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High-Level Production of Astaxanthin by *Xanthophyllomyces dendrorhous* Mutant JH1, Using Chemical and Light Induction

KIM, JEONG-HWAN[1] AND HYO-IHL CHANG[1]*

[1]*Department of Biotechnology, School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Korea*

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Abstract The production of astaxanthin by *Xanthophyllomyces dendrorhous* mutant depended on the culture conditions. Therefore, a cultivation strategy, including effective chemical and light induction, for the high-level production of astaxanthin by *X. dendrorhous* mutant JH1 was explored. Effective chemicals such as ethanol, acetic acid, and hydrogen peroxide, which are known inducers or precursors of astaxanthin synthesis, were investigated for their increase of astaxanthin production. Each of 1.0% ethanol, 1.0% acetic acid, and 1.0% hydrogen peroxide increased the astaxanthin concentration to 49.77 mg/l, 46.33 mg/l, and 45.61 mg/l, respectively. Among these chemicals, 1.0% ethanol showed the best effect on increasing astaxanthin concentration after 48 h of cultivation. Under 1.0% ethanol feeding condition, high light intensity (2,400 lux) stimulated astaxanthin production to 59.67 mg/l, compared with that in the dark-grown cultivation.

Key words: Astaxanthin, chemical induction, high-level production, light induction, *Xanthophyllomyces dendrorhous*

Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is widely distributed in nature and is the principal pigment in crustaceans, salmonoids, and many other organisms [10]. It gives attractive pigmentation to many farmed animals and also contributes to consumer appeal in the marketplace. In aquaculture, it is employed as a source of natural pigmentation and dietary supplement for trout and salmon [7, 9]. Astaxanthin also has important metabolic functions in animals, including its conversion to vitamin A [2], enhancement of immune response [5], and protection against diseases such as cancer by scavenging oxygen radicals [3, 11, 13]. 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 that of α-tocopherol [15, 16, 18]. Therefore, astaxanthin has attracted commercial interest not only as a pigmentation source, but also as a potent antioxidative reagent, and a recent FDA communication permitted the use of astaxanthin as a supplement and listed it for use in salmonoid fish feed.

Astaxanthin has been found in several microorganisms, including bacteria *Mycobacterium lacticola* [23], bacidiomycetous fungus *Peniophora* spp. [6], green algae *Haematococcus pluvialis* [4, 19], and heterobasidiomycetous yeast *Xanthophyllomyces dendrorhous* [8]. Among these microorganisms, only *H. pluvialis* and *X. dendrorhous* are currently considered as sources of astaxanthin for industrial production. The *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 bioreactors.

The aim of this study was to achieve a high-level production of astaxanthin by *X. dendrorhous* mutant JH1 using chemical and light induction. Effective chemicals (ethanol, acetic acid, and hydrogen peroxide) and light induction were investigated to increase the accumulation of astaxanthin in the grown cells.

Materials and Methods

Microorganism

X. dendrorhous ATCC 96594 was provided by Korea Research Institute of Bioscience and Biotechnology. The astaxanthin-overproducing mutant JH1 was derived from *X. dendrorhous* ATCC 96594 by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [12]. The strain was cultivated in YM agar at 22°C for 7 days and stored at −70°C in 30% glycerol.

Culture Conditions

The basal medium (YM) consisted of 1% glucose, 0.3% yeast extract, 0.3% malt extract, and 0.5% peptone, and the

*Corresponding author
Phone: 82-2-3290-3421; Fax: 82-2-923-9923;
E-mail: hichang@korea.ac.kr

pH was adjusted to 7.0. The test medium consisted of 4.14% glucose, 0.34% yeast extract, 0.25% KH_2PO_4, 0.05% $MgSO_4$, 0.02% $MnSO_4$, and 0.01% $CaCl_2$, and the pH was adjusted to 7.0. A colony of yeast was inoculated into a test tube containing 5 ml of YM broth, and then incubated in a rotary shaking incubator at 22°C and 140 rpm for 48 h. A 0.9 ml aliquot of the culture broth was inoculated into a 250-ml baffled flask containing 30 ml of YM broth, and further incubated for 36 h. Seed culture (3%, v/v) was used as inoculum in the main cultures. The main cultures were carried out in 250-ml baffled flasks containing 30 ml of the test medium. Inoculated baffled flasks were incubated in a rotary shaking incubator at 22°C and 140 rpm for 7 days.

Carotenoids and Astaxanthin Analysis

For routine analysis of carotenoids, the washed cell pellets were mixed with dimethyl sulfoxide preheated at 55°C and agitated for 1 min. The broken cells were thoroughly stirred in acetone and centrifuged, and the pigments in the supernatant were transferred to petroleum ether with the addition of 20% NaCl solution [12, 22]. The total carotenoids concentration was determined at 474 nm using a spectrophotometer, based on a previously reported extinction coefficient [1]. Astaxanthin was quantitatively analyzed by high performance liquid chromatography (Waters Co., U.S.A.), using a LUNA C_{18} column (250×4.6 mm; 5 μm, Phenomenex) at 25°C at a flow rate of 1.0 ml/min and HPLC-grade astaxanthin (Sigma Co., U.S.A.) as standards. Samples for HPLC analysis were diluted in the mobile phase (85% methanol, 5% dichloromethane, 5.5% acetonitrile, and 4.5% water), and peaks were measured at 480 nm. Astaxanthin was identified according to its retention time and spectrum by photodiode array detection.

Cell Mass

Dry cell mass was measured gravimetrically. The cells were harvested and washed twice with distilled water. The washed cells were dried in a drying oven at 80°C for 48 h.

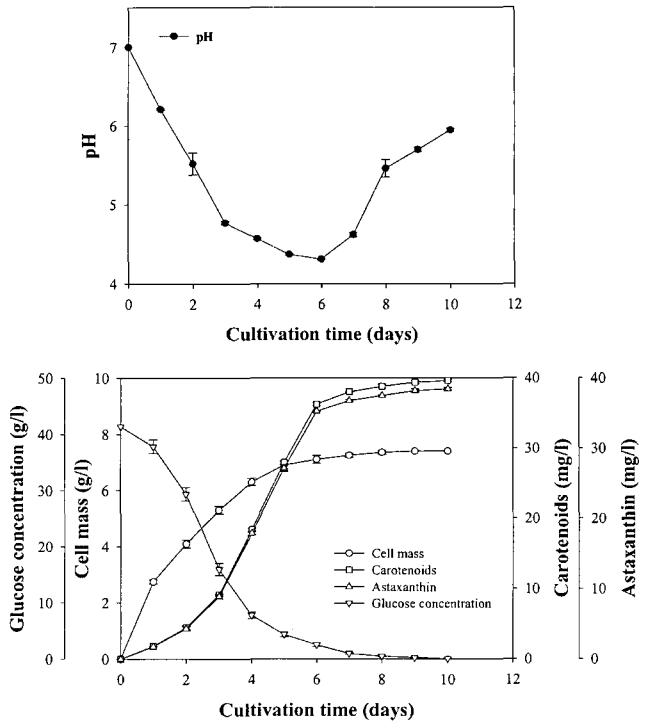

Fig. 1. Time-course profile of the production of cell and astaxanthin by *Xanthophyllomyces dendrorhous* mutant JH1. Experimental conditions: 4.14% glucose, 0.34% yeast extract, 0.25% KH_2PO_4, 0.05% $MgSO_4$, 0.02% $MnSO_4$, and 0.01% $CaCl_2$.

Glucose Analysis

The amount of glucose was measured by using a glucose assay kit (Youngdong Co. Ltd., Korea)

Chemical Induction

Ethanol, acetic acid, and hydrogen peroxide were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). All chemicals were of the highest purity available. These chemicals, in the range of 0–1.5% (v/v), were added to the culture broth when residual glucose was nearly depleted, and then the culture broth was incubated in a rotary

Table 1. Effect of chemicals on cell growth and astaxanthin production.

Chemicals	Concentation (%, v/v)	Cell mass (g/l)	Total carotenoids (mg/l)	Astaxanthin (mg/l)
Control	0	7.35±0.21	39.93±1.86	38.65±2.31
Ethanol	0.5	6.60±0.28	45.96±1.55	42.60±2.06
	1.0	7.20±0.28	52.53±3.03	49.77±1.29
	1.5	5.55±0.35	41.57±1.08	39.33±1.82
Acetic acid	0.5	6.40±0.42	44.23±1.77	41.29±1.27
	1.0	7.30±0.14	49.53±1.89	46.33±0.72
	1.5	5.95±0.50	37.23±1.10	34.49±0.74
H_2O_2	0.5	6.50±0.42	39.59±2.19	37.00±2.29
	1.0	6.70±0.14	49.57±3.03	45.61±2.14
	1.5	5.35±0.35	37.37±1.76	35.49±0.90

Table 2. Effect of chemicals in combination with ethanol on cell growth and astaxanthin production.

Chemicals	Concentration (%, v/v)	Cell mass (g/l)	Total carotenoids (mg/l)	Astaxanthin (mg/l)
Control	0	7.27±0.12	39.24±0.45	37.75±0.44
Ethanol	1.0	7.03±0.21	52.41±1.83	50.38±2.08
Ethanol-Acetic acid (1:1)	1.0	5.40±0.20	30.37±2.77	29.70±2.26
Ethanol-Acetic acid-H_2O_2 (3:4:3)	1.0	4.47±0.31	31.06±2.66	2.88±2.64
Ethanol-H_2O_2 (1:1)	1.0	6.67±0.31	45.01±0.95	42.81±0.98

A total of four sets, including ethanol only, ethanol-acetic acid (1:1), ethanol-acetic acid-hydrogen peroxide (3:4:3), and ethanol-hydrogen peroxide (1:1), were investigated.

shaking incubator at 22°C and 140 rpm. The effect of chemicals on astaxanthin production in combination with ethanol was also investigated. Thus, a total four sets, including ethanol only, ethanol-acetic acid (1:1), ethanol-acetic acid-H_2O_2 (3:4:3), and ethanol-H_2O_2 (1:1), were used. Each (1%, v/v) of these chemicals was added to the culture broth when residual glucose was nearly depleted.

Light Induction

For the light induction experiments, *X. dendrorhous* mutant JH1 was grown in an orbital shaker incubator equipped with two fluorescent tube lamps that provided 2,400 lux at the culture surface. Control flasks were covered with aluminum foil.

RESULTS

Effect of Ethanol, Acetic Acid, and Hydrogen Peroxide on Astaxanthin Production by *Xanthophyllomyces dendrorhous* Mutant JH1

To investigate the effect of chemical induction, each of ethanol, acetic acid, and hydrogen peroxide in the range of 0–1.5% (v/v) was added to the culture broth at day 5 when residual glucose was nearly depleted (Fig. 1). As shown in Table 1, each of 1.0% ethanol, 1.0% acetic acid, and 1.0% hydrogen peroxide increased the astaxanthin concentration to 49.77 mg/l, 46.33 mg/l and 45.61 mg/l, respectively, compared with that in the control without any supplement. However, the addition of more than 1.0% of these chemicals decreased cell and astaxanthin concentrations. This was most likely due to the toxic effect of these chemicals on the yeast cell growth. Among these chemicals, 1.0% ethanol showed the best effect in increasing astaxanthin concentration after 48 h of cultivation. At this concentration, ethanol showed a negligible effect on the cell growth.

Effect of Chemicals in Combination with Ethanol on Astaxanthin Production by *Xanthophyllomyces dendrorhous* Mutant JH1

The effect of chemicals in combination with ethanol on astaxanthin production was also investigated. Thus, a total of four sets, including ethanol only, ethanol-acetic acid (1:1), ethanol-acetic acid-H_2O_2 (3:4:3), and ethanol-H_2O_2 (1:1), were used. As shown in Table 2, the astaxanthin concentration obtained from each culture broth was 50.38 mg/l, 29.70 mg/l, 28.88 mg/l, and 42.81 mg/l, respectively. Among the chemicals tested, 1.0% ethanol only showed the best effect in increasing the astaxanthin concentration after 48 h of cultivation, compared with the others tested.

Effect of Light Induction on Astaxanthin Production by *Xanthophyllomyces dendrorhous* Mutant JH1

To investigate the effect of light induction, *X. dendrorhous* mutant JH1 was grown in the dark or with high light intensity (2,400 lux). Then, chemicals in combination with ethanol were added at day 5. As shown in Table 3, the light-grown cultivation increased the astaxanthin concentration to 59.67 mg/l, 34.01 mg/l, 33.68 mg/l, and 50.58 mg/l, respectively, compared with that in the dark-grown cultivation. Specifically, under the 1.0% ethanol feeding condition, high light intensity (2,400 lux) stimulated astaxanthin

Table 3. Effect of light induction on cell growth and astaxanthin production.

Chemicals	Concentration (%, v/v)	Dark condition			Light condition (2,400 lux)		
		Cell mass (g/l)	Total carotenoids (mg/l)	Astaxanthin (mg/l)	Cell mass (g/l)	Total carotenoids (mg/l)	Astaxanthin (mg/l)
Control	0	6.59±0.14	26.46±2.34	23.87±2.17	7.63±0.16	45.64±1.14	43.56±1.27
Ethanol	1.0	6.40±0.20	38.91±2.06	36.81±1.45	7.42±0.20	61.48±2.05	59.67±1.65
Ethanol-Acetic acid (1:1)	1.0	3.47±0.12	20.36±1.50	17.68±1.58	5.42±0.14	35.73±0.76	34.01±0.86
Ethanol-Acetic acid-H_2O_2 (3:4:3)	1.0	3.44±0.16	20.72±1.69	18.69±1.13	4.41±0.17	36.35±0.68	33.68±0.64
Ethanol-H_2O_2 (1:1)	1.0	5.49±0.11	34.31±1.26	31.45±0.98	6.68±0.14	53.10±1.35	50.58±1.49

Table 4. Comparison of cell mass and astaxanthin production reported in related experiments.

Strain	Cell mass (g/l)	Astaxanthin (mg/l)	Productivity (mg/l·h)	Reference
X. dendrorhous WS-2	7.62	14.90	0.12	Yu and Ryu [25]
X. dendrorhous 25-2	8.20	8.10	0.08	Ramirez *et al.* [21]
X. dendrorhous AH-6-1	7.44	14.46	0.12	Kim *et al.* [14]
X. dendrorhous ATCC 96594	8.80	2.38	0.02	Kim *et al.* [12]
X. dendrorhous JH1	4.80	19.35	0.16	Kim *et al.* [12]
X. dendrorhous JH1	7.42	59.67	0.36	This work

production to 59.67 mg/l, compared with that in the dark-grown cultivation.

DISCUSSION

The use of natural astaxanthin is of great interest in the chemical, pharmaceutical, and alimentary industries because of its several important biological functions [2, 3, 5, 11, 13, 15, 16, 18]. Specifically, there is a strong interest within the aquaculture industry in using natural sources of astaxanthin [17, 20, 24]. Therefore, mass production of astaxanthin constitutes a major commercial interest for industrial application.

The red yeast *X. dendrorhous* appears to be the best candidate for producing a natural astaxanthin among all the strains reported so far, because of its rapid heterotrophic metabolism and production of high cell densities in bioreactors. It could also potentially provide a biological source of astaxanthin for pigmentation and flavor in the aquaculture industry and supply nutrients required for growth of animals.

In the present study, chemical and light induction were used for a high-level production of astaxanthin by *X. dendrorhous* mutant JH1. Ethanol, acetic acid and, hydrogen peroxide, which are known inducers or precursors of astaxanthin synthesis, were found to have a positive effect on astaxanthin concentration and productivity. However, the addition of more than 1.0% of these chemicals decreased the cell and astaxanthin concentrations, since a higher concentration of these chemicals inhibited cell growth. Light was also found to have a positive effect on astaxanthin concentration and productivity. After 1.0% ethanol feeding in the light (2,400 lux)-grown cultivation, the concentration and productivity of astaxanthin by *X. dendrorhous* mutant JH1 were 59.67 mg/l and 0.36 mg/l·h, respectively, and they were compared with those reported for other astaxanthin-producing microorganisms (Table 4). The *X. dendrorhous* mutant JH1 had the highest yield and productivity of astaxanthin among reported values for astaxanthin production.

In conclusion, *X. dendrorhous* mutant JH1 is a potential microorganism for the production of astaxanthin, and chemical and light induction appears to be a promising route for a high-level production of astaxanthin.

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REFERENCES

1. An, G. H., D. B. Schuman, and E. A. Johnson. 1989. Isolation of *Phaffia rhodozyma* mutants with increased astaxanthin content. *Appl. Environ. Microbiol.* **55:** 116–124.
2. Bendich, A. and J. A. Olson. 1989. Biological actions of carotenoids. *FASEB J.* **3:** 1927–1932.
3. Bertram, J. S. 1993. Cancer prevention of carotenoids: Mechanistic studies in cultured cells. *Ann. NY Acad. Sci.* **691:** 177–191.
4. Bubrick, P. 1991. Production of astaxanthin from *Haematococcus*. *Bioresour. Technol.* **38:** 237–239.
5. Chew, B. P. 1993. Role of carotenoids in the immune response. *J. Dairy Sci.* **76:** 2804–2811.
6. Goodwin, T. W. 1980. *The Biochemistry of the Carotenoids*. Second Edition. Chapman & Hall, London, U.K.
7. Johnson, E. A., D. E. Conklin, and M. J. Lewis. 1977. The yeast *Phaffia rhodozyma* as a dietary pigment source for salmonoids and crustaceans. *J. Fish Res. Bd. Canada* **34:** 2417–2421.
8. Johnson, E. A. and M. J. Lewis. 1979. Astaxanthin formation by the yeast *Phaffia rhodozyma*. *J. Gen. Microbiol.* **115:** 173–183.
9. Johnson, E. A. and G. H. An. 1991. Astaxanthin from microbial sources. *Crit. Rev. Biotechnol.* **11:** 297–326.
10. Johnson, E. A. and W. A. Schroeder. 1995. Microbial carotenoids. *Adv. Biochem. Eng.* **53:** 119–178.
11. Jyonouchi, H., S. Sun, K. Lijima, and M. D. Gross. 2000. Antitumor activity of astaxanthin and its mode of action. *Nutr. Cancer* **36:** 59–65.
12. Kim, J. H., C. W. Kim, and H. I. Chang. 2004. Screening and characterization of red yeast *Xanthophyllomyces dendrorhous* mutants. *J. Microbiol. Biotechnol.* **14:** 570–575.
13. Kim, J. H., S. K. Choi, W. J. Lim, and H. I. Chang. 2004. Protective effect of astaxanthin produced by *Xanthophyllomyces dendrorhous* mutant on indomethacin-induced gastric mucosal injury in rats. *J. Microbiol. Biotechnol.* **14:** 996–1003.
14. Kim, S. J., G. J. Kim, D. H. Park, and Y. W. Ryu. 2003. High-level production of astaxanthin by fed-batch culture

of mutant strain *Phaffia rhodozyma* AJ-6-1. *J. Microbiol. Biotechnol.* **13:** 175–181.
15. Krinsky, N. I. 1989. Antioxidant function of carotenoids. *Free Radic. Biol. Med.* **7:** 617–635.
16. Mortensen, A., L. H. Skibsted, and T. Truscott. 2001. The interaction of dietary carotenoids with radical species. *Arch. Biochem. Biophys.* **385:** 13–19.
17. Meyers, S. P. 1993. The biological role of astaxanthin in salmons and other aquatic species. First International Symposium on Natural Colors for Foods, Nutraceuticals, Beverages and Confectionary, Amherst, U.S.A.
18. Naguib, Y. M. 2000. Antioxidant activities of astaxanthin and related carotenoids. *J. Agric. Food Chem.* **48:** 1150–1154.
19. Park, E. K. and C. G. Lee. 2001. Astaxanthin production by *Haematococcus pluvialis* under various light intensities and wavelengths. *J. Microbiol. Biotechnol.* **11:** 1024–1030.
20. Rackham, D. 1988. Salmon and trout marketing, p. 231. *In* Laird, L. and T. Needham. (eds.), *Salmon and Trout Farming.* Ellis Horwood, Chichester, England.
21. Ramirez, J., H. Gutierrez, and A. Gschaedler. 2001. Optimization of astaxanthin production by *Phaffia rhodozyma* through factorial design and response surface methodology. *J. Biotechnol.* **88:** 259–268.
22. Sedmak, J. J., D. K. Weerasinghe, and S. O. Jolly. 1990. Extraction and quantitation of astaxanthin from *Phaffia rhodozyma*. *Biotech. Tech.* **4:** 107–112.
23. Simpson, K. I., T. Katayana, and C. O. Chichester. 1981. Carotenoids from microorganisms. *In* Bauernfeld, J. C. (ed.), *Carotenoids as Colorants and Vitamin A Precursors.* Academic Press, London, U.K.
24. Torissen, O. J. 1989. Pigmentation of salmonids-carotenoid deposition and metabolism. *CRC Crit. Rev. Aquat. Sci.* **1:** 209–225.
25. Yu, S. S. and Y. W. Ryu. 2001. Selection of mutant *Phaffia rhodozyma* and determination of optimum culture conditions for astaxanthin production. *Kor. J. Appl. Microbiol. Biotechnol.* **29:** 96–103.