Journal of Industrial Microbiology, (1995) 14, 502–507
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Carotenoids protect *Phaffia rhodozyma* against singlet oxygen damage

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(Received 18 July 1994; accepted 30 September 1994)

Key words: Phaffia rhodozyma; Astaxanthin; Carotenoids; Yeast; Singlet oxygen

SUMMARY

The only known habitat of the astaxanthin-containing *Phaffia rhodozyma* is in slime fluxes of deciduous trees at high altitudes. In this habitat, the function of carotenoids in *P. rhodozyma* is probably to provide protection against photogenerated antifungal substances in the tree flux such as singlet oxygen ($^1O_2$). To investigate the role of carotenoids in *P. rhodozyma*, genetic selections were employed to determine if carotenogenic yeast strains of *P. rhodozyma* have enhanced ability to quench $^1O_2$. Singlet oxygen was generated in liquid culture by the interaction of visible light ($\lambda = 550$ nm) with the photosensitizer rose bengal or by the activation of α-terthienyl with ultraviolet light ($\lambda = 366$ nm). In each case the treatments selected for growth of pigmented strains of *P. rhodozyma*. Albino (carotenoid-less) or yellow (β-carotene producing) strains grew less well in media containing $^1O_2$. Addition of the $^1O_2$ quencher sodium azide to the medium with α-terthienyl allowed growth of non-pigmented strains. Since the ecological niche of *P. rhodozyma* is highly specific, we investigated whether extracts of birch trees (*Betula*), the original source of *P. rhodozyma*, contained a compound that would select for pigmented populations of the yeast. When *P. rhodozyma* strains were exposed to ethyl acetate extracts of *Betula papyrifera* excited with 366 nm ultraviolet light, only pigmented cells were able to grow. These results suggest that carotenogenesis developed in *P. rhodozyma* in response to the presence of photoactivatable antifungal compounds produced by the host tree.

INTRODUCTION

The heterobasidomycetous yeast *Phaffia rhodozyma* was discovered by Herman Jan Phaff and his colleagues during one of his exploratory trips to discover yeasts with novel properties and to develop an understanding of yeast ecology [25]. Phaff isolated the yeast in the late 1960s from slime exudates of deciduous trees in mountainous regions of Japan and Alaska. While studying the yeast, its novel properties became immediately apparent. On yeast-malt agar, colonies had a pink hue which suggested the presence of carotenoids. The unique characteristic of the yeast was that it fermented sugars producing ethanol and gas, a property not found in other carotenogenic yeasts, all of which have an obligatory respiratory metabolism. Phaff et al. [25] initially named the yeast *'Rhodozyma montanae'*, a single species within a new yeast genus. Miller et al. [22] later demonstrated the heterobasidomycetous nature of the yeast, its unique physiology, and renamed it *Phaffia rhodozmya* in honor of Herman Phaff's many contributions to yeast biology. Arthur Andrewes, while working in Mortimer Starr's laboratory at the University of California, Davis, made the remarkable discovery that the primary carotenoid in *P. rhodozyma* is astaxanthin [2], the characteristic pigment of salmonids, crustacea, and certain birds such as flamingos. He also showed that the yeast astaxanthin has the 3*R*, 3′*R* absolute configuration [3]. This is opposite to most other configurational isomers in nature, and suggested a unique biosynthetic pathway. Andrewes' discovery lent novelty and brought industrial importance to yeast carotenoids.

One of the authors (E.A.J.) was fortunate to study in Professor Michael Lewis's laboratory from 1975–1979 at the University of California at Davis initially as an undergraduate and later as a Master's student. During that time, he was introduced to *Phaffia* by Mary Miranda, Phaff's excellent technician, who tended the yeast collection and identified yeasts as well as helping many students in the department. He became interested in *Phaffia* and, under Phaff and Lewis, began a collaboration to study its industrial potential as a source of pigment for aquaculture. They received a small SeaGrant and investigated the characteristics of the yeast including the biosynthesis of its pigments, cell wall properties, and suitability as a pigment source for many animals including rainbow trout, salmon (unfortunately they did not prosper in the freshwater system at Davis), chicken eggs and skin, quail eggs, and lobsters [11]. They published several papers on *Phaffia* including its suitability as a pigment source for salmonids [13]. On recognizing this ability, they approached the University of California to try to patent the yeast as a pigmenter. However, the University's legal department decided against it because aquaculture was only a fledgling industry. The size of the industry has changed dramatically in the ensuing times. The aquaculture industry is currently the fastest growing sector of agriculture and is expected to be a $40 billion dollar industry by the year 2000. A substantial proportion of this industry involves salmon and shrimp aquaculture, growing from 3–5% of world consumption to 25–30% today [12]. Currently, salmon farming is

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This paper is dedicated to Professor Herman Jan Phaff in honor of his 50 years of active research which still continues.

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prospering. Nearly 270 000 metric tons of salmon were produced worldwide in 1992. The single largest expense in salmon aquaculture is the carotenoid pigment astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione), which accounts for ~10% of feed costs. The current market for astaxanthin is estimated to be about $100 million dollars [10], and several companies have intensely developed *Phaffia* as an industrial pigment source. Among these, Universal Bioventures (Milwaukee, WI, USA) has been successful in developing a *Phaffia* product (Red Star *Phaffia* yeast product). This was successfully introduced to the commercial salmon feed market about 2 years ago and is continually being improved for higher astaxanthin content. The Dutch yeast company Gist-brocades (Delft, Holland) also has a high quality *Phaffia* product (marketed under the name Natupink). Furthermore, astaxanthin is one of the most effective antioxidants in nature, and has been linked to reduced incidence of cancer and other degenerative diseases associated with shortened life expectancy [29–32].

Workers in our laboratory are interested in the physiology of astaxanthin in *Phaffia*, especially the functions of the carotenoids in the yeast. In order to understand these functions of astaxanthin, we have attempted to develop positive genetic selections for carotenogenic strains. Carotenoids, by virtue of their extended polyene chain, are potent antioxidants. They act to quench oxygen catabolites, including products derived from metabolism including H_2O_2, O_2^-, $OH\cdot$, and particularly $^1O_2$ [8]. We showed that *P. rhodozyma* is particularly susceptible to activated oxygen species including hydrogen peroxide (H_2O_2) and superoxide radical (O_2^-) and that carotenoids apparently compensate for this sensitivity [28]. The present study was carried out to determine whether carotenoids also play a role in $^1O_2$ detoxification. In addition, we were interested whether there was a factor unique to the specific habitat of *Phaffia* (wounds in *Betula* and related trees) that might select for the yeast's presence in this habitat. Since examples are known of plant-synthesized antimicrobial and antiparasitic compounds that act through photoactivatable singlet oxygen generation [19], a similar activity was searched for in various *Betula* extracts.

MATERIALS AND METHODS

Chemicals

The following chemicals were obtained from Sigma Chemical Company, St Louis, MO, USA: dimethylsulfoxide (DMSO), rose bengal (RB) and thymol. Petroleum ether, ethyl acetate, hexane, acetone, sodium azide and α-terthienyl (α-T) were from Aldrich Chemical, Milwaukee, WI, USA. Ethanol (200 proof; USP grade) was from Aaper Alcohol and Chemical Co., Shelbyville, KY, USA. All other chemicals used were of analytical grade or the best grade commercially available.

Yeast strains and growth

P. rhodozyma strains 67-385 and AF-1 were described previously [28]. Strains Yan-1 and ENM 5 were derived from 67-385 by NTG mutagenesis (Table 1). Yeasts were maintained on slants of yeast extract/malt extract/peptone/glucose medium (YM broth, Difco Co., Detroit, MI, USA) with 2% agar (YM agar) and refrigerated at 4 °C. Yeast strains were also stored in 40% glycerol/60% YM broth at −70 °C.

TABLE 1

Phaffia rhodozyma strains used in this study

Strain	Pigment content (μg g^{-1})	Characteristics
AF-1	0	albino mutant
Yan-1	100	β-carotene producing mutant
67-385	300	wild-type
ENM 5	2700	carotenoid hyperproducing mutant

Yeast cultures were grown by inoculating 5 ml of YM broth in roller tubes with cells from YM slants and growing them for 2 days. These cultures were then used as inoculum at 1% (v/v) into 30 ml YM broth in 300-ml baffled shake flasks. Cultures were grown for periods of 2 or 5 days depending on the experiment. Dry cell weight, total carotenoid and individual carotenoids were analyzed as described by Schroeder and Johnson [28].

Singlet oxygen selection experiments

For determination of selection of strains differing in carotenoid content, the cultures were mixed such that each strain comprised a defined proportion of the total population as determined by plating and scoring this inoculum. They were then treated by conditions that generated $^1O_2$. After treatment, cultures were diluted in 0.85% NaCl solution and plated on 2% YM agar. After 5 days, the plates were scored for the relative proportions of various strains based on colony color. All experiments were performed in duplicate or triplicate and replicated at least once. The error estimates presented in the text and figures represent one standard deviation from the mean.

Rose bengal was dissolved in water, filter sterilized through a 0.2-μm pore size Acrodisc filter (Gelman Sciences, Ann Arbor, MI, USA), and used from a 1000-fold aqueous stock solution. One milliliter of culture was removed every 24 h and centrifuged to pellet the cells. Culture supernatant fluids were assayed for the amount of rose bengal remaining according to the extinction coefficient (A ~1.551 for a 10-μM solution) and additional rose bengal was added daily to replace that lost through photodegradation.

α-Terthienyl was dissolved in ethanol as a 1000-fold stock solution. To activate $^1O_2$ and expose cells, mixed cultures were diluted 10-fold in fresh YM medium in sterile Pyrex petri dishes containing α-terthienyl, placed in a Chromato-Vue cabinet (Ultraviolet Products, Inc., San Gabriel, CA, USA) and exposed for 5 min to 366 nm light.

Preparation of tree extracts

Betula (birch) extracts were made by extracting 50 g wet weight of *Betula papyrifera* saw dust by three consecutive extractions with 200 ml of various solvents (water, hexane, ethanol and ethyl acetate). The pooled extracts were filtered through glass wool and dried in a rotary evaporator to 15 ml

total volume. For each selection, a similar procedure was followed as described for α-terthienyl except that 250 μl of the extracts were used, in a 10 ml total volume.

RESULTS

Carotenoids are effective quenchers of $^1O_2$ (Fig. 1), and astaxanthin is one of the most efficient [8]. We previously demonstrated that astaxanthin in *P. rhodozyma* protected against killing by H_2O_2 and O_2^- [28]. Since $^1O_2$ can be generated in the dark by decay of peroxides or oxygen radicals, we investigated the resistance to and selective ability of $^1O_2$ challenge in various strains of *P. rhodozyma.*

The food dye rose bengal generates $^1O_2$ when activated by 550 nm light [5]. To investigate inactivation of *P. rhodozyma* by rose bengal and light, an inoculum was prepared consisting of equal proportions of three strains: (a) 67-385 (wild-type; 300 μg total carotenoid g^{-1} DCW), (b) Yan-1 (β-carotene producing mutant, 100 μg g^{-1}), and (c) AF-1 (albino mutant, 0 μg g^{-1}). These strains were incubated in YM broth with rose bengal for 2 days. Light and rose bengal in the medium selected for the astaxanthin-containing strain (Fig. 2). A similar experiment was conducted with the inoculum comprised of equal proportions of the wild-type strain and strain ENM 5 (a carotenoid hyperproducing mutant; ~2700 μg g^{-1}). When the cultures were incubated with increasing quantities of rose bengal, there was an increasingly strong selection for the hyperproducing ENM 5 mutant (Fig. 3).

Inhibitors of the isoprenoid pathway have previously been used to select for *P. rhodozyma* strains with higher levels of astaxanthin [20,34]. We therefore investigated the potential for $^1O_2$ selection using a combination of rose bengal and the carotenogenesis inhibitor thymol. When 6 μM rose bengal was added to the mixed culture, yeasts containing higher levels of carotenoids survived whereas the low producers were selected against. When thymol was used in combination with rose bengal, the selection for ENM 5 was increased from 62.4 (±0.6)% with 6 μM rose bengal to 94.3 (± 5.1)% with rose bengal and 50 μM thymol and 93.3 (± 0.2)% with rose bengal and 100 μM thymol (Fig. 4). The results suggest that existing pools of carotenoids in the overproducer allowed survival in the presence of $^1O_2$.

We also used α-terthienyl to generate $^1O_2$ and examined its selective ability (Fig. 5). α-Terthienyl was originally isolated from marigold petals (*Tagetes patula*) and was identified as a nematocidal compound in which killing is mediated through formation of $^1O_2$ [19]. Exposure of mixed cultures of 67-385 and AF-1 to the combination of α-terthienyl and 366 nm UV light selected for the pigmented strain. *Saccharomyces cerevisiae* was unaffected by the treatment (data not shown). When azide, a quencher of $^1O_2$ [7], was added to the medium, it partially reversed the *P. rhodozyma* selection, probably by detoxifying $^1O_2$.

Since the habitat of *P. rhodozyma* is deciduous trees, we investigated whether a naturally occurring selective agent from birch analogous to α-terthienyl may be present that selected for pigmentation in *P. rhodozyma.* We examined various solvent extracts of *Betula papyrifera*, a genus of deciduous trees known to be one habitat for the yeast. Ethyl acetate extracts exposed to 366 nm ultraviolet light selected for the more highly pigmented strains in a mixed *P. rhodozyma* culture (Fig. 6). Water and ethanol extracts showed no selective activity, while hexane extracts showed a lesser activity than did ethyl acetate (data not shown).

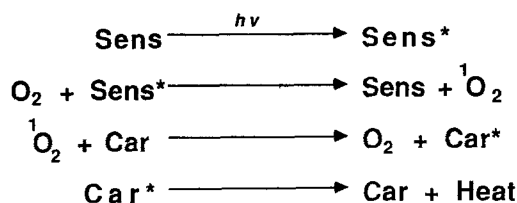

Fig. 1. Reaction scheme for the generation of singlet oxygen ($^1O_2$) by a photosensitizer (Sens), and the detoxification of $^1O_2$ by carotenoids (Car). Asterisks indicate the molecule in an excited energy state.

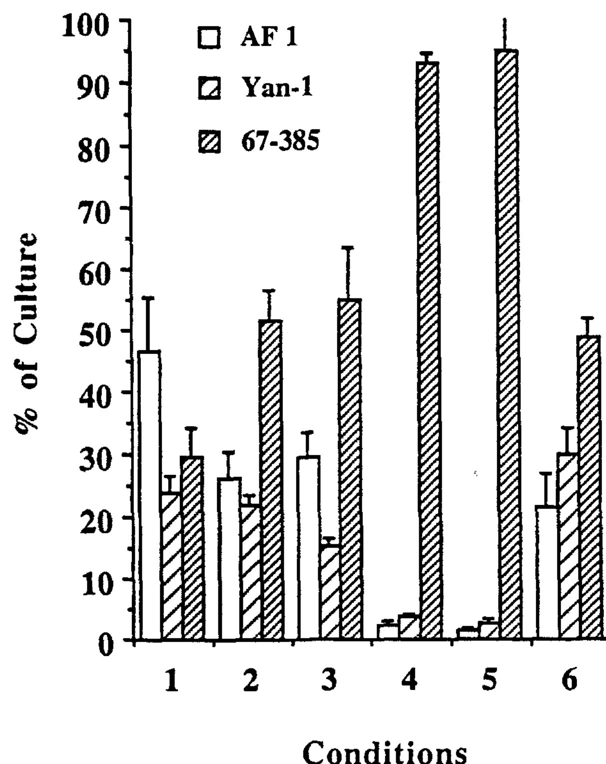

Fig. 2. Growth of wild-type (67-385) *P. rhodozyma* and mutants (AF-1 and Yan-1) with rose bengal and visible light. Cultures were grown for 2 days with the various treatments. Conditions: 1) inoculum, 2) visible light, 3) dark, 4) visible light and 10 μM rose bengal, 5) visible light and 15 μM rose bengal, 6) dark and 15 μM rose bengal.

DISCUSSION

We showed that *P. rhodozyma* is deficient in enzymes involved in detoxification of activated oxygen species such as H_2O_2, O_2^- and OH·. We proposed that carotenoids located in cytosolic lipid globules and membraneous regions of the yeast

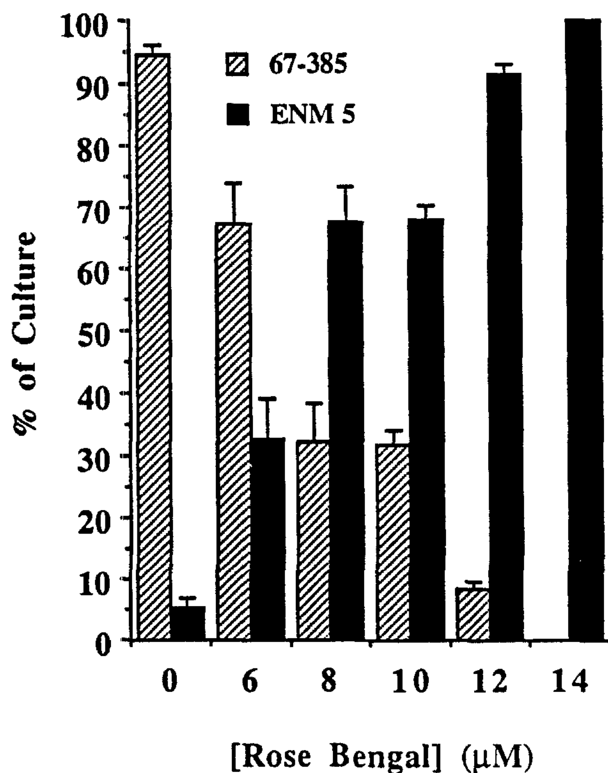

Fig. 3. Selection for carotenoid hyperproducing strain (ENM 5) over wild-type (67-385) strain with rose bengal and visible light. Strains were selected as a function of rose bengal concentration. Cultures were grown for 2 days with the various treatments.

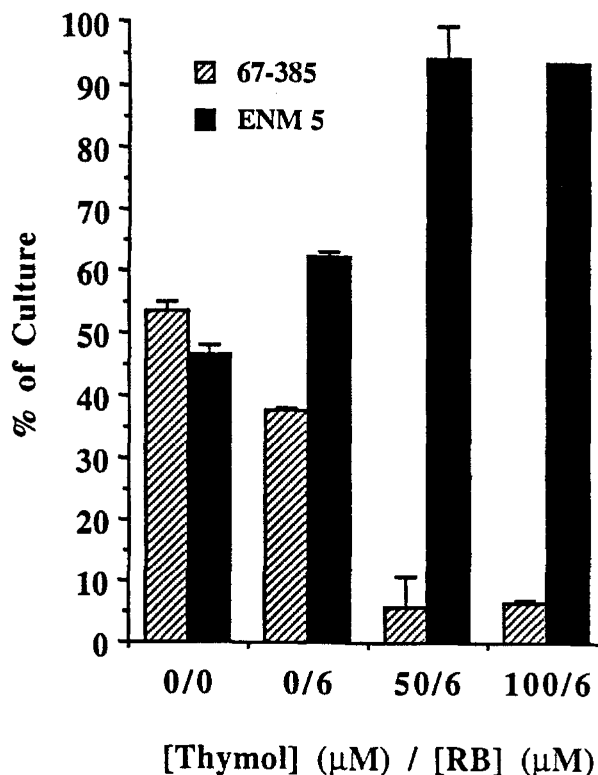

Fig. 4. Enhancement of rose bengal/visible light selection by the carotenogenesis inhibitor thymol. The combinations of thymol/rose bengal (RB) are presented. Cultures were grown for 2 days in the treatments.

may compensate for the lack of antioxidant enzymes [28]. *P. rhodozyma* has only one superoxide dismutase, the Mn-superoxide dismutase, which is probably located in mitochondria where it occurs in other organisms. *P. rhodozyma* has a low content of catalase in the stationary phase compared to *S. cerevisiae* [28]. We observed that *P. rhodozyma* became highly sensitive to hydrogen peroxide exposure in the stationary phase [28]. Aging of fungi is accompanied by increased formation of oxygen radicals and probably an increased redox potential of the cells [24]. Interestingly, the later stages of growth and the stationary phase are associated with an increase in cyanide-insensitive respiration and are the most active period for xanthophyll synthesis in *P. rhodozyma.*

Singlet oxygen is a highly reactive oxygen species which can induce DNA damage and oxidize proteins [29]. Singlet oxygen has been recognized as a product of excited triplet state porphyrin type II photooxidation in photosynthetic systems and to react with carotenoids [16]. The activation of carotenogenesis in *Myxococcus xanthus* by blue light was proposed to be triggered by $^1O_2$ [9]. The authors proposed a model in which blue light photosensitized protoporphyrin IX, generating $^1O_2$. Singlet oxygen subsequently interacted with membrane-bound CarR protein initiating a regulatory cascade resulting in enhanced carotenogenesis. This activation would also lead to enhanced detoxification of $^1O_2$.

Although the significance of light-catalyzed formation of $^1O_2$ has been widely discussed, less attention has been paid to $^1O_2$ generation in the dark. Singlet oxygen can be generated in the dark by the decomposition of endoperoxides and other types of enzymatic and chemical reactions [1,33]. In biological systems, these dark reactions occur by lipid peroxidation and by peroxidase enzymes [6,14]. Such generation of $^1O_2$ in the dark has been identified as the mediator of an immune response in mammals [17]. Krinksy [18] reported that $^1O_2$ can be generated through the spontaneous, non-enzymatic decay of H_2O_2, O_2^- and OH·. Enzymatic removal of H_2O_2 and O_2^- in enzyme-competent organisms containing high levels of catalases, peroxidases, and superoxide dismutases would yield O_2 in the ground state. Since *P. rhodozyma* lacks high levels of these enzymes, it is likely that the yeast also has difficulty in detoxifying $^1O_2$. The importance of resistance to singlet oxygen in *P. rhodozyma* is also supported by its content of lipids. The yeast is high in fat, and oleic and linoleic acids comprise about 70–75% of the total fatty acids [13]. These two fatty acids are among the most reactive with singlet oxygen [5]. Singlet oxygen is relatively long-lived in cells, lasting 2–4 μs in an aqueous environment but 25–10 μs in the lipid phase and the diffusion of singlet oxygen has been estimated to have a radius of 100 Å [23,27]. Since *P. rhodozyma* has a high level of fat, carotenoids which are associated with lipids and the cell membrane may serve as an excellent defense against lipid peroxidation in the cell. Although *S. cerevisiae* is not pigmented, it is highly resistant to $^1O_2$, perhaps because it has high quantities of detoxification enzymes, is low in lipids, and contains predominantly palmitoleic acid which is less reactive with $^1O_2$ [21].

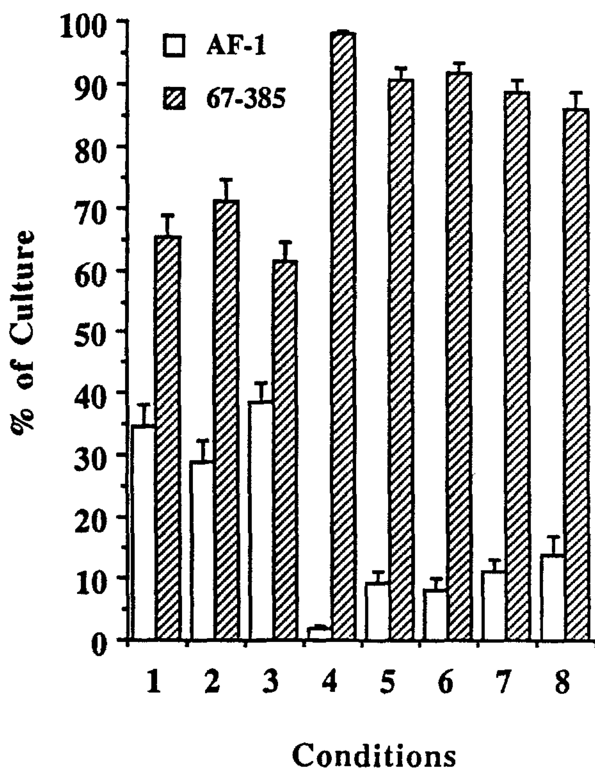

Fig. 5. Selection of wild-type (67-385) strain over an albino strain (AF-1) with 5 μM α-terthienyl and UV light (366 nm), and relief of selective pressure by the $^1O_2$ quencher azide (5 min exposure). Conditions: 1) inoculum, 2) UV light, 3) α-terthienyl, 4) α-terthienyl and UV light, 5) α-terthienyl, UV light and 0.5 mM azide, 6) α-terthienyl, UV light and 1.0 mM azide, 7) α-terthienyl, UV light and 2.5 mM azide, 8) α-terthienyl, UV light and 5.0 mM azide. Cultures were plated immediately following treatment.

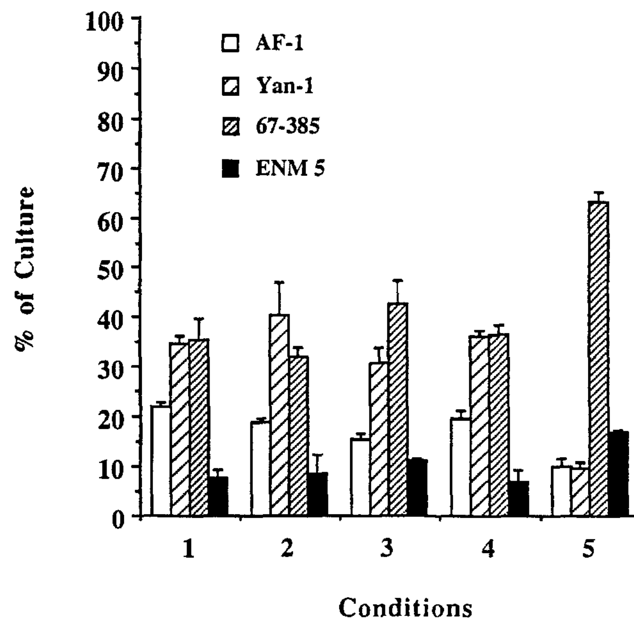

Fig. 6. Selection for pigmented strains of *P. rhodozyma* by birch tree (*Betula papyrifera*) ethyl acetate extracts and UV light (366 nm, 60 min exposure). Conditions: 1) inoculum, 2) ethyl acetate solvent control, 3) ethyl acetate extract, 4) ethyl acetate solvent and UV light, 5) ethyl acetate extract and UV light. Cultures were plated immediately following treatment.

Although most carotenoids have the capability to quench $^1O_2$, this capacity varies among different pigments and in general increases with the number of conjugated double bonds [8]. Even though β-carotene, with 11 double bonds, is very effective, quenching 250–1000 $^1O_2$ molecules, astaxanthin and lycopene are more active than β-carotene and are among the most active of the carotenoids [5,8]. The growing evidence of the ability of carotenoids to prevent degenerative diseases including cancer and cardiovascular disease in animals and humans by virtue of their ability as antioxidants has created considerable excitement in the carotenoid field. Although β-carotene has been proposed as an important dietary anticarcinogen, other carotenoids including α-carotene and astaxanthin are more effective in preventing certain kinds of cancers. It appears that xanthophylls such as astaxanthin have potential as a therapeutic agent to prevent degenerative diseases and increase longevity [31,32].

P. rhodozyma natural isolates have been found only in a specific ecological niche, the wounds of birch trees (*Betula*) and related tree species at high elevations and northerly latitudes [25]. This specificity suggests that there may be unique interactions occurring between trees and the yeast. It is recognized that antimicrobial and antiparasitic compounds are synthesized by plants, especially in response to infection or wounding. α-Terthienyl, which generates $^1O_2$, was isolated from Marigold (*Tagetes patula*) and shown to kill nematodes [19]. This compound together with light selected for pigmented strains of *P. rhodozyma* in this study. Other examples are also known of interactions of plants and fungi. Phenylheptatriyne isolated from *Bidens pilosa* has been shown to photosensitize *Escherichia coli* and *S. cerevisiae* to long wavelength UV radiation [4]. Two species of birch (*Betula pendula* and *B. pubescens*) have shown varying abilities to resist infection by the leaf rust fungus *Melampsoridium betulinum* [26], and decreased phenol levels in birch predisposed tissues to wood rot fungi [15]. Our results in this study suggest that birch has a component that selects for *P. rhodozyma* as a constituent microflora in its sap. It appears that carotenogenesis in *P. rhodozyma* has evolved as a means for the yeast to cope with the host's $^1O_2$-mediated defense mechanism.

It is clear from current commerical interest in *Phaffia* that it is becoming an increasingly important yeast in industrial microbiology. In closing, we hope that Herman Phaff and his colleagues continue their explorations of habitats for new yeasts to provide the scientific, industrial, and medical communities with yeasts with such remarkable attributes as have been demonstrated in *P. rhodozyma*. In addition, the already extensive collection of many unstudied yeasts obtained by Phaff and Miller at UC Davis provides a potential resource for designation of yeasts with valuable properties which should be explored by industry.

ACKNOWLEDGEMENTS

We thank Julie Schmidt for her expert technical assistance and Karl Schroeder for his generous gift of samples of *Betula papyrifera*. This work was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, WI, USA, and by Royal Gist-Brocades NV, Delft, Holland. W.S. was supported by a National Science Foundation Fellowship.

REFERENCES

1 Adam, W. and G. Cilento. 1982. Chemical and Biological Generation of Electronically Excited States. Academic Press, New York.
2 Andrewes, A.G., H.J. Phaff and M.P. Starr. 1976. Carotenoids of *Phaffia rhodozyma*, a red-pigmented fermenting yeast. Phytochemistry 15: 1003–1007.
3 Andrewes, A.G. and M.P. Starr. 1976. (3*R*,3′*R*)-Astaxanthin from the yeast *Phaffia rhodozyma*. Phytochemistry 15: 1009–1011.
4 Arnason, T., C.-K. Wat, K. Downum, E. Yamamoto, E. Graham and G.H.N. Towers. 1980. Photosensitization of *Escherichia coli* and *Saccharomyces cerevisiae* by phenylheptatriyne from *Bidens pilosa*. Can. J. Microbiol. 26: 698–705.
5 Bradley, D.G. and D.B. Min. 1992. Singlet oxygen oxidation of foods. Crit. Rev. Food Sci. Nutr. 31: 211–236.
6 Cadenas, E. and H. Sies. 1984. Low-level chemiluminescence as an indicator of singlet oxygen in biological systems. Meth. Enzymol. 105: 221–231.
7 Hasty, N., P.B. Merkel, P. Radlick and D.R. Kearns. 1972. Role of azide in singlet oxygen reactions: reactions of azide with singlet oxygen. Tetrahedron Lett. 1: 49–52.
8 Hirayama, O., K. Nakamura, S. Hamada and Y. Kobayashi. 1994. Singlet oxygen quenching ability of naturally occurring carotenoids. Lipids 29: 149–150.
9 Hodgson, D.A. and F.J. Murillo. 1993. Genetics of regulation and pathway of synthesis of carotenoids. In: Myxobacteria II (Dworkin, M. and D. Kaiser, eds), pp. 157–181, Amer. Soc. Microbiol. Washington, DC.
10 Johnson, E.A. and G.-H. An. 1991. Astaxanthin from microbial sources. Crit. Rev. Biotechnol. 11: 297–326.
11 Johnson, E.A., M.J. Lewis and C.R. Grau. 1980. Pigmentation of egg yolks with astaxanthin from the yeast *Phaffia rhodozyma*. Poultry Sci. 59: 1777–1782.
12 Johnson, E.A. and W.A. Schroeder. 1994. Microbial carotenoids. Adv. Biochem. Eng. (in press).
13 Johnson, E.A., T.G. Villa and M.J. Lewis. 1980. *Phaffia rhodozyma* as an astaxanthin source in salmonid diets. Aquaculture 20: 123–134.
14 Kanofsky, J.R. 1989. Singlet oxygen production by biological systems. Chem.-Biol. Interact. 70: 1–28.
15 Kauppi, A., K. Paukkonen and H.-L. Tela. The role of phenols in sprouting and wood decay of birches. Can. J. For. Res. 21: 1066–1072.
16 Kautsky, H. and H. de Bruijn. 1931. Die aufklarung der photoluminescenztilgung fluorescierender systeme durch sauerstoff: die bildung aktiver, diffusionsfahiger sauerstoffmolekule durch sensibilisierung. Naturwissenschaften 19: 1043.
17 Krinsky, N.I. 1974. Singlet excited oxygen as a mediator of the antibacterial action of leukocytes. Science 186: 363–365.
18 Krinsky, N.I. 1977. Singlet oxygen in biological systems. TIBS 2: 35–38.
19 Kyo, M., Y. Miyauchi, T. Fujimoto and S. Mayama. 1990. Production of nematocidal compounds by hairy root cultures of *Tagetes patula*. L. Plant Cell Rep. 9: 393–397.
20 Lewis, M.J., N. Ragot, M.C. Berlant and M. Miranda. 1990. Selection of astaxanthin-overproducing mutants of *Phaffia rhodozyma* with β-ionone. Appl. Environ. Microbiol. 56: 2944–2945.
21 Lyons, T.P., K.A. Jacques and K.A. Dawson. 1993. Miscellaneous products from yeast. In: The Yeasts (Rose, A.H. and D.E. Harrison, eds), pp. 293–324, Academic Press, London.
22 Miller, M.W., M. Yoneyama and M. Soneda. 1976. *Phaffia*, a new yeast genus in the *Deuteromycotina* (*Blastomycetes*). Int. J. Syst. Bacteriol. 26: 286–291.
23 Moan, J. 1990. On the diffusion of singlet oxygen in cells and tissues. J. Photochem. Photobiol. 6: 343–344.
24 Munkres, K.D. 1990. Aging of fungi. Rev. Biol. Res. Aging 4: 29–40.
25 Phaff, H.J., M.W. Miller, M. Yoneyama and M. Soneda. 1972. A comparative study of the yeast florae associated with trees on the Japanese Islands and on the west coast of North America. In: Proc. 4th IFS: Ferment. Technol. Today (Terui, G., ed.), pp. 759–774, Kyoto Society of Fermentation Technology, Osaka.
26 Poteri, M. 1992. Screening of clones of *Betula pendula* and *B. pubescens* against two forms of *Melampsoridium betulinum* leaf rust fungus. Eur. J. For. Path. 22: 166–173.
27 Schnuriger, B. and J. Bourdon. 1968. Photosensitized oxidation through stearate mononuclear films. Photochem. Photobiol. 8: 361–368.
28 Schroeder, W.A. and E.A. Johnson. 1993. Antioxidant role of carotenoids in *Phaffia rhodozyma*. J. Gen. Microbiol. 139: 907–912.
29 Sies, H. and C.F.M. Menck. 1992. Singlet oxygen induced DNA damage. Mutat. Res. 275: 367–375.
30 Sohal, R.S., S. Agarwal, A. Dubey and W.C. Orr. 1993. Protein oxidative damage is associated with life expectancy of houseflies. Proc. Natl. Acad. Sci. USA 90: 7255–7259.
31 Tanaka, T., Y. Morishita, M. Suzui, T. Kojima, A. Okumura and H. Mori. 1994. Chemoprevention of mouse urinary bladder carcinogenesis by the naturally occurring carotenoid astaxanthin. Carcinogenesis 15: 15–19.
32 Wagner, J.R., P.A. Motchnik, R. Stocker, H. Sies and B.N. Ames. The oxidation of blood plasma and low density lipoprotein components by chemically generated singlet oxygen. J. Biol. Chem. 268: 18502–18506.
33 Wasserman, H.H. and R.W. Murray. 1979. Singlet Oxygen. Academic Press, New York.
34 Wieth, T. 1991. Development of a yeast product with high astaxanthin content. Ph.D. Thesis. Akademiet for de Tekniske Videnskaber, Copenhagen, Denmark.