Journal of General Microbiology (1979), **115**, 173–183. *Printed in Great Britain*

Astaxanthin Formation by the Yeast *Phaffia rhodozyma*

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(*Received* 5 *January* 1979)

The production of carotenoid pigments by the yeast *Phaffia rhodozyma* depended on the culture conditions. Astaxanthin, the primary carotenoid in this yeast, was produced mainly during the exponential phase of growth. The concentration of carotenes in *P. rhodozyma* remained relatively constant [about 5 μg (g yeast)$^{-1}$] throughout growth in a 1·5% (w/v) glucose medium, but the xanthophyll concentration increased from 90 to 406 μg (g yeast)$^{-1}$ during fermentation. Active xanthophyll synthesis occurred during the period of accelerating growth and after exhaustion of glucose from the growth medium. In media containing more than 1·5% (w/v) glucose, however, yeast and carotenoid yields were considerably reduced. The pH of the medium affected yeast yields and carotenoid production; the optimum pH was 5·0. At pH 3·5, β-zeacarotene accumulated in *P. rhodozyma*. β-Carotene was the primary carotene in the yeast under all other conditions tested. The optimum temperature for yeast growth and pigment formation was 20 to 22 °C and the best carbon source was D-cellobiose. Oxygen was an important substrate for optimum yields of yeast and astaxanthin; under microaerophilic growth conditions, astaxanthin production was drastically decreased and *P. rhodozyma* accumulated β-carotene and the monoketone echinenone.

INTRODUCTION

The red yeasts of the genera *Cryptococcus*, *Rhodotorula*, *Rhodosporidium*, *Sporidiobolus* and *Sporobolomyces* are very similar in their carotenoid composition. They contain β-carotene (β,β-carotene), γ-carotene (β,γ-carotene), torulene (3′,4′-didehydro-β,Ψ-carotene) and torularhodin (3′,4′-didehydro-β,Ψ-carotene-16′-oic acid) as their major pigments (Simpson *et al.*, 1971). Recently, plectaniaxanthin (3′,4′-didehydro-1′,2′-dihydro-β,Ψ-carotene-1′,2′-diol) has been found in *Cryptococcus laurentii* (Bae *et al.*, 1971) and 2-hydroxyplectaniaxanthin in *Rhodotorula aurantiaca* (Liu *et al.*, 1973) which has added some structural diversity to the carotenoids found in this group of fungi. *Phaffia rhodozyma* is a recently discovered yeast (Miller *et al.*, 1976) that is strikingly different from the other pigmented yeasts in producing the carotenoid pigment astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) (Andrewes *et al.*, 1976).

Though astaxanthin is rarely found in the fungi [it has occasionally been isolated from the basidiomycetes *Peniophora auranthica* and *Pe. quercina* of the Aphyllophorales (Goodwin, 1972)], it is common in the animal kingdom. It is conspicuously displayed in the plumage of many birds including flamingoes and the scarlet ibis, in marine invertebrates such as lobsters, crabs and shrimps, and in fishes such as trout and salmon, where astaxanthin is responsible for flesh colour. These fish, when raised in pens, often lack desirable red flesh colour. In an earlier study (Johnson *et al.*, 1977) we found that a preparation of *P. rhodozyma* is a potentially important source of astaxanthin for pen-reared salmonids, as the yeast pigment is rapidly accumulated from the feed and deposited in the flesh of rainbow trout.

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0022-1287/79/0000-8534 $02.00

Though some metabolic characteristics of this yeast have been reported (Phaff *et al.*, 1972; Miller *et al.*, 1976), these were not studied in relation to astaxanthin formation. The purpose of this study was to investigate the effect of culture conditions on astaxanthin formation in *P. rhodozyma* with a view to optimizing pigment production.

METHODS

Yeast and culture conditions. The type strain of *Phaffia rhodozyma* (UCD 67–210) was obtained from the yeast culture collection of this department. The yeast was maintained on slants of yeast extract/malt extract agar (YM agar, Difco) at 4 °C.

Flask cultures were grown on an orbital shaker (Environ-Shaker 3597, Lab Line Instruments) at 22 °C in 500 ml baffled side-arm flasks. All shake flask experiments were performed in triplicate. Yeast extract/malt extract broth (YM broth) supplemented with antifoam (FG-10, Dow Corning) at 0·1 ml l^{-1} was the usual growth medium. A medium of yeast nitrogen base (YNB) broth lacking amino acids and ammonium sulphate (Difco) was used in carbon and nitrogen assimilation experiments; carbon and nitrogen sources were added as required. Shake flasks contained 50 ml medium unless otherwise stated. Media were buffered at pH 5·0 with 0·1 M-potassium hydrogen phthalate buffer.

Fermenter cultures were grown in a Virtis 20 l fermenter (model 43-100, Virtis Co., Gardener, N.Y., U.S.A.) at 20 °C using a 14 l working volume, an air flow rate of 8 l min^{-1} and a stirring rate of 400 rev. min^{-1}. The standard medium contained (per litre): Cerelose (CPC International, Englewood Cliffs, N.J., U.S.A.) 20 g; $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 0·5 g; $CaCl_2.2H_2O$, 0·1 g; yeast extract (Difco), 2 g; antifoam, 0·1 ml. pH was monitored with a sterilizable pH probe and controlled at pH 4·8 by automatic titration with 4 M-KOH.

Shake flasks were inoculated with 1 % (v/v) and fermenters with 2 % (v/v) of a 20 h washed cell suspension containing about 1·5 mg yeast dry wt ml^{-1}. An additional volume (0·02 %, v/v) of sterile antifoam was added near the middle of the exponential phase of growth to prevent foaming. Yeast growth rate is expressed as the specific growth rate μ (h^{-1}), and in flask culture was estimated during the exponential phase of growth by measuring the increase in absorbance of the culture broth at 600 nm in a Klett photometer (Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.). Growth rate in the fermenter was estimated by measuring the increase in cell dry weight as described by Johnson *et al.* (1978). Cultures were harvested by centrifuging after reaching a constant absorbance or dry weight, washed with water, and frozen at −20 °C to await analysis. The yeast yield (Y) is defined as $Y = X_f - X_0$, where X_f and X_0 are the final and initial yeast dry weights (mg ml^{-1}), respectively.

For light induction experiments, *P. rhodozyma* was grown in an orbital shaker incubator equipped with two fluorescent tube lamps which provided 2700 lx at the culture surface. Control flasks were covered with aluminium foil.

Analyses of culture media. Reducing sugar concentration (mg ml^{-1}) in the culture medium was determined with the 3,5-dinitrosalicylic acid reagent (Sumner & Somers, 1949). The rate of dissolution of oxygen into culture media was estimated by the sulphite oxidation method (Cooper *et al.*, 1944). The results are expressed as mmol O_2 dissolved l^{-1} h^{-1}.

Carotenoid extraction and analysis. For routine analyses of astaxanthin, *P. rhodozyma* cell suspensions were mixed with 0·5 mm glass beads, and then vibrated for 3 min in a Braun homogenizer (Bronwill Scientific, Rochester, N.Y., U.S.A.). The broken cells were thoroughly stirred in about 20 vol. acetone, centrifuged, and the pigments in the supernatant were transferred to petroleum ether with the addition of dilute NaCl solution. Astaxanthin concentration in the petroleum ether extract was estimated by measuring the absorbance at λ_{max} (474 nm). The specific absorption coefficient $A^{1\%}_{1cm} = 1600$ (Andrewes *et al.*, 1976) and the formula provided by Davies (1976) allowed the calculation of astaxanthin concentration.

Petroleum ether extracts of carotenoid mixtures to be chromatographed were dried over Na_2SO_4 and concentrated by rotary evaporation at 30 °C in subdued light. The carotenes were separated from the xanthophylls on a MN Kieselgel (Brinkmann Instruments) column by elution with 3 % (v/v) diethyl ether in petroleum ether. Xanthophylls were eluted with acetone. The total concentration of xanthophylls and carotenes in the eluates was estimated by measuring A_{478} for xanthophylls and A_{448} for the carotenes (but A_{428} in cells grown at pH 3·5, see text) using the absorption coefficients $A^{1\%}_{1cm} = 1600$ and 2600, respectively. The individual eluates were sometimes further chromatographed by thin-layer chromatography on aluminium oxide (Alox 25 UV_{254}; Brinkmann Instruments) and silica gel (Silica Gel 60; EM Laboratories) using various combinations of acetone, ethyl ether and petroleum ether as developing solvent mixtures.

Identification of carotenoids. Carotenoids which had been purified to chromatographic homogeneity were characterized by their electronic absorption spectrum, by co-chromatography with identical or related pure carotenoids in two solvent systems and by their mass spectrum (if sufficient material was available).

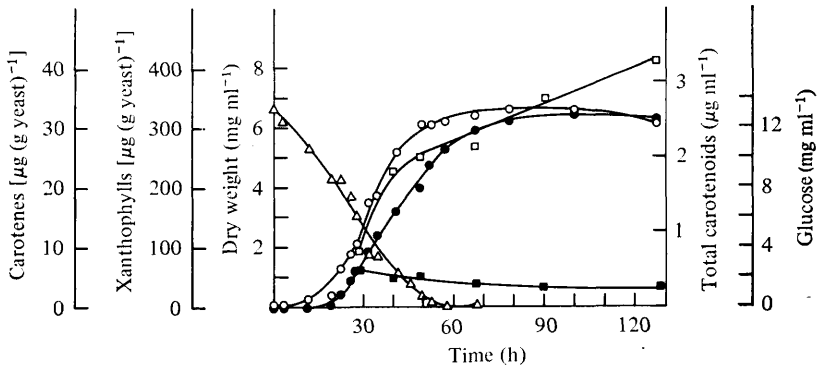


Fig. 1. Production of carotenoids by *P. rhodozyma* in fermenter batch culture. Yeast growth (○), total carotenoid formation (●), xanthophyll (astaxanthin) formation (□), carotene synthesis (■) and glucose utilization (△). The growth medium initially contained 1·5% (w/v) D glucose.

Visible absorption spectra were recorded in acetone, and concentrations of carotenoids were calculated using the specific absorption coefficients provided by Davies (1976). The mass spectra were determined on a Finnegan 3200 instrument at 220 °C with an ionization voltage of 70 eV.

Chemicals. All chemicals were, where possible, of analytical grade. D-Mannitol, L-arabinose and D-xylose were obtained from ICN Pharmaceuticals, Cleveland, Ohio, U.S.A.; cellobiose was from Sigma, gluconō-δ-lactone from Merck; maltose from Calbiochem; sucrose, succinic acid, D-glucose, potassium hydrogen phthalate, phosphate salts and all solvents from Mallinckrodt, St. Louis, Mo., U.S.A.; and 3,5-dinitrosalicylic acid from Eastman Kodak Co.

RESULTS

Growth and astaxanthin production

In fermenter batch culture, growth of *P. rhodozyma* began after a 10 h lag and a constant dry weight of yeast was reached after about 80 h (Fig. 1). Termination of growth coincided with the exhaustion of glucose from the medium. Astaxanthin was found to be the major xanthophyll in all samples of yeast taken during the fermentation and was produced mainly during the exponential growth period. Its production slowed soon after cessation of growth. The concentration of xanthophylls in the cells increased from 92 to 225 μg g^{-1} during the period of exponential growth (30 to 40 h) and then increased only slightly to about 260 μg g^{-1} in the next 20 to 30 h. On exhaustion of glucose, the concentration of xanthophylls increased steadily until 128 h when a final concentration of 406 μg g^{-1} was obtained. In contrast, the concentration of carotenes in *P. rhodozyma* during the lag and exponential phase was fairly constant at 6 μg g^{-1} and decreased to 3 μg g^{-1} in the stationary phase. The primary carotene identified in all samples during growth was β-carotene.

Effect of pH on growth and pigment formation

In a preliminary screening of buffers, it was found that 0·1 M-potassium hydrogen phthalate and 0·1 M-sodium phosphate buffered well over the necessary range of pH values and allowed good growth and pigmentation of *P. rhodozyma*. Citrate buffer was unsatisfactory because it inhibited growth. Lactate buffer was also unsuitable because a rise in pH of the culture broth occurred late in the exponential phase of growth, probably due to the utilization of lactate by *P. rhodozyma* (Miller *et al.*, 1976).

In phthalate or phosphate buffer, the final yield of yeast was only slightly affected by pH in the range 3·8 to 7·5 (Fig. 2) in shake flasks. The growth rate of *P. rhodozyma* was much more affected by pH and was highest at pH 5·8. The yield of astaxanthin was also affected by medium pH; a maximum yield of 2·0 μg ml^{-1} was obtained at pH 5·0 and at this pH the concentration of astaxanthin in *P. rhodozyma* was also highest [510 μg (g yeast)$^{-1}$]. Gener-

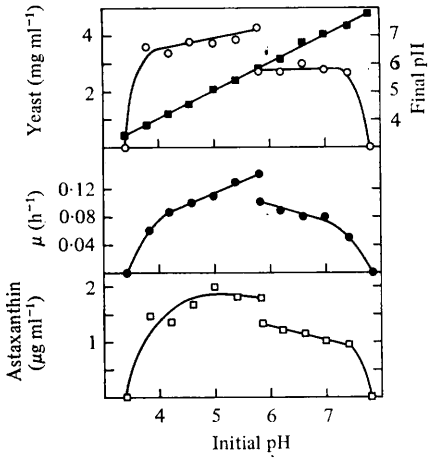


Fig. 2. Effect of pH on carotenoid formation and growth of *P. rhodozyma* in shake flasks buffered with 0·1 M-potassium hydrogen phthalate or 0·1 M-sodium phosphate (pH 5·8 to 7·8). Final yeast yield (○), yeast growth rate (●), astaxanthin yield (□) and final pH (■).

Table. 1. *Effect of pH on growth and astaxanthin formation of P. rhodozyma in fermenter culture*

pH of medium	Yeast yield (mg ml^{-1})	Growth rate, μ (h^{-1})	Astaxanthin [μg (g yeast)$^{-1}$]	Total xanthophylls [μg (g yeast)$^{-1}$]	Total carotenes [μg (g yeast)$^{-1}$]
6·5	6·03	0·14	325	332	11·0
5·5	9·68	0·12	336	339	15·0
4·5	11·79	0·16	387	385	6·5
3·5	5·96	0·09	212	219	15·0

ally within the pH range 4·0 to 7·0 the choice of buffer (potassium hydrogen phthalate or sodium phosphate) had a greater effect on the parameters tested than did pH. Sodium phosphate buffer was slightly inhibitory and the yield of yeast, yeast growth rate and astaxanthin formation were all lower in phosphate buffer than in phthalate buffer at the same pH (5·8).

The influence of pH on carotenoid production was also studied in fermenters since the pH could be controlled by automatic titration so that the effects of buffers were eliminated. Of the four pH values studied (Table 1), the optimum was found to be pH 4·5 where the maximum yield of cells, the highest growth rate and the maximum production of astaxanthin were obtained. Analysis of the pigments showed that astaxanthin was the dominant xanthophyll present at each pH value. However, the absorption spectrum of the carotene fraction from yeast grown at pH 3·5 was strikingly different from that obtained at other pH values (Fig. 3). The primary carotene present at pH 3·5 was β-zeacarotene whereas β-carotene dominated in yeast grown at the other pH values (see Table 2). After purification, these pigments were conclusively identified by their absorption spectra, chromatographic characteristics and mass spectra. β-Zeacarotene could not be found in cells grown at pH 4·5.

Influence of temperature on growth and pigmentation of P. rhodozyma cultured in shake flasks

The final yield of yeast was relatively constant at growth temperatures of 22 °C and below, but the yield decreased considerably at temperatures above 22 °C, the optimum for growth rate (Fig. 4). The highest temperature at which growth was observed was 27·5 °C; at this temperature *P. rhodozyma* increased its mass about twofold after inoculation and

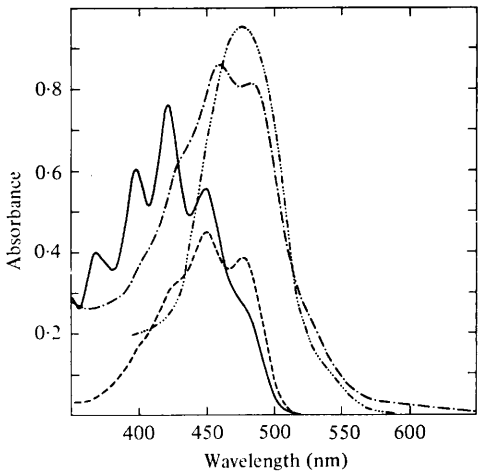


Fig. 3. Visible absorption spectra (in acetone) of the carotenes isolated from *P. rhodozyma* grown at pH 3·5 (———) and pH 4·5 (— — — —) and of the total carotenoid extract of cells grown under aerobic (· · · — · · · —) or microaerophilic (— · — ·) conditions.

Table 2. *Analysis of carotenes isolated from P. rhodozyma grown in a fermenter at pH* 3·5 *or* 4·5

		pH 4·5		pH 3·5	
Carotene	Structure	μg isolated	% of total*	μg isolated	% of total*
β-Carotene	β,β-Carotene	27	77·0	trace	~1
β-Zeacarotene	7′,8′-Dihydro-β,ψ-carotene	0	0	40	90
γ-Carotene	β,γ-Carotene	2·7	7·7	1·5	3·0
Neurosporene	7,8-Dihydro-γ,γ-carotene	1·7	5·0	2·5	5·0
Lycopene	γ,γ-Carotene	3·5	10·0	trace	~1

* Total yield of carotenes: pH 3·5, 15 μg (g yeast)$^{-1}$; pH 4·5, 7 μg (g yeast)$^{-1}$. The concentrations of xanthophylls were 219 and 385 μg (g yeast)$^{-1}$, respectively.

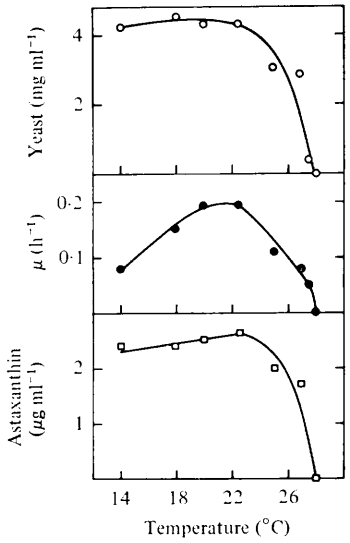


Fig. 4. Effect of temperature on growth and pigmentation of *P. rhodozyma* grown in shake flasks. Symbols as in Fig. 2.

Table 3. *Effect of carbon source on growth and pigmentation of P. rhodozyma in shake flask culture*

The growth medium was 50 ml 0·1 M-phthalate-buffered yeast nitrogen base medium (see Methods), containing 0·6% (w/v) Bacto-peptone and 200 mg carbon (supplied as the various sugars). The values represent the mean of two determinations. Carbon sources were sterilized separately from the basal medium. No growth occurred in the basal medium without the addition of a carbon source.

Carbon source	Growth rate, μ (h^{-1})	Yeast yield (mg ml^{-1})	Yeast yield [mg (mg carbon)$^{-1}$]*	Astaxanthin yield (μg ml^{-1})	Astaxanthin yield [μg (g yeast)$^{-1}$]
D-Maltose	0·14	3·63	0·91	1·86	512
D-Cellobiose	0·10	3·48	0·87	2·27	652
Sucrose	0·19	3·72	0·93	1·89	508
Succinate	0·09	2·66	0·67	1·33	500
D-Mannitol	0·16	3·68	0·92	1·80	489
D-Xylose	0·04	1·21	0·30	0·58	479
L-Arabinose	0·06	3·30	0·83	1·25	379
Glucono-δ-lactone	0·10	1·48	0·37	0·80	541
D-Glucose	0·20	3·85	0·96	1·62	421
D-Glucose†	0·21	6·46	0·81	1·11	171

* Assuming all carbon utilized. † 800 mg carbon [4% (w/v) glucose].

then stopped growing. The astaxanthin concentration in yeast grown at all the temperatures tested was constant (about 480 μg g^{-1}). Insufficient yeast was obtained at 27·5 °C to estimate the concentration of astaxanthin, but the cells were very pale. All the acetone extracts gave visible absorption spectra typical of astaxanthin. The concentrations of carotenes in the yeast also remained constant at about 7 μg g^{-1}.

Growth and pigmentation of P. rhodozyma grown on various carbon sources

Cellobiose supported more pigmentation of *P. rhodozyma* [652 μg (g yeast)$^{-1}$] than any of the other carbon sources tested (Table 3). The other disaccharides, maltose and sucrose, also promoted high pigmentation. Sucrose and glucose promoted more rapid growth of *P. rhodozyma* (μ 0·19 h^{-1}) than the other carbon sources. Although succinate and glucono-δ-lactone supported slow growth and rather sparse yields of yeast, these compounds promoted high concentrations of astaxanthin in *P. rhodozyma*. The sugar alcohol D-mannitol supported good yeast growth and pigmentation. The pentoses L-arabinose and D-xylose were utilized but resulted in slow growth and carotenoid production. D-Glucose supported a high rate of growth but only a moderate yield of astaxanthin, especially when present at a higher concentration (4%, w/v).

Influence of glucose concentration and shaking on growth and pigmentation of P. rhodozyma

As *P. rhodozyma* is a fermentative yeast, growth and pigment production were studied in a wide range of glucose concentrations in YM medium in shake flasks. The final yield of yeast per g glucose utilized decreased significantly with increasing glucose concentrations in shake flasks. The yield of astaxanthin per g yeast followed a remarkably similar pattern (Fig. 5). However, because the total yield of yeast increased substantially in the high glucose media, more astaxanthin (μg ml^{-1}) was produced. At concentrations of glucose above 10 mg ml^{-1}, the efficiency of biomass and astaxanthin production decreased steadily. Above about 40 mg glucose ml^{-1}, the yeast yield per g glucose and astaxanthin yield per g yeast were less affected by increasing glucose concentrations than below 40 mg ml^{-1} (Fig. 5).

The production of carotenoids was also studied in a fermenter in medium containing 5% glucose (Fig. 6). Glucose was not completely utilized after 126 h in this medium; yeast yields were reduced to about half those obtained in the 1·5% glucose fermentation and the concentration of astaxanthin in *P. rhodozyma* decreased to 350 μg (g yeast)$^{-1}$ (compare

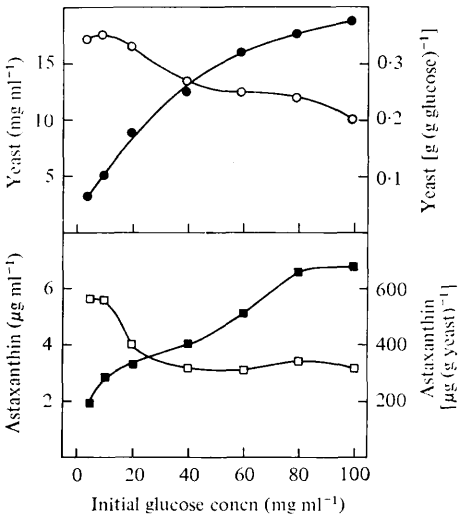


Fig. 5. Effect of glucose concentration on growth and pigmentation of *P. rhodozyma*. Final yeast yield (●), yeast yield per g glucose (○), astaxanthin yield (■) and astaxanthin yield per g yeast (□). Calculations corrected for residual glucose. The growth medium contained (per litre in 0·1 M-phthalate buffer, pH 5·0): 3 g yeast extract, 3 g malt extract, 6 g peptone (YM basal broth) and various concentrations of D-glucose.

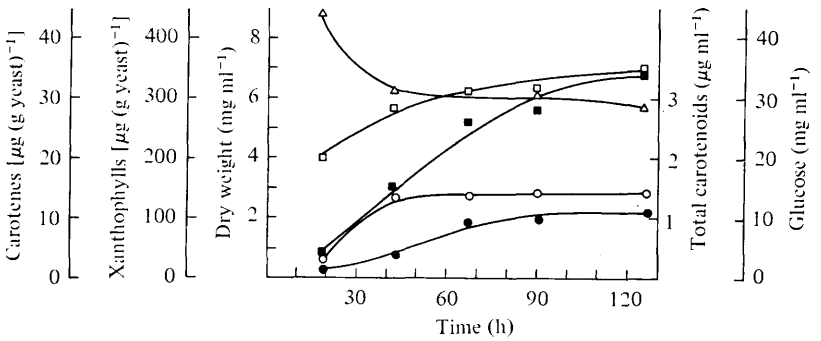


Fig. 6. Effect of 5% glucose on carotenoid formation and growth of *P. rhodozyma* in fermenter batch culture. Yeast growth (○), total carotenoid formation (●), xanthophyll (astaxanthin) formation (□), carotene synthesis (■) and glucose utilization (△).

Fig. 1). However, the concentrations of total carotenoid pigment in the cells grown at the two glucose concentrations were almost the same (about 400 μg g^{-1}); this was because the concentration of carotenes was 12-fold higher in the cells grown in the high-glucose medium. The primary carotene present in yeast grown in the 5% glucose medium, from all fermenter samples, was β-carotene. In addition, approximately 2·5% of the total carotenes in the cells was found to be β-zeacarotene. The absorption spectrum of the xanthophyll fraction, in all samples, was identical to that of astaxanthin and therefore we did not analyse this group of pigments for concentrations of individual carotenoids.

Effects of relative aeration on growth and pigmentation

To study the effects of aeration on growth and carotenoid production in *P. rhodozyma*, we varied the volume of medium in the shake flasks between 25 and 200 ml and their shaking rates from 50 to 200 rev. min^{-1} to produce a range of aeration rates. The dissolution rates of oxygen into a sulphite-containing solution indicated that a wide range of aeration rates was achieved (from 3·6 to 108 mmol O_2 dissolved l^{-1} h^{-1}).

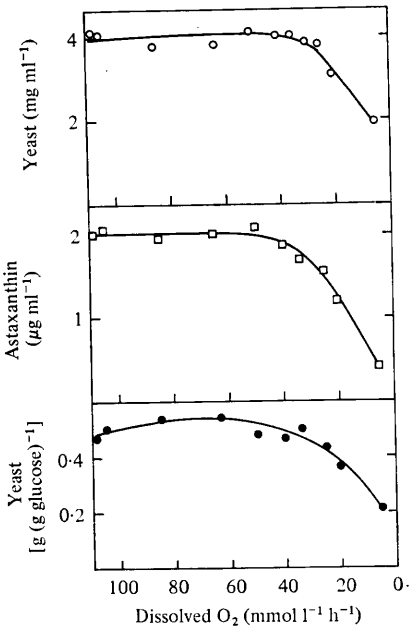


Fig. 7. Effect of aeration on carotenoid formation and growth of *P. rhodozyma*. Final yeast yield (○), yeast yield per g glucose (●) and astaxanthin production (□). The abscissa represents the air supply as mmol O_2 supplied l^{-1} h^{-1}, obtained in 0·5 l baffled shake flasks by using different medium volumes (25, 50, 100 and 200 ml) and shaking speeds (50, 100, 150, 200 rev. min^{-1}).

Table 4. *Carotenoids of P. rhodozyma grown under microaerophilic or aerobic conditions*

Carotenoid	Structure	Individual carotenoids isolated from cells (% of total carotenoid present)*	
		Microaerophilic	Aerobic
Astaxanthin	3,3′-Dihydroxy-β,β-carotene-4,4′-dione	26	87
Phoenicoxanthin	3-Hydroxy-β,β-carotene-4,4′-dione	4	6
3-Hydroxy-3′,4′-didehydro-β,ψ-caroten-7-one	—	9	<1
3-Hydroxyechinenone	3-Hydroxy-β,β-carotene-4-one	<1	2
Echinenone	β,β-Carotene-4-one	27	3
β-Carotene	β,β-Carotene	33	2
Other carotenes	—	<1	<1

* Total carotenoid yield: aerobic, 509 μg (g yeast)$^{-1}$; microaerophilic, 244 μg (g yeast)$^{-1}$.

The yields of cell mass and astaxanthin were fairly independent of oxygen dissolution rates except at the lowest aeration values, i.e. less than 30 mmol O_2 l^{-1} h^{-1} (Fig. 7). Below this level, the yields of yeast and astaxanthin were significantly reduced; at the lowest oxygen dissolution rate (3·6 mmol l^{-1} h^{-1}) the yield of yeast decreased from the usual value of about 4·0 mg ml^{-1} to 2·0 mg ml^{-1} and the yield of astaxanthin decreased from approximately 2·0 to 0·3 μg ml^{-1}.

Because low aeration drastically influenced the concentration of carotenoids in *P. rhodozyma*, we incubated *P. rhodozyma* without an air supply in the fermenter. Under these conditions, the yeast tripled its biomass but then grew no more. Addition of ergosterol and Tween 80 to the medium did not promote growth. The harvested yeast was low in total

carotenoids (244 μg g^{-1}) and the visible absorption spectrum of the total carotenoid extract was not typical of astaxanthin (see Fig. 3). The concentration of xanthophylls was 163 μg g^{-1} and of carotenes 81 μg g^{-1}. The concentrations of individual carotenoids are given in Table 4. Under these microaerophilic conditions, comparatively little astaxanthin was produced; it made up only 26% of the carotenoid mixture compared with nearly 90% under aerobic growth conditions. The primary carotenoid synthesized under anaerobic conditions was β-carotene. Echinenone was also produced in much higher amounts anaerobically than in aerobically grown yeast.

Because low aeration and high glucose in the growth medium caused significant reductions in the efficiency of astaxanthin production, we decided to combine these effects. In a medium containing 4% (w/v) glucose and with O_2 supplied at 5·0 mmol l^{-1} h^{-1}, the specific growth rate of *P. rhodozyma* was 0·1 h^{-1} and the yield of yeast was only 0·05 mg (g glucose)$^{-1}$. The cells were tan rather than pink and contained only 30 μg total carotenoid g^{-1}. On analysis the total pigment extract showed a visible absorption spectrum similar to β-zeacarotene. Chromatography showed that the yeast contained little astaxanthin but proportionately higher concentrations of less polar pigments (probably carotenes) including β-zeacarotene.

Light and carotenoid production

Phaffia rhodozyma was grown in triplicate shake flasks in YM medium in the dark or with high light intensity (2700 lx). The yield of yeast in the dark- and light-grown cultures was 3·7 and 3·5 mg ml^{-1}, respectively. Observation of the shake flasks after growth suggested that the light-grown cultures synthesized more astaxanthin, because the cells had a redder hue. This may have been due to different relative concentrations of the carotenoids present, since the assay of astaxanthin gave only a slightly increased mean, 538 μg g^{-1} compared to 510 μg g^{-1} for the dark-grown culture.

Effect of nitrogen source and complex media on pigmentation in P. rhodozyma

The concentration of ammonium sulphate in the range 0·25 to5 mg ml^{-1} had little effect on the yeast growth rate, final yeast biomass or on carotenoid production in YNB medium supplemented with 1% D-glucose. The substitution of $(NH_4)_2HPO_4$ or peptone for $(NH_4)_2SO_4$ at various concentrations also did not affect these values. However, when increasing concentrations of yeast extract (0·1 to 10 mg ml^{-1}) were added to a vitamin-free medium (vitamin-free yeast base, Difco), there was an increase in pigmentation from 156 to 524 μg g^{-1}. Similarly, when *P. rhodozyma* was grown in fermenter culture on complex media – (i) brewer's malt wort diluted to a specific gravity of 1·020 or (ii) addition of 1·0 l of colourless tomato pressings to 2·0 l of the standard medium – the carotenoid yields were 712 and 814 μg g^{-1}, respectively, after 60 h growth.

DISCUSSION

Astaxanthin formation in *P. rhodozyma* is clearly growth-associated, although its production does not exactly coincide with increase in biomass. The growth-associated production of astaxanthin contrasts with results found with *Sporobolomyces roseus* (Bobkova, 1965) and *Rhodotorula glutinis* (Vecher & Kulikova, 1968), where carotenoid production occurred only after yeast growth had stopped; it is also common in the Phycomycetes (e.g. *Phycomyces blakesleeanus*) for the primary period of carotenoid synthesis to follow cessation of growth.

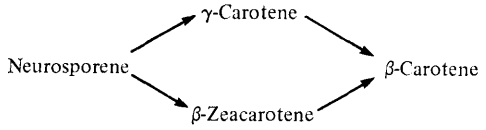
Phaffia rhodozyma is the only carotenogenic yeast that ferments glucose (Miller *et al.*, 1976). It would be expected that growth at low dissolved oxygen concentrations and/or high glucose levels would promote fermentative metabolism and possibly affect carotenoid production. When the supply of oxygen to *P. rhodozyma* was reduced to low levels there

was a decrease in the yield of yeast per g glucose utilized, which suggested that *P. rhodozyma* was fermenting. This was accompanied by a decrease in astaxanthin concentration and an accumulation of β-carotene. Similarly, when *P. rhodozyma* was cultured with increasing concentrations of glucose, fermentative growth was indicated by decreased yields of yeast per g carbon utilized and this was accompanied by decreases in astaxanthin concentration. These results show that astaxanthin production is inhibited under fermentative conditions. This is supported by the fact that glucose, which is readily fermented by *P. rhodozyma*, promoted relatively low levels of astaxanthin production. Cellobiose, however, which can only be used aerobically by *P. rhodozyma* (Phaff *et al.*, 1972), stimulated relatively high astaxanthin production as did succinate, which may be directly utilized aerobically by the tricarboxylic acid cycle.

Changes in the lipid content of aerobically and anaerobically grown *Saccharomyces cerevisiae* have been interpreted as a reflection of the state of mitochondrial development (Jakovicic *et al.*, 1971). The inability to form ergosterol in mutants of *S. cerevisiae* (Bard *et al.*, 1974) is due to lesions in the biosynthesis of porphyrins rather than direct lesions in the synthesis of sterols, which implies the requirement of an active respiratory chain for the synthesis of ergosterol. It is possible that an active respiratory chain is also needed in the biosynthesis of carotenoids, since these share many enzymic steps with sterols in their formation from acetyl-CoA. Exposure of anaerobically grown cells to oxygen causes a rapid induction of the sterol-synthesizing enzyme, 3-hydroxy-3-methyl-glutaryl CoA reductase (Berndt *et al.*, 1973). Further, since carotenoids have been reported to be located primarily in the mitochondria in certain fungi (Heim, 1946), this location may also indicate their site of synthesis.

Very little is known about the formation of xanthophylls in micro-organisms. It is generally assumed that hydroxyl functions at C-3 and C-3′ of the carotenoid skeleton arise from the incorporation of molecular oxygen by carotene hydrocarbons. The evidence for this hypothesis is indirect (see Britton, 1976). Nothing is known about the introduction of oxygen functions at C-4 and C-4′ except that it is likely that oxo groups are formed through the hydroxy intermediates (Britton, 1976). When *P. rhodozyma* was cultured with minimal oxygen supply, the astaxanthin yields were greatly reduced, and the yeast tended to accumulate β-carotene as well as the monoketone echinenone. These results suggest that the hydroxyl functions in astaxanthin are formed only in the presence of oxygen and that carotenes and echinenone are formed under conditions of low aeration. When we aerated anaerobically grown stationary phase cells, there was no detectable change in their astaxanthin content.

β-Zeacarotene accumulated in cells of *P. rhodozyma* grown under adverse environmental conditions. This pigment was not originally detected in the analysis of the pigments of *P. rhodozyma* by Andrewes *et al.* (1976). Its isolation suggests that the well known alternative route for β-carotene synthesis is operative in *P. rhodozyma* thus:



β-Zeacarotene accumulates in cells of Rhodotorula (Simpson *et al.*, 1964) and *Phycomyces blakesleeanus* in the presence of inhibitors. Because β-zeacarotene usually accumulates in cells only under adverse conditions, e.g. in the presence of inhibitors such as diphenylamine or in stressful environments, it may be regarded as an indicator of inefficient carotenoid biosynthesis. Its formation can perhaps be rationalized by a hypothesis of Goodwin and his coworkers, (McDermott *et al.*, 1974) who postulate that the synthesis of zeaxanthin by *Flavobacterium* spp. involves an enzyme complex with two active sites, each of which acts on a carotenoid 'half molecule' in synchrony and with equal efficiency, i.e.

desaturation or cyclization of each half molecule proceeds at the same rate. Under abnormal conditions, however, the individual sites may not act in synchrony and asymmetrical products such as β-zeacarotene may result. Further, genetic evidence has shown that there is an enzyme complex with two cyclases for the formation of β-carotene in Phycomyces (De la Guardia *et al.*, 1971).

In contrast to many other micro-organisms, light does not stimulate carotenogenesis in *P. rhodozyma*. The biosynthesis of astaxanthin was, however, greatly enhanced by growth in the presence of tomato wastes. The high yield of carotenoid obtained with the tomato supplement suggests that carotenoid precursors may enter the cell and enhance carotenoid production. These results indicate the possibility of commercial production of astaxanthin by *P. rhodozyma* using citrus or vegetable wastes as adjuncts in the fermentation.

This research was financially supported by Sea Grant no. NOAA-04-6-158 R/R-11. A traineeship was provided by Sea Grant for E. A. Johnson.

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