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Screening and Characterization of Red Yeast *Xanthophyllomyces dendrorhous* Mutants

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Abstract Three different strains of carotenoid accumulating *Xanthophyllomyces dendrorhous* mutants, JH1, JH2, and JH3, were isolated by NTG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) mutagenesis, which might potentially be useful for animal feed as well as for studies on the regulation and biosynthesis of astaxanthin. Mutants were selected based on the capability of growth and carotenoid production on the YM agar plate containing chemical inhibitor, β-ionone. Astaxanthin-overproducing mutant JH1 produced 4.032 mg astaxanthin/g dry cell weight, and this value was about 15-folds higher than that of wild-type. β-Carotene-overproducing mutant JH2 produced 0.273 mg β-carotene/g dry cell weight, and this was 4-folds increase from that of wild-type. In contrast, JH3 was a white-colored mutant that was unable to produce carotenoid pigment.

Key words: Astaxanthin, β-carotene, β-ionone, NTG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine), mutagenesis, *Xanthophyllomyces dendrorhous*

Astaxanthin (3,3'-dihydroxy-β,β'-carotene-4,4'-dione) has important metabolic functions in animals, including conversion to vitamin A [3], enhancement of immune response [8, 18], and protection against diseases such as cancer by scavenging oxygen radicals [9, 23, 24, 25]. The antioxidant activity of astaxanthin has been reported to be approximately 10 times stronger than that of other carotenoids tested, including zeaxanthin, lutein, canthaxanthin, and β-carotene, and 100 times greater than that of α-tocopherol [11, 16, 17]. These effects are considered to be defense mechanisms from the attack of reactive oxygen species (ROS). Astaxanthin also gives attractive pigmentation to many farmed animals and contributes to consumer appeal in the marketplace. Since animals lack the ability to synthesize astaxanthin, the pigment must be supplemented in feeds, usually at considerable expense to the farmer.

Worldwide production of farmed salmon increased rapidly in the past decade, and more than 200,000 tons have been raised in 1990 (New York Times, 17 August 1987). Currently, chemically synthesized astaxanthins are added to salmonoid feeds as pigmenters, however, there is robust interest within the aquaculture industry to use natural sources of astaxanthin [4, 19].

The principal biological sources of astaxanthin are crustacea and crustacean extracts, the green microalga *Haematococcus pluvialis*, and the yeast *Xanthophyllomyces dendrorhous* [6, 7]. Each natural pigment source has its limitations, and they cannot currently compete economically with the synthetic additive. Crustacean meals have relatively low contents of astaxanthin and high levels of moisture, ash, and chitin. The green microalga *Haematococcus pluvialis* has high concentration of astaxanthin, but industrial application is limited by lengthy autotrophic cultivation in open freshwater ponds and requirement to disrupt the cell wall to liberate the carotenoid. The yeast *X. dendrorhous* has desirable properties and potential commercial value as a dietary source of natural astaxanthin, including rapid heterotrophic metabolism and production of high cell densities in fermentors, but it has relatively low content of astaxanthin and high cost for industrial production.

In the present study, *X. dendrorhous* ATCC 96594 was mutagenized with NTG, and astaxanthin-overproducing mutant was then selected based on the capabilities of growth and carotenoid production on the YM agar plate containing chemical inhibitor, β-ionone, and characterized [1, 13, 27]. β-Carotene-overproducing yellow mutant and carotenoid pigment-lacking white mutant were also selected and characterized.

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MATERIALS AND METHODS

Chemicals and Reagents

β-Ionone, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), dimethyl sulfoxide (DMSO), acetone, sodium chloride, petroleum ether, HPLC grade methanol, acetonitrile, and dichloromethane were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other inorganic chemicals purchased were the highest grades available.

Yeast Strains and Growth Condition

X. dendrorhous ATCC 96594 was kindly provided by the Korea Research Institute of Biotechnology. Yeasts were maintained on slants of 0.3% yeast extract/0.3% malt extract/0.5% peptone/1% glucose medium (YM broth, Difco) with 1.5% (v/v) agar (YM agar) and refrigerated at 4°C. Wild-type and mutants, JH1, JH2, and JH3, were also stored in 40% glycerol/60% YM broth at -70°C. For analysis of pigment production, yeasts were grown for 5 days in 50 ml of YM broth in 250-ml baffled flasks shaken at 140 rpm on an orbital shaker controlled at 22°C. Growth in YM broth was measured by determining optical density at 660 nm.

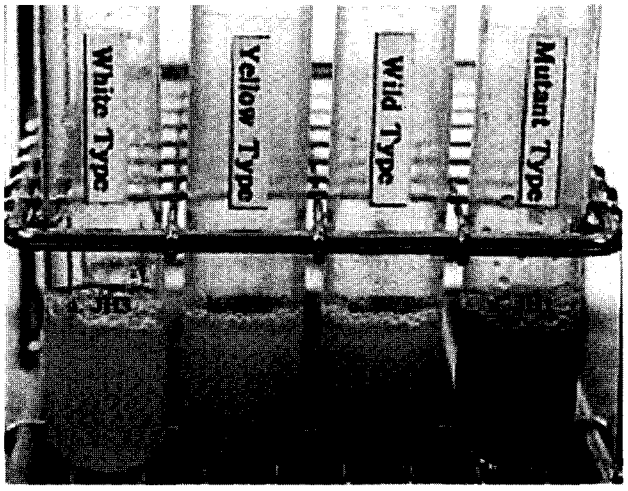
Mutagenesis

Mutagenesis of *X. dendrorhous* was performed with NTG after being grown to early log phase [1, 13, 27]. Freshly grown yeasts were washed twice in 0.1 M sodium citrate (pH 5.5) and suspended to an optical density of 1.5 to 2 (660 nm). A sample of 1 ml of the yeast suspension was pipetted to a test tube, and 0.1 ml of NTG (1 mg/ml in sodium citrate) was added (final concentration of NTG, 0.1 mg/ml), and the suspension was vortexed for 15 to 20 s. The sample was transferred to sterile 1.5-ml test tube, which was incubated for 20 min (more than 95% kill). The tube was centrifuged for 5 min at 3,000 rpm, and the cells were washed with 1 ml of 0.1 M potassium phosphate (pH 7.0). The cells were resuspended in the same buffer and inoculated into YM broth for overnight growth before plating on 10^{-4} M β-ionon-containing selective media. β-Ionon was dissolved in ethanol and added to YM agar just before being poured onto plates. After incubation, the cells were plated on 10^{-4} M β-ionon-containing selective media, and interested mutants, JH1, JH2, and JH3, were selected by the difference in their characteristic color.

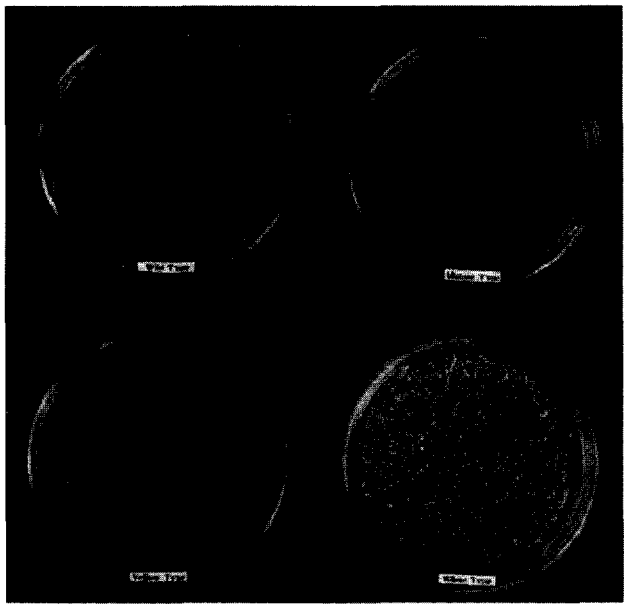
Carotenoid Extraction and Analysis

For routine analyses of astaxanthin, the washed cell pellets of *X. dendrorhous* were mixed with DMSO (Sigma Chemical Co., St. Louis, MO, U.S.A.) preheated at 55°C and then agitated for 1 min. The broken cells were thoroughly stirred in acetone, centrifuged, and the pigments in the supernatant were transferred to petroleum ether with the addition of 20% NaCl solution [7, 20]. Optical density of the petroleum ether phase was measured at 474 nm using a Ultrospec 2100 pro UV/Visible Spectrophotometer, and the total carotenoid composition was calculated by using 1% extinction coefficient of 2,100 by the following formula [1]:

$$\text{Total carotenoids content (mg carotenoids/g of yeasts)} = \frac{(\text{ml of petrol}) \times (A_{474}) \times (100)}{(21) \times (\text{Yeast dry weight})}$$



A



B

Fig. 1. Comparison of wild-type and mutants JH1, JH2, and JH3 in YM broth and YM agar.
(A) *X. dendrorhous* ATCC 96594 and mutants grown at 22°C for 5 days in YM broth. a. JH3 (albino-type), b. JH2 (β-carotene-overproducing mutant), c. ATCC 96594 (wild-type), d. JH1 (astaxanthin-overproducing mutant). (B) *X. dendrorhous* ATCC 96594 and mutants grown at 22°C for 10 days in YM agar. a. ATCC 96594 (wild-type), b. JH1 (astaxanthin-overproducing mutant), c. JH2 (β-carotene-overproducing mutant), d. JH3 (albino-type).

Total carotenoids content (μg carotenoids/ml of sample)
=Yeast dry weight (mg/ml)
×total carotenoids content (mg carotenoids/g of yeasts)

To obtain carotenoid extracts, the petroleum ether phase was then dried and concentrated by rotary evaporation. Growth and extraction of yeast strains were repeated at least twice independently to accurately assess carotenoid production.

Carotenoids were analyzed by high-performance liquid chromatography (HPLC) on LUNA C_{18} column (250×4.6 mm; 5 μm, Phenomenex) at 25°C at a flow rate of 1.0 ml/min with two 510 pumps and 996 photodiode array detector [2, 20, 28]. The mobile phase consisted of methanol (85%), dichloromethane (5%), acetonitrile (5.5%), and water (4.5%). Samples for HPLC analysis were diluted in the mobile phase, and peaks were measured at 480 nm. Astaxanthin was identified according to its retention time and spectrum by photodiode array detection [28]: Co-chromatography with standards (astaxanthin and β-carotene) was used for identification of the pigments.

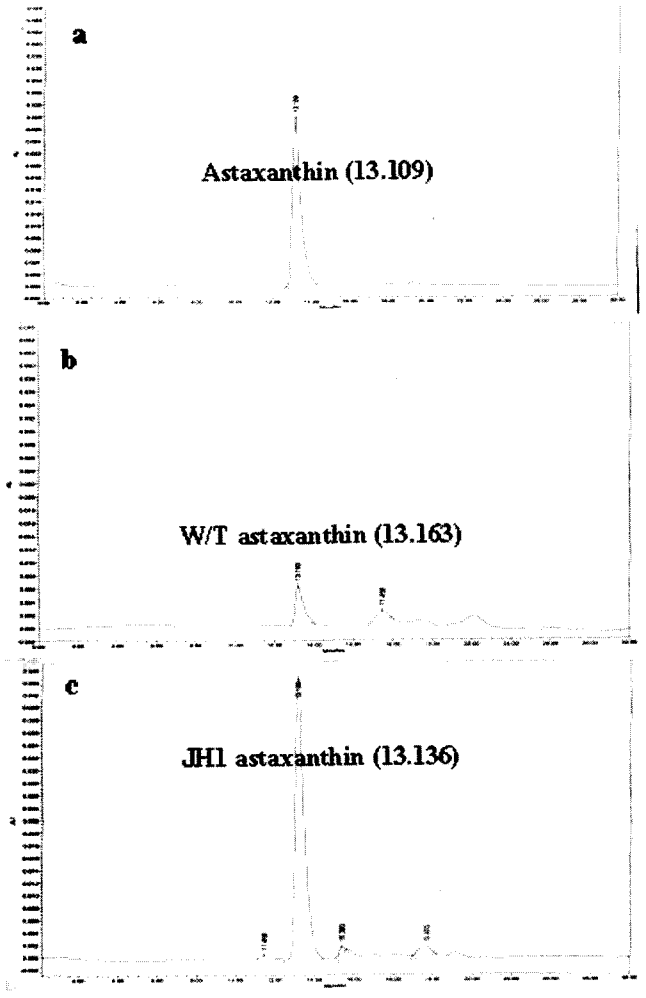


Fig. 2. HPLC chromatograms of wild-type and astaxanthin-overproducing mutant JH1.
a. Astaxanthin standard, b. ATCC 96594 (wild-type), c. JH1 (astaxanthin-overproducing mutant).

RESULTS

Isolation of *X. dendrorhous* Mutants

To isolate *X. dendrorhous* mutants producing a large amount of astaxanthin or β-carotene, the wild-type was serially mutated using NTG. After mutagenesis of wild-type, it was plated on 10^{-4} M β-ionone-containing-YM agar plates, and mutant strains were selected by screening for resistance to β-ionone. Isolated strains often revert at high frequency, but stable clones can be obtained by repeated mutagenesis with NTG. Among the mutagenized population of wild-type, red, yellow, and white mutants were selected by their unique color (Fig. 1). Astaxanthin-overproducing mutant JH1 was selected by its intense red color on β-ionone-containing-YM agar plates, and it was also demonstrated to be an astaxanthin-overproducing mutant by HPLC chromatograms (Fig. 2). Also, β-carotene-overproducing mutant JH2 was selected by its intense yellow color on β-ionone-containing-YM agar plates. Finally, white-colored mutant JH3 was selected by its white color on β-ionone-containing-YM agar plates. This albino-type mutant was unable to produce carotenoids due to deficiency of pigment production ability. The three mutant strains,

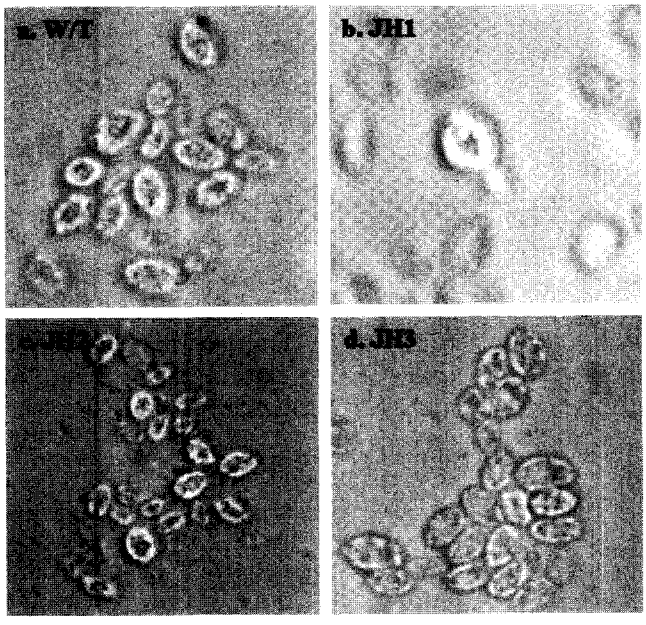


Fig. 3. Light microscopic images (×1,000) of wild-type and mutants JH1, JH2, and JH3.
a. ATCC 96594 (wild-type), b. JH1 (astaxanthin-overproducing mutant), c. JH2 (β-carotene-overproducing mutant), d. JH3 (albino-type).

JH1, JH2, and JH3, had morphology slightly different from that of wild-type strain; cell size and cell surface (Fig. 3).

Growth Kinetics and Total Carotenoids Content of Wild-Type and Mutant Strains

Growth kinetics of mutant strains JH1, JH2, and JH3 were compared to that of wild-type (Fig. 4). Figure 4e shows the growth curve of wild-type and mutant strains. Wild-type and JH2 reached the exponential phase at 13 h, while JH1 reached it at 30 h. JH3 reached it at 9 h, which grew the fastest of all strains. While wild-type reached the stationary phase at 40 h, JH1 and JH2 reached it at 50 h and JH3 at 24 h. This result indicates that wild-type yeast cells grow slightly faster than mutants JH1 and JH2. However, mutant JH3 grows faster than wild-type. Figure 4f shows the change of total carotenoids content of wild-type and mutant strains during cultivation. The carotenoids formation by wild-type and mutants JH1 and JH2 increased rapidly at 50 h and reached the stationary phase at 120 h. Compared to the growth curve, the total carotenoids content of wild-

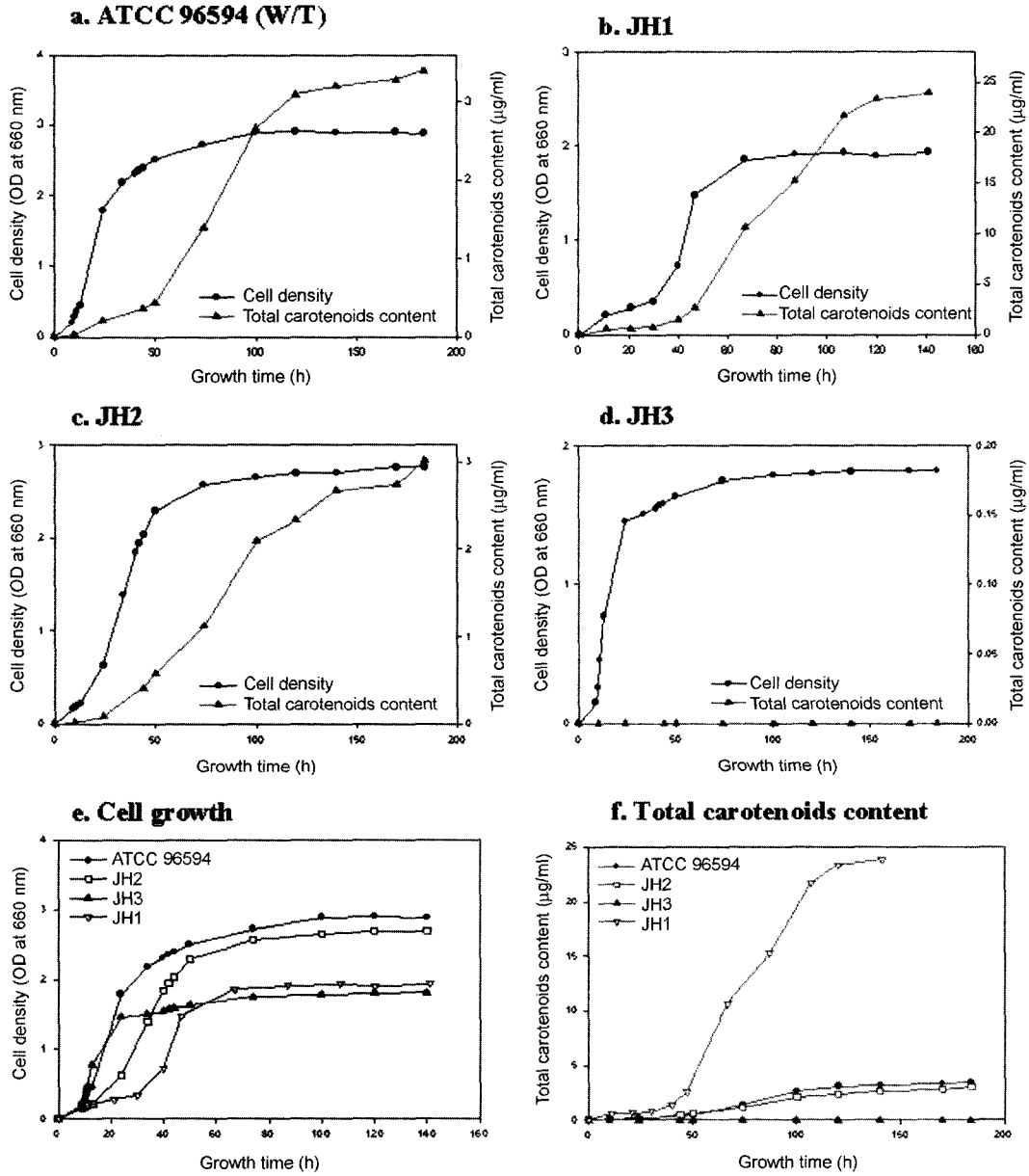


Fig. 4. Growth curve and change of total carotenoids content of wild-type and mutants JH1, JH2, and JH3.
a. Growth curve (●) and change of total carotenoids content (▲) of ATCC 96594; b. Growth curve (●) and change of total carotenoids content (▲) of JH1; c. Growth curve (●) and change of total carotenoids content (▲) of JH2; d. Growth curve (●) and change of total carotenoids content (▲) of JH3; e. Comparison of cell growth of wild-type and mutants; f. Comparison of total carotenoids content of wild-type and mutants.

Table 1. Cell mass and total carotenoids content of wild-type and mutants JH1, JH2, and JH3.

Strain	Cell mass (mg/ml)	Total carotenoids	
		(mg/g yeast)	(μg/ml)
ATCC 96594	8.80	0.351	3.088
JH1	4.80	4.862	23.337
JH2	7.10	0.329	2.335
JH3	1.30	0	0

type and mutants JH1 and JH2 increased very robustly during the exponential phase. Furthermore, in spite of the stationary phase, it continued to increase at a slow rate (Fig. 4a-c).

The total carotenoids content of mutant strains was quantified by the methods of An *et al.* [1] and compared to that of wild-type (Table 1). In spite of slow growth due to mutation, the red mutant JH1 produced 4.862 mg carotenoids/g dry cell weight in 120 h and this was about 14-folds higher than that of wild-type. However, the total carotenoids content of JH2 in 120 h was lower than that of wild-type. JH3 was incapable of producing carotenoids pigment, even though its growth rate was the fastest among all the strains.

Yield of Astaxanthin and β-Carotene

To measure the yields of astaxanthin and β-carotene from the wild-type and mutant strains, individual carotenoids were extracted from all strains and quantitatively measured by HPLC. Tables 2 and 3 show the astaxanthin and β-carotene content of JH1 and JH2, compared with wild-type, respectively. These results showed that astaxanthin-overproducing mutant JH1 produced 4.032 mg astaxanthin/g dry cell weight in 120 h, and this was about 15-folds higher than that of wild-type. β-Carotene-overproducing mutant JH2 produced 0.273 mg β-carotene/g dry cell weight in 120 h, and this was 4-folds higher than that of wild-type.

Discussion

Since astaxanthin (3,3'-dihydroxy-β,β'-carotene-4,4'-dione) has several important biological functions [3], including protection against UV light effects [5, 12, 14, 21], immune response [8, 18], communication, pigmentation, reproductive behavior, and improved reproduction, it provides important

Table 2. Cell mass and astaxanthin content of wild-type and astaxanthin-overproducing mutant JH1.

Strain	Cell mass (mg/ml)	Total carotenoids (mg/g yeast)	Astaxanthin content	
			(mg/g yeast)	(μg/ml)
ATCC 96594	8.80	0.351	0.271	2.384
JH1	4.80	4.862	4.032	19.353

Table 3. Cell mass and β-carotene content of wild-type and β-carotene-overproducing mutant JH2.

Strain	Cell mass (mg/ml)	Total carotenoids (mg/g yeast)	β-carotene content	
			(mg/g yeast)	(μg/ml)
ATCC 96594	8.80	0.351	0.066	0.580
JH2	7.10	0.329	0.273	1.938

applications in the nutraceutical, cosmetics, food, and feed industries. Therefore, a large-scale production of astaxanthin constitutes major commercial interest for industrial application. In particular, there is a strong interest within the aquaculture industry in using a natural source of astaxanthin [4, 15, 19, 26]. However, the biosynthesis of astaxanthin as a dietary source is limited to a few species of microorganisms [6, 7]. Some pigment sources such as shrimp shells or krill may also have detrimental properties such as high ash and bulk, and pigments are an expensive component of the aquaculture industry.

Recently, there has been considerable commercial interest in using *X. dendrorhous* as a dietary source of natural astaxanthin for aquacultured animals, because of its rapid heterotrophic metabolism and production of high cell densities in fermentors. *X. dendrorhous* could also potentially provide a biological source of astaxanthin for pigmentation and flavor in the aquaculture industry and supply nutrients required for growth of the animals. However, wild-type strains of the yeast *X. dendrorhous* are uneconomical for industrial application, because of their low astaxanthin production and high production cost. Consequently, mutant strains of *X. dendrorhous* with increased contents of astaxanthin are needed to provide an economical source of astaxanthin [10, 22].

In conclusion, in the present study, astaxanthin-overproducing mutant JH1 was developed from *X. dendrorhous* ATCC 96594 by mutagenesis with NTG and screening for resistance to β-ionone [1, 13, 27]: β-Ionone blocks the astaxanthin biosynthetic pathway at the β-carotene level. β-Carotene-overproducing mutant JH2 and white mutant JH3 were also derived from *X. dendrorhous* ATCC 96594 during the mutagenesis process with NTG. Selection of JH2 and JH3 is important for studies on the astaxanthin biosynthetic pathway and regulation of *X. dendrorhous*. Furthermore, these mutants are expected to have significant contribution to the later study of the genes related to the astaxanthin biosynthetic pathway in developing an astaxanthin-overproducing recombinant strain.

The yields of astaxanthin and β-carotene isolated from wild-type and mutant yeasts have been compared in the present study. In spite of slow growth due to mutation, mutants JH1 and JH2 mass-produced astaxanthin and β-carotene, respectively. The yield of astaxanthin from mutant JH1 was 15-folds higher than that from wild-type after mutagenesis with NTG, and the yield of β-carotene

from mutant JH2 was 4-folds higher than that from wild-type after mutagenesis with NTG. These results indicate that the amount of astaxanthin and β-carotene had been increased by mutation with NTG, resulting in higher yields of astaxanthin and β-carotene with several common biological functions such as photoprotection [5, 12, 14, 21], antioxidant effects [11, 16, 17], immunomodulation, and anticancer activity [9, 23, 24, 25].

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