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β-Carotene producing mutants of *Phaffia rhodozyma*

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Abstract Like other carotenoid-producing organisms, *Phaffia rhodozyma*, a red astaxanthin-producing yeast, is supposed to synthesize carotenoids by the following steps: formation of phytoene from geranylgeranyl pyrophosphate, dehydrogenation of phytoene to lycopene, cyclization of lycopene to β-carotene and oxidation of the latter to astaxanthin. Mutagenic treatments generated in *P. rhodozyma* a wide diversity of colour variants ranging from white to dark red. The identification of the corresponding carotenoid compounds revealed the occurrence of β-carotene-accumulating strains, phytoene-accumulating strains, and strains lacking any carotenoid compound. These classes of strains are likely to result from alterations in, respectively, the oxidation of β-carotene, phytoene dehydrogenation and the phytoene synthetase step. Except for the cyclization of lycopene to β-carotene, all the steps of carotenogenesis in *P. rhodozyma* are represented by the above mutants. Furthermore, astaxanthin-overproducing mutants were also selected; they are likely to be affected in some upstream step, and certainly before β-carotene, as after an additional mutagenesis they generated oxidaseless strains that, in this case, overproduce β-carotene. The latter strains appear very promising for biotechnological production of natural β-carotene.

Introduction

Interest in biotechnological production of β-carotene lies not only in its function as provitamin A and use as a food colorant (for a review, see Bauernfeind 1981), but also, and still more excitingly, in its antioxidant properties. There is increasing evidence that carotenoids, and especially β-carotene, can function directly as antioxidants by reacting with active oxygen species (Burton and Ingold 1984; Krinsky 1989), thereby acting as defence systems against genotoxic damage and displaying anticarcinogenic properties (Bendich 1991).

Although widely distributed in plants and animals, carotenoids are synthesized only by plants and micro-organisms, which start a food chain leading to the pigmentation of certain animals. Among the carotenoid-producing micro-organisms, bacteria, filamentous fungi and unicellular algae have been extensively examined in order to evaluate their possible industrial interest (for a review, see Armstrong et al. 1990). However, only the halophilic alga *Dunaliella* is so far commercially produced as a source of β-carotene (Ben-Amotz and Avron 1980). Relatively abundant production of β-carotene has been achieved also with the phycomycete fungi *Blakeslea trispora* and *Phycomyces blakesleeanus* (Ninet and Renaut 1979; Weete 1980).

In contrast, carotenoid biosynthesis is unfortunately seldom found in yeasts, which are among the most efficient tools for natural molecule production by biotechnology. Certain yeast species of the genera *Rhodotorula*, *Cryptococcus*, *Sporobolomyces* and *Phaffia* do, however, produce characteristic carotenoids such as torulene and torularrhodin, the main carotenoids of *R. glutinis* (Simpson et al. 1964) and astaxanthin, the major pigment synthesized by *P. rhodozyma* (Andrewes et al. 1976).

Up to now, the only economically valuable carotenoid production process using yeasts is the production of astaxanthin by *P. rhodozyma* (Johnson et al. 1991; Villadsen 1992). Moreover, an enhancement of the metabolic flow of carotenogenesis, resulting in higher yields of astaxanthin was obtained in *P. rhodozyma* by mutagenesis (An et al. 1989).

The biosynthetic pathway, in all the carotenoid-producing organisms so far examined, probably follows

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similar routes (Goodwin 1968) with minor variations in the final steps leading to characteristic carotenoids. The pathway proposed for astaxanthin biosynthesis in *P. rhodozyma* is summarized in Fig. 1, based upon the work of Andrewes et al. (1976). Despite its occurrence in carotenoid-producing yeasts, there is no report so far of β-carotene-accumulating strains providing economical sources of β-carotene. The minor production of β-carotene by *R. aurea* (Ceigler 1965) seems unsuitable for further developments, whereas the mutants of *P. rhodozyma* blocked at the β-carotene step mentioned by An et al. (1989) were not characterized further. In this report we describe the selection of carotenoid mutants of *P. rhodozyma*, some of which are very efficient β-carotene producers.

Materials and methods

Yeast strains

All the mutant strains investigated in this work derived from *P. rhodozyma* PR-219 (= CBS 6938).

Media, growth conditions and mutagenesis

Complete media consisted of 1% yeast extract, 1% peptone and either 2% glucose (YPG20), 10% glucose (YPG100), 3% (v/v) glycerol (YPGly). Minimal medium (SD) contained 0.67% Bacto-Yeast Nitrogen Base without amino acids (Difco) and 2% glucose. When needed, media were solidified with 2% agar. Strains were maintained on YPG20 slants and stored at − 80°C in 25% glycerol–75% YPG20. Routine 100-ml liquid cultures were performed at 25°C in 1-l baffled flasks, shaken at 150 rpm. Mutagenesis was carried out as described by An et al. (1989); ultraviolet (UV) and ethyl-methane sulphonate (EMS) treatments gave respectively 10% and 20% survival. Colour variants were selected by visual examination of YPG20 plates and auxotrophic strains were recovered after an enrichment cycle (Littlewood 1975).

Protoplast fusion

Cells grown in YPG20 were harvested in mid-log phase and washed twice in distilled water. Subsequently, all the buffers and media were supplemented with 0.6 M KCl. Cells (10^9) were resuspended in 5 ml TEM buffer [50 mM TRIS-HCl, pH 7.5, 40 mM ethylenediaminetetraacetic acid (EDTA), 0.5% (v/v) β-mercaptoethanol] and incubated for 1 h at 25°C. Pretreated cells washed in CPM buffer [20 mM citrate-phosphate, pH 5.6, 0.5% (v/v) β-mercaptoethanol], were resuspended in 5 ml CPM buffer containing 8 mg/ml of Novozym SP299 (Novo Nordisk) and incubated at 25°C for 2.5 h. The protoplasts were harvested by centrifugation, washed in TC buffer (50 mM TRIS-HCl, pH 7.5, 10 mM $CaCl_2$), and resuspended in 250 µl TC buffer. Protoplasts from each of the two strains were mixed in a 1:1 ratio, to give a total of about 10^7 protoplasts and pelleted by centrifugation. The pellet was resuspended in 2 ml of 30% (w/v) polyethylene glycol, 50 mM $CaCl_2$ in 50 mM TRIS, pH 8.0 and incubated for 1 h at 25°C, then 2 ml of 50 mM TRIS-HCl buffer, pH 7.5, was added. After centrifugation, the pellet was resuspended in 5 ml 50 mM TRIS-HCl buffer, pH 7.5. Aliquots of this suspension were mixed with 5 ml soft agar [1% agarose; Low Gel Temperature, Bio-Rad Laboratories in 20 mM citrate-phosphate buffer, pH 5.6]. The suspension was, after a gentle shaking, overlaid onto SD plates and incubated at 25°C. Regenerated prototrophic fusants were observed after 7–8 days.

Carotenoid extraction

Cells were harvested by centrifugation, washed in distilled water and lyophilized. Lyophilized cells (100 mg) were put in a 2-ml microfuge tube and 600 µl dimethyl sulphoxide (DMSO) was added. The suspension was vortexed for 1 min, and then allowed to stand for 20 min at room temperature in the dark. After this chemical disruption, the cells were centrifuged at 14,000 *g* for 1 min and the supernatant was poured in a 10-ml tube flushed with N_2 and kept on ice. The pellet was then extracted five times with 500 µl acetone until it became white. The combined acetone and DMSO extracts were centrifuged at 30,000 *g* for 20 min at 4°C. The clear supernatant was filtered on glass wool. Petroleum ether (500 µl) was added as well as the necessary amount of saturated NaCl solution to ensure the phase separation. The resulting emulsion was broken by centrifugation at 5,000 *g* for 5 min. The non-aqueous phase was recovered and the aqueous phase was extracted twice more with 500 µl petroleum ether. The three petroleum ether fractions were pooled, dried on anhydrous sodium sulphate and finally evaporated to dryness under a stream of N_2. The residue was redissolved in 600 µl petroleum ether and analysed by HPLC.

Chromatographic analysis

The analytical technique developed for the separation and identification of carotenoids found in various *P. rhodozyma* strains is based upon previously reported methods (Khachik and Beecher 1987; Khachik et al. 1989; Rouseff et al. 1992).

An HP1090 high performance liquid chromatograph (HPLC) coupled with an Analytichem C18 reversed phase HPLC column (Waters µBondapak, 300 mm length × 3.9 mm i.d., 10 µm particles with a pore size of 125 Å) was used. The isocratic mobile phase consisted of acetonitrile (65%), methanol (30%) and methylene chloride (5%) and then modified by adding 1% octanol-2 in order to obtain the best separation of β-carotene and phytoene. All solvents were of chromatographic grade from Merck (Lichrosolv); the flow rate was 0.6 $ml \cdot min^{-1}$. The separation was carried out at ambient temperature. The injection volume was 25 µl. The photodiode array detector used to monitor the chromatographic effluent acquired absorbance spectra from 200 to 600 nm, the detections of carotenoids were made at 286 nm (phytoene), 350 nm (phytofluene), 400 nm (ζ-carotene and β-zeacarotene) and 478 nm (lycopene, β-carotene, echinenone, hydroxyechinenone and astaxanthin).

Standards of echinenone, hydroxyechinenone, β-zeacarotene and phytofluene were obtained from Hoffmann-La Roche (Basel, Switzerland); β-carotene and lycopene were purchased from Extrasynthèse (France). Astaxanthin and phoenicoxanthin were identified by their absorbance maxima as indicated by Andrewes et al. (1976). Since standards for phytoene, ζ-carotene, neurosporene and γ-carotene were not commercially available, these carotenoids were identified by reference to natural sources known to contain them; for this purpose, hexane extracts of red grapefruit and fresh pumpkin were run in parallel and carotenoids were identified by their retention times and spectral data as reported by Khachik and Beecher (1987), Khachik et al. (1989) and Rouseff et al. (1992).

Quantitative determination

When the strains produced astaxanthin, total carotenoids were calculated as described by An et al. (1989). In strains producing β-carotene as the major carotenoid, total carotenoids were determined using a 1% extinction coefficient of 2,600 based on determinations with synthetic β-carotene. When the strain produced

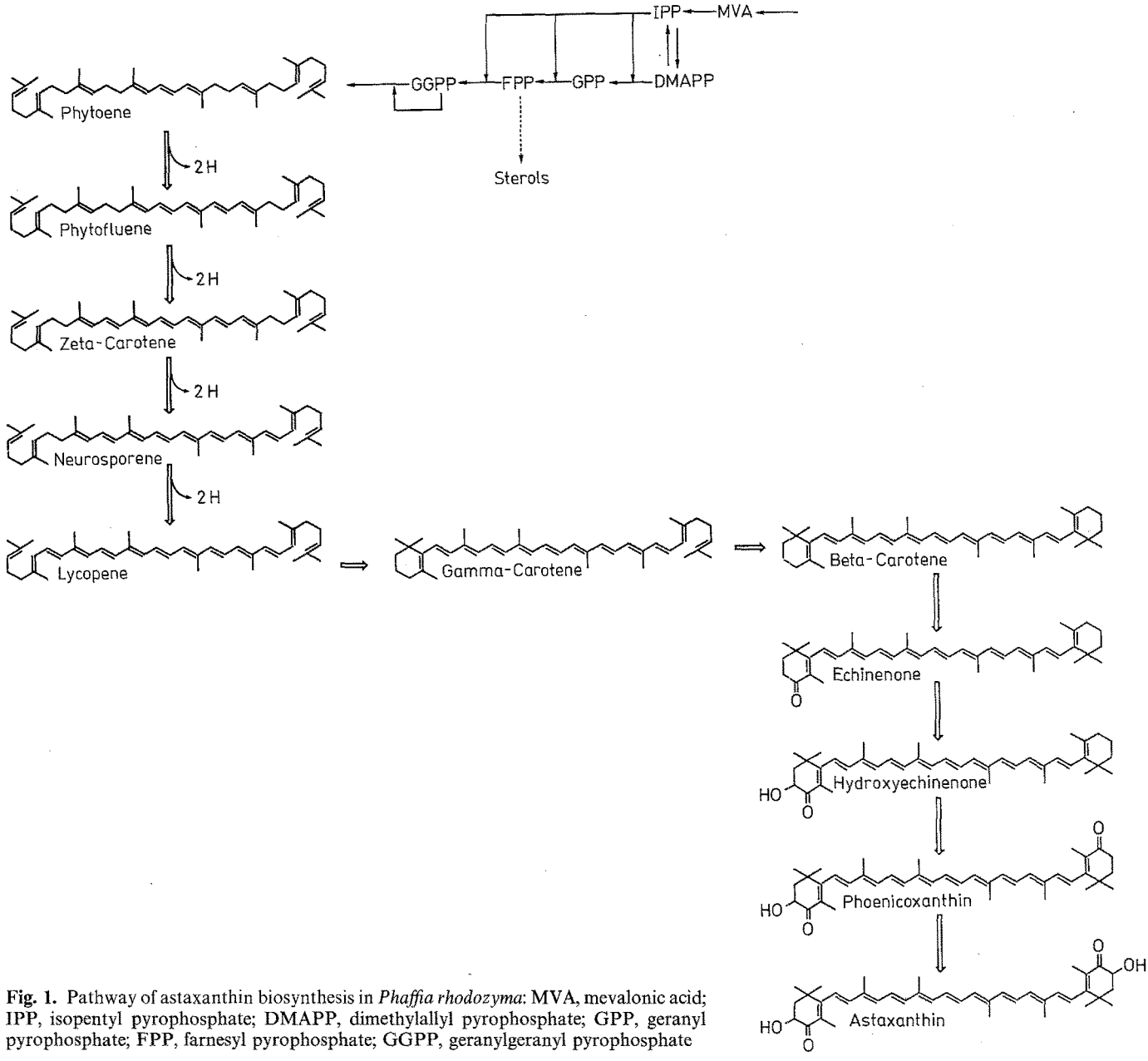

Fig. 1. Pathway of astaxanthin biosynthesis in *Phaffia rhodozyma*: MVA, mevalonic acid; IPP, isopentyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate

exclusively phytoene, the eluted HPLC fraction containing this colorless product was recovered, the solvent evaporated to dryness under a stream of N_2 and the residue redissolved in 1 ml petroleum ether; absorbance at 286 nm was measured and the carotenoid concentrations calculated using a 1% extinction coefficient of 1,250 according to Britton (1985).

Results

Colour variants

Screening by visual examination of isolated colonies after UV or EMS mutagenesis of strain PR-219 revealed the occurrence of a great number of colour

Table 1. Colour variants produced from *Phaffia rhodozyma* PR-219 by mutagenesis

Colour	Mutagen	
	UV (number of clones)	EMS (number of clones)
Dark red	5	2
Dark orange	7	2
Pink	12	2
Pink orange	8	2
Yellow orange	1	3
Yellow	3	18
Cream	1	3
White	7	13

UV, Ultraviolet light; EMS, Ethyl-methane sulphonate

variants ranging from white to intense red; all of them displayed a remarkable colour stability upon successive subculturing. Such colour variants were never generated spontaneously by the original strain.

Among 89 colonies distinguishable by their pigmentation, several distinct classes were apparent (Table 1). In addition, both UV and EMS treatments quite frequently yielded sectored colonies of different patterns, the origin of which was not investigated in this study. Moreover, after mutagenesis we recovered 15 auxotrophs identified as either Met^-, Leu^-, Trp^- or Arg^- (data not shown).

Carotenoid compounds identified in wild-type and colour variants of *P. rhodozyma*

Twenty-eight colour mutants belonging to different pigmentation classes were analysed by HPLC and the corresponding carotenoids identified (Table 2). A compilation of the results obtained with the original wild-type strain PR-219 and the different mutants indicates the occurrence in *P. rhodozyma* of almost all of the known intermediates of the carotenoid pathway: phytoene, phytofluene, ζ-carotene, neurosporene, β-carotene, as well as all of the oxycarotenoids leading in *P. rhodozyma* to astaxanthin. For phytoene and β-carotene the major isomer was *trans*, but traces of the *cis* isomers were also detected. In addition, an unidentified oxycarotenoid was observed in all of the astaxanthin-producing mutants.

However, neither in the wild-type strain, nor in any colour mutant, were we able to detect lycopene or γ-carotene. As the absence of these intermediates was likely to suggest an unorthodox mechanism for the cyclization leading to β-carotene, we analysed the carotenoids produced in the presence of 10 mM nicotine, known to specifically inhibit the cyclization step. As shown in Fig. 2, in the absence of cyclase activity, the major accumulated precursor was indeed lycopene, but γ-carotene also occurred in significant amounts.

Another point to be mentioned concerns the occurrence in certain mutants of β-zeacarotene, the position of which in the carotenoid pathway in *P. rhodozyma* is rather puzzling (see Discussion).

Table 2. Carotenoid composition of *P. rhodozyma* PR-219 and of the colour mutants investigated in this study

Carotenoid	219	100	171	111	166	106	137	119	126	168	181	165	104	160	162
cis-Astaxanthin	109		143	67		3	69	11	3	24					
trans-Astaxanthin	303	69	507	380	275	3	182	60		77					
Phoenicoxanthin		6		97				8	5	37					
Unknown	30	4	66	73	27	33	20		37	30					
Hydroxyechinenone	25	6	17	36	28	5	19	8	132	125					
Echinenone	10	2	4	10	13		7	4	87	55					
Neurosporene												13		4	
γ-Carotene											19	12	11		
ζ-Carotene									27	12				11	
β-Zeacarotene														12	
Phytofluene														8	
β-Carotene	30	6	9	14	40		18	5	421	101	382	416	532	135	28
Phytoene	8	3		10			4	35						78	
Total carotenoids	515	96	746	687	383	44	319	131	712	461	401	441	543	148	28

Carotenoid	163	155	108	130	132	141	104-1	104-2	104-3	104-4	167	102	120	139
cis-Astaxanthin														
trans-Astaxanthin														
Phoenicoxanthin														
Unknown														
Hydroxyechinenone														
Echinenone														
Neurosporene														
γ-Carotene														
ζ-Carotene														
β-Zeacarotene														
Phytofluene														
β-Carotene	5	18												
Phytoene		31	84	73	59	73	128	243	41	68	175			
Total carotenoids	5	49	84	73	59	73	128	243	41	68	175	0	0	0

Cultures 120 h old were used and values are in $\mu g \cdot g^{-1}$ carotenoid dry wt.

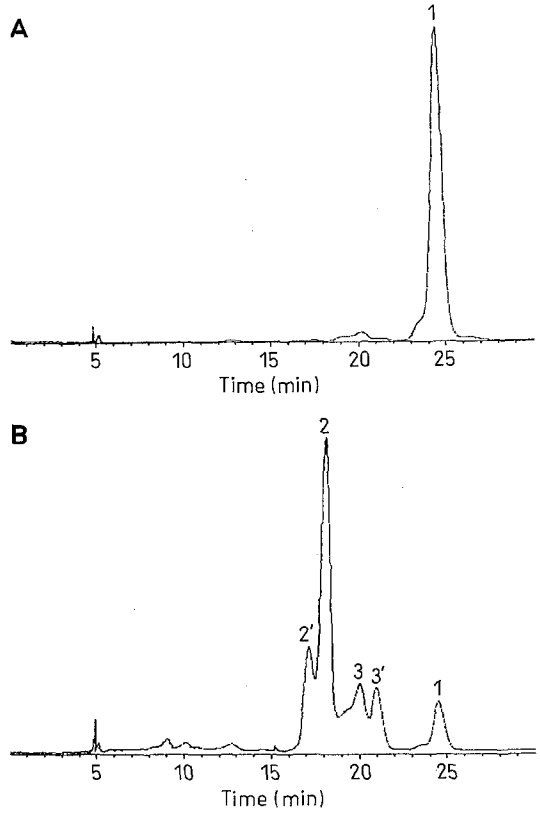

Fig. 2A, B. HPLC chromatograms of 120-h-old cultures *P. rhodozyma* PR-1-104 grown in YPGly (see Materials and methods) under standard conditions (**A**) or in the presence of 10 mM of nicotine (**B**): *1*, β-carotene; *2*, lycopene, *2'*, *cis*-lycopene; *3*, *cis*-γ-carotene; *3'*, *trans*-γ-carotene

Classes of mutants

An examination of Table 2 reveals that among the 28 mutants investigated, two distinct types of alterations are apparent: 1. Putative blocks in particular steps of the carotenogenesis, resulting in accumulations of specific intermediates. 2. Alterations of the metabolic flow of the carotenoid pathway resulting in significant differences in the total carotenoid content.

As a rule, the white mutants so far examined belong to two categories:

1. Mutants accumulating solely phytoene and supposed to be affected in the phytoene dehydrogenase steps (PR-1-108, PR-1-130, PR-1-132, PR-1-141, PR-2-104-1, PR-2-104-2, PR-2-104-3, PR-2-104-4, PR-2-167).

2. Mutants completely devoid of carotenoid intermediates (PR-1-102, PR-1-120, PR-1-139), which are in all likelihood affected in the phytoene synthetase.

β-Carotene is the major carotenoid in three yellow mutants (PR-1-104, PR-2-165, PR-2-181) representing, respectively, 97.9%, 94.3%, and 95.2% of the total carotenoids. In PR-1-104, PR-2-165 and PR-2-181 trace amounts of other intermediates prior to β-carotene also occur. The remaining yellow mutant, PR-2-160, differs from the previous ones by a marked slowing down of carotenoid biosynthesis; moreover, β-carotene represents in this mutant only 54.4% of the total carotenoids, as significant amounts of phytoene are also produced (31.5%). Furthermore, neurosporene was also found only in the yellow mutants PR-2-160 and PR-2-165. Interestingly enough, the yellow mutant PR-2-160, unlike all the other colour mutants investigated in this study, always produces small amounts of β-zeacarotene.

Table 3. Carotenoid composition of fusion products formed in the cross PR-1-102 × PR-1-104

Carotenoid ($\mu g \cdot g^{-1}$ dry wt.)	Fusion products			Parental strains		WT
	F1	F2	F3	102	104	219
cis-Astaxanthin	95	88	105			109
trans-Astaxanthin	298	304	306			303
Phoenicoxanthin						
Unknown	18	28	32			30
Hydroxyechinenone	24	31	42			25
Echinenone	7	12	6			10
Neurosporene						
γ-Carotene					11	
ζ-Carotene						
β-Zeacarotene						
Phytofluene						
β-Carotene	29	43	37		532	30
Phytoene	5	11	9			8
Total carotenoids	476	517	537	0	543	515

WT, Wild type

As revealed by their carotenoid profiles, yellow and white mutants are obviously affected in distinct steps of the carotenoid pathway. Evidence for this was provided by complementation after protoplast fusion between yellow and white mutants. We used auxotropic derivatives of strains PR-1-102 (white, Met^-) and PR-1-104 (Yellow Arg^-) obtained by nystatin enrichment after EMS mutagenesis. Red fusants were isolated on SD medium. The frequency of fusions with respect to the mixed protoplast populations was about 10^{-7}. Three independent fusion products were analysed with respect to the carotenoid profile (Table 3). A comparison with PR-1-102 and PR-1-104 (Table 2) reveals that the integrality of the carotenoid pathway was restored in the fusant and its operation results in the formation of astaxanthin in amounts similar to those found in PR-219.

In addition to the mutants representative of particular steps in carotenogenesis, a certain number of mutants distinguishable from the wild-type strain by their colour, turned out to be significantly affected in the overall efficiency of the carotenoid pathway. All of them synthesized astaxanthin, yet over a range as wide as 3 to 650 $\mu g \cdot g^{-1}$ dry weight. It should be noted that the variations in total carotenoids, ranging from 44 to 687 $\mu g \cdot g^{-1}$ dry weight, are not always superimposable

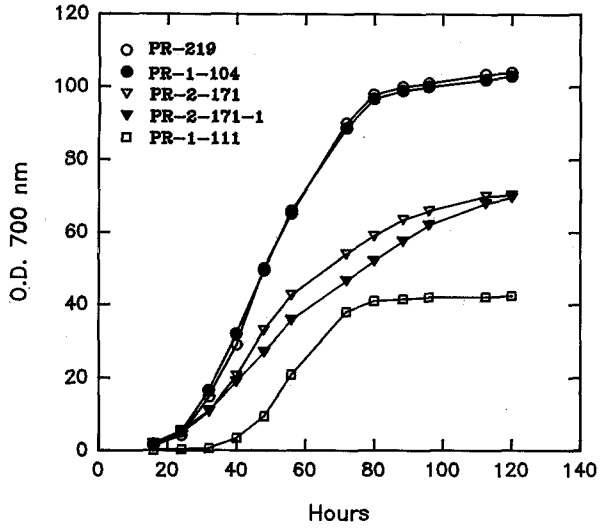

Fig. 3. Growth, measured as optical density (O.D.) at 700 nm, of *P. rhodozyma* PR-219 (○), of the astaxanthin-overproducing mutants PR-2-171 (▽) and PR-1-111 (□) and of two yellow derivatives: ●, PR-1-104; ▼, PR-2-171-1

upon those of astaxanthin, as other carotenoids may be synthesized instead of astaxanthin (e.g. β-carotene in R-1-126 and PR-2-168 and the unknown compound in PR-1-106). The red pigmentation intensity seems to be related to the amount of astaxanthin or to the combination of different carotenoids. As the astaxanthin content decreases, colonies become pale red (PR-1-100, PR-2-166, PR-1-137), pink (PR-1-106) or cream (PR-1-119). In spite of the presence of astaxanthin in only trace amounts in strain PR-1-126, colonies are deep-orange due to the high content of β-carotene.

Carotenoid overproduction

Two dark red mutants PR-2-171 and PR-1-111, owe their pigmentation to an overproduction of astaxanthin. It should be noted that both of them display slower growth and, especially PR-1-111, low biomass production (Fig. 3). On the other hand, unlike the mutation resulting in astaxanthin overproduction, the alterations generating yellow mutants do not affect growth.

Assuming that the high astaxanthin content in strain PR-2-171 is due to some upstream mutation, responsible for the higher efficiency of carotenogenesis, we isolated a yellow oxidase mutant PR-2-171-1 after EMS mutagenesis of strain PR-2-171, in order to take advantage of the remarkable characteristic of this strain. A fivefold increase in β-carotene accumulation, as compared to the yellow mutants issued directly from PR-219, was indeed found with mutant PR-2-171-1 (Fig. 4), which therefore appears as a suitable candidate for further experiments on β-carotene production with *P. rhodozyma*. Carotenoid production greatly depends on the composition of the growth medium. As can be seen in Fig. 4, the maximum yield is found in minimal

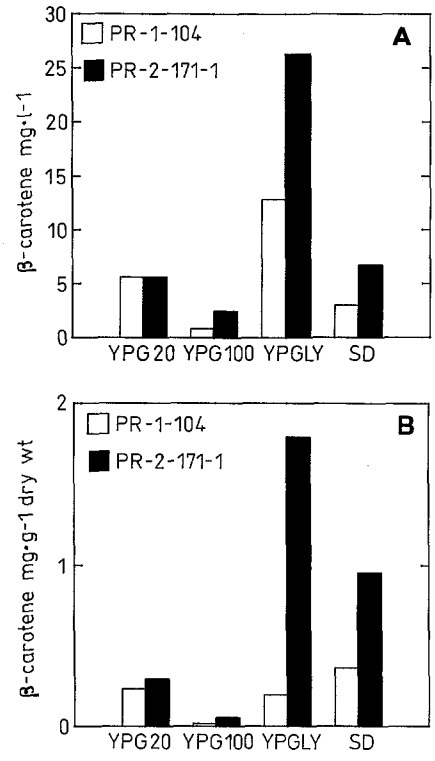

Fig. 4A, B. β-Carotene production in $mg \cdot l^{-1}$ (**A**), and on a dry weight basis (**B**), with the yellow mutant PR-1-104 (□) and a derivative of the overproducing strain PR-2-171 (■) in different growth media (see Materials and methods)

medium and in the presence of non-fermentable carbon sources such as glycerol.

Discussion

A comprehensive inspection of the carotenoid compounds in all the strains examined as well as in nicotine-treated cells, allowed the identification of most of the carotenoids previously described in *P. rhodozyma* (Andrewes et al. 1976; An et al. 1989). The original strain used in this study, PR-219, while producing astaxanthin as the major carotenoid, is nevertheless slightly different from the *P. rhodozyma* strains studied by other authors. As compared to the type strain UCD-FST 67-210 studied by Andrewes et al. (1976), PR-219 displays a simpler profile, with only small amounts of phytoene, β-carotene, echinenone and hydroxyechinenone, accompanying the major peak of astaxanthin. Unlike UCD-FST 67-210, in which phoenicoxanthin is the second predominant carotenoid, in PR-219 no phoenicoxanthin was detected. An et al. (1989) reported a similar discrepancy with the strain UCD-FST 67-385. Phoenicoxanthin was however present in certain mutants in amounts up to 14.1% of total carotenoids. Likewise, neurosporene, ζ-carotene and phytofluene were detected only in mutants.

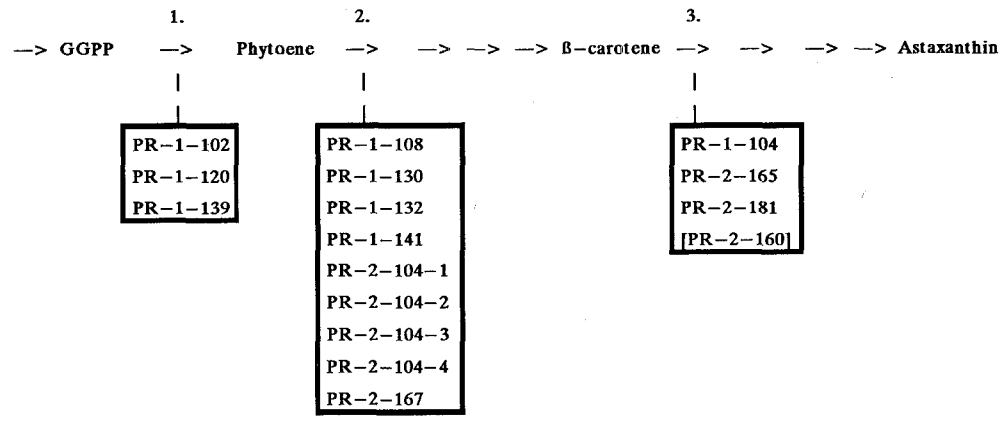

Fig. 5. Classes of carotenoid mutants of *P. rhodozyma* according to the accumulated intermediates: *1*, phytoene synthetase; *2*, phytoene dehydrogenase; *3*, oxidase

The unknown carotenoid occurring in significant amounts both in PR-219 and in certain mutants could be tentatively considered as 3-hydroxy-3′, 4′-didehydro-β-ψ-caroten-one (HDCO) according to its spectral and polarity characteristics. If so, the relatively high content observed in the low-astaxanthin-producing strain PR-1-106 (pink colonies) should originate in a mechanism different from that underlying the antimycin-susceptible mutants described by An et al. (1989), which show simultaneously increased concentrations of both astaxanthin and HDCO.

Lycopene and γ-carotene were never detected in the strains examined, unless nicotine was used to inhibit the cyclase step. The result of this experiment demonstrates that lycopene and γ-carotene are indeed intermediates of the carotenoid pathway in *P. rhodozyma*. Thus, the sequence of compounds, as revealed by accumulations in blocked pathways is consistent with the biosynthetic pathway proposed by Andrewes et al. (1976) (Fig. 1).

The enzymes involved in carotenogenesis have not been studied up to now. The mutants described in this study clearly correspond to distinct steps of the carotenoid pathway, i.e. phytoene synthetase, phytoene dehydrogenase, oxidase (Fig. 5). Concerning the formation of xanthophylls, our results do not throw light on the steps between β-carotene and astaxanthin; alternative pathways might operate, as suggested by the occurrence of β-zeacarotene and the putative HDCO, which may be transformed to astaxanthin (An et al. 1990). In contrast, the mutants described in this study allow a better understanding of the early steps of carotenogenesis in *P. rhodozyma*.

The different classes of mutants are in many respects reminiscent of the carotene mutants of the zygomycete fungus *Phycomyces blakesleeanus* (for a review, see Cerdá-Olmedo 1985). Like most of the white mutants of *Phaffia rhodozyma*, certain white mutants of *Phycomyces blakesleeanus* contain large amounts of phytoene and practically no other carotenoid; they are affected in the phytoene dehydrogenase, the enzyme responsible for the four successive dehydrogenations of phytoene leading to lycopene. Nevertheless, in *P. blakesleeanus* no mutant blocked before the formation of phytoene was isolated, whereas in *Phaffia rhodozyma* three of the white mutants (PR-1-102, PR-1-120 and PR-1-139) are in all likelihood blocked in the phytoene synthetase step. In these mutants, carotenogenesis is abolished without apparent damage to viability. The protoplast fusion experiment, in which strain PR-1-102 was used as one of the parents, supports evidence that at least the β-carotene oxidation steps, absent in the yellow parent, are functional in this type of mutant. As we did not look for the possible occurrence of geranylgeranyl pyrophosphate (GGPP), a block in GGPP synthetase could not be a priori ruled out, however unlikely, as GGPP is involved in other polyprenol biosynthetic pathways.

It is interesting to note that so far we have failed to isolate ergosterol auxotrophs of *P. rhodozyma*, either in the early steps of the ergosterol pathway (expected to be carotenoidless mutants) or in the steps between farnesyl pyrophosphate (FPP) and ergosterol, which are supposed to display an altered flow of carotenogenesis. The next step in the carotenoid pathway, consecutive to the dehydrogenations of phytoene is the formation of cyclic carotenoids containing cyclohexylidene rings, such as β-carotene. The accumulation of lycopene and γ-carotene in the presence of nicotine strongly suggests that in *P. rhodozyma* the cyclohexylidene ring formation proceeds by the sequence lycopene → γ-carotene → β-carotene. That is, lycopene, rather than neurosporene acts as the substrate for the cyclization reaction, which is consistent with the proposed pathway in mycobacteria (Howes and Batra 1970).

So far, we have been unable to isolate lycopene-accumulating strains even among colour variants displaying different reddish shades, although inhibition of lycopene cyclase by nicotine is not deleterious to the cell up to 20 mM. Lycopene accumulated in the presence of nicotine is readily cyclized when the alkaloid is removed; nicotine, apparently does not act at the transcription level (Howes and Batra 1970). The failure to

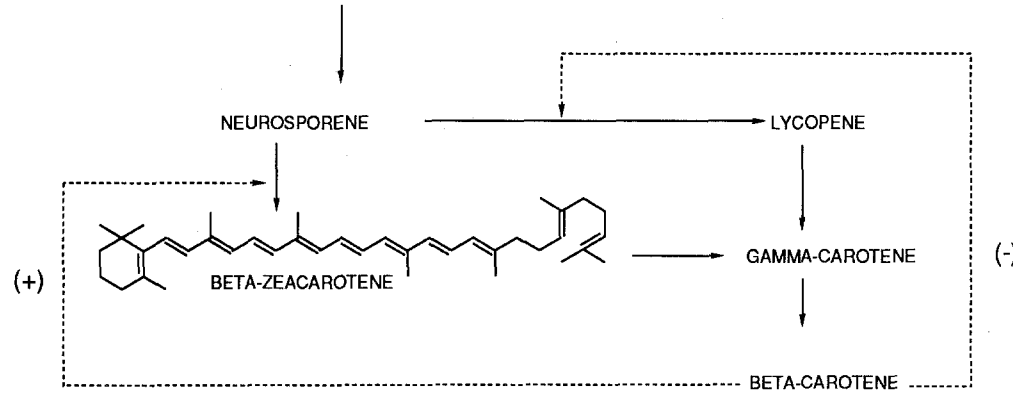

Fig. 6. Alternative cyclization mechanism that may account for the occurrence of β-zeacarotene in yellow strains (after Howes and Batra 1970)

obtain cyclase mutants is intriguing; if it is somehow related to the functions of the enzyme, one might reasonably assume that cyclase plays an additional role, essential for the cell and unaffected by nicotine; of course, the presence of two or more copies of the gene cannot be ruled out.

All of the yellow mutants examined are likely to be blocked in the oxidase step and therefore unable to perform the conversion of β-carotene to echinenone. The pathway is thus abolished beyond β-carotene, which thus may be reasonably considered as the final product of carotenoid biosynthesis in these mutants, especially since in some yellow mutants the amount of total carotenoids nearly approximates that found in the wild-type yeast. Concerning the atypical yellow mutant PR-2-160, which accumulates phytoene in addition to β-carotene while displaying a lower flow of carotenogenesis, it is not clear whether an additional leaky mutation affecting phytoene dehydrogenase is associated with the oxidase deficiency in this mutant.

In other respects, certain β-carotene-accumulating strains remarkably contain small amounts of neurosporene and β-zeacarotene, both absent in all the other strains. Considering the possible alternative pathway of the cyclization reaction (Fig. 6) suggested by Howes and Batra (1970), it is conceivable that the side reactions that lead to γ-carotene via β-zeacarotene are induced, possibly as a result of the accumulation of β-carotene.

Concerning the carotenoid-overproducing mutants reported in this study, no indication on their nature is available. Astaxanthin-overproducing mutants were previously isolated (An et al. 1989) on the basis of their increased sensitivity to antimycin and tentatively accounted for by alteration of cytochrome b or cytochrome P-450 components. Like the antimycin-sensitive mutants, our astaxanthin-overproducing strains grow more slowly and have a lower cell yield as compared to the original isolate.

Surprisingly, the yellow derivatives obtained after an additional mutagenesis treatment of these strains still overproduce carotenoids, namely β-carotene and grow more slowly. Obviously the mutation resulting in carotenoid overproduction affects the overall flow of the pathway and possibly even upstream steps in other polyprenol pathways. The nature of this mutational alteration in *P. rhodozyma* remains obscure at present. A similar mechanism was proposed in the case of β-carotene overproducing mutants of *Dunaliella bardawil* (Shaish et al. 1991), in which alterations in metabolic steps preceding GGPP were postulated to account for the activation of the carotene biosynthetic pathway.

In view of the high β-carotene content of certain oxidase mutants of *P. rhodozyma* described in this paper, the biotechnological production of natural β-carotene using this yeast seems to be reasonably feasible. Our results indicate that carotenoid biosynthesis in *P. rhodozyma* is decreased by glucose, which is in agreement with the conclusion of Johnson and Lewis (1979). Therefore, further investigations are necessary for optimization of culture media and scaling up.

In other respects, the characterization of carotenoid mutants in *P. rhodozyma* has to overcome the lack of knowledge concerning the life cycle of this organism, which hinders genetic analysis. The recent demonstration of a sexual life cycle in *P. rhodozyma* (Villadsen 1992) does not yet allow routine analyses of complementation groups and linkage relationships. Our collection of mutants blocked in particular steps of the carotenoid pathway appears to be a very useful tool for cloning and characterization of carotenoid genes.

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