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An Efficient Method for the Extraction of Astaxanthin from the Red Yeast *Xanthophyllomyces dendrorhous*

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Abstract This study investigated an efficient method for the extraction of astaxanthin from the red yeast *Xanthophyllomyces dendrorhous*. The extraction process comprised three steps: 1) cultivating the yeast; 2) treating the yeast culture suspension with microwaves to destroy the cell walls and microbodies; and 3) drying the yeast and extracting the astaxanthin pigment using ethanol, methanol, acetone, or a mixture of the three as the extraction solvent. Ultimately, various treatment tests were performed to determine the conditions for optimal pigment extraction, and the total carotenoid and astaxanthin contents were quantified. A frequency of 2,450 MHz, an output of 500 watts, and irradiation time of 60 s were the most optimum conditions for yeast cell wall destruction. Furthermore, optimal pigment extraction occurred when using a cell density of 10 g/l at 30°C over 24 h, with a 10% volume of ethanol.

Keywords: Astaxanthin, microwave, extraction, *Xanthophyllomyces dendrorhous*

Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is an oxycarotenoid with the molecular formular $C_{40}H_{52}O_4$ and molecular weight of 596.86 [4]. Astaxanthin has two asymmetric carbon atoms at the 3 and 3' position and can exist in four configurations [21]. Its melting point is approximately 224°C. It is insoluble in aqueous solutions and most organic solvents, but can be dissolved at room temperature in dichloromethane, chloroform, acetone, dimethylsulfoxide, and other nonpolar solvents. Its absorption spectrum represents a conjugated polyene structure, λ_{max}= 489 nm in chloroform, 478 nm in ethanol, and 480 nm in acetone [9]. It is commonly obtained from microorganisms such as the red yeast *Xanthophyllomyces dendrorhous* [15], the green algae *Haematococcus pluvialis* [13], and *Brevibacterium* [8]. It offers various functions including the color and flavor enhancements of food, anti-aging and anticancer activities by the removal of oxygen free-radicals, and the activation of immunity [2, 12]. The antioxidant activity of astaxanthin has been reported to be approximately 10 times stronger than that of other carotenoids such as zeaxanthin, lutein, canthaxanthin, and β-carotene; and 100 times greater than the antioxidant activity of α-tocopherol [14, 16, 19]. Therefore, in addition to functioning as an edible pigment, it is believed that astaxanthin has importance for medical uses [10], and a recent FDA communication allowed the use of astaxanthin as a supplement and salmonoid fish feed [17, 18].

The extraction of astaxanthin from crustacea such as shrimp or crawfish is rarely implemented because they contain only a small amount of the pigment. Furthermore, the extraction of astaxanthin from *X. dendrorhous* has not been well-utilized since its cell walls are too hard to efficiently extract the pigment from them [8].

To obtain astaxanthin from *X. dendrorhous*, the development of an efficient method for destroying the cell walls is required [7]. A number of methods have been developed and tried, such as chemical methods using acid; physical methods using a French pressure cell press, a Braun homogenizer, or a microfluidizer; and biochemical methods using digestive enzymes such as cellulase, hemicellulase, and pectinase [11, 20]. However, these processes have low yields of astaxanthin because of the destruction of the pigment during extraction. The direct extraction of astaxanthin using solvents such as ethanol or acetone also has problems owing to the high cost and low yield. Microwave was also used for cell-wall destruction. When the *E. coli* cell suspension was exposed to microwave, the cell wall

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destruction rate and the temperature were increased [6]. In addition *S. aureus*, *P. fluorescens*, and *B. cereus* were also destructed by exposing to microwave [5]. An external magnetic field can induce a higher transmembrane electric potential than that of a normal cell. This abnormal transmembrane electric potential causes enough voltage drop for the cell-wall destruction.

To address these problems, we developed a microwave treatment method for destroying the cell walls of *X. dendrorhous*, allowing for the efficient extraction of astaxanthin. We also designed a vessel and tube to be used during this microwave irradiation process.

MATERIALS AND METHODS

Microorganism

X. dendrorhous ATCC 96594 was obtained from the Korea Research Institute of Bioscience and Biotechnology. The strain was cultivated in YM broth at 22°C and 270–330 rpm for 4–6 days, and stored at −70°C in 30% glycerol.

Evaluation of the Pigment Extraction Yield Utilizing a Continuous or Fixed Microwave Treatment System: Test 1

The extraction yield of astaxanthin was measured after microwave illumination under the following conditions: a microwave output of 50–1,000 watts, a frequency of 916 or 2,450 MHz, and an irradiation time of 10–500 s. The cultivated yeast cells (10 g/l) were treated with microwaves *via* a continuous microwave treatment system and a fixed microwave treatment system as shown in Fig. 1. In the continuous system, a Teflon tube was inserted into the microwave oven (Model: Goldstar, ER-646JB, Seoul, Korea) (Fig. 1A). The length and diameter of the tube were designed according to the microwave holding time and the microwave treatment capacity. The cultivated suspension was then passed through the tube using a variable speed peristaltic pump (Model: AS-90361, Won Corporation, Korea). For the fixed system (Fig. 1B), the Teflon extraction vessel was laid in the center of the microwave oven and the cultivated suspension was placed in the vessel. In this system, the Teflon tube and pump were not required. Although the fixed system is limited to its cell-wall destruction capacity, the continuous system is not limited to its cell-wall destruction capacity because the cultivated suspension can be continuously passed through the Teflon tube. A 10% (by volume) amount of ethanol was added to a 1% (by volume) amount of the cultivated yeast suspension. After extraction at 30°C for 24 h, the extract was placed at −20°C for more than 30 min to remove the lipid. After centrifugation (10,000 ×*g*), the astaxanthin extract was obtained by concentrating the supernatant at reduced pressure. After the addition of acetone, we measured the extraction yield of the pigment by UV/VIS spectrophotometry (Pharmacia, LKB Biochrom 4060, NJ, U.S.A.) at 478 nm. The pigment extraction yield was calculated by the following equation [1, 3].

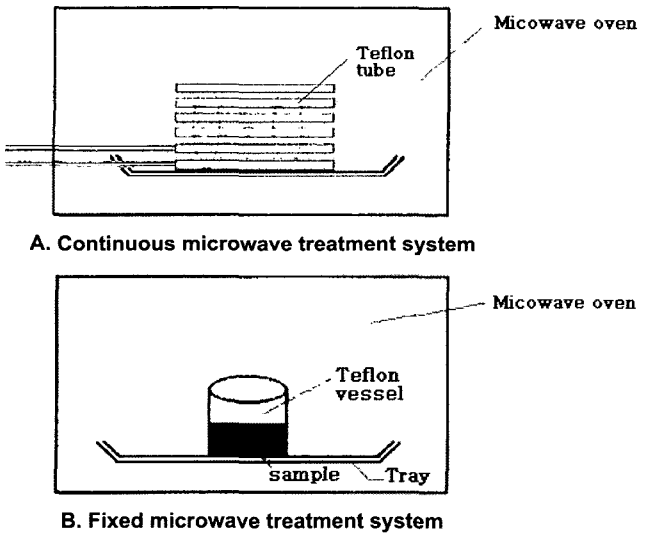

Fig. 1. A schematic view of the continuous and fixed microwave treatment systems.
A. A schematic view of the continuous microwave treatment system using a Teflon tube to the suspension. **B.** A schematic view of the fixed microwave treatment system using a Teflon extraction vessel to the suspension.

$$\text{Pigment extraction yield (\%)} = B/A \times 100$$
$$\text{Pigment destruction rate} = \{A-(B+C)\}/A \times 100$$

A: The absorbance of the control group after destroying the cell walls using DMSO at 478 nm.
B: The absorbance of the soluble matter for the experimental group after microwave irradiation at 478 nm, and treatment with an organic solvent.
C: The absorbance of the insoluble matter for the experimental group after microwave irradiation at 478 nm, and treatment with an organic solvent.

Evaluation of the Pigment Extraction Yield Based on the Extraction Solvent and the Extraction Time following a Microwave Irradiation Treatment: Test 2

According to data we acquired in Test 1, the conditions for Test 2 were adjusted as follows: a frequency output of 2,450 MHz, a microwave output of 500 watts, and an irradiation time of 60 s; the cultivation suspension had a cell density of 10 g/l. The solvents used for the pigment extraction were ethanol, methanol, and acetone. The volume of the extraction solvent was 10% of the yeast suspension volume. The extraction temperature was set at 30°C. The astaxanthin extract was obtained by using a rotary vacuum evaporator followed by agitation in a dark room. After adding acetone, the absorbance of the pigment extract was measured at 478 nm.

Evaluation of the Pigment Extraction Yield Based on the Cultivation Suspension Cell Density at the Time of Microwave Irradiation: Test 3

This experiment was carried out in the same manner as Test 2, except it used varied cell densities (5–200 g/l). The density of the cell cultivation suspension was evaluated by the dried mass of the yeast cells per liter. The pigment extraction yield and the pigment destruction rate were measured using UV/VIS spectrophotometry.

Evaluation of the Pigment Extraction Yield Based on the Extraction Temperature and Extraction Time Following a Microwave Irradiation Treatment: Test 4

This experiment was also carried out in the same manner as Test 2, but it used varied extraction temperatures (20–80°C). The pigment extraction yield was measured using UV/VIS Spectrophotometry.

Evaluation of the Pigment Extraction Yield Based on the Extraction Solvent Volume Following a Microwave Irradiation Treatment: Test 5

Test 5 was carried out in the same manner as Test 2, except the extraction solvent volumes were varied. The extraction time was 22 h and the extraction temperature was 30°C. Again, the pigment extraction yield was measured using UV/VIS spectrophotometry.

Carotenoid and Astaxanthin Analyses

The total carotenoid content was measured according to the following method: 1 ml of the cultivated suspension was centrifuged and the precipitate was washed twice with sterile water. Then, 1 ml of a preheated (55°C) DMSO stock solution was added and the mixture was vigorously agitated to fully destroy the yeast cells. To extract the pigment, 1 ml of petroleum ether, followed by 1 ml of sodium chloride (20%), were added. After a storage period in the refrigerator, the solution was centrifuged at 10,000 ×*g* for 5 min. The absorbance of the supernatant was measured at 474 nm [3, 20].

Using 1 ml of the pigment extract solution from Test 5, the solvent was evaporated at reduced pressure. Then, 1 ml of petroleum ether was added and the absorbance was measured at 474 nm. The extraction yield of the pigment was measured by comparing it with the pigment amount that was extracted by DMSO (100%).

Total carotenoid content was estimated using the following equation, which incorporates a 1% extinction coefficient (2,100) and the mass of the dried cell contents. The measured astaxanthin pigment was acquired from the ethanol extract after the evaporation extraction of the solvent under reduced pressure. The obtained pigment was dissolved in chloroform before analysis by HPLC (Waters 486, U.S.A.). The analytical conditions for HPLC were as follows: i) mobile phase=n-hexane/acetone (8:2); ii) stationary phase=silica (4.0×250 mm); iii) solvent=chloroform; iv) flow rate=1 ml/min; v) wavelength=476 nm; and vi) standard=astaxanthin (Sigma, 98% up) dissolved in chloroform. The astaxanthin content of the total carotenoid portion was measured according to a standard quantification curve prepared from the measurement of a standard astaxanthin concentration [20, 22].

$$\text{Total carotenoid contents (mg/g of yeast cell dry weight)} = (A \times B \times 100)/(21 \times D)$$

A: The absorbance of the pigment at 474 nm.
B: The amount of solvent used for extraction (ml).
D: The dry mass of the *Phaffia rhodozyma* cells.
21: 1% of the extinction coefficient from the mass of the cells=2,100.

RESULTS

To evaluate the pigment extraction yield of astaxanthin, we employed both continuous and fixed microwave treatment systems under the following conditions: a microwave output of 50–1,000 watts at a frequency of 916 or 2,450 MHz for

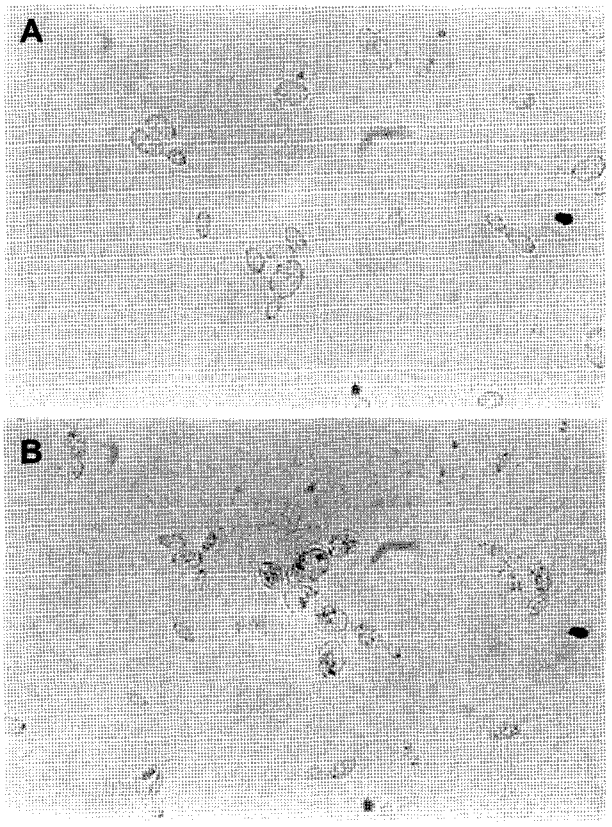

Fig. 2. Photomicrographs of the yeast cells treated with microwave illumination.
A. A view of the yeast cells not treated with microwave irradiation. **B.** A view of the yeast cells treated with microwave irradiation.

Table 1. The effects of the frequency, irradiation time, and output on the pigment extraction.

Frequency (MHz)	Time (s)	Output (Watt)			
		50	100	500	1,000
916	10	12(2)	14(2)	18(2)	22(3)
	30	19(2)	23(3)	54(4)	52(5)
	60	51(3)	53(5)	87(5)	88(7)
	120	51(5)	57(5)	86(7)	85(11)
	240	53(9)	69(7)	84(9)	83(12)
	500	55(6)	70(9)	82(12)	81(13)
2450	10	13(2)	15(2)	19(2)	23(3)
	30	25(5)	37(5)	53(4)	55(5)
	60	52(4)	79(6)	95(4)	90(4)
	120	51(6)	77(7)	90(7)	88(9)
	240	54(7)	75(8)	87(7)	84(11)
	500	61(6)	78(8)	85(10)	80(12)

The extraction yield of pigment was measured after microwave illumination in a continuous or fixed microwave treatment system. All results are the mean values of three repeated experiments.
( ): destruction rate (%).

an irradiation time of 10–500 s. The yeast cells treated with microwaves are shown in Fig. 2. The results for the pigment extraction yield and the pigment destruction rate based on variations in the frequency, time, and output are shown in Table 1. A frequency of 2,450 MHz, an output of 500 watts, and an irradiation time of 60 s were found to be the most favorable extraction conditions.

To evaluate the pigment extraction yield based on the extraction solvent used and the extraction time following microwave irradiation, various solvents (ethanol, methanol, and acetone) and times were investigated (Table 2). The control group data indicate the pigment absorbance following ethanol extraction without a microwave treatment of the cells. Overall, the most favorable pigment extraction occurred using ethanol as the solvent and an extraction time of 24 h.

The results for the pigment extraction yield based on the cell suspension density are shown in Table 3. For this

Table 2. The effects of the extraction solvent and time on the pigment extraction.

Extraction solvent	Time(h)				
	0	6	12	18	24
Control	2(1)	8(2)	11(2)	13(3)	15(4)
Ethanol	25(2)	57(3)	79(3)	90(4)	95(4)
Methanol	27(2)	63(4)	81(5)	82(7)	82(8)
Acetone	8(2)	52(5)	63(6)	75(6)	77(8)

The extraction yield of pigment based on the extraction solvent and time was measured. The solvents used for the pigment extraction were ethanol, methanol, and acetone. The control group data indicate the pigment absorbance following ethanol extraction without a microwave treatment of the cells. All results are the mean values of three repeated experiments.
( ): destruction rate (%).

Table 3. The effects of the cell density on the pigment extraction.

Cell density (g/l)	Extraction rate (%)
5	94(3)
10	95(4)
50	91(7)
100	86(9)
200	90(7)

The extraction yield of pigment based on cell density was measured. This experiment was carried out in the same manner as Test 2, except it used varied cell densities. All results are the mean values of three repeated experiments.
( ): destruction rate (%).

experiment, we followed the method of Test 2; however, cell densities ranged from 5–200 g/l at the time of microwave irradiation. The best pigment extraction occurred with a cell density of 10 g/l.

Table 4 shows the resultant pigment extraction yields and pigment destruction rates based on variations in the extraction temperatures (20–80°C) and extraction times. Here, an optimal pigment extraction occurred at 30°C over 24 h.

The results of the pigment extraction yield based on variations in the extraction solvent volumes are shown in Table 5. An optimal pigment extraction occurred in an ethanol volume that was 10% of the yeast cell suspension volume.

The total carotenoid content and the amount of astaxanthin it contained are shown in Table 6. Following microwave destruction, the carotenoid content obtained from the yeast cells was 4.06 mg/g. This was an extraction of 95% compared with the extraction found after DMSO destruction. The results of the HPLC indicated that 90% of the total carotenoid content was astaxanthin (Fig. 3).

DISCUSSION

When the yeast cells underwent microwave irradiation, the free water and other dipoles in the cells were rotated

Table 4. The effects of the temperature and time on the pigment extraction.

Temperature (°C)	Time (h)					
	0	6	12	18	24	48
20	25(2)	32(2)	55(2)	56(3)	56(3)	55(5)
30		57(3)	79(3)	90(4)	95(4)	89(7)
40		57(3)	76(3)	79(5)	90(6)	85(9)
50		58(4)	77(7)	80(9)	82(11)	76(12)
80		62(8)	69(8)	74(12)	75(13)	70(13)

The extraction yield of pigment based on extraction temperature and time was measured. This experiment was carried out in the same manner as Test 2, except it used varied extraction temperatures. All results are the mean values of three repeated experiments.
( ): destruction rate (%).

Table 5. The effects of the solvent volume on the pigment extraction.

Ethanol:Cell suspension (v/v)	Extraction rate (%)
1:1	10(5)
2:1	39(6)
5:1	67(7)
10:1	95(4)
20:1	94(4)

The extraction yield of pigment based on the solvent volume was measured. The test was carried out in the same manner as Test 2, except the extraction solvent volumes were varied. All results are the mean values of three repeated experiments.
( ): destruction rate (%).

according to the alternating electric field, which converted microwave energy into thermal energy. The cell walls and microbodies (*i.e.*, nucleus, mitochondria, golgi apparatus) in the cells were destroyed as a result of an elevated internal pressure caused by the internal heating. Therefore, microwave treatment allowed for the astaxanthin pigment to be extracted without physically destroying the cell walls. Furthermore, the microwave treatment allowed the organic solvent to easily diffuse into the cells, improving the pigment extraction.

In this study, we applied the microwave irradiation method to *X. dendrorhous,* which has hard and thick cell walls composed of α-1,3-glucan. This treatment allowed for the destruction of the α-glucan cell walls, and the efficient extraction of the astaxanthin pigment contained inside. A frequency of 2,450 MHz, an output of 500 watts, and an irradiation time of 60 s were the most appropriate irradiation conditions. By performing various extraction tests after the microwave treatments, we found that optimal pigment extraction occurred using a cell density of 10 g/l at 30°C over 24 h, with a 10% volume of ethanol. The total carotenoid content extracted with this method was 4.06 mg/g of yeast, which was a 90% extraction compared with that using a DMSO destruction method. The amount of extracted astaxanthin was 3.65 mg/g of yeast.

These results suggest that a microwave destruction method can be applied effectively to *X. dendrorhous* for an efficient extraction of astaxanthin. Based on these study findings, our future research objective is to provide a method for better utilization of the astaxanthin pigment in cosmetic, food additive, and animal feed applications.

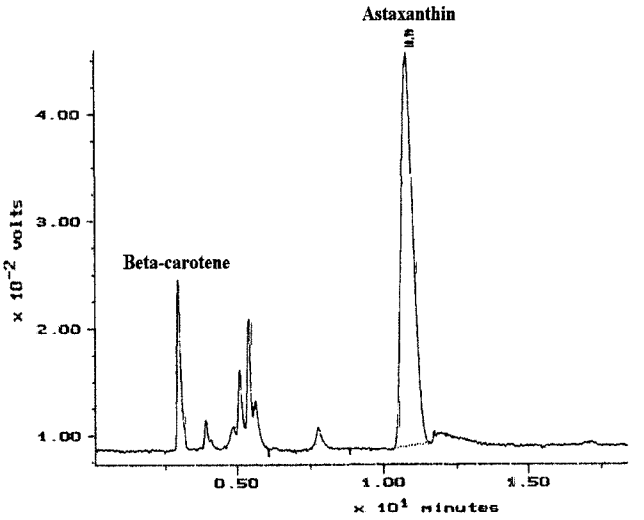

Fig. 3. HPLC analysis of pigment extract.
The HPLC data of the extract obtained following i) the cultivation of *X. dendrorhous*, ii) a microwave treatment, and iii) ethanol extraction.

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Table 6. The analysis of total carotenoids and total astaxanthin.

Method	DMSO destruction		Microwave destruction			
	Carotenoids (mg/g yeast)	Extraction rate (%)	Carotenoids (mg/g yeast)	Extraction rate (%)	Astaxanthin (mg/g yeast)	Extraction rate (%)
	4.27	100	4.06	95	3.65	90

The total carotenoids and total astaxanthin contents using the microwave destruction system was compared with that of the DMSO destruction system. All results are the mean values of three repeated experiments.

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