

PHAFFIA RHODOZYMA AND XANTHOPHYLLOMYCES DENDRORHOUS: ASTAXANTHIN-PRODUCING YEASTS OF BIOTECHNOLOGICAL IMPORTANCE

A MINIREVIEW

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The nutritional value of carotenoids such as α - and β -carotene, β -cryptoxanthin and astaxanthin has been known for many years, and their antioxidant properties and their efficiency in the prevention of certain human diseases have also been claimed. Accordingly, interest in these compounds from a nutritional aspect has increased substantially. *Phaffia rhodozyma* and *Xanthophyllomyces dendrorhous* are the most promising industrial sources of astaxanthin. Since *Phaffia* was described in 1972, many studies of the phylogenesis, and biochemical and biotechnological properties of this red-pigmented yeast have been performed. The commercial demand for astaxanthin is increasing and, although biological production is still not economical, progress is being made in strain improvement and in fermentation methods.

Keywords: astaxanthin, *Phaffia rhodozyma*, *Xanthophyllomyces dendrorhous*

1. Description and characteristics of *Phaffia rhodozyma*

In the 1960s, Herman Jan Phaff isolated *Phaffia rhodozyma*, a carotenoid-producing orange-red budding yeast (PHAFF et al., 1972). The isolates were initially obtained from slime exudates of deciduous trees from Japan, and from the Pacific Northwest region of North America. Later, other strains were isolated in Russia, Finland, Chile and in other regions of the United States. In contrast with *Rhodotorula rubra*, which is a non-fermenting yeast, these yeast strains ferment glucose, sucrose, raffinose and maltose. The intensity of their colour depends on the strain and on the maturation of the culture (MILLER et al., 1976; JOHNSON, 2003). In 1976, ANDREWES and co-workers suggested a possible biosynthetic pathway for the formation of astaxanthin in *Phaffia rhodozyma*, astaxanthin surprisingly being the principal carotenoid in this species; moreover the 3R,3'R configuration of this astaxanthin was the opposite of that of astaxanthin from other sources.

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The genus *Phaffia* was proposed at this time (MILLER et al., 1976). Its morphology, multilayered cell wall, heterobasidiomycetes yeast-like, enteroblastic bud formation, metabolic properties and pigmentation suggested that it was a basidiomycetous fungus, although MILLER and co-workers (1976) did not observe a sexual life cycle. Hence, it was classified as fermentative yeast of the *Deuteromycotina* (*Blastomycetes*). GOLUBEV (1995) subsequently detected sexual activity, involving homothallic mating between the mother cell and her bud, and eventually observed long holobasidia with terminal basidiospores in certain strains. The teleomorph was designated as a new species *Xanthophyllomyces dendrorhous*, and *P. rhodozyma* CBS 5905 was considered to be an anamorph of *X. dendrorhous*. 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*).

In the 1990s, different molecular approaches were utilized to acquire further data for the taxonomic, phylogenetic and biochemical characterization of *Phaffia/Xanthophyllomyces* isolates. The electrophoretic karyotype results obtained with a contour-clamped homogeneous electric field (NAGY et al., 1994; ADRIO et al., 1995) and orthogonal field alternation gel electrophoresis (NAGY et al., 1995a; 1995b) revealed the existence of substantial chromosomal length polymorphisms among the strains. The correct number of chromosomes per haploid cell is difficult to determine as a consequence of the chromosomal DNA co-migration, nevertheless under optimized separation conditions, a better resolution could be achieved, and a more exact chromosome number could be claimed (PALÁGYI et al., 1996a; 1999). A different approach, radiation-induced chromosomal rearrangements, has also been applied to gain exact data on the genome size (NAGY et al., 1996; 1997). In these experiments, 7–17 distinct chromosomal bands were resolved, and the estimated total genome size of the strains varied between 15.4 and 23.2 Mbp. Surprisingly, some strains previously considered to be identical in origin exhibited differences in their electrophoretic karyotypes, and the type strain of *P. rhodozyma* (CBS 5905) displayed a substantially different chromosomal number and genome size.

VARGA and co-workers (1995a; 1995b) compared the isoenzyme profiles of various *P. rhodozyma* strains and concluded that isoenzyme markers were more useful than RAPD or ribosomal DNA analysis. Their rDNA analyses revealed only a limited degree of intraspecific variation. The carbon-source assimilation patterns of wild-type isolates were determined by PALÁGYI and co-workers (1998; 2001). In a structural and phylogenetic analysis with the actin gene of *P. rhodozyma* (WERY et al., 1996), the architecture of *P. rhodozyma* introns proved similar to those of the basidiomycete *Filobasidiella neoformans* (perfect stage of *Cryptococcus neoformans*), and the *P. rhodozyma* introns could not be spliced in the case of the ascomycetous yeast, *Saccharomyces cerevisiae*.

CASTILLO and CIFUENTES (1994) detected double-stranded RNA (dsRNA) and virus-like particles (VLPs) in certain *P. rhodozyma* isolates, while PFEIFFER and

co-workers (1996) found variability among *Phaffia* strains as concerns both the number and the length of the harboured dsRNAs. They also observed the transmission of VLPs through the basidiospores during the sexual cycle.

Other molecular techniques have likewise been used successfully to characterize *P. rhodozyma* strains. PALÁGYI and co-workers (2004) performed random amplified polymorphic DNA (RAPD) analysis involving 13 *Phaffia/Xanthophyllomyces* yeast strains, and made use of the RAPD data for cluster analysis. They demonstrated a correlation with the results of earlier studies: one of the clusters contained isolates (including the type strain CBS 5905), which could not sporulate, but were able to produce respiratory-deficient petite mutants (KUCSERA et al., 1998); these isolates have a genome size in excess of 20 Mbp (NAGY et al., 1994). The other cluster comprised *Xanthophyllomyces* strains with sporulation capability, a petite-negative character and a genome size of about 16 Mbp. In this study, some markers allowed the differentiation of *Xanthophyllomyces* strains. This high intraspecific genetic variability might be explained by the aneuploid or polyploid state of the strain. A higher than haploid ploidy state for *Phaffia/Xanthophyllomyces* was suggested by several independent studies; NAGY and co-workers (1997) concluded that *X. dendrorhous* is aneuploid, while HERMOSILLA and co-workers (2003) suggested a diploid, and MEDWID (1998) a polyploid state.

2. The biosynthesis of astaxanthin

Astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione) is the principal pigment in salmonids, crustaceans and various other organisms (STEVEN, 1948; TORRISSEN et al., 1989). Carotenoids are of importance in animals and humans, including enhancement of the immune response, conversion to vitamin A, and the scavenging of oxygen radicals. Animals cannot synthesize carotenoids, and these pigments must therefore be added to the feeds of farmed species, including aquacultured salmon (GOODWIN, 1984, 1986; BENDICH & OLSON, 1989). The colour of the meat of salmon and trout is an essential demand for customers (BAKER & GUNTHER, 2004), and for this reason the aquaculture industry requires substantial amounts of carotenoids as animal feed additives per year. The yeasts *Phaffia/Xanthophyllomyces* and the green microalga *Haematococcus pluvialis* are the currently known microbial systems with commercial value for astaxanthin production. These yeasts are also potentially useful dietary supplements because of their high contents of unsaturated fats, vitamins and minerals (JOHNSON et al., 1980a).

The strong cell wall of *P. rhodozyma* impedes the absorption of astaxanthin from the gut of animals, but if its cells are ruptured by mechanical treatment, the carotenoid is deposited in the flesh of trout or salmon (JOHNSON et al., 1977; 1980a; 1980b; GENTLES & HAARD, 1991). Enzyme treatment can also be effective: OKAGBUE and LEWIS (1983) co-cultured the yeast with a cell wall-lysing enzyme-producing *Bacillus circulans* to increase extractability.

In 1976 ANDREWES and co-workers suggested the first scheme for astaxanthin biosynthesis; production of the tetraterpenoid astaxanthin through the acetate-mevalonate pathway. The first committed step of isoprenoid biosynthesis is mevalonate formation. This involves two consecutive reactions: the synthesis of hydroxymethylglutaric (HMG) acid from acetyl CoA and acetoacetyl CoA (catalyzed by HMG CoA synthase) and the reduction of HMG CoA to mevalonic acid (catalyzed by HMG CoA reductase). Mevalonate is the precursor of the 5-carbon isoprene unit, the precursor of isopentenyl pyrophosphate, geranyl pyrophosphate and farnesyl pyrophosphate. Phytoene is synthesized from these units, leading through many steps and intermediates including lycopene, β -carotene and echinenone to astaxanthin. However, ANDREWES and co-workers (1976) additionally detected 3-hydroxy-3,4'-didehydro- β,ψ -caroten-4-one, but could not explain its biosynthesis in terms of the above scheme; hence, they postulated that β -carotene is first converted to echinenone, which is then hydroxylated. The resulting 3-hydroxyechinenone (3-hydroxy- β,β -caroten-4-one) is next oxidized to phenicoxanthin (3-hydroxy- β,β -caroten-4,4'-dione), which is hydroxylated to astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione). Later, other intermediates were detected including β -zeacarotene (JOHNSON & LEWIS, 1979), 3,3'-dihydroxy- β,γ -carotene-4,4'-dione (DCD) and torulene (AN et al., 1989), which indicated a bypathway through β -zeacarotene $\rightarrow$ torulene $\rightarrow$ 3-hydroxy-3',4'-didehydro- β,ϕ -caroten-4-one (HDCO) $\rightarrow$ DCD to *trans*-astaxanthin.

3. Improvement of astaxanthin production

For *Phaffia/Xanthophyllomyces* to be of commercial value as an astaxanthin source, it is important both to optimise the fermentation parameters and to improve the astaxanthin-producing ability of the industrially used wild-type yeast strains.

JOHNSON and LEWIS (1979) determined some of the basic fermentation parameters, including the pH, the aeration rate and the nature and concentration of the carbon source. Considerable efforts have been made to use certain cheap food-processing waste products as low-cost culturing media. There have been studies on alfalfa residual juice (OKAGBUE & LEWIS, 1984), cane molasses (HAARD, 1988), sugar cane juice (FONTANA et al., 1996), corn wet-milling co-products (HAYMAN et al., 1995), grape juice (MEYER & DU PREEZ, 1994), hydrolysed peat (MARTIN et al., 1993; ACHEAMPONG & MARTIN, 1995), raw coconut milk (DOMÍNGUEZ-BOCANEGRA & TORRES-MUÑOZ, 2004) and vegetable oils (LUKÁCS et al., 2005). Nutrient-rich culture media and the presence of astaxanthin precursors have generally been found to be favourable for increased astaxanthin production.

Carotenogenesis in many organisms is regulated by light. AN and JOHNSON (1990) observed increased growth and carotenoid formation in *P. rhodozyma* when it was exposed to light and antimycin, a respiratory chain inhibitor. It was suggested that carotenoids are formed in response to oxygen radical generation in the cells. Extracts from deciduous trees contain substances lethal to many organisms and it is presumed

that the high level of astaxanthin in these organisms enables them to live in slime exudates. AN and co-workers (1996) investigated the effect of oxygen radical and aeration on carotenogenesis and growth of *Phaffia rhodozyma*.

Astaxanthin is synthesised during and after the growth of the yeast, and the presence of a suitable carbon source is important for astaxanthin biosynthesis during the non-growth phase (JOHNSON & LEWIS, 1979; AN et al., 1989; 1991). It has been stated that *P. rhodozyma* excretes an alcohol, or acetic acid, or citric acid cycle intermediates as carbon-containing intermediates during growth, and through reassimilation it stimulates carotenogenesis (JOHNSON & AN, 1991). This has led to several studies of the effects of ethanol (GU et al., 1997) and different monoterpenes (MEYER et al., 1994) on carotenoid production: ethanol and α -pinene were found to be stimulatory at low concentrations. It was also observed that, when *P. rhodozyma* was grown on succinate, certain micronutrients (manganese and iron) stimulated carotenoid synthesis, possibly by the exacerbation of oxygen radical formation (JOHNSON & AN, 1991). SANPIETRO and KULA (1998) investigated the effects of low temperature (4 °C) and various inhibitors (nicotine and diphenylamine) on astaxanthin biosynthesis. One of the difficulties in the industrial utilization of these yeasts is that they cannot grow above 27 °C: the optimum growth temperature for *P. rhodozyma* is 22.5 °C (JOHNSON & LEWIS, 1979). Efforts to obtain strains with significantly increased optimum growth temperatures have not been successful to date.

Although *Phaffia* is the best available natural source of astaxanthin, wild-type strains produce only a maximum of ~500 µg of total carotenoids per gram of yeast (JOHNSON & AN, 1991). Accordingly, a number of strain improvement approaches have been made to increase the level of astaxanthin production of wild-type isolates. Mutagenic treatment with antimycin, N-methyl-N'-nitro-N-nitrosoguanidine, UV light, H₂O₂, β -ionone, γ -irradiation, ethidium bromide and acriflavin have been used successfully to isolate various hyperproducing strains (AN et al., 1989; LEWIS et al., 1990; AN et al., 1991; FANG & CHENG, 1993; MEYER et al., 1993; SCHROEDER & JOHNSON, 1993; PALÁGYI et al., 1995; SCHROEDER et al., 1996; PALÁGYI et al., 1996b; BON et al., 1997; KIM et al., 2004; SUN et al., 2004; PALÁGYI et al., 2005).

In an attempt to increase the astaxanthin content of *Phaffia*, CHUN and co-workers (1992) isolated carotenoid-hyperproducing hybrids (>2000 µg of carotenoid/g of yeast) after the PEG-Ca-mediated protoplast fusion of double auxotrophic mutants of the parental strains. They confirmed the karyogamy by the isolation of recombinants after mitotic segregation of parental genetic markers. NAGY and co-workers (1996) also obtained hybrids with increased carotenoid content: in this case, the fusion event was confirmed by PFGE analysis. Despite the success of these efforts, mutagen treatment generally proved to be more effective in increasing the carotenoid content of these yeasts.

The third potential approach is cloning and overexpression of carotenoid biosynthetic genes of *Phaffia/Xanthophyllomyces* strains. An efficient transformation system for *X. dendrorhous*, which is required for such experiments, was developed and optimized by WERY and co-workers (1998). The first cloned carotenoid biosynthetic gene of *X. dendrorhous* was phytoene desaturase (VERDOES et al., 1999b). Later, genes

encoding isopentenyl diphosphate isomerase and epoxide hydrolase were also isolated, and successfully expressed in *Escherichia coli*. The lycopene production of this organism through heterologous expression was increased 3-fold (VERDOES et al., 1999a; 1999b). Specific carotenoid biosynthetic mutants were constructed, targeting the *crtYB* locus (encoding the chimeric phytoene synthase-lycopene cyclase). Overexpression of these genes resulted in transformants with higher carotenoid content. The increase was mainly due to elevated β -carotene and echinone contents, the astaxanthin content remaining unaffected or proving even lower (VERDOES et al., 2003).

Carotenoid-hyperproducing mutants can be selected by visual examination on the basis of the intensity of the red colour of the colonies. A more powerful method for the screening or enrichment of carotenoid-overproducing cells after mutagenesis is flow cytometry based on the autofluorescence of certain metabolites, including carotenoids (AN et al., 1991). Another approach is to use certain inhibitors of the carotene biosynthetic pathway, as an example CHUMPOLKULWONG and co-workers (1997) used diphenylamine to select astaxanthin-overproducing strains.

4. Conclusion

The red-pigmented yeast *P. rhodozyma*, first isolated in the 1960s, has generated considerable biotechnological interest because of its principal carotenoid astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione). It serves as a natural astaxanthin source in animal diets for colouring salmonids, lobsters, and the egg yolk of poultry and quail. The excellent antioxidant properties of astaxanthin are also attractive commercially for the pharmaceutical, cosmetics and food industries (MELENDEZ-MARTINEZ et al., 2004; KIM et al., 2005). Although all-trans astaxanthin can be manufactured synthetically, the interest in *Phaffia/Xanthophyllomyces* as the most promising natural source of this pigment will continue in the future.

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