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Hydrogen peroxide-induced astaxanthin biosynthesis and catalase activity in *Xanthophyllomyces dendrorhous*

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Abstract *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*) in shake-flask cultures was exposed to 10–20 mmol/L H_2O_2 at various culture stages, and the astaxanthin production was significantly increased by H_2O_2 fed at 0 or 24 h (exponential phase), but only slightly at 48 h (near stationary phase). The astaxanthin production was enhanced most significantly with double feeding of 10 mmol/L H_2O_2 at 0 and 24 h, reaching a cellular content of 1.30 mg/g cell and a volumetric yield of 10.4 mg/L, which were 83 and 65% higher, respectively, than those of the control (0.71 mg/g cell and 6.3 mg/L). The intracellular catalase (CAT) activity was also increased after H_2O_2 treatment. The increases in CAT and astaxanthin of cells could be detected within 4 h of H_2O_2 treatment. The increase in the astaxanthin content of cells was concomitant with a notable decrease in the β-carotene content. The older yeast cells at late culture stage (120 h), due perhaps in part to their higher astaxanthin contents, were more tolerant to H_2O_2 toxicity than the younger cells (24 h). No enhancement of the astaxanthin biosynthesis was attained when H_2O_2 was added to the yeast culture together with a sufficient amount of exogenous CAT. The results suggest that astaxanthin biosynthesis in *X. dendrorhous* can be stimulated by H_2O_2 as an antioxidative response.

Keywords *Xanthophyllomyces dendrorhous* · Astaxanthin · β-carotene · Hydrogen peroxide · Catalase · Antioxidant

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Introduction

Astaxanthin (3,3′-dihidroxy-β,β-carotene-4,4′-dione) is a carotenoid pigment which confers a characteristic coloration to some birds, crustaceans, and salmons. It is a potential functional food and pharmaceutical supplement because of its excellent antioxidant activity (Johnson and Schroeder 1995; Kobayashi et al. 1997). *Xanthophyllomyces dendrorhous*, previously known as *Phaffia rhodozyma*, is an excellent astaxanthin-producing yeast and has been regarded as a potential source of dietary astaxanthin (Andrewes et al. 1976; Johnson and Schroeder 1995). However, commercial application of *X. dendrorhous* fermentation for astaxanthin production has been hampered by the low product yield in the yeast cells. Understanding and effectively manipulating the major physiological factors regulating the carotenogenesis and astaxanthin biosynthesis may be one of the most fruitful approaches to improve the production.

Carotenoid pigments in many microorganisms are secondary metabolites which usually accumulate in the organisms during exposure to biotic and abiotic stresses. Astaxanthin and related carotenoids such as β-carotene, zeaxanthin, and canthaxanthin are potent antioxidants that may have protective effects on the microorganisms against oxidative damage (Schroeder and Johnson 1993; Kobayashi et al. 1997). The stimulated carotenoid biosynthesis by oxidative stress created by reactive oxygen species (ROS) has been observed in various carotenoid-producing microorganisms including the green algae (Kobayashi et al. 1993; Ma and Chen 2001), *X. dendrorhous* (Schroeder and Johnson 1995), and several other microbial species (Manjula Rao and Sureshkumar 2001; Marova et al. 2004; Iigusa et al. 2005). Therefore, the feeding of various ROS agents to the culture media has been considered as a possible measure for

improving carotenoid production by these microorganisms. However, this stimulus has not been evaluated for the improvement of astaxanthin production in *X. dendrorhous* yeast cultures.

This work was performed to explore the stimulating effect of oxidative stress imposed by hydrogen peroxide (H_2O_2) on astaxanthin production in liquid cultures of *X. dendrorhous* yeast and to examine the antioxidant role of astaxanthin in defending the yeast cells against the oxidative stress and toxic effect of H_2O_2. In addition, the effects of H_2O_2 on the intracellular activity of catalase (CAT, H_2O_2 scavenger) and the content of β-carotene were also evaluated.

Materials and methods

Microorganism and culture conditions

The *X. dendrorhous* used in this study was the *P. rhodozyma* strain ENM 5 obtained from E.A. Johnson (University of Wisconsin, Madison), which was stored at −80 °C. The medium for liquid culture of the yeast was made of 25 g glucose, 3.0 g $(NH_4)_2SO_4$, 1.5 g KH_2PO_4, 1.5 g $MgSO_47H_2O$, 1.5 g yeast extract, and 10 g corn steep liquor (per liter). The medium was adjusted to pH5.0 and sterilized by autoclaving at 121 °C for 20 min. Liquid culture was maintained in shake-flasks with 250-ml Erlenmeyer flasks on an orbital shaker at 250 rpm and 20 °C. Each flask was inoculated with 6% starter culture broth into a volume of fresh medium to make up the desired liquid volume. The starter culture was prepared by preculture of the yeast in shake-flask culture for 2 days (48 h). The H_2O_2 solution, external CAT, and carotenoid standards used in this study were obtained from Sigma (St. Louis, USA).

All experiments in this study were carried out in shake-flask cultures of 250-ml flasks with 50 ml liquid. The overall culture period was 120 h unless otherwise specified. All tests were run in triplicate and repeated at least once, and the results were expressed by their averages.

Test of H_2O_2 effects in *X. dendrorhous* cultures

To test the effects of H_2O_2 on the yeast growth and carotenoid production, H_2O_2 (30% w/w) was added to the culture broth in shake-flasks to a final concentration of 10 or 20 mmol/L at 0, 24, or 48 h postinoculation. The flasks were harvested at selected time intervals for measurement of the biomass, carotenoid contents, and CAT activity.

For the test of cell tolerance to H_2O_2 damage or toxicity, the yeast cells were harvested aseptically from the shake-flasks at 24 and 120 h by centrifugation at 1,600×*g* and 4 °C for 1 min and washed twice in ice-cold, 100-mmol/L phosphate buffer (pH7.0). Each 10 mg of the cell mass was resuspended in 2 ml buffer in a centrifuge tube and kept at 4 °C for 12 h. The cell suspension was then fed with 50– 400 mmol/L of H_2O_2 and incubated on a shaker (20 °C, 250 rpm) for 12 h. The H_2O_2-treated cells were transferred to the normal culture medium in shake-flasks and cultured for 48 h. The survival ratio was the percentage of yeast biomass in the H_2O_2-treated culture relative to that in the control.

Measurement of yeast biomass and carotenoid contents

Yeast cells were separated from the liquid medium by centrifugation and rinsed twice with double distilled water and then dried at 105 °C overnight to constant dry weight (dw). The carotenoid pigments were extracted from the yeast cells (disrupted with DMSO at 55 °C) with hexane–ethyl acetate at 50:50 (v/v) as described previously (Liu et al. 2006). The contents of astaxanthin and other carotenoids were determined using high-performance liquid chromatography with an Alltech Econosphere reversed-phase C18 column (5 μm, 250×4.6 mm) (Alltech Associates, Deerfield, USA). The mobile phase consisted of acetonitrile (A) and methanol (B) run at 1.0 ml/min in a gradient scheme (10% B from 0 to 5 min, linear gradient of 10–30% B from 5 to 8 min, and 30% B thereafter). The carotenoid peaks were detected with a UV detector at 478 nm and confirmed and quantified by cochromatography with carotenoid standards from Sigma (β-carotene at 7.0 min elution time and astaxanthin at 11.0 min elution time).

Measurement of CAT activity

The yeast cells were suspended in 100 mmol/L phosphate buffer (pH 7.0) (~10 g fresh weight cell/L) and homogenized in a French press at 2,500 psi and 0 °C. The cell homogenate was centrifuged at 20,000×*g* and 0 °C for 20 min, yielding a cell-free enzyme extract for the CAT assay. The enzyme extract (1.0 ml) was added to ice-cooled 10 ml of 100 mmol/L phosphate buffer (pH7.0) containing 10 mmol/L H_2O_2, and the decreasing absorbance at 240 nm was recorded over 30 s on a spectrophotometer. One CAT unit (U) was defined as the decomposition of 1 μmol substrate per minute. Total protein content of extract was determined by the Bradford method using bovine serum albumin as a standard (Bradford 1976).

Results

Effects of H_2O_2 on cell growth, CAT activity, astaxanthin, and β-carotene production

Figure 1 shows the time courses of biomass (yeast dw), astaxanthin yield, intracellular CAT activity, and astaxanthin

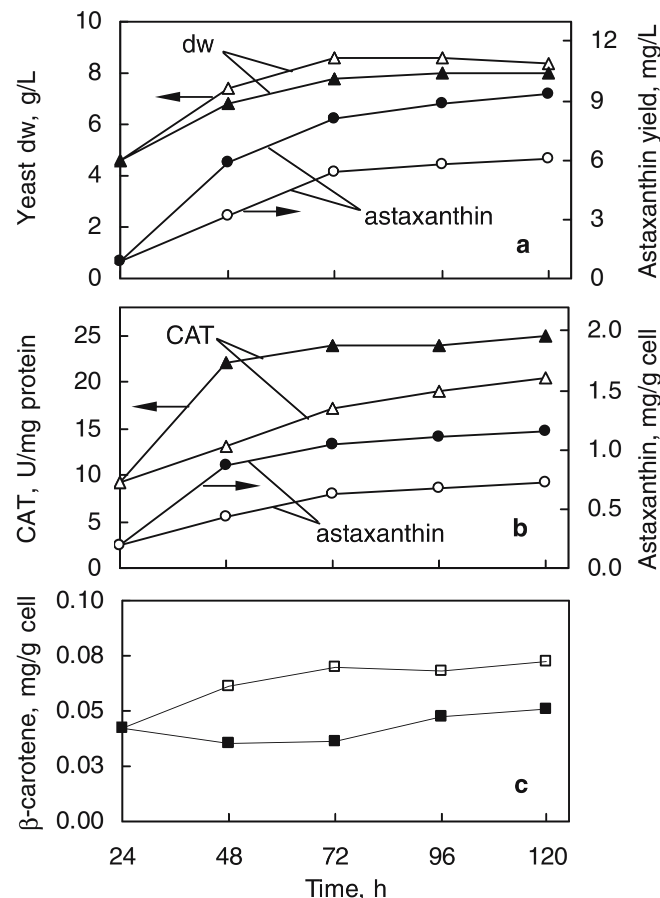

Fig. 1 Time courses of biomass, astaxanthin volumetric yield, intracellular CAT activity, and astaxanthin and β-carotene contents in the control culture (*open symbols*) and the culture fed with 10 mmol/L H_2O_2 at 24 h (*solid symbols*) of *X. dendrorhous* in shake-flasks (data representing the mean of triplicate measurements; maximum SE relative to the mean, <5% for yeast weight, <10% for CAT, astaxanthin, and β-carotene)

and β-carotene contents of yeast cells in the control and the culture exposed to 10 mmol/L H_2O_2 at 24 h of culture. The H_2O_2 exposure caused a slightly lower cell growth (yeast dw) but a significantly higher astaxanthin yield than those of the control culture (Fig. 1a). The H_2O_2-treated yeast cells also had significantly higher intracellular CAT activity and astaxanthin content (Fig. 1b). At the end of the culture period (120 h), the CAT activity was 30% higher (26 vs 20 U/mg protein) and the astaxanthin content was 60% higher (1.16 vs 0.73 mg/g cell) than those of the control, respectively. However, the β-carotene content of H_2O_2-treated yeast cells was notably lower than that of the control (Fig. 1c). The H_2O_2-induced increases in astaxanthin content and CAT activity, as well as the decrease in β-carotene content, were mostly achieved within 24 h of exposure (at 48 h).

Effects of H_2O_2 feeding time and dosage on cell growth and astaxanthin production

The effects of H_2O_2 on the yeast cell growth and astaxanthin production depended on both the time and dose of H_2O_2 feeding to the culture (Table 1). The suppression of cell growth (yeast dw) by H_2O_2 was more notable at a higher dose (20 mmol/L) and earlier feeding time (0 h) and was negligible by late feeding at 48 h. The significant stimulation of astaxanthin production was only attained with earlier feeding of H_2O_2 at 0 and 24 h, but not with late feeding at 48 h. The astaxanthin content of cells was increased with the increase of H_2O_2 dose from 10 to 20 mmol/L fed at 0 or 24 h, but more significantly at 0 h. The volumetric astaxanthin yield did not follow a consistent trend with dose 10–20 mmol/L and feeding time 0–24 h, due to the negative effect of H_2O_2 on the cell growth. The volumetric astaxanthin yield was increased most dramatically with twice feeding of 10 mmol/L H_2O_2 at 0 and 24 h during the culture period by about 65% more than that of the control (10.4 vs 6.3 mg/L). The corresponding astaxanthin content was 83% higher than that of the control culture (1.30 vs 0.71 mg/g cell).

Tolerance of yeast cells to H_2O_2 toxicity

Figure 2 shows the survival ratio (percentage) of the yeast cells at two culture ages (24 h in the exponential growth

Table 1 Effects of H_2O_2 feeding time and dosage on *X. dendrorhous* yeast cell growth and astaxanthin production in shake-flask cultures (error bars=SE, *n*=3)

H_2O_2 feeding time, dose[a]	Yeast dw (g/L)	Astaxanthin content (mg/g cell)	Astaxanthin yield (mg/L)
None (control)	8.8±0.2	0.71±0.03	6.3±0.4
0 h, 10 mM	8.2±0.4	1.16±0.04	9.5±0.6
0 h, 20 mM	6.8±0.3	1.31±0.05	8.9±0.7
24 h, 10 mM	8.6±0.4	1.09±0.04	9.4±0.5
24 h, 20 mM	8.2±0.3	1.19±0.06	9.8±0.8
0 h, 10 mM; 24 h, 10 mM	8.0±0.4	1.30±0.05	10.4±0.6
48 h, 10 mM	8.7±0.3	0.82±0.04	7.1±0.2
48 h, 20 mM	8.4±0.2	0.86±0.03	7.2±0.5

[a]mM=mmol/L

Table 2 Effects of external CAT on H_2O_2 induction of astaxanthin accumulation and intracellular CAT activity

Additive and dose[a]	CAT[b] (U/mg protein)	Astaxanthin[b] (mg/g cell)
None (control)	9.6±0.7	0.24±0.015
H_2O_2 (10 mM)	13.5±1.3	0.31±0.022
CAT (30 U/mg cell)	9.1±1.9	0.17±0.013
H_2O_2 (10 mM)+CAT (30 U/mg cell)	9.5±1.6	0.22±0.018

[a]Addition of external CAT and H_2O_2 to culture at 24 h, and measurements taken 4 h later (28 h during culture); at 24 h, intracellular CAT=9.1±0.7 U/mg protein and astaxanthin=0.20±0.011 mg/g cell
[b]All values represent mean±SE, *n*=3

phase and 120 h in the stationary phase) after exposure to various doses of H_2O_2. The survival ratio of cells at both cell ages decreased with the increase of H_2O_2 dose, and more sharply for 24-h-old cells. The growth of the 24-h-old cells (in the exponential phase) was completely arrested with a close-to-zero survival ratio at 200 mmol/L H_2O_2. In comparison, the 120-h-old cells (in the stationary phase) were more resistant to H_2O_2 and could maintain a 75% survival ratio at 200 mmol/L H_2O_2.

Effects of exogenous CAT on H_2O_2 induction of astaxanthin biosynthesis

To verify the function of H_2O_2-induced astaxanthin biosynthesis as an antioxidant response of the yeast cells, we tested the effect of exogenously supplied CAT on H_2O_2 induction of astaxanthin production (Table 2). Without the exogenous CAT, the addition of 10 mmol/L H_2O_2 at 24 h postinoculation resulted in higher intracellular CAT activity (13.5 U/mg protein) and astaxanthin content (0.31 mg/g cell) than those of the control cultures (9.6 U/mg protein and 0.24 mg/g cell) within 4 h (at 28 h). The addition of CAT (30 U/mg cell) together with 10 mmol/L H_2O_2 resulted in no increase in the astaxanthin content of cells compared with the control (0.22 vs 0.24 mg/g cell). This result may be explained as that, as a specific H_2O_2 scavenger, the exogenous CAT in the culture medium completely scavenged the H_2O_2 added to the culture, preventing the H_2O_2 stress and the induction of astaxanthin biosynthesis. In addition, the exogenous CAT may also be sufficient to scavenge the H_2O_2, which may be generated endogenously from cell metabolism and diffused into the culture medium. This could be a cause for the lower astaxanthin content (0.17 mg/g cell) with the addition of CAT alone (without H_2O_2) to the culture than that of the control (0.24 mg/g cell). In addition, intracellular CAT production was slightly decreased by external CAT compared to the control. The results here support that H_2O_2-induced astaxanthin biosynthesis, as well as the CAT activity, is a defense response of the yeast cells to the oxidative stress induced by H_2O_2.

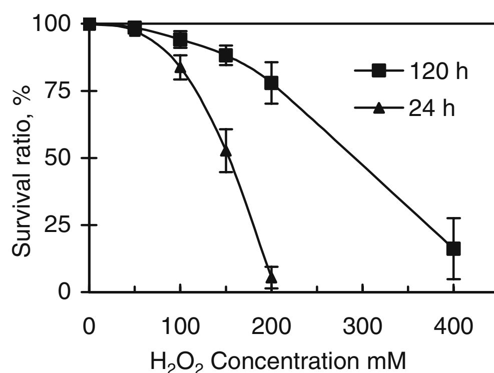

Fig. 2 Survival ratio of *X. dendrorhous* cells at two culture ages exposed to large doses of H_2O_2 (initial cell concentration in the treatment, 5 g fresh weight/L; *error bars*=SE, *n*=3)

Effect of H_2O_2 on carotenoid composition

The astaxanthin and β-carotene contents of cells were affected oppositely by H_2O_2 treatment, with the astaxanthin content increasing but the β-carotene decreasing with the H_2O_2 dose (Fig. 3). At the largest dose of 80 mmol/L H_2O_2 applied, the astaxanthin content was nearly 100% higher and the β-carotene content was about 50% lower than those of the control. With or without the H_2O_2 treatment, the astaxanthin content was much higher than the β-carotene content in the yeast.

Discussion

The experimental results have shown that the astaxanthin biosynthesis of *X. dendrorhous* yeast can be strongly enhanced by exposing to suitable doses of H_2O_2 in the

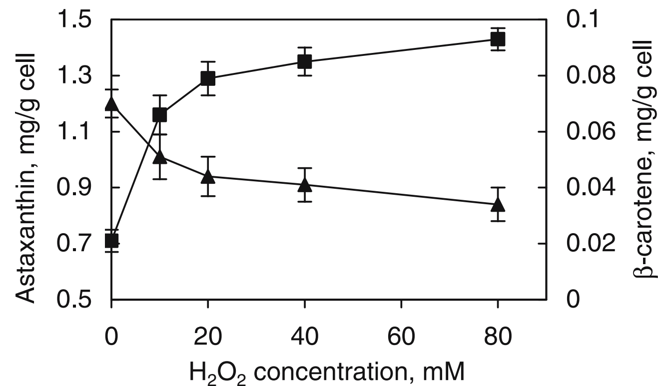

Fig. 3 Astaxanthin and β-carotene contents of *X. dendrorhous* yeast cells subjected to different doses of H_2O_2 (added at 24 h and analyzed at 120 h postinoculation; *error bars*=SE, *n*=3)

early stage of culture. The induction of astaxanthin biosynthesis by H_2O_2 was completely blocked by exogenous H_2O_2 scavenger CAT. Similarly, the astaxanthin production was enhanced by oxidative stress created by H_2O_2 and other ROS species in green microalgae *Haematococcus pluvialis* (Kobayashi et al. 1993) and *Chlorococcum* sp. (Ma and Chen 2001), and the stimulation was blocked by specific radical scavengers, such as KI. As suggested by these authors, the possible causes for the enhancement are that the oxidative stress induced the activation of the carotenogenic enzymes (Ma and Chen 2001) and that some ROS species, such as HO·, directly participated in the carotenogenic reaction.

The increasing astaxanthin content of yeast cells in parallel with the decreasing β-carotene content with H_2O_2 dose (Fig. 3) suggests that some other carotenoids, for example, β-carotene, may be converted into astaxanthin under the effect of H_2O_2. In another *X. dendrorhous* yeast culture, dosing of duroquinone, a superoxide anion (O_2^-) generator, increased the proportions of major xanthophylls, such as astaxanthin, but decreased the proportions of carotenes (β-carotene and γ-carotene) (Schroeder and Johnson 1993). An et al. (1989) also have suggested that some carotenoids may serve as precursors for astaxanthin biosynthesis in antimycin mutants of *X. dendrorhous* yeast. In microalgae *Chlorococcum* sp. cultures, Ma and Chen (2001) have suggested that ROS may promote the activity of β-carotene hydroxylase, the enzyme responsible for the conversion of canthaxanthin to astaxanthin. However, there is still no experimental evidence showing that ROS promote the activity of β-carotene hydroxylase. Another possible cause is that astaxanthin is a more potent antioxidant than β-carotene and its production would be more favorable for the cells to defend against the oxidative stress and the reactive species (Palozza and Krinsky 1992). Conversely, the exposure of a *X. dendrorhous* yeast strain to a peroxyl radical generator *t*-butylhydroperoxide or H_2O_2 resulted in decreasing astaxanthin but increasing other carotenoids, including β-carotene (Schroeder and Johnson 1995). Schroeder and Johnson suggested that astaxanthin was degraded by peroxyl radicals into other carotenoids to relieve the feedback inhibition of carotenoid biosynthesis by astaxanthin. According to our tests on the possible reaction of H_2O_2 with pure astaxanthin and β-carotene (data not shown), however, none of the two carotenoids was degraded by H_2O_2 alone, though both were degraded to a similar extent by H_2O_2+Fe^{2+} (to generate hydroxyl radicals). As H_2O_2 was used without Fe^{2+} in their experiments, the decrease of astaxanthin content in the yeast by degradation was unlikely. In a later study, An et al. (1996) showed that both astaxanthin and β-carotene increased slightly in *X. dendrorhous* strain 67–385 but decreased significantly in strain ant-1 after H_2O_2 exposure. Therefore, it appears that the different effects of H_2O_2 or other oxidative species on astaxanthin and β-carotene (positive or negative) between our and previous studies may be attributed to the different strains and other unknown culture factors, which remain to be identified.

The H_2O_2-induced astaxanthin accumulation in the yeast cells was in concomitant with a moderate increase in the intracellular CAT activity. The higher resistance of the older cells to larger H_2O_2 doses may be attributed partially to their higher astaxanthin content and intracellular CAT activity than the younger cells. However, CAT activity should not be a major contributor to the H_2O_2 tolerance of *X. dendrorhous* yeasts, which have usually very low CAT activity compared with other yeast species such as *S. cerevisiae* (Schroeder and Johnson 1993). Ducrey Santopietro et al. (1998) have reported that high carotenoid-producing mutants of *X. dendrorhous* were more resistant to free radicals generated by H_2O_2 and Fe^{2+} than the unpigmented mutants. In view of the astaxanthin enhancement by H_2O_2 and the relatively low CAT activity in *X. dendrorhous*, we may hypothesize that astaxanthin may be a supplement to CAT deficiency in this strain for the defense against H_2O_2-induced oxidative stress. This

may also serve as an explanation for the more significant increase in the astaxanthin content with earlier H_2O_2 addition (at 0 h) than with later additions (at 24 or 48 h) (Table 1), as the yeast cells at the early culture stage had a lower CAT activity and would synthesize more astaxanthin to fortify their antioxidative defense in response to H_2O_2 stimulation. Another possible reason for the higher resistance of the aged cells to H_2O_2 is that more astaxanthin in the aged cells moved to the cell membrane, the front line of H_2O_2 invasion of the cell. According to Johnson and An (1991), the synthesis of carotenoids in *X. dendrorhous* cells may be initially associated with the mitochondria, but the final-product carotenoids are mainly located in lipid globules, which are dispersed to the plasma membrane as the cells age.

The use of molecular biology and genomic approaches may help us to understand the mechanisms of ROS stimulation of astaxanthin biosynthesis and the physiological role of astaxanthin in the *X. dendrorhous* cells under oxidative stress. The stress-induced accumulation of secondary metabolites in the microorganisms is often the result of increased transcription of the genes coding for the biosynthetic enzymes. Iigusa et al. (2005) have indicated that ROS such as H_2O_2 can induce the expression of carotenogenic gene *al*-1 in the fungus *Neurospora crassa*. In the green alga *H. pluvialis*, however, Steinbrenner and Linder (2001) found that ROS did not increase the transcript levels of the two genes coding for phytoene synthase and β-carotene hydroxylase, the two key enzymes for astaxanthin biosynthesis. The authors suggested that ROS regulation of the carotenoid synthesis in the alga might be at the posttranscription level. Although there is still no experimental evidence for the ROS regulation of carotenogenic genes in the yeast *X. dendrorhous*, Schroeder and Johnson (1995) have suggested that singlet oxygen $^1O_2$ may induce carotenoid synthesis in *X. dendrorhous* by gene activation or protein regulation. Moreover, carotenoid biosynthesis in various microorganisms may be regulated at multiple levels, including transcription, translation, and enzyme activity, and limited by multiple control points in the pathways. In this regard, the transcriptomic and metabolomic profiles of the secondary metabolism process will be useful for elucidating the global responses of the microorganisms to the oxidative stress, as well as for systematic and effective manipulation of the carotenoid production.

In conclusion, astaxanthin biosynthesis in *X. dendrorhous* can be stimulated by H_2O_2, and the phenomenon may represent an antioxidative response of the yeast cells. Significant improvement of astaxanthin production in the *X. dendrorhous* culture was achieved by feeding suitable doses of H_2O_2 during the early days of culture. Therefore, the feeding of H_2O_2 may be a simple and effective means for enhancing astaxanthin production in *X. dendrorhous* fermentation processes. The astaxanthin yield could be increased further with the optimization of the H_2O_2 dosage and feeding scheme.

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