The Journal of Microbiology, April 2007, p. 128-132
Vol. 45, No. 2
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Increased Carotenoid Production in *Xanthophyllomyces dendrorhous* G276 Using Plant Extracts

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(Received March 5, 2007 / Accepted March 28, 2007)

The red yeast *Xanthophyllomyces dendrorhous* (previously named *Phaffia rhodozyma*) produces astaxanthin pigment among many carotenoids. The mutant *X. dendrorhous* G276 was isolated by chemical mutagenesis. The mutant produced about 2.0 mg of carotenoid per g of yeast cell dry weight and 8.0 mg/L of carotenoid after 5 days batch culture with YM media; in comparison, the parent strain produced 0.66 mg/g of yeast cell dry weight and a carotenoid concentration of 4.5 mg/L. We characterized the utilization of carbon sources by the mutant strain and screened various edible plant extracts to enhance the carotenoid production. The addition of *Perilla frutescens* (final concentration, 5%) or *Allium fistulosum* extracts (final concentration, 1%) enhanced the pigment production to about 32 mg/L. In a batch fermentor, addition of *Perilla frutescens* extract reduced the cultivation time by two days compared to control (no extract), which usually required five-day incubation to fully produce astaxanthin. The results suggest that plant extracts such as *Perilla frutescens* can effectively enhance astaxanthin production.

Keywords: *Xanthophyllomyces dendrorhous*, carotenoid, astaxanthin, plant extract, *Perilla frutescens*

Astaxanthin (3,3'-dihydroxy-β,β'-carotene-4,4'-dione) is composed of eight isoprenoid units and dicyclic rings that contain oxygen (Ducrey Sanpietro and Kula, 1998). This pigment is not only a useful feed additive for poultry, trout, and salmon, but also a strong antioxidant that protects the phospholipid cell membrane and other lipid components by removing singlet oxygen and inhibiting free radicals (Schroeder and Johnson, 1995; Schroeder *et al.*, 1996; An, 1997). Astaxanthin has considerable potential and promising applications in human health and nutrition in the prevention of cancer, inflammation, and diabetes (Guerin *et al.*, 2003; Hussein *et al.*, 2006). Extensive applications in pharmaceuticals, human dietary supplements, and cosmetics, as well as in feed additives, will cause rapid growth in its industrial market share in the future. Synthetic astaxanthin is also available in the aquaculture and poultry industries.

Natural astaxanthin from microorganisms is mainly supplied by the red yeast *Xanthophyllomyces dendrorhous* and the green algae *Heamatococcus pluvialis*. *Xanthophyllomyces dendrorhous* is basidiomycetous yeast, the teleomorphic state of *Phaffia rhodozyma* (Golubev, 1995). In particular, astaxanthin isolated from *X. dendrorhous* has the 3*R*, 3'*R* configuration and represents more than 80% of the carotenoid produced (Andrewes *et al.*, 1976; Andrewes and Starr, 1976). Mutation technologies for strain improvement and better fermentation processes have been developed to increase production of the pigment (An *et al.*, 1989; Fang and Cheng, 1993; Yamane *et al.*, 1997; Florencio *et al.*, 1998; Kim *et al.*, 2003; Kim *et al.*, 2004). In spite of these efforts, the development of the red yeast *X. dendrorhous* with increased astaxanthin content is needed for commercial users.

In this study, *X. dendrorhous* was mutated with a chemical mutagen and screened to find cells that produced high levels of carotenoid. With the increased carotenoid production in the mutant, we also report that plant extracts such as *Perilla frutescens* or *Allium fistulosum* could enhance the pigment production. The wild-type strain *X. dendrorhous* ATCC 66272 was used in this study. Cells were grown in YM media (3 g yeast extract, 3 g malt extract, 5 g Bactopeptone, and 1% glucose per liter, initial pH = 5.0) at 20°C for 5 days.

Carotenoid was measured as described previously (An *et al.*, 1996). Briefly, 1 ml of culture broth was sampled and centrifuged, and the supernatant was removed. Then DMSO, acetone, petroleum ether, and 20% of NaCl solution were added serially to the pellet and vortexed vigorously for 20-30 sec. After separating the solvent layers, the absorbance of the supernatant layer was measured at 478 nm by UV spectrophotometer (Shimazu Co., Japan). Total carotenoid was calculated with the following equation:

$$\text{Total carotenoid [mg/g of yeast]} = \frac{(ml\ of\ petrol) \times A_{478} \times 100}{21 \times (yeast\ dry\ weight)}$$

Mutagenesis of *X. dendrorhous* was performed using *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG). Cells were washed twice in 0.1 M sodium citrate buffer (pH 5.5) and resuspended in the same solution. The suspension was treated with NTG at 20°C for 20 min at final concentrations ranging from

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2-20 μg/ml, respectively. Cells were washed twice by suspension in 1 ml of 0.1 M potassium phosphate buffer (pH 7.0) to remove the mutagen and inoculated into 3 ml of YM broth. Overnight cultures were plated on YM agar media, and colonies were screened visually for enhanced red color after incubating for 5-7 days.

The mutant *X. dendrorhous* G276 exhibited the greatest increase of about 3 times (2.0 mg/g of yeasts) that of the parent strain, *X. dendrorhous* ATCC 66272 (0.66 mg/g of yeasts) carotenoid content (Table 1). *X. dendrorhous* G276 also produced about 1.8 times larger carotenoid concentration (8.0 mg/L) than the parent strain (4.5 mg/L), although it contained less dry cell weight (4.0 g/L) than the parent strain (6.8 g/L). The G268, G274, and G278 strains also produced above two-fold greater carotenoid content, but only a small increase in carotenoid concentration compared to the parent strain.

Table 1. Carotenoid production of *X. dendrorhous* mutants after NTG treatment

Strain[a]	Dry cell weight (g/L)	Carotenoid concentration (mg/L)	Carotenoid content (mg/g)
Parent[b]	6.8	4.5	0.66
G219	3.5	5.0	1.44
G266	4.5	5.1	1.13
G268	4.4	5.6	1.26
G274	4.0	5.4	1.34
G276	4.0	8.0	2.00
G278	3.3	5.5	1.67

[a] Cells were grown in YM broth for 5 days and assayed for carotenoid production.
[b] *X. dendrorhous* ATCC 66272.

We tested for astaxanthin production in the rich YM-based media to determine whether *X. dendrorhous* G276 can utilize various carbon sources (final concentration, 1%) for carotenogenesis (Fig. 1). As previously reported (Fang *et al.*, 1993), *X. dendrorhous* G276 contained a high cell concentration when using glucose, fructose, and sucrose. Sucrose was an effective carbon source for producing carotenoid concentration (9.7 mg/L) and carotenoid content (1.8 mg/g yeast). Carbon sources such as rhamnose, κ-gluconate, pyruvate, and acetate were also tested in this report, but resulted in much less pigmentation, indicating that these carbon sources were not effective for carotenoid production. In media containing gluconate as a carbon source, carotenoid production was low despite the highest observed cell mass (8.2 mg/L), suggesting that gluconate or its derivatives may inhibit carotenogenesis. *X. dendrorhous* G276 grew less in media containing pyruvate or acetate. Glycerol significantly increased carotenoid production although cell growth was slow and attenuated. In glycerol-containing media, *X. dendrorhous* G276 had the highest carotenoid content (3.17 mg/g yeasts) with a carotenoid concentration of 9.5 mg/L. Kusdiyantini *et al.* (1998) also showed that glycerol may be co-utilized with other nutrients for astaxanthin production.

Using *X. dendrorhous* G276 strain, we screened some plant extracts to find those that effectively enhance astaxanthin production. Various readily available vegetables were selected, and their leaves were squeezed. Each extract was filtered using a 0.45 μm pore size syringe filter. *X. dendrorhous* G276 was then inoculated to YM broth (pH 5.0) containing each extract (final concentration, 0.1%). The effects of adding various vegetable extracts are shown in Table 2. Of the 11 plant species, both *Allium fistulosum* and *Perilla frutescens* enhanced the carotenoid concentration (9.61 mg/L and

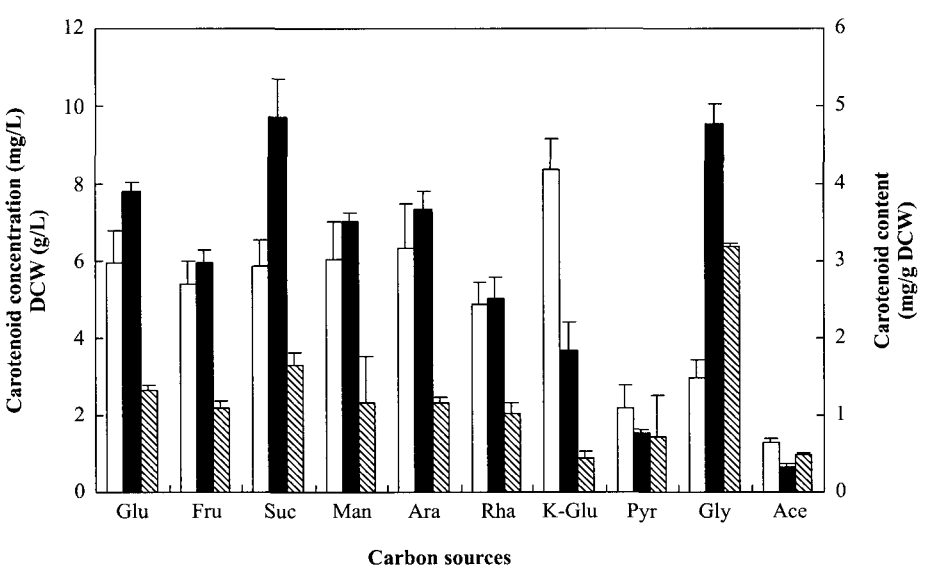

Fig. 1. Effect of carbon sources on carotenoid production in *X. dendrorhous* G276. Dry cell weight (DCW) (g/L) (□); Carotenoid concentration (mg/L) (■); Carotenoid content (mg/g DCW) (▨); Glu, glucose; Fru, D-fructose; Suc, sucrose; Man, D-mannose; Ara, L-arabinose; Rha, L-rhamnose; K-Glu, κ-gluconate; Pyr, sodium pyruvate; Gly, glycerol; Ace, potassium acetate. Each point represents mean±SD (standard deviation) of three independent experiments. 1% (final concentration) of each carbon source was used in YM-based media. Cells were cultured in 5 ml of YM-based media with 1% (final concentration) of each carbon source for 5 days.

Table 2. Effect of various vegetable extracts on carotenoid production of *X. dendrorhous* G276

Scientific name of plant	Common name of plant	Dry cell weight[a] (g/L)	Carotenoid concentration (mg/L)	Carotenoid content (mg/g)
No addition	-	4.97	8.01	1.61
Lactuca sativa	lettuce	6.37	8.90	1.40
Chrysanthemum coronarium	crown daisy	5.10	5.90	1.16
Allium fistulosum	green onion	6.73	9.61	1.43
Lavendula officinalis	lavender	5.80	9.17	1.58
Allium cepa	onion	6.10	8.95	1.47
Perilla frutescens	sesame leaf	11.20	11.26	1.01
Rosemarinus officinalis	rosemary	6.13	8.33	1.36
Angelica utilis	angelica	6.53	8.82	1.35
Capsicum annuum	pepper	6.43	8.93	1.39
Brassica oleracea var. *acephala*	kale	6.00	8.97	1.50
Brassica oleracea var. *capitata*	cabbage	6.57	8.76	1.33

[a] *X. dendrorhous* G276 cells were incubated for 5 days in YM broth with 0.1% of each plant extract and assayed for carotenoid production.

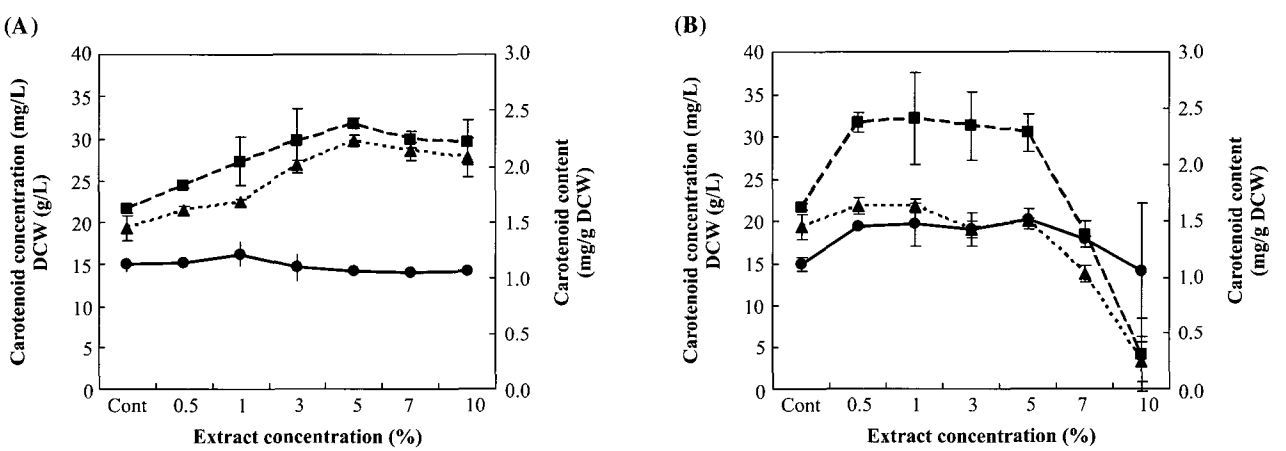

Fig. 2. Effect of plant extract concentration on carotenoid production of *X. dendrorhous* G276. (A) *Perilla frutescens*; (B) *Allium fistulosum*; Dry cell weight (DCW) (g/L) (•); Carotenoid concentration (mg/L) (■); Carotenoid content (mg/g DCW) (▲). "Cont" indicates control (no added plant extract). Each point represents mean±SD (standard deviation) of three independent experiments. *X. dendrorhous* G276 cells were incubated for 5 days in 5 ml of YP media with various concentrations of each plant extract.

11.26 mg/L, respectively) and dry cell weight (6.73 g/L and 11.20 g/L, respectively) compared to no added plant extract (carotenoid concentration 8.01 mg/L and dry cell weight 4.97 g/L). The addition of *Chrysanthemum coronarium* extract resulted in lower carotenoid concentration (5.9 mg/L) than control, although the cell growth was almost the same as the control. The remaining plant extracts elevated the carotenoid concentration slightly with increased cell mass. However, addition of all plant extracts decreased the carotenoid content compared to control (1.61 mg/g). The carotenoid concentration is generally more important than carotenoid content for industrial astaxanthin production.

To determine the appropriate concentration of plant extract, various levels of *Perilla frutescens* and *Allium fistulosum* extracts were tested in YP media (10 g/L of yeast extract, 3 g/L of malt extract, 10 g/L of peptone, and 5% sucrose). In the test of optimal medium composition, the carotenoid concentration in YP media (27 mg/L) was about 1.4-fold higher than that (19 mg/L) in YM-based media (3 g/L of yeast extract, 3 g/L of malt extract, 5 g/L of peptone, and 5% sucrose). Carotenoid production increased continuously to 5% *Perilla frutescens* extract, reaching a concentration of 31.8 mg/L (Fig. 2A). When the plant extract concentration exceeded 5%, carotenoid productivity decreased. It should be noted that cell growth of *X. dendrorhous* G276 did not change much in proportion to the changes in *Perilla frutescens* extract concentration. The same tendency was also observed when adding *Allium fistulosum* extract (Fig. 2B). These results suggest that *Perilla frutescens* and *Allium fistulosum* extracts may contain carotenogenesis inducer (s) since these extracts elevated carotenoid concentration without changing cell growth. If the plant extracts simply acted as nutrient sources for the culture without enhancing carotenoid production, there should be considerable differences in cell growth. There

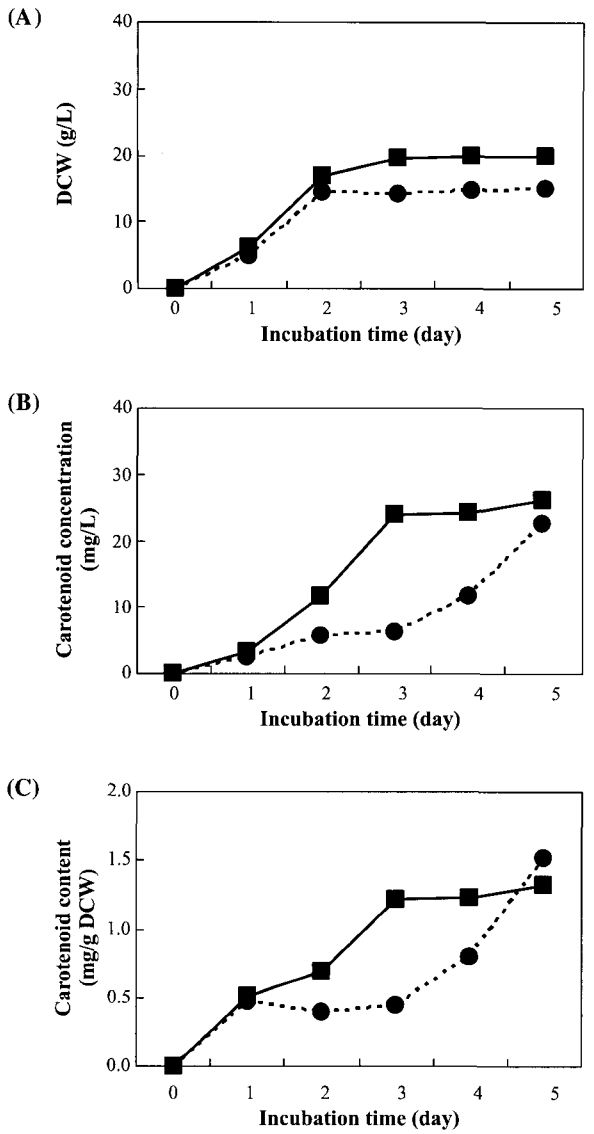

Fig. 3. Cell growth and carotenoid production of *X. dendrorhous* G276 in batch fermentor. (A) Dry cell weight (DCW) (g/L); (B) Carotenoid concentration (mg/L); (C) Carotenoid content (mg/g DCW); No addition (•); 5% *Perilla frutescens* extract (■).

may be a possibility that intermediate carotenoids such as lycopene, β-carotene, canthaxanthin, or zeaxanthin derived from plant extracts will also enhance astaxanthin production. In the case of *Allium fistulosum*, the carotenoid concentration (32 mg/L) reached the highest level with 0.5-1% extract addition (Fig. 2B). A significant decrease in carotenoid production was observed with the addition of concentrations greater than 5% extract, suggesting that high concentrations of *Allium fistulosum* extract could inhibit cell growth and carotenogenesis.

Batch culture of *X. dendrorhous* G276 was performed in a 7 L fermentor (Best Korea, Co. Ltd.) with or without the addition of 5% *Perilla frutescens* extract. *Perilla frutescens* extract (5% v/v) was initially added to the YP media. Inoculum size, temperature, initial pH, and agitation were 3%, 20°C, pH 5.0, and 350 rpm, respectively. Air was delivered to the culture through a sterilized air filter (1.5 v/v/m). No difference in cell growth was observed over the first 2 days, but the addition of *Perilla frutescens* extracts increased cell mass slightly (19.8 g/L) after 2 days compared to no extract (14.9 g/L) (Fig. 3A). Carotenoid production increased rapidly between the late exponential phase and the early stationary phase with added *Perilla frutescens* extract, but increased slowly for 5 days when no extract was added. After 3 days incubation, the carotenoid concentration (24.0 mg/L) in the presence of *Perilla frutescens* extract was about five-fold higher than the concentration (5.0 mg/L) in the absence of extract (Fig. 3B). After 5 days incubation, the carotenoid concentration was 26.2 mg/L with added *Perilla frutescens* extract. The slightly lower concentration in the fermentor culture compared to that (32 mg/L) in test tube batch culture may be improved by optimal fermentation condition. The carotenoid concentration (24.0 mg/L) with *Perilla frutescens* extract after 3 days incubation should be noted and was higher than the control, in which no plant extract was added, after 5 days incubation (22.6 mg/L). That carotenoid accumulation could increase rapidly with addition of *Perilla frutescens* extract was also clearly verified in batch fermentor as well as batch culture. The same tendency was also observed in the measurement of carotenoid content (Fig. 3C). After 3 days incubation, carotenoid content (1.25 mg/g of yeast) with added *Perilla frutescens* extract was about three-fold higher than control (0.45 mg/g of yeasts). The results suggest that plant extracts such as *Perilla frutescens* can enhance pigment production and shorten the cultivation time from the normal 5 days to 3 days.

To improve astaxanthin yield, the replacement of carbon source or other nutrients, and the addition of astaxanthin precursors were reported (Nelis and De Leenheer, 1991; Florencio *et al.*, 1998; Vazquez and Martin, 1998; Flores-Cotera *et al.*, 2001). However, the addition of various plant extracts was first tested in this study to determine whether plant extracts can enhance carotenoid production in *X. dendrorhous*. *Perilla frutescens* extract enhanced the carotenoid production and shortened the cultivation time, suggesting that the addition of *Perilla frutescens* extract will trim costs for industrial carotenoid production. The high cost of astaxanthin production from natural sources limits its usage in chicken, as well as salmon and trout, farming to enhance pigmentation. The addition of plant extract such as *Perilla frutescens* or *Allium fistulosum* should provide an economical method to enhance carotenoid production in *X. dendrorhous*. Inducers involved with the enhancement of carotenoid production would be identified from *Perilla frutescens* and other plant extracts in the future.

Acknowledgement

This work was supported by the Faculty Research Fund of Konkuk University in 2002.

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