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REVIEW ARTICLE

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Phaffia rhodozyma: colorful odyssey

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Abstract *Phaffia rhodozyma* was isolated by Herman Phaff in the 1960s, during his pioneering studies of yeast ecology. Initially, the yeast was isolated from limited geographical regions, but isolates were subsequently obtained from Russia, Chile, Finland, and the United States. The biological diversity of the yeast is more extensive than originally envisioned by Phaff and his collaborators, and at least two species appear to exist, including the anamorph *Phaffia rhodozyma* and the teleomorph *Xanthophyllomyces dendrorhous*. The yeast has attracted considerable biotechnological interest because of its ability to synthesize the economically important carotenoid astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) as its major pigment. This property has stimulated research on the biology of the yeast as well as development of the yeast as an industrial microorganism for astaxanthin production by fermentation. Our laboratory has isolated several mutants of the yeast affected in carotenogenesis, giving colonies a vivid array of pigmentation. We have found that nutritional and environmental conditions regulate astaxanthin biosynthesis in the yeast, and have demonstrated that astaxanthin protects *P. rhodozyma* from damage by reactive oxygen species. We proposed in the 1970s that *P. rhodozyma* could serve as an economically important pigment source in animal diets including salmonids, lobsters, and the egg yolks of chickens and quail, in order to impart characteristic and desirable colors. Although *P. rhodozyma*/*Xanthomyces dendrorhous* has been studied by various researchers for nearly 30 years, it still attracts interest from yeast biologists and biotechnologists. There is a bright and colorful outlook for *P. rhodozyma*/*X. dendrorhous* from fundamental and applied research perspectives.

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Introduction

Herman Jan Phaff began his illustrious career in microbiology by helping as a young man in the operations of a small fruit winery owned by his family in the Netherlands, watching the fermentation and observing the budding and growing of the yeast cells in the wine-making process [29]. Herman later attended the Technical University of Delft in the chemical engineering curriculum, where he studied under the eminent A. J. Kluyver, Professor of General and Applied Microbiology, whose famous predecessor had been Martinus Beijerinck. These early days of exposure to industrial and food microbiology had a profound impact on his career in microbiology [29] and likely contributed to his appreciation of both fundamental and applied microbiology throughout his career.

I had the great fortune of attending the University of California at Davis and studying with both Michael Lewis and Herman Phaff from 1974 to 1978, during the period when Herman was increasingly focusing much of his work on the ecological and taxonomic aspects of yeasts [29]. During this period he isolated a unique yeast that was initially designated "Rhodozyma montanae", and later *Phaffia rhodozyma* by certain of his coworkers in recognition of Herman's valuable contributions to yeast ecology and taxonomy.

Because of *P. rhodozyma*'s ability to synthesize the industrially important carotenoid astaxanthin, there has been considerable commercial interest in the development of the yeast as a biological source of carotenoids. There has also been renewed activity in the taxonomy and physiology of the yeast. In this review, salient properties of *P. rhodozyma* are described with an emphasis on my studies of the yeast at the University of

California,-Davis and later in my laboratory at the University of Wisconsin.

Isolation, identification, and properties of *Phaffia rhodozyma*

In the late 1960s, Hermazn began to reduce his program on yeast cell wall biochemistry and expand on his "adventures" in yeast ecology [29]. During this time he collaborated with Martin W. Miller and two Japanese scientists, Minoru Yoneyama and Masumi Soneda, in studies of yeast florae associated with trees on the Japanese Islands and on the West Coast of North America (Phaff et al. 1972). The purpose of these studies was to expand the knowledge of yeasts associated with slime exudates or insect frass occurring in trees. Their survey of yeasts on the Japanese islands was made from April through June of 1967 and in northwestern regions of North America during June and July of 1968. The great majority of samples were taken from active slime fluxes (exudates) occurring on tree stumps of broad-leaved trees remaining after logging operations (Japan) and from natural slime fluxes from standing trees. A variety of ascosporogenous and basidiomycetous yeasts were obtained, but perhaps the most interesting isolates were a group of imperfect yeasts, notably an unusual imperfect yeast that formed colonies with a distinct orange-red color [29]. Ten strains of this yeast were isolated, nine from various broad-leaved trees in Japan [the source trees included *Alnus* (1 strain), *Betula* (3), *Cornus* (3), *Fagus* (1), and *Ulmus* (1)], and one strain of this yeast was later isolated from *Betula* in Alaska. Phaff and collaborators found that this organism represented a new yeast genus, which they initially designated "Rhodozyma montanae" due to its orange-red color and its having been isolated from mountainous regions. The most striking feature of the yeast was the orange-red color of the colonies, which was determined to be due to the presence of carotenoid pigments and which varied in intensity depending on the strain. The yeast also fermented a number of sugars including glucose, maltose, sucrose, and raffinose. A new genus, *Phaffia*, with one species *P. rhodozyma*, was validly described in 1976 [27]. The yeast was recognized to be of basidiomycetous origin based on its morphology, cell wall properties, mode of bud formation, pigmentation, and metabolic properties, but despite attempts for several years to mate the various strains and observe a dikaryotic mycelium and teliophore formation, a sexual life cycle was not found. The type strain of the anamorphic yeast was designated as *P. rhodozyma* UCD 67-210, which was later deposited as the teleomorphic strain CBS 5905 (ATCC 24202) (*Xanthophyllomyces dendrorhous*).

P. rhodozyma was most unusual in being the only known carotenoid-containing species of yeast that had the ability to vigorously ferment sugars. Until this observation was made, all carotenoid-containing yeasts were known to have a strictly respiratory metabolism. It was fortuitous that during this time Arthur Andrewes, an expert in carotenoid chemistry, was studying as a postdoctoral fellow in Mortimer Starr's laboratory, which was a short walk from Phaff's laboratory in Cruess Hall. I visited Art's lab frequently and watched him purify and collect the colorful carotenoids from a variety of organisms using various columns and solvent extractions, usually with a home-rolled and lighted cigarette dangling from his mouth as he worked. Art Andrewes made the highly surprising discovery that the principal carotenoid in *P. rhodozyma* was astaxanthin, which is a common carotenoid in the marine environment and provides the characteristic and vivid pigmentation of certain animals, including salmon, lobsters and shrimp, and certain birds, such as varieties of flamingos and the scarlet ibis. Andrewes also made the remarkable finding that astaxanthin isolated from *P. rhodozyma* had the 3R,3′R-configuration, which is opposite to that of astaxanthin from other sources that had been investigated [5]. This was the first example of a naturally occurring carotenoid biosynthesized in different optical forms, and it raised interesting questions regarding the properties of the enzymes involved in the apparently distinct *R*-astaxanthin biosynthetic pathways in *P. rhodozyma* compared to those of other organisms that contained astaxanthin with the *S* configuration [5].

During this period, from approximately 1974 to 1979, at Davis, I also became acquainted with many of the yeast researchers of past and present in Herman's mecca of yeast research, including Tomás G. Villa, Mary Miranda, Tom Starmer, and the extraordinarily talented Marc-Andre' Lachance. Mary Miranda, Tomas, and Andre took considerable interest in "my project" on *Phaffia*, and we collaborated in many aspects, including running fermentations in Bacteriology's 100 l fermentor, attempting to obtain protoplasts in *Phaffia* using *Bacillus circulans* lytic enzymes, and conducting many other experiments.

As mentioned above, elucidating the sexual cycle of *P. rhodozyma* was initially a frustrating and unsuccessful endeavor. Phaff and other yeast biologists searched for the teleomorphic state of *P. rhodozyma* for many years using the ten early isolates, but they were not successful. Since most basidiomycetous yeasts are heterothallic, sexual activity usually requires mating of compatible strains [10]. Meanwhile, additional isolates were being collected in Finland, Russia [10, 11] and other geographic regions of the world during the 1970s through 1990s. In 1995, Golubev, in Russia, found a sexual cycle in certain strains isolated near Moscow as well as in the type strain CBS 5905 (ATCC 24202; UCD 67-210) [10]. Sexual activity involved homothallic mating between the mother cell and her bud (pedogamy) under specialized conditions, and eventually long holobasidia with terminal basidiospores were observed (Fig. 1). Golubev designated the teleomorph as the new species *Xanthophyllomyces dendrorhous*, and *P. rhodozyma* CBS 5905 was considered to be the anamorph of *X. dendrorhous* [10, 28]. Although it has been commonly assumed that

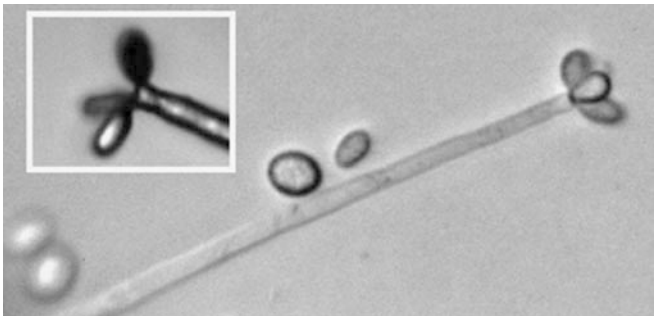

Fig. 1 Basidia with basidiospores formed by conjugated mother cell and a bud. (Photograph courtesy of Carlos Echavarri-Erasun, University of Wisconsin)

all strains of this yeast should be designated as *X. dendrorhous*, Kucsera et al. [24] reevaluated the life cycle of certain strains and reported that the distinct anamorphic species *P. rhodozyma* and teleomorphic *X. dendrorhous* existed based on the inability of certain strains to form basidia and basidiospores. Fell and Blatt [9] concluded from sequence analysis of rDNA IGS and ITS regions that *P. rhodozyma*, isolated from a beech (*Fagus*) tree (CBS 5905), was a distinct species from five strains isolated from other deciduous trees. It is becoming apparent that *P. rhodozyma*/*X. dendrorhous* has a wider habitat and greater biological diversity than originally believed, and different species and lineages of this group of related yeasts correspond to different geographic location and host specificity as well as to other biological factors [9, 10]. Golubev [10, 11] suggested that the anamorph *P. rhodozyma* be designated "Rhodomyces dendrorhous", since it apparently resembled yeasts described by Ludwig in the late 1800s, but these strains are no longer available and this suggestion has been refuted [7].

Regulation of carotenoids and their function in *P. rhodozyma*/*X. dendrorhous*

During studies at UC-Davis, and since becoming a professor at the University of Wisconsin in 1985, my laboratory has been interested in the physiology of biosynthesis and function of astaxanthin in *P. rhodozyma*. During graduate studies at UC-Davis in the laboratory of Professor Michael Lewis, I demonstrated that astaxanthin is produced as a secondary metabolite in *P. rhodozyma*, and that certain nutritional conditions affected its biosynthesis [18]. In particular, excessive levels of glucose and semi-anaerobic conditions markedly decreased carotenoid formation. Under semi-anaerobic conditions or in high glucose media, not only was the level of carotenoids in the yeast reduced, but the carotenoid composition consisted of carotenes and the characteristic xanthophylls (oxygenated carotenoids including astaxanthin) were absent or present at very low concentrations. Evidence was also found which suggested that the nitrogen source and rate of nitrogen utilization affected astaxanthin biosynthesis [3, 18].

Initial studies conducted at Davis suggested that light did not influence carotenoid formation and composition in *P. rhodozyma* [18], but a later study showed that light could markedly affect yeast pigmentation, particularly at high light fluencies and in the presence of photosensitizers [2]. These studies on dramatic changes in pigmentation by light and respiratory inhibitors provoked us to evaluate various metabolic perturbants and conditions that may affect astaxanthin formation. We found that certain respiratory inhibitors such as antimycin A strongly modulated astaxanthin biosynthesis [3]. In an interesting series of experiments, it was found that plating of *P. rhodozyma* onto yeast-malt agar containing 50 μM antimycin A gave rise to colonies of quite unusual morphology, characterized by a nonpigmented lower smooth surface, that developed highly pigmented papillae after 1–2 months of incubation. Isolation and purification of these papillae showed the development of mutant strains with a two- to five-fold increase in pigment content compared with the parental strain UCD 67-385. Surprisingly, these hyperproducer strains were more sensitive to antimycin and other respiratory inhibitors in liquid media and buffers, despite being isolated as resistant colonies on antimycin plates. These strains used certain nitrogen sources more slowly and were more rapidly killed by hydrogen peroxide. The carotenoid composition of the strains was also distinct from the parent, containing highly oxygenated carotenoids not normally observed in the parent, and these chemical properties of the unknown carotenoids suggested that the strains isolated on antimycin agar tended to oxygenate and desaturate carotene precursors to a greater extent than the parent. The physiology of the antimycin isolates led us to hypothesize that alteration of cytochrome B with spillover of reactive oxygen species (ROS) stimulated carotenoid production. In 1989, we also hypothesized that cytochrome P-450 components may be involved in oxygenation and desaturation of carotenes and increased astaxanthin production [3]. To my knowledge, this was the first postulate advanced that cytochrome P-450 systems are involved in oxygenation of carotenoids during biosynthesis. The actual enzymes involved in astaxanthin biosynthesis have yet to be elucidated, but transformation of a β-carotene mutant isolated in our laboratory with a gene coding for a cytochrome P-450 suggested that such an enzyme system is involved in the enzymatic conversion of β-carotene to astaxanthin [15]. If a cytochrome P-450 is indeed responsible for β-carotene conversion to astaxanthin, this would be one of the most complex reaction sequences carried out by the P-450 superfamily (C. Jefcoate, personal communication). The proof for this hypothesis should become available when the enzymes responsible for biosynthesis of astaxanthin are actually isolated in vitro and characterized. Recent studies in our laboratory have shown that the β-carotene mutant used for transformation may actually be a regulatory mutant,

which leads to some question regarding the sole involvement of a cytochrome P-450 in oxygenation of carotene precursors to astaxanthin (Echavarri-Erasun and Johnson, unpublished data).

The studies involving respiratory inhibitors led to the hypothesis that a function of astaxanthin and perhaps other carotenoids in *P. rhodozyma*/*X. dendrorhous* is to provide protection against ROS and accompanying cellular damage. 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 $^1O_2$ [14, 20]. We showed that *P. rhodozyma*/*X. dendrorhous* is particularly susceptible to activated oxygen species including H_2O_2 and O_2^- and that carotenoids possibly compensate for this sensitivity [30, 32, 33]. The yeast appears to have an unusual physiology for toleration of carotenoid, including low levels of certain enzymes such as catalase, and it possesses Mn-superoxide dismutase but completely lacks other classes that are present in many other yeasts [30]. We also provided intriguing evidence that the astaxanthin biosynthesis level may be controlled at the transcriptional level in part by the amount of ROS in the cell [32].

The observations that ROS are involved in the regulation of carotenoid biosynthesis and that carotenoids may protect against cellular injury and death suggest that *P. rhodozyma*/*X. dendrorhous* could serve as an excellent single-celled eukaryotic model to study the molecular biology of aging and degenerative diseases in eukaryotes [6, 8, 31] as related to damage and pathology by ROS. Most studies of cellular aging with fungi have used *Saccharomyces cerevisiae* since there are powerful genetic tools and a genome sequence available for studies in *S. cerevisiae*. However, evolutionary studies have shown that basidiomycetous fungi are more closely related to humans than are ascomycetous fungi, and thus *P. rhodozyma*/*X. dendrorhous* may be a more appropriate model system Furthermore, it is interesting that ethanol strongly increases carotenoid formation in *P. rhodozyma*/*X. dendrorhous* [12], and studies in a simple eukaryote could provide insights into the effect of ethanol on eukaryotic cells.

Biotechnology of astaxanthin

Since animals cannot synthesize carotenoids, these must be provided into their feed for deposition into the flesh, carapace, or plumage. Astaxanthin is valuable commercially mainly for pigmentation of salmonids raised in aquaculture. Salmon farming is a multibillion dollar industry that is growing and gradually replacing the world's wild salmon fisheries [13, 26]. The most expensive ingredient in salmonid feeds is astaxanthin, and though the actual revenues are privately held, it has been estimated that the market for astaxanthin is > US $100 million per year. This market is supplied primarily by chemically synthesized astaxanthin. However, since the synthesis is relatively difficult and expensive compared to simpler carotenoids such as β-carotene, several companies and academic laboratories have investigated biological sources of astaxanthin. Among these sources, *P. rhodozyma*/*X. dendrorhous* and the microalga *Haematococcus pluvialis* have attracted much interest because of their ability to synthesize high levels of astaxanthin and because *P. rhodozyma*/*X. dendrorhous* can be grown in fermentor culture to very high cell densities (> 50 g dry cell weight per liter) [8, 16, 20]. We first suggested in 1977 that *P. rhodozyma* could be a source of astaxanthin for salmonids raised in aquaculture [17] and as a carotenoid source for other animals [18, 21].

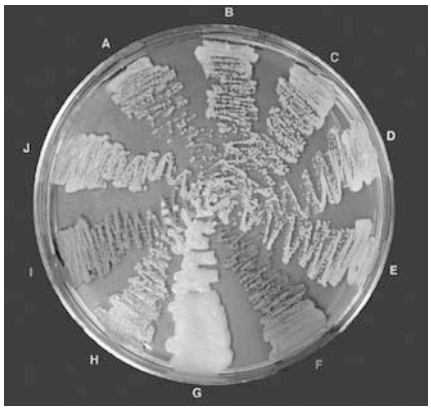

Fig. 2 Color mutants of *Xanthophyllomyces dendrorhous* isolated in our laboratory (see cover of this issue for the colors). The parental strain was UCD 67-385, and mutants were mainly obtained by mutagenesis using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) as described [3]. *A* UCD 67-385 (*X. dendrorhous*), *B* CBS5909 (*Phaffia rhodozyma*), *C* Y174-30 (isolate of *X. dendrorhous* from Michigan; courtesy of Clete Kurtzman), *D* AF-1 (albino mutant of UCD 67-385), *E* YAN-1 (β-carotene mutant), *F* CAX (hyperproducer), *G* YAN-1-OP (lycopene mutant from UCD 67-385), *H* YAN-1-OO (β-carotene hyperproducer), *I* CAX-HP20 (astaxanthin hyperproducer), *J* YAN-1-HP (β-carotene hyperproducer). (Photograph courtesy of Carlos Echavarri-Erasun)

The primary limitations to utilizing *P. rhodozyma*/*X. dendrorhous* as a commercial astaxanthin source is the low astaxanthin levels found in wild-type isolates and the thick cell wall and capsule of the yeast which apparently hinders astaxanthin uptake [8,16, 20]. Several companies have developed astaxanthin-hyperproducing strains that produce > 10,000 μg per g yeast in industrial fermentors. My laboratory has also developed innovative selection and screening strategies for mutant isolation, including ROS selection [33], ethanol treatment [12], and flow cytometry and cell sorting [1]. We have isolated a variety of mutants affected in carotenogenesis (Fig. 2). These mutants have been valuable as lead strains for hyperproducers, as strains for the development of genetic tools, and for the elucidation of genes involved in astaxanthin biosynthesis. Most commercial mutants have been isolated by chemical mutagenesis and random screening, but this approach has considerable limitations. Our laboratory has attempted to elucidate the functions of astaxanthin in the yeast to develop

positive selections for higher pigmentation [33]. Other laboratories have isolated the genes responsible for astaxanthin biosynthesis and have developed genetic tools such as transformation to assist in the isolation of mutants [34]. The second limitation impacting the industrial utility of *P. rhodozyma*/*X. dendrorhous* has been hindered absorption of astaxanthin due to the yeast's thick cell wall. At UC-Davis, we developed an interesting process in which *Bacillus circulans* was inoculated into fermentors of growing *P. rhodozyma*, and the bacillus secreted lytic enzymes that partially hydrolyzed the cell wall and facilitated uptake of the pigment by rainbow trout [17, 18, 21]. Mechanical breakage also facilitated uptake of the astaxanthin in the flesh of rainbow trout [17, 21]. The biotechnology industry has developed other means of pigment liberation by the yeast including optimization of drying conditions, mechanical breakage, and enzyme treatment. Considerable efforts have been undertaken to optimize fermentation conditions for enhanced astaxanthin production, such as the development of specialized media, including fed-batch processes, and stimulation of carotenoid production by physical and chemical manipulations of the fermentation. Lastly, the enhanced understanding of the molecular biology of *P. rhodozyma*/*X. dendrorhous* and the genes and enzymes involved in astaxanthin biosynthesis and its regulation [25, 34; Echavarri-Erasun, Bradshaw, Johnson, unpublished results] will have a marked impact on the development of improved strains and industrial production processes.

Interaction of *P. rhodozyma*/*X. dendrorhous* with biotic factors in its habitat

I would like to conclude this article with a brief discussion of observations made in our laboratory of possible factors in the ecological niche of *P. rhodozyma* that would contribute to the yeast's ability to colonize and thrive in its habitat. I feel that this subject would be of most interest to Herman Phaff, due to his deep appreciation of the ecology of yeasts. We postulated that deciduous trees may contain a substance that would be lethal to many yeasts but could be tolerated by *P. rhodozyma*/*X. dendrorhous* due its unique physiology and carotenoid content [30, 31, 32]. The yeast also may biosynthesize astaxanthin to protect against ROS produced by the reaction of ozone with plant compounds or photosensitizers [23, 33], including compounds extracted in ethyl acetate from *Betula* that generate ROS when activated by ultraviolet light [32]. In addition, we recently observed that carotenoid production by *P. rhodozyma*/*X. dendrorhous* was markedly increased in colonies in the vicinity of a fortuitous fungal contaminant on a yeast malt plate (Echavarri-Erasun and Johnson, manuscript submitted). The fungus was isolated and identified as *Epicoccum nigrum*, and extracts from the fungus were demonstrated to enhance carotenoid formation by various strains of *P. rhodozyma*/*X. dendrorhous* grown in liquid culture, including an albino mutant and a β-carotene mutant. Although the compound(s) responsible for enhancement of astaxanthin biosynthesis from *E. nigrum* have not been isolated and characterized, it is intriguing that *E. nigrum* is a well documented plant pathogen that produces compounds deleterious to plants and competitor fungi, including ROS as well as organic antimicrobials, and thus carotenoid biosynthesis in *P. rhodozyma* may function to avoid killing by the noxious compounds produced by the fungus.

Conclusion

In conclusion, the contributions of Herman Jan Phaff to yeast ecology are unparalleled. In addition to his deep appreciation for yeasts and their biology, Herman was a wonderful mentor and teacher. His perseverance, integrity and honesty in science are particularly vivid in my memory. Although often not considered a "colorful" and boisterous individual, his quiet encouragement was of tremendous importance and offered me the opportunity to be involved in a colorful odyssey with a yeast discovered by him.

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