Selection for carotenogenesis in the yeast *Phaffia rhodozyma* by dark-generated singlet oxygen

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Selection for carotenogenesis in *Phaffia rhodozyma* was achieved by exposure of yeast strains to dark chemical reactions that generate singlet oxygen. Incubation of a mixture of *P. rhodozyma* strains containing varying levels of carotenoids in hypochlorous acid or hydrogen peroxide resulted in weak selection for pigmented strains. However, the combination of hydrogen peroxide and hypochlorous acid was strongly selective for carotenogenesis and gave a monoculture of a carotenoid-hyperproducer. Exposure of the yeast to ozone for 10 to 20 min also selected for a hyperproducing strain. These selections were relieved by 1,4-diazabicyclo[2.2.2]-octane, a specific quencher of singlet oxygen or by L-ascorbic acid. Continuous growth of *P. rhodozyma* on agar plates in an ozone/air atmosphere for 5 d decreased astaxanthin and total carotenoid levels and increased the levels of carotenoid biosynthetic intermediates. Repeated rounds of random mutagenesis followed by ozone exposure yielded mutant strains with higher pigmentation than control cultures. Our results support the hypothesis that a primary function of carotenoids in *P. rhodozyma* is to protect against singlet oxygen generated in the natural environment of the yeast and that a practical method for preventing strain degeneration during industrial fermentations may be achieved by generation of singlet oxygen using simple chemical supplements or by bubbling ozone through *P. rhodozyma* cultures during fermentation.

Keywords: *Phaffia rhodozyma*, astaxanthin, carotenoids, heterobasidiomycetous yeast

INTRODUCTION

The carotenoid pigment astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione) is responsible for the attractive coloration in many birds, crustaceans and fish. In addition to its ornamental appeal and possible biological functions as an antioxidant (Burton & Ingold, 1984; Gerster, 1993; Hirayama *et al.*, 1994; Johnson & Schroeder, 1996), astaxanthin has considerable economic value as an integral component of salmon feed. Salmon farming is a multi-billion dollar industry that is rapidly growing and gradually replacing the world's wild salmon fisheries (Meyers, 1994). The most expensive ingredient in salmon feed is astaxanthin, and though the actual revenues are

privately held, it has been estimated that the market for astaxanthin is > US\$80 million per year. This market is supplied chiefly by synthetic astaxanthin. However, since this synthesis is relatively expensive, and many consumers prefer natural food ingredients, several companies have investigated biological sources of astaxanthin. Among these, the heterobasidiomycetous yeast *Phaffia rhodozyma* has attracted much interest because of several desirable properties (Johnson & An, 1991; Johnson & Schroeder, 1996).

The only known natural habitat of *P. rhodozyma* is the slime fluxes of trees of the birch family Betulaceae in high altitude mountainous regions of Japan, Alaska and Russia (Phaff *et al.*, 1972; Golubev, 1995). This limited ecological niche suggests that environmental factors present in this habitat select for survival of *P. rhodozyma*. The successful colonization of *P. rhodozyma* in sap fluxes appears to be

Abbreviation: DABCO, 1,4-diazabicyclo[2.2.2]-octane.

related to the yeast's carotenoid composition. Our laboratory showed that the North American birch tree *Betula papyrifera* contains a compound(s) that, when activated by 366 nm UV light, killed albino or poorly pigmented strains of *Phaffia*, but allowed growth of pigmented strains. This selective mechanism appears to involve singlet oxygen ($^1\text{O}_2$) (Schroeder & Johnson, 1995a). Carotenoids have been demonstrated to effectively quench $^1\text{O}_2$ (Oliveros *et al.*, 1992) and astaxanthin is one of the most effective $^1\text{O}_2$ quenchers in the carotenoid family (Hirayama *et al.*, 1994). The unique ability of *P. rhodozyma* to synthesize astaxanthin probably contributes to successful colonization of sap fluxes at high altitudes and high incidental UV irradiation.

One of the major limitations in utilizing *P. rhodozyma* as a commercial astaxanthin source is the low astaxanthin levels found in wild-type isolates and several companies have developed astaxanthin-hyperproducing strains (Johnson & Schroeder, 1996). These strains have been derived mainly by repeated random mutagenesis and visual screening. Although pigmentation is enhanced, these strains have certain drawbacks including slow growth, genetic instability and accumulation of precursors of astaxanthin or by-products of the astaxanthin pathway (Johnson & Schroeder, 1996). Furthermore, discrimination of the carotenoid content of yeast colonies by the human eye becomes very difficult at relatively high pigment levels ($\sim 1500 \mu\text{g}$ astaxanthin g^{-1} yeast) (Wieth, 1991). These limitations in random mutagenesis and visual screening has led our laboratory to try to elucidate the physiological roles of carotenogenesis in *P. rhodozyma* (Schroeder & Johnson, 1995a) and to develop positive genetic selection for higher pigmentation. In this study, we describe a positive genetic selection involving dark generation of $^1\text{O}_2$ from inexpensive chemicals and gases which could be applicable in high cell density fermentations as carried out in industry.

METHODS

Chemicals. Sodium hypochlorite, hydrogen peroxide (3%), NTG and potassium hydrogen phthalate were from Sigma. 1,4-Diazabicyclo[2.2.2]-octane (DABCO), sodium phosphate monohydrate (monobasic), sodium phosphate heptahydrate (dibasic), sodium hydroxide, L-ascorbic acid and sodium chloride were from Aldrich. All other chemicals used were of analytical grade or the highest grade commercially available. Ozone (O_3) was generated by a UV-275 ozone generator (Clearwater Tech. Inc.).

Yeast strains and growth. *P. rhodozyma* strains 67-385, AF-1, Yan-1 and ENM 5 were described previously (Schroeder & Johnson, 1995a). Briefly, the phenotypes are: 67-385, wild-type, $\sim 300 \mu\text{g}$ total carotenoid (g yeast) $^{-1}$; AF-1, albino mutant, $0 \mu\text{g}$ total carotenoid g^{-1} ; Yan-1, β -carotene-producing mutant, $\sim 100 \mu\text{g}$ β -carotene g^{-1} ; ENM 5, astaxanthin-hyperproducing mutant, $\sim 2700 \mu\text{g}$ total carotenoid g^{-1} . Astaxanthin-hyperproducing strain anF-1P ($\sim 800 \mu\text{g}$ total carotenoid g^{-1}) was generated by NTG mutagenesis followed by screening by Fluorescence Activated Cell Sorting (FACS) as described by An *et al.*, (1991). These individual strains are readily distinguished by colony coloration on yeast extract/malt extract/peptone/

glucose medium (YM broth, Difco) with 2% (w/v) agar (YM agar; Difco). Yeasts were maintained at 4 °C on slants of YM agar and were also stored in 40% (v/v) glycerol/60% (v/v) YM broth at -70 °C.

Yeasts were grown as described previously (Schroeder & Johnson, 1993, 1995a). Briefly, 5 ml YM broth was inoculated with cells from YM agar slants. The yeasts were grown aerobically on a roller drum at 20 °C for 5 d. To prepare strain mixtures, equal volumes of the 5-d cultures were combined, the combined strains harvested by centrifugation and the cell pellet washed twice with 0.2 M phthalate-buffered YM broth (pH 5.0). The cells were resuspended at $\sim 0.5 \text{ mg (ml yeast)}^{-1}$ (10-fold dilution) in ice-cold 0.2 M PBS (0.85% NaCl, pH 7.4) and plated on YM agar plates to determine the relative numbers of the different strains in the initial culture. The strain mixture was then dispensed into sterile 13 × 100 mm borosilicate tubes and subjected to various treatments with hypochlorous acid (HOCl), H_2O_2 and DABCO. After treatment, the yeasts were diluted in ice-cold PBS and plated on YM agar. Samples were also diluted in ice-cold tryptic soy broth, which has been shown to quench HOCl (Mustapha & Liewen, 1989). The recovery of the individual strains did not differ with the two dilution buffers (data not shown). Agar plates were incubated at 20 °C for 7 d. They were then scored for survival of each strain.

Exposure to HOCl and H_2O_2 . Stock solutions of H_2O_2 and HOCl were prepared from a 3% H_2O_2 (v/v) stock or sodium hypochlorite ($\sim 4\%$ available chlorine) by diluting in sterile ice-cold water. These solutions were kept on ice until addition to the cultures. The volumes of the cultures were kept constant by adding the appropriate quantity of sterile water. The HOCl concentration of the stock solution was titrated by the available chlorine method (Casse *et al.*, 1985).

Exposure to O_3 . A mixed culture (50 ml) of *P. rhodozyma* was placed in a 100 ml glass bottle and bubbled with an O_3 /air mixture through a ground glass sparging stone while being continuously stirred. The diffusion rate of the O_3 into the media was estimated as $72.6 \pm 0.2 \mu\text{g l}^{-1} \text{ s}^{-1}$ by the indigo colorimetric method of Bader & Hoigne (1981). Culture samples were taken at various time intervals depending on the individual experiment, diluted in ice-cold PBS and plated on YM agar to determine survival of individual strains.

Effect of O_3 on carotenoid content. Since O_3 could not be continuously generated in shake flask culture, the influence of O_3 on carotenoid composition was determined by incubation of yeasts grown confluent on YM agar plates in anaerobic jars containing an O_3 /air mixture. After 5 d, one-half of the plates were removed and the cells harvested and assayed for pigment content. The other half of the plates were incubated in an air atmosphere for an additional 5 d after which they were harvested and analysed for carotenoid content. Control plates were also grown for 5 and 10 d in air prior to analysis. Extraction and purification of carotenoids by HPLC was performed using a silica stationary phase and isocratic elution using a mobile phase of ethyl acetate/hexane (1:1) (Calo *et al.*, 1995).

Generation of carotenoid-hyperproducing mutant strains. A 2-d-old culture of *P. rhodozyma* strain 67-385 (100 ml) was subjected to mutagenesis by NTG according to the method of An *et al.* (1989). Following mutagenesis, the cells were

resuspended in 100 ml Yeast Nitrogen Base broth (YN broth; Difco) + 1% glucose, divided into two 50 ml portions and incubated for 24 h for phenotypic expression. One culture was then bubbled with O₃ for 20 min. Both the O₃-treated and control cultures were diluted in ice-cold 0.2 M PBS (pH 7.4), plated on YM agar and incubated at 20 °C for 7 d. Twelve colonies were picked from both cultures, pooled and grown into 50 ml YM broth. These cultures were then subjected to repeated rounds of this mutagenesis and O₃ selection. After 10 rounds, five colonies were randomly chosen from the control and O₃-treated cultures, grown individually in YM broth for 7 d and assayed for total carotenoid content.

Statistical validation of results. All experiments were performed in duplicate or triplicate and replicated at least once. All platings for scoring of the different strains were done in triplicate. The error estimates presented in the text and figures represent one SD from the mean.

RESULTS

Selection for carotenogenesis in *P. rhodozyma* by ¹O₂ generated from HOCl and H₂O₂

Previous studies from another laboratory (Khan & Kasha, 1994) indicated that acidified hypochlorite spontaneously decomposes generating ¹O₂ as a reaction product:

Although ¹O₂ may be formed from decomposition of HOCl, its yield is low (Khan & Kasha, 1994). To increase the yield of ¹O₂, the more efficient reaction of HOCl with H₂O₂ was also performed:

This reaction generates ¹O₂ with > 3-fold higher quantum yield than HOCl and also proceeds at a much faster rate (Khan & Kasha, 1994). To investigate whether ¹O₂ generated solely by HOCl decomposition or whether H₂O₂ + HOCl was more effective in selecting for enhanced carotenogenesis in *P. rhodozyma*, a mixed culture consisting of four differently pigmented strains of *P. rhodozyma* was exposed to varying concentrations of HOCl/H₂O₂ in YM broth buffered with 0.1 M phthalate (pH 5.0) (Fig. 1). Although there was a small and reproducible increase in the proportion of the hyper-producing strain (ENM 5) in the mixed cell population, the selection was relatively small and it did not improve with increasing incubation time (data not shown). Surprisingly, a β-carotene-producing strain, Yan-1, became the dominant strain in the culture after 60 min exposure to HOCl (data not shown).

In contrast, exposure of mixed cultures to increasing equimolar concentrations of HOCl/H₂O₂ for 5 min effectively selected for the astaxanthin-hyperproducer ENM 5 (Fig. 1a). This selection did not significantly decrease the viability of the hyper-producing strain even when subjected to the strongest treatment (Fig. 1a). Increasing the exposure time beyond 5 min did not affect the selection. Previous work in this laboratory (Schroeder & Johnson, 1993) showed that exposure of the yeast to

Fig. 1. Selection for carotenoid-hyperproducing strains ENM 5 (a) and anF-1P (b) by ¹O₂ generated from HOCl (pH 5.0) or the reaction of equimolar amounts of HOCl and H₂O₂. Data are presented as relative composition of culture following 5 min treatment. □, AF-1; ▨, Yan-1; ▩, 67-385; ■, ENM 5 (a), anF-1P (b). Survival of the respective hyper-producing strain during selection is also presented (○).

H₂O₂ resulted in a slight selection for an astaxanthin-hyper-producing strain. However, exposure to H₂O₂ alone did not give a strong selection (data not shown).

To determine if the increased resistance to HOCl/H₂O₂ of the hyperproducer ENM 5 was strain-specific, a second hyper-producing strain (anF-1P) was also evaluated for its resistance to HOCl/H₂O₂ (Fig. 1b). Like ENM 5, the hyper-producing strain anF-1P was found to be more resistant to the treatment than the wild-type, β-carotene or albino strains. However, it was more sensitive to the treatment than ENM 5. This may be due to the different carotenoid compositions of the two hyperproducer strains. Although anF-1P produces significantly more carotenoid than the wild-type strain [800 compared to 300 μg total carotenoid (g yeast)⁻¹], it produces significantly less pigment than ENM 5 [2700 μg total carotenoid (g yeast)⁻¹].

Addition of the ¹O₂ quencher DABCO at increasing concentrations to the HOCl/H₂O₂ system progressively

Fig. 2. The relief of $^1\text{O}_2$ selection pressure by the $^1\text{O}_2$ quencher DABCO. Singlet oxygen was generated by 3.5 mM HOCl/3.5 mM H_2O_2 . Data are presented as relative composition of culture following 5 min treatment. $\square$, AF-1; $\square$, Yan-1; $\square$, 67-385; $\blacksquare$, ENM 5. Survival of hyperproducing strain ENM 5 during selection is also presented ($\circ$). Conditions: 1, inoculum; 2, HOCl/ H_2O_2 + 1 mM DABCO; 3, HOCl/ H_2O_2 + 5 mM DABCO; 4, HOCl/ H_2O_2 + 10 mM DABCO.

relieved the selection for the hyperproducer (Fig. 2). However, even at 10 mM, DABCO did not completely abolish the preferential growth of the hyperproducing strain. This partial relief of selection may have resulted from incomplete quenching owing to the large size of the DABCO molecule and its inability to penetrate the cell wall at a rate to efficiently quench cell-associated $^1\text{O}_2$ (Kanofsky & Sima, 1991).

Selection for carotenogenesis by O_3

Although the HOCl/ H_2O_2 system selected for enhanced carotenogenesis in *P. rhodozyma*, we explored alternative methods for enhancing carotenogenesis because of the potential health risks of residual HOCl/ H_2O_2 in an animal feed. An alternative method of effectively generating $^1\text{O}_2$ is by using O_3 . O_3 decomposes spontaneously and rapidly with biological reducing agents and its decomposition generates $^1\text{O}_2$ (Kanofsky & Sima, 1991). To determine if O_3 would select for carotenogenesis in *P. rhodozyma*, O_3 gas was bubbled ($\sim 72 \mu\text{g s}^{-1} \text{l}^{-1}$) into 0.1 M phthalate buffer (pH 5.0) containing the four differently pigmented strains of yeast. The hyperproducing strain (ENM 5) rapidly became the dominant yeast in the culture. However, under these conditions, viability of the hyperproducing strain rapidly dropped to $\sim 23\%$ after 2 min and $\sim 1.5\%$ after 5 min (Fig. 3a). Exposure of the complete strain mixture to this level of O_3 for 10 min led to complete inactivation of viable yeasts. To determine if the O_3 selection acted by generation of $^1\text{O}_2$, the $^1\text{O}_2$ quencher DABCO was added to the cell suspension which was then bubbled with O_3 and air (Fig. 3b). Supplementation with DABCO significantly relieved the selection pressure, although it did not completely abolish the selection.

Fig. 3. Selection for enhanced carotenoid content by O_3 . (a) Time course of selection in 0.1 M phthalate buffer (pH 5.0); (b) relief of selective pressure by the $^1\text{O}_2$ quencher DABCO; (c) relief of selective pressure by the reducing agent L-ascorbic acid (vitamin C). Data are presented as relative composition of culture following treatment. $\square$, AF-1; $\square$, Yan-1; $\square$, 67-385; $\blacksquare$, ENM 5. Survival of hyperproducing strain ENM 5 during selection is also presented ($\circ$).

Earlier studies suggested that biological reducing agents will convert O_3 to $^1\text{O}_2$ in high yields (Kanofsky & Sima, 1991, 1995a,b). To determine whether reducing agents could enhance the O_3 selection, 10 mM ascorbic acid was added to the cell suspension which was then bubbled with

Fig. 4. Selection for enhanced carotenoid levels by O_3 in 0.1 M phthalate-buffered YN broth (pH 5.0). Data are presented as relative composition of culture following treatment. □, AF-1; ▨, Yan-1; ■, 67-385; ■, ENM 5. Survival of hyperproducing strain ENM 5 during selection is also presented (○).

Fig. 5. Generation of carotenoid-hyperproducing mutants from wild-type *P. rhodozyma* strain 67-385 by random mutagenesis and O_3 treatment.

O_3 (Fig. 3c). Surprisingly, ascorbic acid initially impeded the selection and promoted growth of the β -carotene-producing strain. However, on continued incubation the

astaxanthin-hyperproducer became the predominant strain (Fig. 3c). The hyperproducing strain ENM 5 was inactivated under these conditions, but it was significantly more resistant to this treatment than strains with less carotenoid. Since several media components, including amino acids, are also effective reducing agents, the O_3 selection was carried out in YN broth buffered with 0.1 M phthalate (pH 5.0) (Fig. 4). In this system, selection was evident after 20 min of bubbling, comparable to the selection in buffer alone after 2 min. This indicates that the selection could be achieved even in the presence of complex nutrients.

O_3 treatment was also used in combination with random mutagenesis by NTG in the development of new carotenoid-hyperproducing mutants of *Phaffia rhodozyma*. Ten rounds of random mutagenesis/ O_3 treatment resulted in the isolation of mutants with significantly higher pigmentation than mutagenized control cultures (Fig. 5).

We next examined whether yeasts cultured in O_3 had an altered content of carotenoids. *P. rhodozyma* wild-type (67-385) grown for 5 d in the presence of O_3 had a low total carotenoid content, a low concentration of astaxanthin and relatively high levels of β -carotene and echinenone (Table 1). When the yeasts were removed from the O_3 atmosphere and cultured for 5 d, total carotenoid content as well as the specific content of astaxanthin was increased. After this recovery period, the carotenoid composition was similar to that of control cultures which had not been exposed to O_3 . The levels of β -carotene and echinenone decreased during this continued incubation. The same pattern of carotenoid changes was observed with the astaxanthin-hyperproducer (data not shown).

DISCUSSION

Our laboratory previously demonstrated that carotenogenesis in *P. rhodozyma* protects the yeast against reactive oxygen species, particularly 1O_2 (Schroeder & Johnson, 1995a) and possibly other oxygen species such as H_2O_2 , O_2^- or $OH^\cdot$ (Schroeder & Johnson, 1993, 1995b). The astaxanthin pathway has evolved to protect the yeast by at least two major mechanisms: (1) by detoxification of reactive oxygen species by carotenoids; and (2) by rapid synthesis of carotenoids through relief of end-product inhibition and possibly specific induction of the pathway mediated by 1O_2 (Schroeder & Johnson, 1995b). The antioxidant function of astaxanthin is understandable in

Table 1. Carotenoid composition of wild-type *P. rhodozyma* placed in an O_3 /air atmosphere followed by placement in air

Treatment	Carotenoid [$\mu\text{g (g yeast)}^{-1}$]					Total carotenoid [$\mu\text{g (g yeast)}^{-1}$]	
	<i>cis</i> - Astaxanthin	<i>trans</i> - Astaxanthin	Phoenicoxanthin	3-Hydroxy- echinenone	Echinenone β -Carotene		
5 d O ₃ exposure	1	58	12	34	22	63	190
5 d O ₃ exposure + 5 d recovery	2	182	14	40	9	21	268

light of the very specific habitat of *P. rhodozyma* of sap fluxes of *Betula* and related genera at high elevations (Phaff *et al.*, 1972; Golubev, 1995). In this habitat, high UV irradiance and high levels of O_3 would be encountered. A *Phaffia* host plant, *Betula papyrifera*, was found to contain a compound(s) that generates 1O_2 when activated by long wavelength UV light (Schroeder & Johnson, 1995a). The presence of carotenoids in the yeast probably enables the yeast to survive in the face of the high levels of reactive oxygen species present in this habitat.

Our hypothesis that carotenoids are essential for survival of yeasts on specific trees at high elevations may have general ecological relevance. Duckmanton & Widden (1994) have suggested that environments rich in O_3 may render plants more susceptible to fungal invasion. However, the present study has demonstrated that high O_3 levels associated with the ecological niche of *P. rhodozyma* are particularly detrimental to unpigmented strains of this heterobasidiomycetous yeast. The reaction between O_3 and emulsions of unsaturated fatty acids such as oleic, linoleic and linolenic acids results in the formation of H_2O_2 and peroxy radicals which promote oxidative reactions (Pryor *et al.*, 1991; Bradley & Min, 1992). These fatty acids are known to be predominant in *P. rhodozyma* and carotenoids could protect them from oxidation. High levels of O_3 and 1O_2 present in the environment may provide a selective pressure in nature for the evolution of carotenoid biosynthesis.

Yeast ecologists have noted that yeasts isolated from plants at high altitudes are commonly pigmented (H. J. Phaff, personal communication). The ecological role of increased oxidative stress on cell physiology and evolution has not been studied in depth. In a study of the influence of high elevations and northerly latitudes on *Picea abies* (Norway spruce), Polle *et al.* (1992) suggested that high UV and O_3 exposure increased oxidative stress and that the increased ratio of carotenoids to chlorophyll was an adaptive response to this oxidative stress. Increased carotenoid synthesis in *Populus nigra* (poplar) was also noted by Ballach *et al.* (1992) in response to increased O_3 levels and exposure to air pollutants. Increased carotenogenesis may represent one of several cellular antioxidant defenses. The mixture of reactive oxygen species involved in generating stress is complex and not well defined. Although Wingsle *et al.* (1992) suggested that O_3 decomposed to form $OH^\cdot$, O_2^- and H_2O_2 , they found no correlation between O_3 damage and the activities of antioxidant enzymes including superoxide dismutase and glutathione reductase in *Pinus sylvestris*. This argues against these species being the key oxidative species involved. In the present study, the 1O_2 quencher DABCO only partially abolished selection, suggesting that oxidized species besides 1O_2 may serve as stress signals.

In this study, we have attempted to develop positive genetic selections for carotenogenesis based on our understanding of the functions of carotenoids in *P. rhodozyma*. Several previous studies have attempted to utilize efficient screening and/or selection schemes to isolate highly pigmented strains of *P. rhodozyma*. These

schemes have included resistance to carotenogenesis inhibitors (Lewis, *et al.*, 1990; Wieth, 1991; Fang & Cheng, 1993), cell sorting based on autofluorescence of carotenoids (An *et al.*, 1991), resistance to respiratory inhibitors (An *et al.*, 1989), resistance to reduced oxygen species such as $OH^\cdot$, O_2^- and H_2O_2 . (Schroeder & Johnson, 1993), resistance to high light intensities (An & Johnson, 1990; Meyer & DuPreez, 1994) and resistance to photogenerated 1O_2 (Schroeder & Johnson, 1995a). These treatments have shown varying degrees of success in strain development. However, these methods cannot practically be used for strain selection during large-scale production in fermenters. It would be valuable to apply continued selection for carotenogenesis during industrial fermentations by dark-generation of 1O_2 . This could be accomplished by the feeding of HOCl and H_2O_2 , although residues of these compounds or breakdown products could present potential health risks in a feed yeast. It is interesting that O_3 has also been claimed to increase cell proliferation in *Dunaliella salina* fermentations (Mitsubishi, 1994). Although not stated in this patent, growth of *D. salina* may be favoured in open-pond algal fermentations since this organism has high concentrations of carotenoids and competitive organisms lacking pigments would be more susceptible to inactivation by O_3 . Use of O_3 as a selective agent could overcome the limitation presented by the use of HOCl and H_2O_2 since O_3 has a short half-life. O_3 is currently under investigation as a direct food additive (Anonymous, 1991).

In conclusion, high levels of oxidative stress present in the environment of *P. rhodozyma* has contributed to the development of carotenogenesis in the yeast. More research is needed to define precisely the oxygen species involved and the mechanism(s) of their detoxification by carotenoids. Elucidation of the physiological response to oxidative stress could provide practical methods to increase yields of carotenoids in industrial fermentations.

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