Analysis of Astaxanthin and Other Carotenoids from Several *Phaffia rhodozyma* Mutants

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Astaxanthin and other carotenoids from 29 mutant strains of the yeast *Phaffia rhodozyma* obtained by means of benomyl and/or ethyl-methanesulfonate treatment and extracted with dimethyl sulfoxide (DMSO) were separated by high-performance liquid chromatography. Detection at 474 nm revealed variations in the pigment content of the different mutant strains. Hypopigmented mutants showed higher β-carotene contents than the wild type, whereas hyperpigmented mutants exhibited considerable increases (up to 232%) in astaxanthin contents. Furthermore, and contrary to the wild type, the pigments in some of the mutants could be directly extracted with ethanol, and although the yield of pigment decreased in relation to DMSO, the content of astaxanthin increased up to 80%.

Keywords: *Carotenoids; astaxanthin; benomyl; Phaffia rhodozyma*

INTRODUCTION

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is a carotenoid pigment that provides the natural pink color of salmonids, boiled crustaceans, and flamingoes; these animals are unable to direct astaxanthin biosynthesis and have to incorporate it from their diets. Wild salmonids and flamingoes have natural sources of pigment (mainly microalgae), but in fisheries it is necessary to add feed complements containing astaxanthin to obtain the desired degree of pigmentation (An *et al.*, 1989). In the past, the source of astaxanthin pigment for industrial purposes was crustacean debris, while more recently cantaxanthin and astaxanthin have been extensively used. Most recently, however, it has been shown that the use of astaxanthin from natural sources, especially from microorganisms, is preferable. Of these, only a few can carry out astaxanthin biosynthesis, including the yeast *Phaffia rhodozyma* (Johnson and Lewis, 1979).

This yeast was isolated from exudates of deciduous trees in Japan and Alaska (Miller *et al.*, 1976), and the biosynthetic pathway for astaxanthin was first suggested by Andrewes *et al.* (1976) and later by Johnson (1992).

The basic structure of carotenoids is composed of eight isoprene units. The structural formula of all carotenoids comes from that of lycopene, starting with different structural modifications (Goodwin, 1980). Natural carotenoids are generally present in the most stable structural form, *all-trans*, in which all of the double bonds are in the *trans* configuration (Lesellier and Tchapla, 1993). *Cis* isomers of carotenoid pigments are less stable to light and may undergo oxidation more rapidly than the *trans* compounds (Zechmeister, 1962).

The method most frequently used for carotene separations is thin-layer chromatography (TLC). The main advantages of high-performance liquid chromatography (HPLC) are the easier quantification of products and the speed of analysis, which in turn reduces photochemical or oxidative degradation. Such analyses are carried out on supports classically used for thin-layer pigment separation (Lesellier and Tchapla, 1993). The properties of the supports used together with the efficiency of HPLC explain the excellent results obtained in the separation of positional *trans*–*cis* isomers.

The purpose of this study was to characterize the pigment extracted with dimethyl sulfoxide (DMSO) and ethanol from several mutant strains of *P. rhodozyma*.

EXPERIMENTAL PROCEDURES

Yeast Strains. The *P. rhodozyma* strains used were the natural isolates UCD-FST-67-210 (Miller *et al.*, 1976) and several mutants generated from this strain in our laboratory. The medium used for their growth was YM (malt extract, 3 g/L; yeast extract, 3 g/L; peptone, 5 g/L; glucose, 10 g/L; and 3% agar for solid media). All strains were stored at −75 °C in 40% YM medium supplemented with 40% glycerol.

Reagents and Chemicals. Ethyl methanesulfonate (EMS), DMSO, and β-carotene were obtained from Sigma Chemical Co. (St. Louis, MO); methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate (benomyl) (analytical grade) was from DuPont (Wilmington, DE). All solvents were of HPLC grade from Romil Chemicals (Leics, U.K.).

Mutagenesis. To obtain different mutant strains of the yeast *P. rhodozyma*, as a first approach we used the fungicide benomyl, which is widely used as a mutagenic agent in *Saccharomyces cerevisiae* genetics for gene mitotic mapping. The mutants thus obtained were then mutated with EMS. For benomyl mutagenesis, the fungicide was dissolved in DMSO (Merck) and dispensed into the YM medium at 100, 50, 25, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, and 0.005 mg/mL final concentrations to determine the minimal inhibitory concentration (MIC) and the ED_{50} (concentration inhibiting growth by 50%) (Edgington *et al.*, 1970). Growth was measured and compared with untreated controls. EMS mutagenesis was done with cells grown in liquid YM medium to an optical density (600 nm) of 0.3–0.4. Cells were suspended in 0.96 mL of phosphate buffer

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(pH 7.0) in 10 mL bottles. EMS was added to give a 4% concentration, and the cell suspension was incubated for 10 min (>95% kill). Cells were then washed three times in buffer and grown for 24 h in liquid YM medium before plating onto YM plates (Sherman *et al.*, 1979; An *et al.*, 1989).

Sample Preparation for HPLC. Strains of *P. rhodozyma* were grown in YM broth. After 5 days, cells were harvested by centrifugation, washed with sterile water, and dried in an oven at 50 °C. Then, 0.2 g of dried yeast was resuspended in 5 mL of DMSO (preheated to 55 °C) and vortexed for 30 s. Afterward, 0.5 mL of 0.01 M sodium phosphate (pH 7.0) and 3 mL of hexane fraction from petroleum were added to the tube and vortexed for an additional 30 s to mix the aqueous and the organic phases; both phases were then separated by centrifugation (Sedmak *et al.*, 1990), and the pigment-containing upper hexane phase was recovered. Finally, the samples were filtered through Teflon membranes and stored at −20 °C until analysis.

Carotenoid Analysis. Total carotenoids were estimated by visible spectrophotometry at 474 nm. Individual carotenoid determinations were carried out by high-performance liquid chromatography (HPLC). Chromatographic separations were performed on a Beckman Ultrasphere silica 5 μm, 250 × 4.6 mm high-performance column with an Ultrasphere silica 5 μm, 45 × 4.6 mm guard column. The eluting solvent was the hexane fraction from petroleum/ethyl acetate 1/1 (v/v), flow rate being 1 mL/min and pressure 0.36 psi. The eluant was monitored at 476 nm.

Carotenoid Standards. Standard stock solutions of β-carotene (Sigma) were diluted in the hexane fraction from petroleum. The concentrations of the rest of the carotenoids were measured by area comparison with this compound. β-Carotene displayed linear calibration curves (peak area *vs* concentration) through the origin.

RESULTS AND DISCUSSION

Different strains of *P. rhodozyma* inoculated onto Petri dishes containing benomyl concentrations varying from 0.005 to 100 mg/mL grew when the compound concentration was equal to or less than 1 mg/mL. Growth was measured and compared with untreated wild yeast to calculate the percentage of inhibition of growth. The experimental value for the ED_{50} was *ca.* 0.01 mg/mL which, according to the classification of Edgington *et al.* (1970), means that the yeast *P. rhodozyma* is highly sensitive to this fungicide.

Cells of the wild strain UCD-FST-67-210 of *P. rhodozyma* were grown to an optical density of 0.4 at 660 nm and spread onto YM plates containing 1 mg/L benomyl fungicide. Colonies started to appear after 4 days of incubation and continued to grow up to the 12th day. Naked-eye observation revealed the presence of hypo- and hyperpigmented colonies after this simple treatment. From these experiments an albino strain (B1) and a hyperpigmented one (B10) were selected and their pigments studied by HPLC. The results of these analyses evidenced that strain B1, visually classified as hypopigmented, produced less *trans*-astaxanthin (66.1 μg/g of dry weight of yeast) than the wild type (265.9 μg/g), as it can be observed in Table 1. On the other hand, strain B10, visually classified as hyperpigmented, accumulated more *trans*-astaxanthin (374.5 μg/g dry weight of yeast) than the referred *P. rhodozyma* wild-type strain.

At the same time, standard mutations with EMS were done on the wild-type strain, these giving rise to strains E2, E3, E4, and E7; these four are hypoproducers of *trans*-astaxanthin as compared to the wild-type strain (Table 1). Finally, mutant B10, obtained by treatment with benomyl and later mutated by EMS, gave rise to hypo- (BE1, BE2, and BE4) and hyperpigmented (BE3, BE5, BE6, BE9, BE10, and BE11) strains (Table 1). Figure 1 summarizes the typical chromatograms obtained with the hypo- and hyperpigmented strains.

Table 1. Carotenoid Content (Micrograms per Gram of Dry Weight of Yeast) of Wild-Type and Several *P. rhodozyma* Strains Obtained with Benomyl and/or EMS[a]

	retention time t_r							
strain	3.12 min	3.60 min	4.19 min	5.32 min	6.41 min	6.62 min	8.35 min	total
67-210	22.2	5.7	55.5	5.6	265.9	0	2.7	357.9
E7	49.5	33.3	55.5	12.0	79.5	0	1.4	238.9
E2	51.2	28.3	48.3	12.2	94.5	0	3.1	239.5
B1	90.0	32.2	46.7	7.8	66.1	0	0.5	244.3
BE2	55.3	16.7	41.1	15.0	116.0	0	1.9	246.7
E3	55.6	48.3	92.8	26.1	203.4	0	4.4	452.3
BE7	43.0	22.2	158.4	9.4	215.0	0	1.3	450.1
E4	140.5	90.6	74.5	19.4	133.4	0	4.2	514.6
BE1	307.3	174.6	66.0	11.0	18.9	0	0	660.9
BE4	358.9	81.7	105.3	18.3	169.5	0	2.8	768.5
B10	97.8	72.2	92.8	52.2	374.5	0	8.3	750.2
BE10	139.5	73.3	120.0	17.0	338.9	0	8.1	724.1
BE6	237.5	68.6	177.8	24.2	449.0	0	9.5	1019.9
BE3	98.7	55.6	258.2	5.3	500.1	0	17.9	1005.6
BE11	111.1	44.6	166.5	17.8	323.9	190.6	11.1	912.6
BE5	87.5	48.3	175.9	34.4	378.9	232.3	22.2	1089.2
BE9	87.2	56.4	187.8	41.7	619.6	0	15.6	1034.1

[a] The retention time of 3.12 min corresponds to β-carotene, 4.19 min to 3-hydroxyechinenone, 6.41 min to *trans*-astaxanthin, and 6.62 min to *cis*-astaxanthin.

It should be pointed out that strains originated by mutation with EMS after treatment with benomyl are by far more sensitive to the mutagenic agent (>98% cells killed after 2 min of exposure) than the wild-type strains. This, in turn, was taken as a good indication that benomyl was originating aneuploids, these being far less resistant to the alkylating agent than the wild types (Quinlan *et al.*, 1980).

According to their carotenoid profiles and contents, all of these strains could be classified into five groups (Table 1): (1) hypopigmented strains (E7, E2, B1, and BE2) producing lower concentrations of total carotenoids but higher amounts of β-carotene than the wild-type strain of *P. rhodozyma* and considerably reduced *trans*-astaxanthin content; (2) hypopigmented strains (E3 and BE7) producing higher concentrations of total carotenoids than the wild-type strain (this increase was due, however, to increases in the intermediate carotenoids, while the *trans*-astaxanthin content decreased as compared to the wild-type strain); (3) hypopigmented strains (E4, BE1, and BE4) with a remarkable decrease in the production of *trans*-astaxanthin but an overaccumulation of β-carotene, which increased the total carotenoids content; (4) hyperpigmented strains (B10, BE10, BE6, BE3, BE11, and BE5) exhibiting an increase in total carotenoids as compared to the wild-type strain due to higher amounts of intermediate carotenoids as well as *trans*-astaxanthin (two of these strains (BE5 and BE11) produced high levels of *cis*-astaxanthin); (5) hyperpigmented strain BE9 showing higher amounts of total carotenoids than the wild strain, 60% being *trans*-astaxanthin.

Strain BE2 was further treated with benomyl, allowing colonies to grow for a month in the presence of the carbamate. After this time had elapsed, the colonies had lost their color but had grown red papillae. These were subsequently harvested and spread onto fresh YM medium. These papillae showed greater genetic stability than the other mutants as far as the ability of pigment accumulation was concerned (reversion $<10^{-3}$). The pigment content of these papillae was characterized

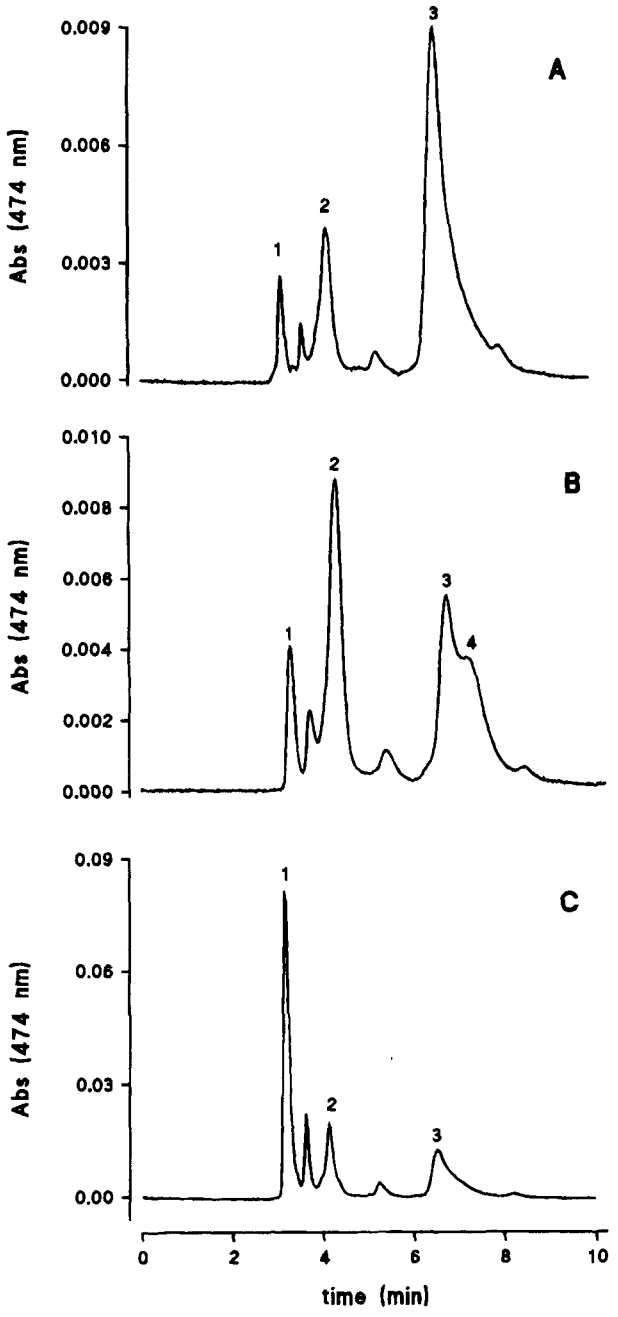

Figure 1. Typical chromatograms of a pigment extract from (A) wild-type strain UCD-FST-67-210 of *P. rhodozyma*, (B) hyperpigmented mutant strain BE5, and (C) hypopigmented mutant strain E7. Peaks: (1) β-carotene; (2) 3-hydroxyechinenone; (3) *trans*-astaxanthin; (4) *cis*-astaxanthin.

by HPLC, as before; the results are summarized in Table 2. Again, all of these new strains could be classified into five groups: (1) hyperpigmented strains (PAP1, PAP3, PAP13, and PAP14) that showed an increased production of total carotenoids, in which the *trans*-astaxanthin concentration compared to that of the wild-type strain was the same and in which intermediate carotenoid concentrations were far higher; (2) hyperpigmented strains (PAP9, PAP11, PAP12, PAP15, and PAP5) that showed an increased production of total carotenoids, mainly due to the overproduction of *trans*-astaxanthin; (3) hyperpigmented strains (PAP10 and PAP4) with a higher content of carotenoids than the wild-type strain, due to the hyperproduction of both β-carotene and *trans*-astaxanthin; (4) hyperpigmented strain PAP6 with an increased production of total carotenoids from which nearly 70% was *trans*-astaxanthin; (5) hyperpigmented strain PAP8 that produced high levels of *cis*-astaxanthin (Table 2).

Table 2. Carotenoid Content of Different Papillae Derived from Strain BE2 (Micrograms per Gram of Dry Weight of Yeast)[a]

	retention time t_r							
strain	3.12 min	3.60 min	4.19 min	5.32 min	6.41 min	6.62 min	8.35 min	total
67-210	22.2	5.7	55.5	5.6	265.9	0	2.7	357.9
BE2	55.3	16.7	41.1	15.0	116.0	0	1.9	246.7
PAP1	58.1	10.5	115.6	3.3	260.3	0	2.5	451.7
PAP3	96.5	30.4	228.4	3.9	273.4	0	3.7	636.8
PAP13	32.7	18.8	133.9	7.3	277.3	0	2.1	472.9
PAP14	33.3	18.1	150.0	5.4	295.6	0	3.1	505.5
PAP9	42.6	12.8	172.8	7.2	536.2	0	4.7	781.9
PAP11	41.7	22.8	160.0	12.2	483.4	0	2.8	724.6
PAP12	63.9	35.6	110.6	13.3	477.3	0	4.3	742.4
PAP15	19.3	17.7	158.9	11.1	507.3	0	2.9	718.5
PAP5	23.7	5.5	76.7	7.8	415.1	0	3.1	971.9
PAP10	205.6	70.6	331.7	12.8	547.4	0	7.2	1176.4
PAP4	244.5	63.6	489.0	12.2	661.3	0	8.3	1481.5
PAP6	106.1	42.8	213.9	15.5	706.3	0	6.1	1097.5
PAP8	56.7	21.1	257.3	9.3	539.0	38.9	6.4	971.9

[a] For retention times see Table 1.

Table 3. Carotenoid Content of Different Papillae Derived from Strain BE2 (Micrograms per Gram of Dry Weight of Yeast) Extracted with Ethanol[a]

	retention time t_r								
strain	3.12 min	3.60 min	4.19 min	5.32 min	6.41 min	6.62 min	8.35 min	total	extraction[b]
67-210	0	0	0	0	0	0	0	0	0
BE2	0	0	0	0	0	0	0	0	0
PAP1	13.3	2.7	27.6	1.7	204.5	0	1.7	253.4	56.0
PAP3	14.5	3.6	30.0	1.7	155.6	0	1.1	207.3	32.5
PAP13	27.2	6.7	61.1	3.9	248.9	0	1.1	348.9	73.8
PAP14	7.2	1.4	13.3	1.1	83.3	0	0.5	108.9	21.5
PAP9	13.2	2.2	35.0	0.5	116.6	0	0.1	167.8	21.5
PAP11	10.5	1.4	19.4	0.9	72.8	0	0.4	105.6	14.6
PAP12	15.6	3.9	35.6	2.8	207.8	0	0.7	266.7	35.9
PAP15	11.7	20.6	22.8	0.5	142.5	0	0.9	180.6	25.1
PAP5	0.4	0.5	0.6		8.3	0		9.8	1.0
PAP10	1.5	0.3	2.2		10.4	0		14.4	0.9
PAP6	20.0	5.0	41.7	2.8	183.9	0	1.6	255.6	23.3
PAP8	27.2	6.1	49.4	3.3	296.2	0	2.5	387.3	39.8

[a] For retention times see Table 1. [b] Percentage of extraction of total carotenoids with ethanol with respect to DMSO.

With regard to direct pigment extractability with ethanol from intact *P. rhodozyma* cell biomass, it was found that some of the papillae mutants possibly have modified cell walls and/or membranes and hence their pigment could be extracted with ethanol. Table 3 shows the results obtained in the HPLC testing of 12 papillae mutants, when the carotenoids from these strains were directly ethanol-extracted. The results evidenced the remarkable efficiency of ethanol in the pigment extraction from some of the papillae, with respect to the strains from which they were derived (wild-type strain and mutant strain BE2). Thus, the yield of pigment extraction was higher than 30% for strains PAP3, PAP8, and PAP12, higher than 50% for strain PAP1, and rose to 73.8% for strain PAP13. On the other hand, this percentage was lower than 25% in strains PAP5, PAP9, PAP10, PAP11, and PAP14. It should also be noted that the percentage of *trans*-astaxanthin extracted with ethanol was higher than 70% in all cases and rose to 84.7% for strain PAP5 with respect to the total carotenoids extracted with this solvent. This result would indicate a higher solubility of *trans*-astaxanthin in ethanol than the rest of the carotenoids contained in the sample, thus making ethanol extraction more selec-

tive for *trans*-astaxanthin. The employment of ethanol in the extraction of the pigment displays several advantages derived from (i) its lower toxicity as compared to other solvents employed in carotenoid extraction; (ii) the prevention of toxic residue dumping (that would be derived from the use of acetone); and (iii) the prevention of the risk of animal intoxication due to residual acetone or DMSO that in turn may remain in the feeding pigment.

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