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Biotechnological potential of *Phaffia rhodozyma*

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Abstract
Pigments, anthocyanin and carotenoids are natural compounds that are attractive in color and easy to extract. Astaxanthin is the principal carotenoid pigment responsible for the distinctive orange red pigmentation in marine invertebrates, fish, birds, salmonids and crustaceans. These animals obtain the pigment through their diet, which includes various astaxanthin producing microorganisms. Among such microorganisms *Phaffia rhodozyma* (red yeast), produces astaxanthin as the major carotenoid. In addition to being used as food colorant, there is a growing interest in using astaxanthin as a fish feed supplement. This review examines the potential of using *P. rhodozyma* for large-scale production of astaxanthin.

Key words: Phaffia rhodozyma; astaxanthin; natural colorant; antioxidant; strain development; commercial production.

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INTRODUCTION
From time immemorial, color has been an important criterion for acceptability of products like textiles, cosmetics, food and other items. Synthetic colorants become undesirable due to their hazardous effects to health and environment (Carvalho *et al*., 2005). Presently, the number of permitted artificial colors has reduced considerably, and as a consequence, the interest in natural colorants has increased significantly (Carvalho *et al*., 2003; Joshi *et al*., 2003). Anthocyanin and carotenoids, which provide attractive colors and are safe for consumption, have become the major alternatives to synthetic colorants. Interestingly, the pigment from *Phaffia rhodozyma* is responsible for the pink-to-red color of marine animals, e.g. salmon, lobster and shrimp (Johnson & Lewis, 1979; Johnson *et al*., 1980) and certain birds (Johnson, 2003). These animals cannot synthesize astaxanthin but rather they consume it through the ingestion of microorganisms that synthesize the compound (Bon *et al*., 1997).

The use of astaxanthin as a colorant in the aquaculture industry has been increasing (Johnson et al., 1980). Fish feed is commonly supplemented with astaxanthin so that fish acquire the appropriate degree/tone/shade/hew of color and hence increase their appeal, e.g. salmonids, rainbow trout and kuruma prawn (Johnson et al., 1977). In early 1990s, the price of synthetic astaxanthin was $ 2,000/kg and production of the natural counter part is much more expensive (Meyer & du Preez, 1994). Thus, if astaxanthin could be available from a dependable natural source, and at a competitive price, it would have a ready

market. Besides *P. rhodozyma*, only a few other microorganisms produce astaxanthin(Andrews et al., 1976). These include *Brevibacterium* sp., *Mycobacterium lacticola* (Nelis & de Leenheer, 1989), *Agrobacterium auratium* (Yokoyama & Miki, 1995), and *Haematococus pluvialis* (Lotan & Hirschberg, 1995).

However, *Phaffia* is the most promising for commercial production of astaxanthin because this yeast produces astaxanthin as the primary carotenoid pigment and can be grown in an economically feasible process (Yamane et al., 1997a). The work done so far to promote industrial production of astaxanthin from *P. rhodozyma* is discussed herein.

Astaxanthin chemistry

Astaxanthin is an oxygenated carotenoid that is chemically known as 3,3"-dihydroxy-4,4'-diketo-β-carotene (Fig. 1). Carotenoids are chemical derivatives of 40-carbon polyene chain, considered as the backbone of the molecule. The chains are terminated by cyclic end groups. The hydrocarbon carotenoids are known as carotenes while oxygenated derivatives are xanthophylls. Astaxanthin is synthesized *de novo* only by microorganisms and plants.

Phaffia features

In 1960, Herman Phaff Phaff and his collaborators, while working on ecology of yeast flora associated with trees of Japanese Islands and West Coast of North America, identified *P. rhodozyma* (Phaff, 1986). A variety of ascosporogenous and basidiomycetous yeasts were isolated and most interesting among them was a group of imperfect fungi, including an unusual imperfect yeast that formed colonies with a distinct orange red color. The organism was found to represent a new yeast genus, which was initially designated as *Rhodozyma montanae,* due to its orange-red color and report from mountain regions (Johnson, 2003).

Phaffia, which is commonly known as red yeast since it produces the red colored pigment – astaxanthin, belongs to basidiomycetes (Miller et al., 1976) and has no sexual cycle. However, the formation of holobasidia with terminal basidiospores after mother daughter cell conjugation has been described. The perfect stage is designated as *Xanthophyllomyces dendrorhous* (Johnson, 2003). Its growth is associated with enzymes like invertase and urease. Astaxanthin, the major carotenoid pigment of *P. rhodozyma*, has 3R,3R'-configuration which differs from that obtained from other sources (Andrews & Starr, 1976). Astaxanthin (Fig. 1) is the first example of a naturally occurring carotenoid biosynthesized in different optical forms (Johnson, 2003). Cell morphology is presented in Fig. 2 (www.ASM MicrobeLibrary.org).

Production conditions

Medium: Astaxanthin is produced as a secondary metabolite and specific nutritional conditions are required for its biosynthesis (Johnson & Lewis, 1979). Commonly, astaxanthin production by *P. rhodozyma* utilizes inexpensive substrates, e.g. corn wet-milling and co-products (Hayman et al., 1995), molasses (Haard, 1988) or sugarcane juice (Florencio et al., 1998). *Phaffia* is capable of utilizing as many as 99 compounds as sole source of carbon (Vagvolgy et al., 1996). In industry, inexpensive substrates (Kesava et al., 1998) such as corn wet-milling (Hayman et al., 1995) and peat hydrolyzates (Vazquez & Martin, 1998) are used as substrates for growth and carotenoid production by *P. rhodozyma*. Grape juice has also been tested as a medium for *P. rhodozyma* (Meyer & du Preez, 1994). A medium containing corn starch hydrolysate (hydrol), corn steep liquor (CSL), urea and potassium phosphate have been devised for large-scale production of astaxanthin by *P. rhodozyma* (Kesava et al., 1998). Less expensive molasses (grade B or C) favour high yield of astaxanthin (Haard, 1988). Sugarcane juice, which is available at much lower price in some countries, is used as substrate due to its sucrose content (Fontana et al., 1996; Florencio et al., 1998).

Cultural aspects: Checking of C/N ratio is performed following a two-stage fed-batch culture system. In the first stage, cell growth is enhanced by maintaining low C/N ratio, while in the second step a high C/N ratio enhances astaxanthin production. Thus, a low initial C/N ratio of the medium supports cell growth but decreases astaxanthin production. Studies have shown that among other carbon sources, ethanol enhances

astaxanthin content, but decreases cell growth (Gu et al., 1997). Higher amounts of glucose, nitrogen and micro-aerophilic conditions decrease carotenoid formation markedly (Johnson, 2003). Fungal elicitors have been found to influence the growth as well as astaxanthin production (Wang et al., 2006). Experiments on optimization of astaxanthin yield by *P. rhodozyma*, in factorial design including various combinations of pH, temperature, inoculum volume, and carbon and nitrogen concentration have been carried out (Ramirez et al., 2001). Fermentation in medium with high concentration of ammonium and phosphate supported good pigment production, hence, astaxanthin yield (Flores-Cotera et al., 2001). Carbon, temperature and pH optima for growth and astaxanthin production by *P. rhodozyma* are presented in Table 1 and 2.

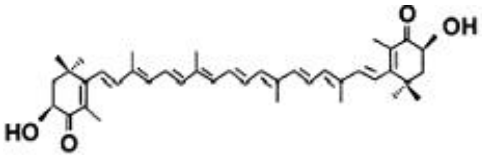

Figure 1: Chemical structure of astaxanthin.

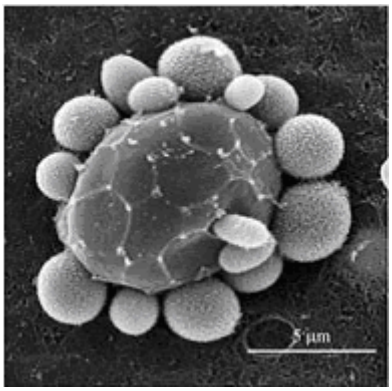

Figure 2: SEM showing cell morphology of *P. rhodozyma* (= *X. dendrorhous).*

Supplementation: Medium supplementation can increase astaxanthin production. Ethanol (0.2%) increases the specific rate of carotenogenesis (Gu et al., 1997) while mevalonic acid enhances astaxanthin biosynthesis in wild type *P. rhodozyma* (Calo et al., 1995). Monoterpenes like pinane, when added to medium, increase total carotenoid as well as astaxanthin production by *P. rhodozyma* (Meyer et al., 1994) while citrate supplementation (28 mM or more) increases pigment content of the cells (Flores et al., 2001). Addition of glutamate, glutamine, asparagine and proline increase biomass but decrease carotenoid formation. The TCA cycle intermediates are more useful as they repress carotenogenesis to a lesser degree (An, 2001). While medium supplemented with copper (>3.2 µM) increases astaxanthin content but decreases growth (from 11.3 to 10.2 mg/ml), iron (>1 µM) decreases both growth and astaxanthin production (Flores-Cotera & Sanchez, 2001).

Influence of light: Exposure of the agar plates to high light intensity inhibits growth and carotenogenesis of *P. rhodozyma.* Wild type strains treated with antimycin and grown in liquid medium regained both growth and carotenoid synthesis on exposure to light, which indicates a light mediated stimulation of alternate oxidase system, while the main respiratory chain is inhibited by antimycin (An & Johnson, 1990).

Fed-batch: Studies have been done on the effect of different feeding methods, e.g. constant feeding, exponential feeding, DO-stat and pH-stat fed-batch cultures on astaxanthin production by *P. rhodozyma.* The Highest carotenoid production was obtained from exponential fed-batch culture, although it is associated with a low biomass yield (Ho et al., 1999). Thus, to allow high cell mass as well as high carotene content a combination of glucose fed-batch culture with pH-stat and ethanol feeding based on DO-stat was performed. This two-stage fed-batch cultivation resulted in cell mass of 30 g/l (dry weight) with astaxanthin content of 0.72 mg/g (Yamane et al., 1997b).

Table 1: Carbon suitability for growth and astaxanthin production by *Phaffia rhodozyma.*

Carbon Source	Growth (g/l)	Total Astaxanthin (mg/l)	Astaxanthin Content (mg/g)	References
Xylose	3.8	-	-	Yamane et al, 1997a
	1.8	1.47	0.82	Fang & Cheng, 1993
Glucose	5.7	1.71	0.30	Yamane et al, 1997a
	4.8	1.81	1.63	Fang & Cheng, 1993
	4.7	-	1.40	Meyer et al, 1993
	-	-	1.90	Meyer & du Preez, 1994
Fructose	5.7	2.14	0.28	Yamane et al, 1997a
	4.9	7.15	1.45	Fang & Cheng, 1993
Mannitol	4.7	-	2.0	Meyer et al, 1993
	2.5	2.46	1.02	Fang & Cheng, 1993
Maltose	6.1	1.50	0.25	Yamane et al, 1997a
	4.05	5.33	1.32	Fang & Cheng, 1993
Sucrose	4.60	7.80	1.70	Fang & Cheng, 1993
	6.7	2.09	0.32	Yamane et al, 1997a
Molasses	-	0.015	-	Haard, 1988
	3.8	4.0	1.04	Fang & Cheng, 1993

Table 2: Temperature and pH suitability for astaxanthin production by *Phaffia rhodozyma.*

Temperature (^{0}C)	pH	Astaxanthin Production (mgL^{-1})	References
15	5.0	9.28	Fang & Cheng, 1993
20	4.5	2.14	Yamane et al, 1997b
22	5.0	3.75	Meyer & du Preez, 1994
			Johnson & Lewis, 1979

Strain development

Thick cell walls and capsule of the yeast apparently hinder diffusion of astaxanthin into the medium. Screening and selection strategies for mutants, including ROS selection (Schroeder et al., 1996), ethanol treatment (Gu, 1998), flow cytometry and cell sorting (An et al., 1991) have been attempted in strain development.

Strain development by mutation is the traditionally preferred practice as it is not restricted by the stringent biosafety legislations of genetically engineered strains. UV-treatment has not been effective in producing auxotrophic *P. rhodozyma* mutants, though auxotrophic mutants were produced with high efficiency through nystatin enrichment technique using N-methyl-N'-Nitro-N-nitrosoguanidine (NTG), resulting into 0.12% survivors (Retamales et al., 1998). Mutants generated by NTG overproduced astaxanthin (1.7mg/g dry biomass), in a medium containing glucose, mannitol or succinate as carbon source (Meyer et al., 1993). Astaxanthin overproducing mutants have also been developed using 1-methyl-3-nitro-1-nitrosoguanidine (MNNG), under corn based fuel ethanol spillage (Bon et al., 1997).

Random screening after chemical mutagenesis is a common practice for isolation of commercially utilizable mutants. However, modern biotechnological tools may overcome the limitations of chemical mutagenesis (Johnson, 2003). Among the various approaches that have been used to enhance astaxanthin yield by *P. rhodozyma*, genetic manipulation and protoplast fusion are the upcoming techniques (Meyer et al., 1993, 1994; Visser et al., 2003).

Recent efforts have focused on elucidating the functions of astaxanthin in yeast so as to develop better selection criteria for higher carotenoid producers (Schroeder et al., 1996). Isolation of the genes that are responsible for astaxanthin biosynthesis, and development of genetic tools, such as transformation, can lead to more rapid development of superior isolates (Visser et al, 2003).

Therapeutic role of astaxanthin

Active oxygen species including O_2, H_2O_2, and OH are suggested to be biological reactants that delay or prevent degenerative diseases like cancer, arteriosclerosis, cataract and aging (Ames et al., 1993). Several studies have indicated the role of carotenoids as antioxidants (An, 1991; Schroeder & Johnson, 1993, 1995). Astaxanthin provides a defense against death caused by O_2, which is important for survival of yeasts in natural habitats. Thus, astaxanthin biosynthesis by *P. rhodozyma* is regulated by singlet oxygen primarily by gene activation (Schroeder & Johnson, 1995). During the exponential phase, exposure to H_2O_2 (10 - 20 mM) enhanced astaxanthin production under shake-flask culture (Liu & Wu, 2006).

Limitations

A major limitation in the industrial utilisation of *P. rhodozyma* has been due to its thick cell wall, which reduces the availability of astaxanthin for absorption by aquatic animals (Johnson, 2003). A method has been developed at the University of California-Davis, USA, where *Bacillus circulans* was introduced into a culture of *P. rhodozyma*, to partially hydrolyse the yeast cell wall (which can also be done mechanically), and thus facilitate (Johnson et al., 1977, 1980). Carotenoid is highly sensitive to changes in pH, which is a major challenge in replacing synthetic colorants (Joshi et al., 2003).

Conclusion

Astaxanthin has immense potential in food, feed, pharmaceutical and cosmetic industries. This potential is ever increasing due to the role of the coumpound in preventing or delaying degenerative diseases (Johnson, 2003). Although most studies on cellular aging that are carried out with fungi have used *Saccharomyces cerevisiae*, but evolutionary studies have shown that basidiomycetous fungi are more closely related to human beings than other groups, which makes *P. rhodozyma* a more appropriate single-celled eukaryotic model system for studying the molecular biology of aging and degenerative diseases (Bauldaff & Palmer, 1993; Schroeder & Johnson, 1995). Industrial production of microbial pigments for use as natural colorants is considered to be still at an infant stage. Although several mutants of suitable isolates have been developed, further research in process development is required to improve the cost-effectiveness of pigment production. Genetic engineering, which has introduced new tools and opportunities for developing suitable strains, needs to be supported by designing of suitable fermenters.

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