasaki,[†] and Tsutomu Hirose[†]

Department of Applied Chemistry and Biochemistry, Kumamoto University, Kurokami 2-39-1, Kumamoto 860-8555, Japan, and Chemical Engineering Department, Chulalongkron University, Phayathai Road, Patumwan, Bangkok 10330, Thailand

This study deals with supercritical CO_2 extraction of astaxanthin from *Haematococcus pluvialis* with ethanol as an entrainer. The effects of pressure, temperature, CO_2 flow rate, and the existence of the entrainer concentration on the amount of total extract, the amount of astaxanthin extracted, and the astaxanthin content in the extract were studied. Extractions were carried out at the pressures of 20−55 MPa, temperatures of 313−353 K, CO_2 flow rates of 2−4 mL/min, and the ethanol entrainer concentrations of 1.67−7.5% (v/v). The amount of the total extract, astaxanthin extracted, and astaxanthin content in the extract increased with increasing temperature and pressure. With higher CO_2 flow rate, the amount of the total extract could be increased while the amount of astaxanthin extracted and the astaxanthin content in the extract almost did not change. By using ethanol as an entrainer, a higher amount of astaxanthin extracted (80.6%) could be obtained at moderate pressure and temperature, and with the same conditions, the addition of the ethanol entrainer could more than twice enhance the amount of astaxanthin extracted. Without the ethanol entrainer, the highest astaxanthin extracted and astaxanthin content in the extract were 77.9 and 12.3%, respectively, and were obtained at high pressure and temperature.

Introduction

Astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) is a vibrantly red ketocarotenoid which has been used as a pigmentation source in poultry and aquaculture industries. In addition to pigmentation, astaxanthin has several key biological functions in these farmed animals. It serves as a precursor of vitamin A and is associated with cell reproduction and embryo development, as well as with cell protection against oxidative damage. Due to its superior antioxidant activity compared with α-carotene, β-carotene, lutein, lycopene, cantaxanthin, and vitamin E, astaxanthin has been gaining widespread popularity as a human dietary supplement.[1−4] Currently, several astaxanthin products derived are available in the marketplace and being promoted as anticancer and antiinflammatory agents as well as immunostimulants.[5,6]

Astaxanthin may be of synthetic origin or obtained from natural sources, e.g. microalgae, yeast, or crustacean byproducts. Nevertheless, natural astaxanthin exhibits greater stability than the synthetic compound. Of all the astaxanthin-producing microorganisms, the chlorophyte alga *Haematococcus pluvialis* (*H. pluvialis*) is believed to accumulate the highest levels of astaxanthin. Commercially grown *H. pluvialis* contains between 1.5 and 3.0% astaxanthin, which consists approximately of 70% monoesters, 25% diesters, and 5% free form. All of the free astaxanthin and its monoesters and diesters have optically pure 3*S*,3′*S* chirality.[7]

Supercritical fluids are now widely accepted for extraction, purification, recrystallization, and fractionation operations in many industries. The technology is used to process hundreds of millions of pounds of coffee, tea, and hops annually, and it is increasingly becoming of common use in pharmaceutical industries for purification and nanoparticle formation.[8−10] Supercritical fluid processing is also gaining more popularity in the botanicals, vitamins, and supplements industries, in which they are becoming synonymous with the highest purity and quality.

Supercritical fluids have been known to be more efficient extraction fluids than the traditional liquid solvents. By adjusting the pressure and temperature, they act like liquid solvents, but with selective dissolving powers. Supercritical CO_2 (SC-CO_2) is by far the most common supercritical fluid used in extraction of natural compounds and food processing. The extract obtained using SC-CO_2 extraction is highly concentrated as CO_2 can be readily separated by process depressurization. This leaves no harsh organic chemicals or residues in the product. Furthermore, the resulting CO_2 gas stream can be recycled, making SC-CO_2 extraction an environmentally friendly process.[11]

Numerous studies have been conducted on extraction of carotenoids from natural plant and marine materials.[7,12,13] SC-CO_2 extractions of astaxanthin from red yeast *Phaffia rhodozyma* and crustacean sources have been reported.[14,15] However, only one published study in the literature was found to involve SC-CO_2 extraction of astaxanthin from *H. pluvialis*.[16] In their paper, Valderrama et al. (2003) conducted SC-CO_2 extraction of astaxanthin from the microalgae at the operating temperature of 60 °C and the pressure of 30 MPa and demonstrated the importance of algae cellular wall breaking and the use of ethanol entrainer on extraction efficiency.

This study was designed to investigate the effect of process variables such as pressure, temperature, and SC-CO_2 flow rate on the amount of total extract, the amount of astaxanthin extracted, and the astaxanthin content of the extract from *H. pluvialis*, as well as the effect of the concentrations of ethanol entrainer.

Materials and Experimental Procedures

Materials and Chemicals. Dried samples of microalgae *H. pluvialis* (maximum of 5% astaxanthin feed grade powder) were

* Corresponding author. Tel: +81-96-342-3664. Fax: +81-96-342-3679. E-mail: mgoto@kumamoto-u.ac.jp.
[†] Kumamoto University.
[‡] Chulalongkron University.

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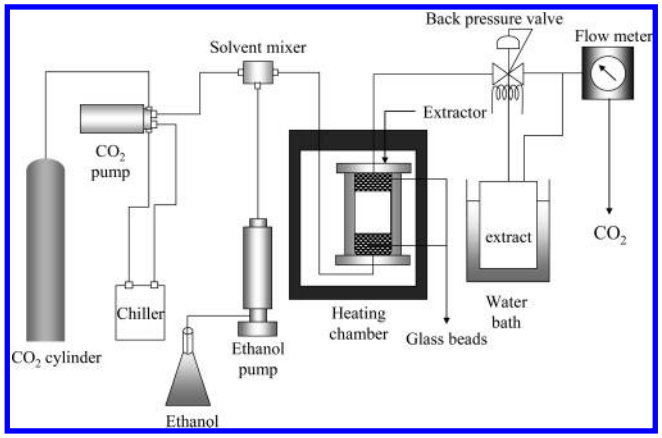

Figure 1. Schematic diagram of supercritical extraction of astaxanthin.

purchased from Cyanotech Corp., Hawaii Ocean, Science Technology Park, USA. To prevent degradation, the samples were stored at 277 K in a tightly sealed aluminum bag until use. Standard astaxanthin with purity of 94% and the HPLC-grade solvents acetone, methanol, acetonitrile, dichloromethane, and water, used for solvent extraction and for the HPLC analysis, were purchased from Wako Pure Chemical Industries, Ltd., Japan. CO_2 was obtained from Uchimura Co., Japan.

SC-CO_2 Extraction. Figure 1 shows a schematic diagram of a continuous-flow SC-CO_2 extraction apparatus. The apparatus includes a chiller (Sibata, Coolman C-560, Japan), a high-pressure pump for CO_2 (Jasco PU-2080-100 MPa, Japan), an ethanol entrainer pump (Syringe pump Model 260D, ISCO, Japan), a heating chamber (ST-110, ESPEC, Japan), a 50 mL extraction vessel (Thar Tech, Inc., USA; 50 mL in volume), back-pressure regulator (AKICO Co., Japan), a number of collection vials, and a wet gas meter (Sinagawa Co., Japan). Astaxanthin were extracted from *H. pluvialis* under various temperatures (313−353 K), pressures (20−55 MPa), CO_2 flow rates (2−4 mL/min), and ethanol concentrations (E/S = 1.67−7.5%) in order to determine the effect of these factors on the total extract, the amount of astaxanthin extracted, and the astaxanthin content in the extract. Specifically, the total amount of the extract is described as the mass of extract divided by mass of dry sample. The amount of astaxanthin extracted is defined as the mass of astaxanthin in the extract divided by the mass of astaxanthin in the feed. The astaxanthin content in the extract is expressed as the mass of astaxanthin in the extract divided by the mass of the total extract. In each experiment, approximately 7 g of *H. pluvialis* was loaded into the extraction vessel and the remaining volume was filled with glass beads at the bottom and the top of the cell. The cell was placed in the heating chamber to maintain the operating temperature. The extract was collected in a collection vessel at every 30−60 min for 4 h and weighed immediately after the collection, then stored at −20 °C in the dark until the analysis. In the case of using ethanol as entrainer, the total extract was not weighed. The extract with entrainer was diluted by the solvent and immediately analyzed using HPLC. Each experiment was conducted in duplicates/triplicates and cited as mean ± standard deviation. To determine the total amount of astaxanthin present in the extract, 6 g of microalgae *H. pluvialis* was extracted with 200 mL of dichloromethane using a Soxhlet apparatus according to modification of a previously published procedure[17] for 6 h until the color of the condensed solvent in the top of apparatus was clear.

Analysis of Astaxanthin. Standard astaxanthin and astaxanthin in the extract were analyzed by a high-performance liquid chromatograph LC-10AD, equipped with diode array detector SPD-M10A (Shimadzu, Japan). The standard and extract solutions diluted with dichloromethane: methanol (1:3, v/v) were injected through a 20 μL loop and separated with a reversed-phase 5C18-MS Waters column (5 μm; 4.6 × 150 mm; Nacalai Tesque, Inc., Japan) at 303 K. Isocratic elution was performed with a methanol:acetonitrile:dichloromethane:water (85:5:5:5, v/v) mobile phase at a flow rate of 1.2 mL/min, and the detection wavelength was kept at 480 nm.[18] For determination of astaxanthin in the extract, the astaxanthin concentration in the extract was estimated on the basis of peak area. All samples were analyzed in duplicate. The amount of astaxanthin in the extract is cited as mean ± standard deviation.

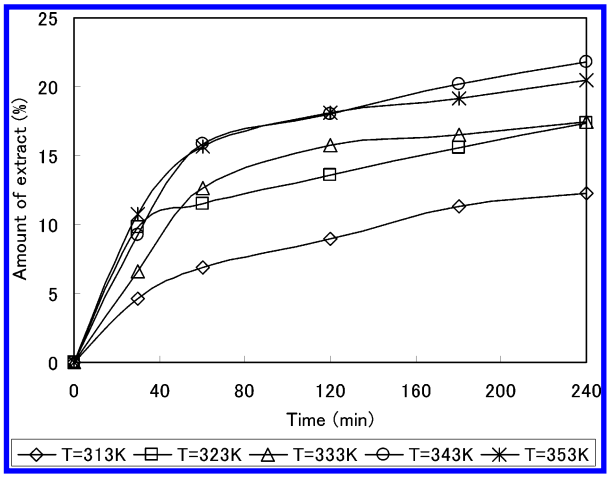

Figure 2. Effect of temperature on the amount of extract as a function of time at 55 MPa and 3 mL/min.

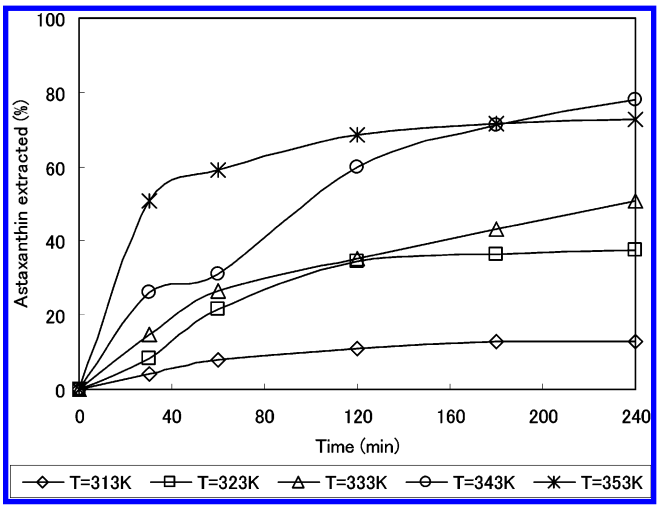

Figure 3. Effect of temperature on the astaxanthin extracted as a function of time at 55 MPa and 3 mL/min.

Results and Discussion

Initially, SC-CO_2 extraction experiments were carried out without ethanol entrainer to determine the effect of temperature, pressure, and CO_2 flow rate. Generally, the extract obtained was dark-red lipid which solidified at lower temperature. On the basis of the Soxhlet extraction, the total amount of astaxanthin in *H. pluvialis* algae was 34.3 mg/g or 3.43% in weight. The amount of astaxanthin extracted was calculated on the basis of this total amount.

Effect of Temperature. Figures 2−4 show the effect of temperature on the amount of extract, the astaxanthin extracted, and the astaxanthin content in the extract, respectively, as a

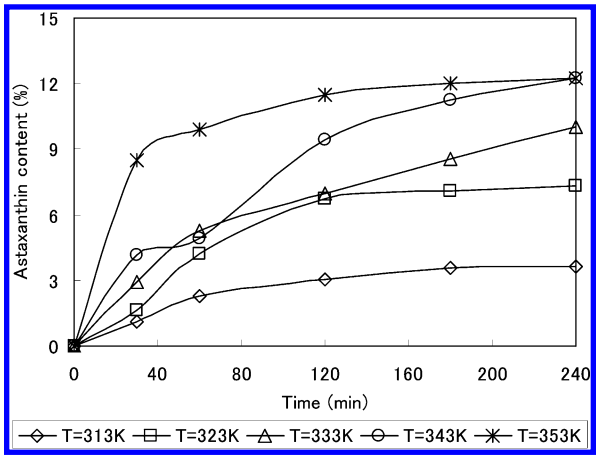

Figure 4. Effect of temperature on the astaxanthin content in the extract as a function of time at 55 MPa and 3 mL/min.

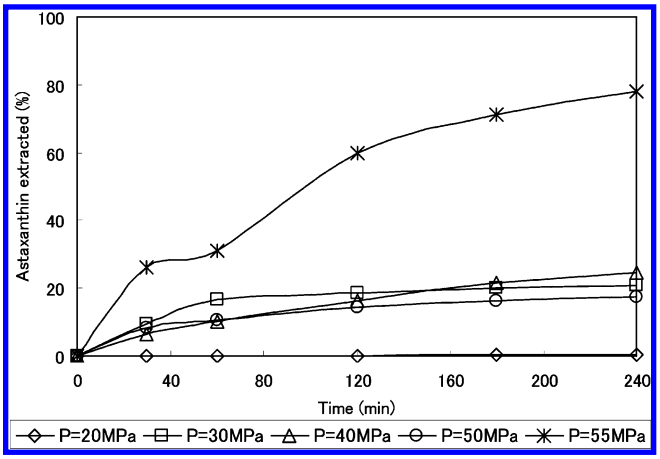

Figure 6. Effect of pressure on the astaxanthin extracted as a function of time at 343 K and 3 mL/min.

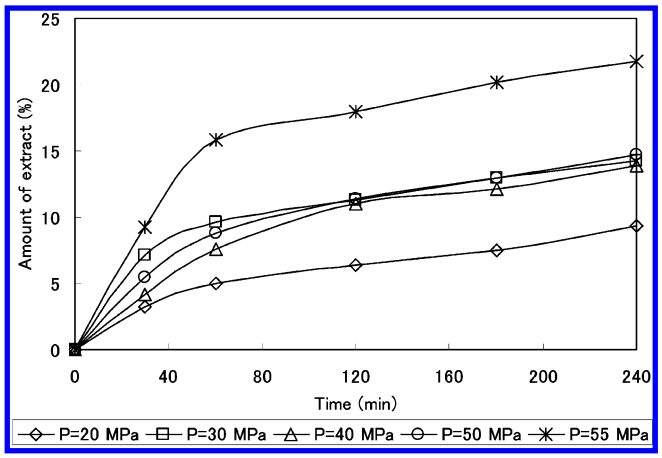

Figure 5. Effect of pressure on the amount of extract as a function of time at 343 K and 3 mL/min.

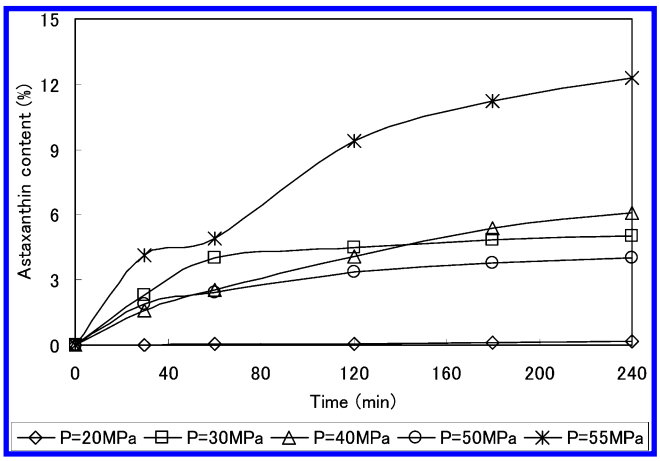

Figure 7. Effect of pressure on the astaxanthin content in the extract as a function of time at 343 K and 3 mL/min.

function of time at the pressure of 55 MPa and the CO_2 flow rate of 3 mL/min. The amount of total extract, the amount of astaxanthin extracted, and the astaxanthin content in the extract increased with an increase in temperature. These results indicate that the total extract and the astaxanthin extraction are dependent on solute vapor pressure which increased with an increase in temperature.[19] Instead of that, the increasing temperature contributed to the decomposition of cell walls, and as a result astaxanthin and extractable compounds availability for extraction was increased. The degree in which the total amount of extract increased was, however, smaller than those of the astaxanthin extracted and the astaxanthin content which increased more dramatically with increasing temperature. These results are basically in agreement with those of other workers for other carotenoids (e.g. lycopene) in the temperature range of 313−353 K.[20,21] The increase in temperature from 343 to 353 K, however, does not increase the amount of astaxanthin extracted and its content in the extract. At 55 MPa, the highest amount of the total extract, the amount of astaxanthin extracted, and the astaxanthin content in the extract were 21.8, 77.9, and 12.3%, respectively, and were obtained at 343 K.

Effect of Pressure. The effect of pressure on the amount of extract, the astaxanthin extracted, and the astaxanthin content in the extract using supercritical CO_2 at temperature of 343 K and CO_2 flow rate of 3 mL/min are shown in Figures 5−7. Generally, the amount of the extract increased with increasing pressure. However, the influence of pressure from 30 to 50 MPa was not significant at the end of the extraction. This result is in agreement with those of other workers for β-carotene and lipid extraction.[21] At the beginning of the process, the amount of extract at 30 MPa was rather higher than that at 40 and 50 MPa. It is evident that an increase in pressure had a small effect on the solubility. Studies by Sakaki[22] showed that an increase in temperature had a stronger effect on the solubility than an increase in pressure. The total extract and the astaxanthin extracted were not significantly affected by the increasing pressure at 30−50 MPa, and after which the dramatic increase was observed at the pressure of 55 MPa. The dependency on the pressure was expected as the CO_2 density increases at higher pressure, and therefore the solvent power to dissolve the substances increases.

Effect of CO_2 Flow Rate. The effect of CO_2 flow rate on the amount of extract, the astaxanthin extracted, and the astaxanthin content in the extract using supercritical CO_2 was studied at a pressure of 50 MPa and a temperature of 323 K. Figures 8−10 show the effect of the CO_2 flow rate on the amount of extract, the amount of astaxanthin extracted, and the astaxanthin content in the extract, respectively, as a function of CO_2 consumption. As seen in Figures 8 and 9, the amount of the total extract and the amount of astaxanthin extracted slightly increased with increasing CO_2 flow rate and tended toward the same value at higher CO_2 consumption. The increasing CO_2 flow rate not only caused the shorter residence time, but also the increase in the number of CO_2 molecules contacting with the solute, thus increasing intermolecular interaction between CO_2 and the solute, thus increasing the solute dissolution. For this condition, mass transfer was influenced by the increasing CO_2 flow rate and the intraparticle diffusion resistance was

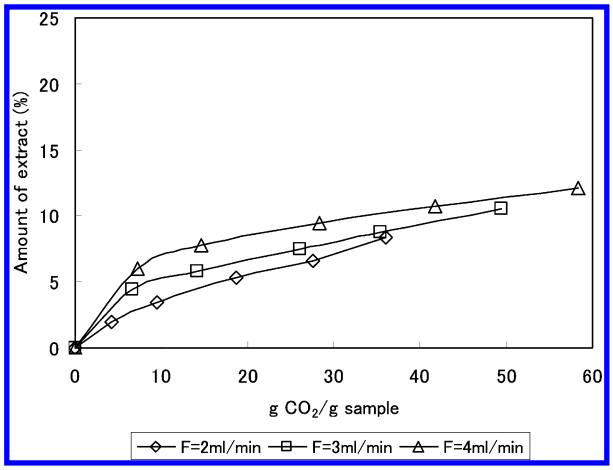

Figure 8. Effect of CO_2 flow rate on the amount of extract as a function of CO_2 consumption at 50 MPa and 323 K for 4 h.

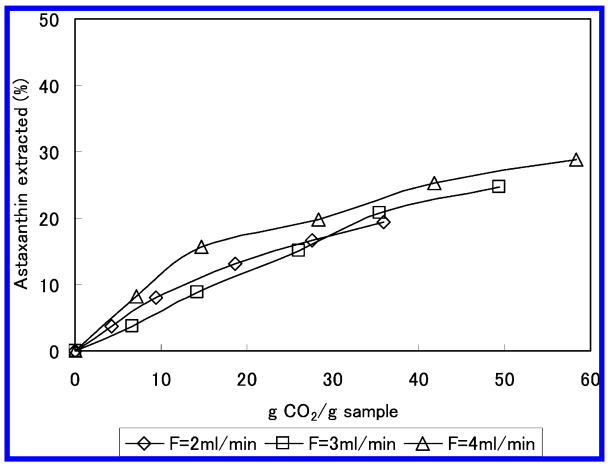

Figure 9. Effect of CO_2 flow rate on the astaxanthin extracted as a function of CO_2 consumption at 50 MPa and 323 K for 4 h.

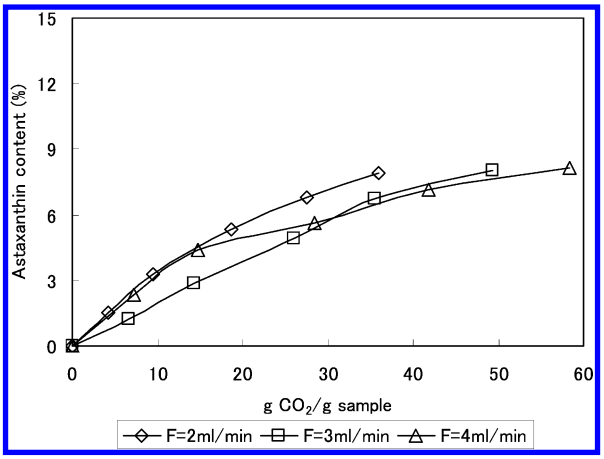

Figure 10. Effect of CO_2 flow rate on the astaxanthin content in the extract as a function of CO_2 consumption at 50 MPa and 323 K for 4 h.

dominant.[23] Figure 10 however shows that CO_2 flow rate does not have a significant effect on the astaxanthin content in the extract as the various flow rates yield almost the same contents. The small effect of flow rate on the extraction process may be caused by the fact that, at these rates, the CO_2 was not able to be distributed evenly throughout the extractor. Furthermore, the experiments were conducted at lower temperature, and the extraction may be highly influenced by the solubility limitation. Therefore, increasing the CO_2 flow rate may not significantly enhance the extraction rate and the yield. Instead of that, internal mass-transfer resistance may also dominate the extraction process; thus, the CO_2 flow rate has no great effect on the extraction performance. At this condition, the highest amount of the extract, the astaxanthin extracted, and the astaxanthin content in the extract obtained were only 12.2, 29, and 8.1%, respectively.

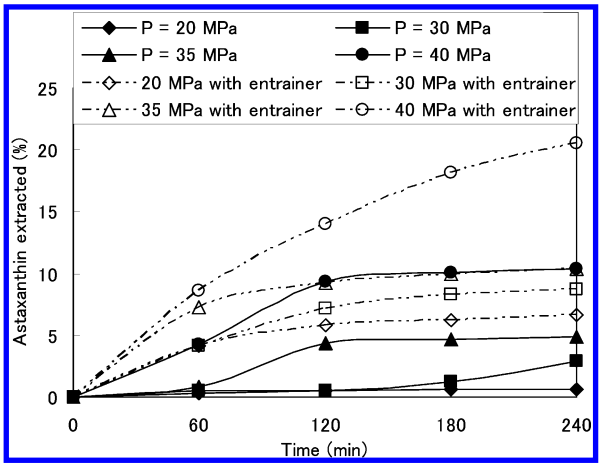

Figure 11. Comparison of SC-CO_2 extraction without and with 1.67% entrainer at various pressures, at 313 K, and at 3 mL/min.

Effect of Addition of Ethanol Entrainer. The amounts of astaxanthin extracted by SC-CO_2 extraction with and without ethanol as entrainer were compared, and the results are shown in Figure 11 for extractions under various pressures at the temperature of 313 K and the CO_2 flow rate of 3 mL/min. In this work, the effect of entrainer addition was studied at low temperature because at that condition the extraction rate was low. As a consideration, if there was an effect at a low extraction rate, it might be applied for a higher extraction rate. The experiments were conducted up to 40 MPa because the maximum capacity of the entrainer pump was 50 MPa. The pressure of the entrainer pump needed higher pressure than the CO_2 pump; thus, if the experiment is conducted at 50 MPa, the entrainer pump capacity must be higher than 50 MPa.

In this experiment, the concentration of 1.67% ethanol was used and was found to enhance the amount of astaxanthin extracted by more than 2-fold. As shown in Figure 11, at 40 MPa the amount of astaxanthin extracted was enhanced from 10 to 20.5% and at low pressure (20 MPa) the enhancement of astaxanthin extracted could reach 10-fold (from 0.6 to 6.6%). This considerable increase in extraction efficiency was because the ethanol added could enhance the solvent power of SC-CO_2 and caused swelling of the matrix, thus increasing the internal volume and the surface area for the contact with SC-CO_2.[14] The addition of ethanol in SC-CO_2 also could cause decomposition of the cellular wall, with the result that the astaxanthin availability for extraction increased.

The effect of CO_2 flow rate on astaxanthin for the case of SC-CO_2 extraction with the entrainer was found to be dramatic compared to that of the extraction without the entrainer. Figure 12 shows the effect of CO_2 flow rate on astaxanthin extracted with 1.67% ethanol entrainer at the pressure of 40 MPa and the temperature of 313 K as a function of CO_2 consumption. The dramatic increase in the amount of astaxanthin extracted at lower flow rate observed here, but not when extraction was conducted without the entrainer, was possibly because the addition of ethanol significantly enhances the solvent power of SC-CO_2 to extract astaxanthin, removing it from being a controlling factor for extraction. Thus, increasing the residence time by lowering the flow rate allows more effective contact between solvent and astaxanthin. This dramatic increase in the

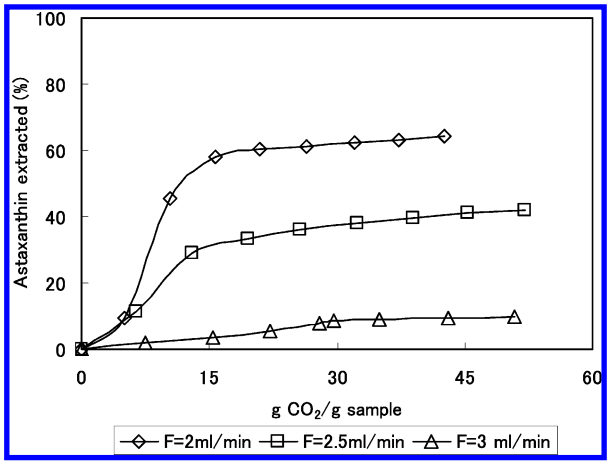

Figure 12. Effect of CO_2 flow rate on the astaxanthin extracted with 1.67% of entrainer concentration as a function of CO_2 consumption at 40 MPa and 313 K for 4 h.

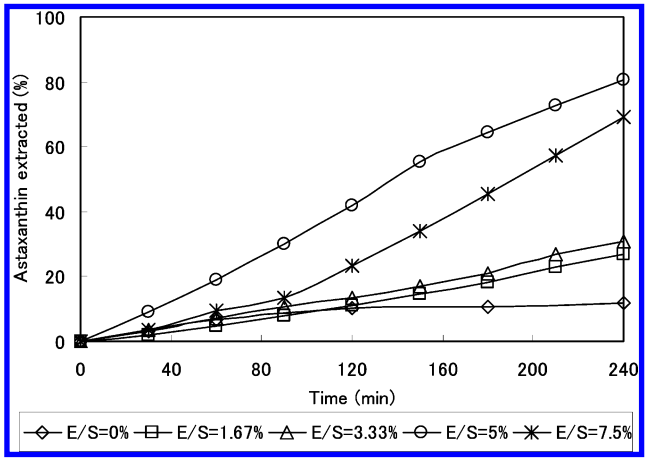

Figure 13. Effect of entrainer concentration on the astaxanthin extracted as a function of time at 40 MPa and 343 K.

amount of astaxanthin extracted at lower flow rates suggests the importance of internal mass diffusion in the extraction of astaxanthin in the *H. pluvialis*. This corresponds with the previous studies which demonstrated that crushing of the algae cells prior to extraction contributes highly to the success of extraction.[13,16] By addition of 1.67% ethanol entrainer, the astaxanthin extracted could reach 66.2% at 2 mL/min of CO_2 flow rate.

Effect of Ethanol Entrainer Concentration. The effect of entrainer concentration in CO_2 (*E*/*S*) on the astaxanthin extracted was studied at the pressure of 40 MPa and the temperature of 343 K, and the results are shown in Figure 13. The astaxanthin extracted increased with increasing ethanol concentration up to 5% (v/v) ethanol. At this condition, more than 80% of astaxanthin could be extracted. For larger entrainer concentration of 7.5% (v/v), however, the astaxanthin extracted was lower. At higher concentration, the amount of astaxanthin extracted was lower because very high entrainer concentrations lower the density of the supercritical fluid.[11] In some cases it creates a two-phases system (liquid and supercritical fluid). Instead of that the selectivity of astaxanthin may also become smaller as other components in the feed could be easily extracted. It is evident that the selectivity can be influenced by the concentration of entrainer.[11]

Conclusions

Extractions of astaxanthin from *Haematococcus pluvialis* using supercritical CO_2 without and with ethanol as entrainer were conducted. Without ethanol entrainer, the amount of the total extract, the amount of astaxanthin extracted, and the astaxanthin content in the extract generally increased with an increase in temperature and pressure. The amount of the total extract and the amount of astaxanthin extracted slightly increased with increasing CO_2 flow rate, while the astaxanthin content in the extract was not changed. The highest astaxanthin extracted and astaxanthin content in the extract were 77.9 and 12.3%, respectively, and were obtained at 55 MPa, at 343 K, and at the CO_2 flow rate of 3 mL/min. In SC-CO_2 extraction with ethanol entrainer, the amount of astaxanthin extracted could be more than twice enhanced, and effective extraction could be achieved at more moderate pressure (40 MPa). The amount of astaxanthin extracted was found to increase with increasing entrainer concentration up to 5% (v/v) ethanol. In addition, when the entrainer was used, the CO_2 flow rate considerably affected the astaxanthin extracted and a higher amount of astaxanthin was extracted as the CO_2 flow rate decreased.

Acknowledgment

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