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Effect of Culture Conditions on Astaxanthin Formation in Red Yeast *Xanthophyllomyces dendrorhous* Mutant JH1

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Abstract The formation of astaxanthin by *Xanthophyllomyces dendrorhous* mutant JH1 depends on the culture conditions. Therefore, the effects of inoculation rate (1–5%, v/v) and medium compositions (various carbon and nitrogen sources) on cell growth and astaxanthin formation in *X. dendrorhous* mutant JH1 were investigated. Inoculation at 3% (v/v) was optimal for cell growth and astaxanthin formation. The most effective carbon source for cell growth and astaxanthin formation was glucose, and the best nitrogen source was yeast extract. The 3% (w/v) glucose and 0.2% (w/v) yeast extract showed the best effect on cell growth and astaxanthin formation, compared with others tested. The 3% glucose, 0.2% yeast extract, 0.15% KH_2PO_4, 0.05% $MgSO_4$, 0.01% $MnSO_4$, and 0.01% $CaCl_2$ were selected for cell growth and astaxanthin formation. Under the conditions selected, the maximum concentrations of cell and astaxanthin obtained after 168 h of cultivation were 5.43 g/l and 28.20 mg/l, respectively.

Key words: Astaxanthin, carbon source, culture conditions, nitrogen source, *Xanthophyllomyces dendrorhous*

As a lipid-soluble pigment, astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is widely distributed in nature and is the major pigment in crustaceans, salmonoids, and many other organisms [9]. It gives attractive pigmentation to many farmed animals and contributes to consumer appeal in the marketplace [6, 8]. Astaxanthin also has a higher antioxidative activity than β-carotene and α-tocopherol [15–17]. Therefore, astaxanthin has attracted commercial interest not only as a pigmentation source, but also as a potent antioxidative reagent, and a recent FDA communication allowed the use of astaxanthin as a supplement and salmonoid fish feed.

Astaxanthin has been found in several microorganisms, including bacteria *Mycobacterium lacticola* [20], bacidiomycetous fungus *Peniophora* spp. [5], green algae *Haematococcus pluvialis* [3, 18], and heterobasidiomycetous yeast *Xanthophyllomyces dendrorhous* [7]. Among these microorganisms, only *X. dendrorhous* is currently considered as a source 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. It is a carotenoid-producing yeast, which synthesizes astaxanthin as its main carotenoid [7]. However, wild-type *X. dendrorhous* is uneconomical, because of its low astaxanthin content and high production cost. Therefore, astaxanthin-overproducing *X. dendrorhous* mutant JH1 developed in our laboratory was used [11]. It produced 22.49 mg astaxanthin/l, and this value was about 9-folds higher than that of wild-type.

Since the formation of astaxanthin by *X. dendrorhous* mutant JH1 depends on the culture conditions, the aim of this study was to investigate the effect of inoculation rate (1–5%, v/v) and medium components (various carbon and nitrogen sources) on cell growth and astaxanthin formation in this mutant.

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*-

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Table 1. Experimental design for determination of test medium composition.

Source (%, w/v)	Test 1	Test 2	Test 3	Test 4	Test 5
Glucose	1	2	3	4	5
Yeast extract	0.2	0.2	0.2	0.2	0.2
$(NH_4)_2SO_4$	0.2	0.2	0.2	0.2	0.2
KH_2PO_4	0.15	0.15	0.15	0.15	0.15
$MgSO_4$	0.05	0.05	0.05	0.05	0.05
$MnSO_4$	0.01	0.01	0.01	0.01	0.01
$CaCl_2$	0.01	0.01	0.01	0.01	0.01
Source (%, w/v)	Test 6	Test 7	Test 8	Test 9	Test 10
Glucose	1	2	3	4	5
Yeast extract	0.2	0.2	0.2	0.2	0.2
$(NH_4)_2SO_4$	0.5	0.5	0.5	0.5	0.5
KH_2PO_4	0.15	0.15	0.15	0.15	0.15
$MgSO_4$	0.05	0.05	0.05	0.05	0.05
$MnSO_4$	0.01	0.01	0.01	0.01	0.01
$CaCl_2$	0.01	0.01	0.01	0.01	0.01

pH 7.0

methyl-*N'*-nitro-*N*-nitrosoguanidine [11]. The strain was cultivated in YM agar at 22°C for 168 h and stored at −70°C in 30% glycerol.

Media and Culture Conditions

The basal medium (YM) consisted of 1% glucose, 0.3% yeast extract, 0.3% malt extract, and 0.5% peptone, and the pH was adjusted to 7.0. The individual test media were made according to experimental design (Table 1), and the pHs of these media were 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 media. Inoculated baffled flasks were incubated in a rotary shaking incubator at 22°C and 140 rpm for 168 h. All experiments were performed in triplicate. All values are represented as mean±S.E.M.

Astaxanthin Analysis

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 [11, 19]. Astaxanthin was quantitatively analyzed by high performance liquid chromatography (HPLC; Waters Co., U.S.A.), using a LUNA C_{18} column (250×4.6 mm; 5 mm, 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 [11].

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.

RESULTS

Selection of Test Medium Composition

The seven ingredients [glucose, yeast extract, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4$, $MnSO_4$, and $CaCl_2$] that play the most important role in *X. dendrorhous* cultivation were selected (Table 1), and the effects of inoculation rate (1–5%, v/v), glucose concentration (1–5%, w/v), and $(NH_4)_2SO_4$ concentration (0.2%, 0.5%, w/v) on cell growth and astaxanthin formation in *X. dendrorhous* mutant JH1 were investigated. As shown in Test 3 of Table 2, cell growth and astaxanthin concentration increased, compared with the others tested. Therefore, the 3% glucose, 0.2% yeast extract, 0.2% $(NH_4)_2SO_4$, 0.15% KH_2PO_4, 0.05% $MgSO_4$, 0.01% $MnSO_4$, and 0.01% $CaCl_2$ were selected for cell growth and astaxanthin formation. Under the conditions selected, 3% (v/v) inoculation was optimal for cell growth and astaxanthin formation (3%> 5%>4%>2%>1%, Fig. 2). Figure 1 shows the time-course profile of the production of cell and astaxanthin by *X. dendrorhous* mutant JH1: Cell mass increased highly during 120 h of cultivation and astaxanthin concentration dramatically increased during 168 h. The highest concentrations of cell and astaxanthin obtained after 168 h of cultivation

Table 2. Results of experimental design for determination of test medium composition.

	pH (Initial pH 7.0)	Cell mass (g/l)	Astaxanthin	
			(mg/g yeast)	(mg/l)
Test 1	6.38±0.14	2.74±0.43	5.04±0.42	13.88±1.24
Test 2	3.06±0.12	4.46±0.12	4.89±0.14	21.79±1.48
Test 3	2.55±0.03	4.88±0.20	5.10±0.22	24.90±2.52
Test 4	2.46±0.21	4.51±0.22	4.73±0.24	21.30±2.24
Test 5	2.57±0.18	4.37±0.24	4.84±0.18	21.16±1.08
Test 6	5.88±0.24	2.36±0.21	4.61±0.22	10.87±0.81
Test 7	3.05±0.15	3.77±0.25	4.04±0.31	15.21±0.87
Test 8	2.81±0.21	3.21±0.13	6.63±0.15	21.27±1.26
Test 9	2.66±0.25	3.69±0.12	5.88±0.12	21.69±1.12
Test 10	2.81±0.31	3.40±0.08	6.30±0.16	21.37±1.36

JH1 was cultivated at 22°C and 140 rpm in Test broth for 168 h with inoculation of 5% (v/v) seed culture. All experiments were performed in triplicate, and all values are represented as mean±S.E.M.

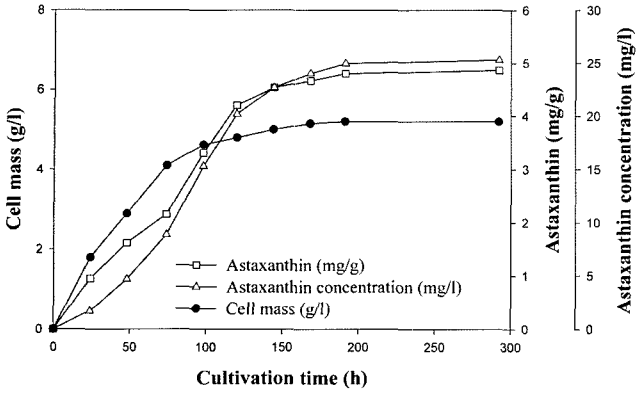

Fig. 1. Time-course profile of the production of cell and astaxanthin by *Xanthophyllomyces dendrorhous* mutant JH1.
Experimental conditions: 3% glucose, 0.2% yeast extract, 0.2% $(NH_4)_2SO_4$, 0.15% KH_2PO_4, 0.05% $MgSO_4$, 0.01% $MnSO_4$, and 0.01% $CaCl_2$.

were 4.88 g/l and 24.90 mg/l, respectively. These results suggest that astaxanthin formation in *X. dendrorhous* mutant JH1 is clearly growth-associated, although its production does not exactly coincide with the increase of cell mass.

Effect of Various Carbon Sources on Cell Growth and Astaxanthin Formation in *Xanthophyllomyces dendrorhous* Mutant JH1

To investigate the effect of various carbon sources on cell growth and astaxanthin formation, each (3%, w/v) of glucose, maltose, lactose, sucrose, arabinose, galactose, and fructose was tested. As shown in Fig. 3, glucose showed the best

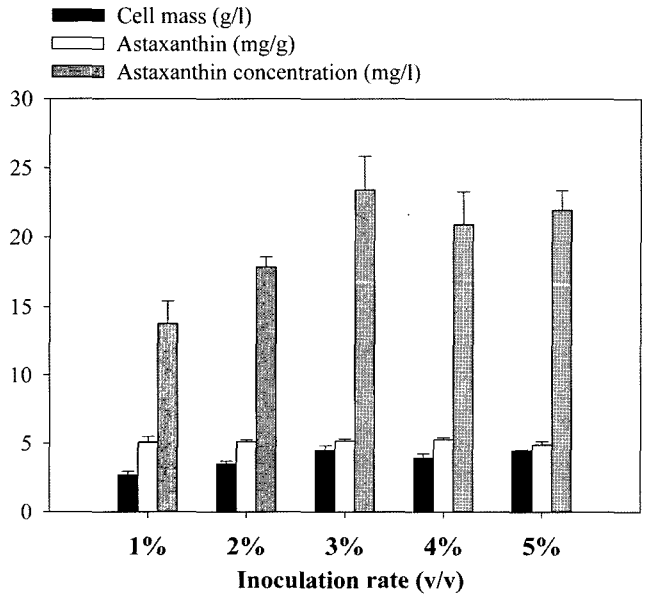

Fig. 2. Effect of inoculation rate on cell growth and astaxanthin formation of mutant JH1 after 168 h of cultivation.
All experiments were performed in triplicate, and all values are represented as mean±S.E.M.

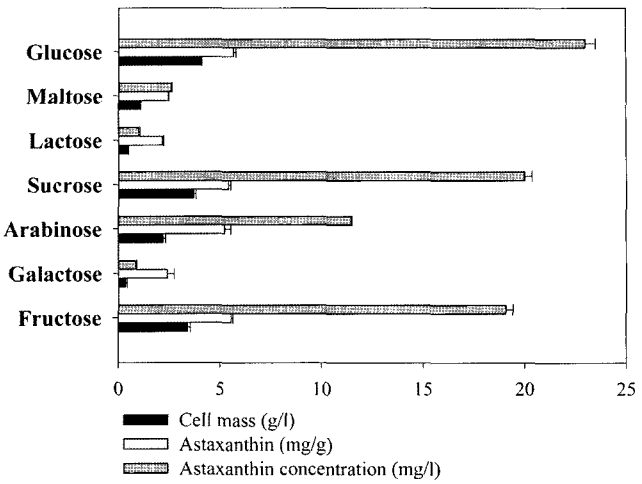

Fig. 3. Effect of various 3% (w/v) carbon sources on cell growth and astaxanthin formation of mutant JH1 after 168 h of cultivation.
JH1 was cultivated at 22°C and 140 rpm for 168 h with inoculation of 3% (v/v) seed culture. All experiments were performed in triplicate, and all values are represented as mean±S.E.M.

effect on cell growth and astaxanthin formation, compared with the others tested. The other disaccharides, sucrose and fructose, were also effective. Maltose, lactose, and galactose showed the lowest effect on cell and astaxanthin concentrations (Glucose>Sucrose>Fructose>Arabinose>Maltose>Lactose >Galactose, Fig. 3).

Effect of Various Nitrogen Sources on Cell Growth and Astaxanthin Formation in *Xanthophyllomyces dendrorhous* Mutant JH1

To investigate the effect of various nitrogen sources on cell growth and astaxanthin formation, each (0.5%, w/v) of yeast extract, bacto-peptone, $(NH_4)_2SO_4$, corn steep liquor, industrial yeast extract, casamino acid, and beef extract was tested. As shown in Fig. 4, industrial yeast extract, corn steep liquor, and yeast extract showed the best effect on cell growth and astaxanthin formation, compared with the others tested [Industrial yeast extract>Corn steep liquor> Yeast extract>Beef extract>Casamino acid>Bacto-peptone >$(NH_4)_2SO_4$, Fig. 4]. Therefore, the effect of the industrial yeast extract, corn steep liquor, and yeast extract on cell growth and astaxanthin formation was investigated. Each of 0.2% yeast extract, 0.2% yeast extract-0.2% $(NH_4)_2SO_4$, 0.5% yeast extract, 0.5% yeast extract-0.2% $(NH_4)_2SO_4$, 0.2% industrial yeast extract, 0.5% industrial yeast, 0.2% corn steep liquor, and 0.5% corn steep liquor was tested. As shown in Fig. 5, 0.2% yeast extract showed the best effect on cell growth and astaxanthin formation, compared with the others tested. $(NH_4)_2SO_4$ showed the lowest effect on cell and astaxanthin concentration (Fig. 4). $(NH_4)_2SO_4$ in combination with 0.2% yeast extract and 0.5% yeast extract also showed the lowest values of cell and astaxanthin concentration [0.2% Yeast extract>0.5% Industrial yeast

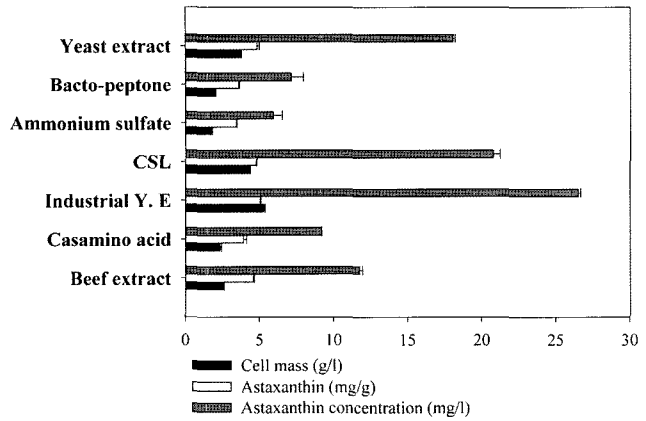

Fig. 4. Effect of various 0.5% (w/v) nitrogen sources on cell growth and astaxanthin formation of mutant JH1 after 168 h of cultivation.

JH1 was cultivated at 22°C and 140 rpm for 168 h with inoculation of 3% (v/v) seed culture. All experiments were performed in triplicate, and all values are represented as mean±S.E.M.

extract>0.2% Yeast extract-0.2% $(NH_4)_2SO_4$>0.5% Corn steep liquor>0.2% Industrial yeast extract>0.5% Yeast extract>0.2% Corn steep liquor>0.5% Yeast extract-0.2% $(NH_4)_2SO_4$, Fig. 5].

DISCUSSION

The use of natural astaxanthin is of great interest in the chemical, pharmaceutical, and alimentary industries owing to its several essential biological functions [1, 2, 4, 10, 12–17]. The red yeast *X. dendrorhous* appears to be the best candidate for producing 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.

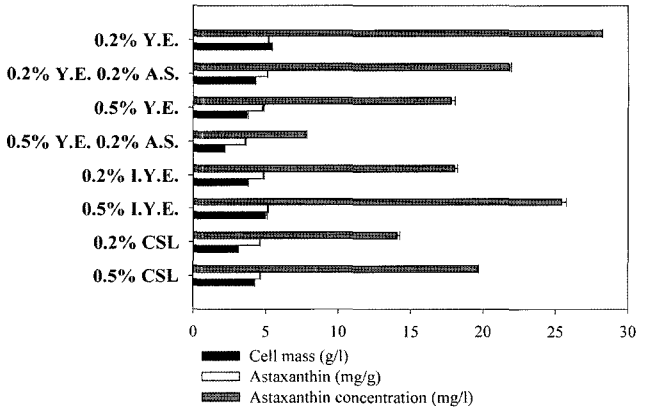

Fig. 5. Effect of various nitrogen sources concentrations on cell growth and astaxanthin formation of mutant JH1 after 168 h of cultivation.

JH1 was cultivated at 22°C and 140 rpm for 168 h with inoculation of 3% (v/v) seed culture. All experiments were performed in triplicate and all values are represented as mean±S.E.M.

Since the formation of astaxanthin by *X. dendrorhous* mutant JH1 depends on the culture conditions, this study was undertaken to investigate the effect of inoculation rate (1–5%, v/v) and medium components (various carbon and nitrogen sources) on cell growth and astaxanthin formation in *X. dendrorhous* mutant JH1. Inoculation rate and medium compositions were optimized for cell growth and astaxanthin formation using the traditional "one-factor-at-a-time" technique for optimization. This method consists of varying one factor while keeping the other factors at a constant level. The test medium consisted of 3% glucose, 0.2% yeast extract, 0.2% $(NH_4)_2SO_4$, 0.15% KH_2PO_4, 0.05% $MgSO_4$, 0.01% $MnSO_4$, and 0.01% $CaCl_2$, and the pHs were adjusted to 7.0. Under the selected conditions, 3% (v/v) inoculation was optimal for cell growth and astaxanthin formation.

In the present study, the most effective carbon source for cell growth and astaxanthin formation was glucose, and the best nitrogen source was yeast extract. Among various carbon sources tested, 3% glucose showed the best effect on cell growth and astaxanthin formation after 168 h of cultivation, compared with the others tested (Glucose>Sucrose>Fructose>Arabinose>Maltose>Lactose>Galactose, Fig. 3). However, as reported elsewhere, the growth of *X. dendrorhous* was inhibited when glucose concentration was more than 1.5% (w/v). Moreover, the lag time and astaxanthin production were also influenced by high glucose concentrations [7]. On the other hand, a high glucose concentration of 3% (w/v) increased cell growth and astaxanthin formation of *X. dendrorhous* mutant JH1 in the present study.

Among the various nitrogen sources tested, 0.2% yeast extract showed the best effect on cell growth and astaxanthin formation after 168 h of cultivation, compared with the others tested [0.2% Yeast extract>0.5% Industrial yeast extract>0.2% Yeast extract-0.2% $(NH_4)_2SO_4$>0.5% Corn steep liquor>0.2% Industrial yeast extract>0.5% Yeast extract>0.2% Corn steep liquor>0.5% Yeast extract-0.2% $(NH_4)_2SO_4$, Fig. 5]. Industrial yeast extract and corn steep liquor as nitrogen sources also showed the best effect on cell growth and astaxanthin formation, compared with the others tested. As commercially available nitrogen sources, industrial yeast extract and corn steep liquor were first used for the production of astaxanthin, and they have already been successfully used as the nitrogen source in batch cultures. However, poor solubility of the suspended insoluble particles limits the use of industrial yeast extract and corn steep liquor as the nitrogen sources.

In conclusion, *X. dendrorhous* mutant JH1 is a potential microorganism for the formation of astaxanthin, and 3% glucose, 0.2% yeast extract, 0.15% KH_2PO_4, 0.05% $MgSO_4$,

0.01% $MnSO_4$, and 0.01% $CaCl_2$ were finally selected for its cell growth and astaxanthin formation.

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