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Biosynthesis of the xanthophyll plectaniaxanthin as a stress response in the red yeast *Dioszegia* (Tremellales, Heterobasidiomycetes, Fungi)

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Abstract

Carotenoid biosynthesis was examined in a phylloplane yeast identified by ITS, 18S and 28S rDNA analysis as a *Dioszegia* sp. close to *D. takashimae*. In well-aerated flask or fermentor cultures, this strain produced essentially a single pigment confirmed as the xanthophyll plectaniaxanthin by NMR analysis, at concentrations of 103–175 μg g^{-1} biomass dry weight. Detailed studies showed increases in plectaniaxanthin concentrations in the presence of 5 mM hydrogen peroxide (1.8-fold), 50 and 100 μM duroquinone (3.1- and 3.7-fold, respectively), and 2% ethanol (4.9-fold). Whereas oxidative stress is known to enhance the biosynthesis of torularhodin or astaxanthin in other red yeasts where they are associated with an antioxidant function, this is the first report implicating plectaniaxanthin in a similar role. At reduced aeration, biosynthesis of plectaniaxanthin was suppressed and its putative precursor γ-carotene accumulated. The carotenoid cyclase inhibitor nicotine (5–20 mM) inhibited plectaniaxanthin formation, with lycopene accumulating stoichiometrically. Hydroxy groups at C-1′ and C-2′ therefore seem to be introduced late in plectaniaxanthin biosynthesis, following cyclization of the β-ionone ring.

Keywords: *Dioszegia*; Antioxidant; Biosynthesis; Carotenoids; Oxidative stress; Phylloplane yeasts; Plectaniaxanthin

1. Introduction

Red yeasts are so called because of their high carotenoid content. With the exception of the yeast states of the Archiascomycetes *Taphrina* and *Protomyces* (van Eijk and Roeymans, 1982), all red yeasts described to date seem to belong to three broad classes of the Basidiomycota, viz. the Heterobasidiomycetes, Urediniomycetes and Ustilaginomycetes (Fell et al., 2001). Their habitats include the phylloplane which is exposed to high UV irradiation, and other associations with plants where these yeasts may be subjected to radical-generating substances. For instance, the astaxanthin-producing species *Phaffia rhodozyma* M.W. Miller et al. has been isolated from the slime flux of broad-leaved trees (notably *Betula* spp.) thought to contain an unidentified photosensitizing substance inducible by UV light (Schroeder and Johnson, 1995a). Consequently, the physiological role of carotenoids in red yeasts may be as antioxidants in situations of high oxidative stress (Johnson, 2003; Bhosale, 2004). One class of substances particularly sensitive to oxygen radicals are polyunsaturated fatty acids, and it is interesting in this context that carotenoids are accumulated in lipid droplets in yeasts and other fungi (Davoli and Weber, 2002a,b; Weber and Davoli, 2002). Further, under conditions of oxidative stress the carotenoid composition of red yeasts may change, and pigments with superior antioxidant properties may be synthesized in preference to β-carotene (Schroeder and Johnson, 1993; Sakaki et al., 2001; Davoli et al., 2004).

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Thirdly, mutants deficient in carotenogenesis show limited viability in the presence of oxygen radicals or high UV irradiation (Schroeder and Johnson, 1995a), whereas carotenoid-overproducing mutants may show enhanced viability (Sakaki et al., 2000).

A diversity of carotenoids has been characterized in basidiomycete yeasts, whereby the monocyclic γ-carotene with its oxidized derivatives torulene and torularhodin, and the bicyclic β-carotene are the most common. The oxidized bicyclic ketocarotenoids canthaxanthin and astaxanthin are much less frequent in red yeasts, having been described so far only from *Phaffia rhodozyma* and a related teleomorphic form, *Xanthophyllomyces dendrorhous* Golubev. Both pigments, but especially astaxanthin, are of economic importance as food and feed colourants and antioxidants, particularly in the farming of salmonid fish (Johnson and An, 1991).

Basidiomycete yeasts represent a major pool of unexplored biodiversity, with as little as 1% of the existing species thought to have been discovered and described as yet (Fell et al., 2000). In the course of our work on fungal pigments, we have isolated and characterized several basidiomycete yeasts with unusual carotenoid spectra. Here we report the presence of plectaniaxanthin (**1**; see Fig. 4), an uncommon 1′,2′-dihydroxylated monocyclic carotenoid, in a yeast assigned to the genus *Dioszegia* on the basis of DNA sequence data, C and N utilization spectra, and microscopic observations. The biosynthesis of **1**, like that of other xanthophylls in different basidiomycete yeasts, was found to be stimulated by oxidative stress, hinting at a similar antioxidant function in the producer.

2. Results and discussion

2.1. Production and identification of *1*

Strain CAR034 produced only one major pigment (**1**) when grown in a range of media under well-aerated conditions both in baffled shaken flasks and in aerated fermentors. A typical fermentation curve of CAR034 in 15 l YG medium in a 20-l stirred aerated fermentor (Fig. 1) showed a steep increase in biomass accompanied by rapid assimilation of glucose from the medium within the first 48 h of cultivation, followed by a stationary phase. Carotenoid production increased as the cells entered the stationary phase. Biomass was harvested after 6 days fermentation for purification of the major pigment.

Purified compound **1** had the following spectral properties. UV/vis: $\lambda_{max} = 447$, 469 and 499 nm in methanol and *n*-hexane. IR (KBr): $\nu_{max} = 3425$, 2955, 1460, 1370, 1070, 1040, 970 and 835 cm^{-1}. APCI–MS indicated a molecular weight of 568 (NI *m/z* 568, 550; PI *m/z* 551 and 533, corresponding to a double loss of water), which would be compatible with the formula $C_{40}H_{56}O_2$ and the presence of two hydroxy groups in the molecule. In addition, fragment peaks at *m/z* 511 and 508 were detected in the APCI-PI spectrum, corresponding to a loss of acetone [M − 58] and isopropanol [M − 60], respectively, and suggesting the presence of a $-C(CH_3)_2OH$ moiety (Enzell et al., 1969). These values matched those reported for plectaniaxanthin (3′,4′-didehydro-1′,2′-dihydro-β,ψ-caroten-1′,2′-diol) in the literature (Arpin and Liaaen-Jensen, 1967; Bae et al., 1971; Rønneberg et al., 1982; Britton et al., 2004). Identification was confirmed unambiguously by NMR spectroscopy, and two-dimensional techniques (gHMQC, gCOSY, gTOCSY and *J*-resolved) were also applied for the safe assignment of ^1H and ^{13}C signals. The structure elucidation of pigment **1** from strain CAR034 is described below.

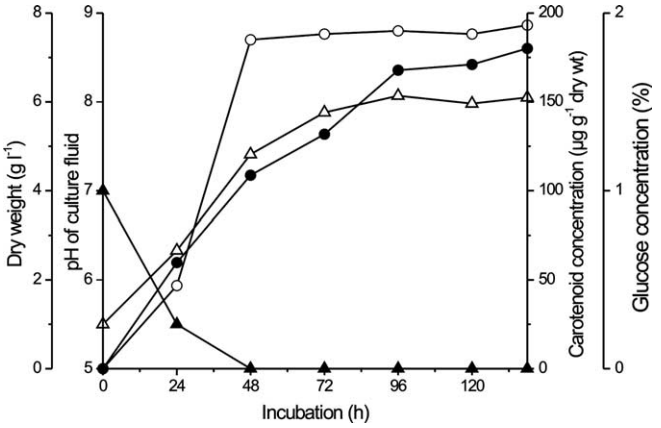

Fig. 1. Growth curve of *Dioszegia* CAR034 in a stirred aerated fermentor (15 l YG medium) at 21 °C. The pH of the culture (△), glucose levels in percent (▲), biomass as g dry weight l^{-1} (○) and carotenoid concentrations as μg g^{-1} dry weight (●) are shown.

Resonances belonging to a carotenoid were immediately recognized in the ^1H NMR spectrum of pigment **1**. Two distinct groups of signals were present in the olefinic region, namely between 6.1–6.4 ppm and 6.5–6.7 ppm, accounting for 11 and 5 protons, respectively. gHMQC, *J*-resolved and gCOSY experiments permitted their identification as olefinic protons belonging to an unsymmetrical all-*trans* conjugated polyene chain of isoprenoid origin; these protons were assigned accordingly. More upfield, an isolated doublet of doublets (*dd*) at 5.71 ppm ($^3J = 7.6$, 15.9 Hz) featured as a characteristic signal in the ^1H spectrum and corresponded well to an olefinic proton adjacent to an oxygen-bearing aliphatic carbon atom, i.e., H-3′. Likewise, a doublet (actually a *dd* from *J*-resolved spectroscopy) at 4.00 ppm with $^3J = 7.6$ Hz coupled with the proton at 5.71 ppm and correlating with a carbon at 80.1 ppm in the gHMQC experiment was diagnostic for the presence of an hydroxy group at C-2′. The non-equivalence of the geminal methyl groups at C-1′ resonating as singlets at 1.18 and 1.23 ppm (δ_C 23.9 and 26.6 ppm, respectively) was in perfect agreement with literature data for plectaniaxanthin (Bae et al., 1971; Dumont and Pfander, 1984; Englert, 1995) and further corroborated the localization of the two hydroxy functions at positions 1′ and 2′ within the acyclic end group of the carotenoid molecule. Examination of the aliphatic region in the ^1H

spectrum revealed signals belonging to CH_3 protons (1.92 and 1.98 ppm) adjacent to olefinic carbon atoms and showing gHMQC correlations with carbons at 13.0 and 12.7 ppm, respectively, which well accounted for methyl groups attached to a polyene chain. An additional singlet at 1.72 ppm (δ_C 21.8) for which gTOCSY showed long-range correlations to methylene protons at 1.47, 1.60 and 2.02 ppm (H-2, H-3 and H-4, respectively), as well as to olefinic protons at 6.14 and 6.17 ppm (H-8 and H-7), was strongly suggestive of a β end group. Comparison of δ_C values for C-2–C-4 atoms assigned through gHMQC (39.7, 19.4 and 33.2 ppm, respectively) with those compiled by Englert (1995) safely established the β-ionone cyclic nature of the second end group. ^{1}H NMR spectral data of the major pigment from strain CAR034 are summarized in Table 1 and are in perfect agreement with those reported for natural (Bae et al., 1971) and synthetic (Dumont and Pfander, 1984) plectaniaxanthin. Similarly, our ^{13}C chemical shifts (Table 1) show an excellent match with published data for the two end groups that are found in plectaniaxanthin and thus expand the partial set of assigned δ_C values for **1** so far available in the literature (Englert, 1995).

Table 1
^{1}H and ^{13}C NMR spectral data (400.13 and 100.61 MHz, respectively) of plectaniaxanthin (**1**) from *Dioszegia* CAR034 in $CDCl_3$ solution (δ in ppm downfield from TMS as reference; $J_{H,H}$ in Hz)

Position	δ_H ($J_{H,H}$)	δ_C
1	–	n.d.
2	1.47 *m*	39.7
3	1.60 *m*	19.4
4	2.02 *m*	33.2
5	–	n.d.
6	–	n.d.
7	6.17 *d* (16.4)	126.7
8	6.14 *d* (16.4)	137.8
9	–	n.d.
10	6.15 *d* (11.2)	130.8
11	6.66 *dd* (11.2, 14.9)	124.7
12	6.35 *d* (14.9)	137.2
13	–	n.d.
14	6.25 *d* (11.8)	133.1
15	6.64 *m*	130
16	1.03 *s*	28.8
17	1.03 *s*	28.8
18	1.72 *s*	21.8
19	1.98 *s*	12.7
20	1.98 *s*	12.7
1′	–	n.d.
2′	4.00 *dd* (1.5, 7.6)	80.1
3′	5.71 *dd* (7.6, 15.9)	126.3
4′	6.38 *d* (15.9)	138.1
5′	–	n.d.
6′	6.20 *d* (11.6)	133.0
7′	6.59 *dd* (11.6, 14.9)	124.2
8′	6.39 *d* (14.9)	139.0
9′	–	n.d.
10′	6.25 *d* (11.6)	133.1
11′	6.63 *dd* (11.6, 15.0)	125.1
12′	6.39 *d* (15.0)	139.0
13′	–	n.d.
14′	6.28 *d* (11.6)	131.9
15′	6.64 *m*	130
16′	1.18 *s*	23.9
17′	1.23 *s*	26.6
18′	1.92 *s*	13.0
19′	1.98 *s*	12.7
20′	1.98 *s*	12.7

n.d. = not detected.

2.2. Identification of the producing strain

Strain CAR034 was isolated from a phylloplane habitat exposed to high solar irradiation, viz. leaves of *Crataegus* sp. forming a major component of a mixed shrub/grassland vegetation on the south-facing slope of Old Winchester Hill (Hants., UK), descending towards the Solent. On agar media, this yeast grew as intensely orange-pigmented, smooth colonies with a sharply delimited margin. After 7 days on YES agar, YM agar, YG agar or 2% malt extract (ME) agar with or without 20% sucrose, budding cells were globose or subglobose, 5–6 μm diameter, with buds arising

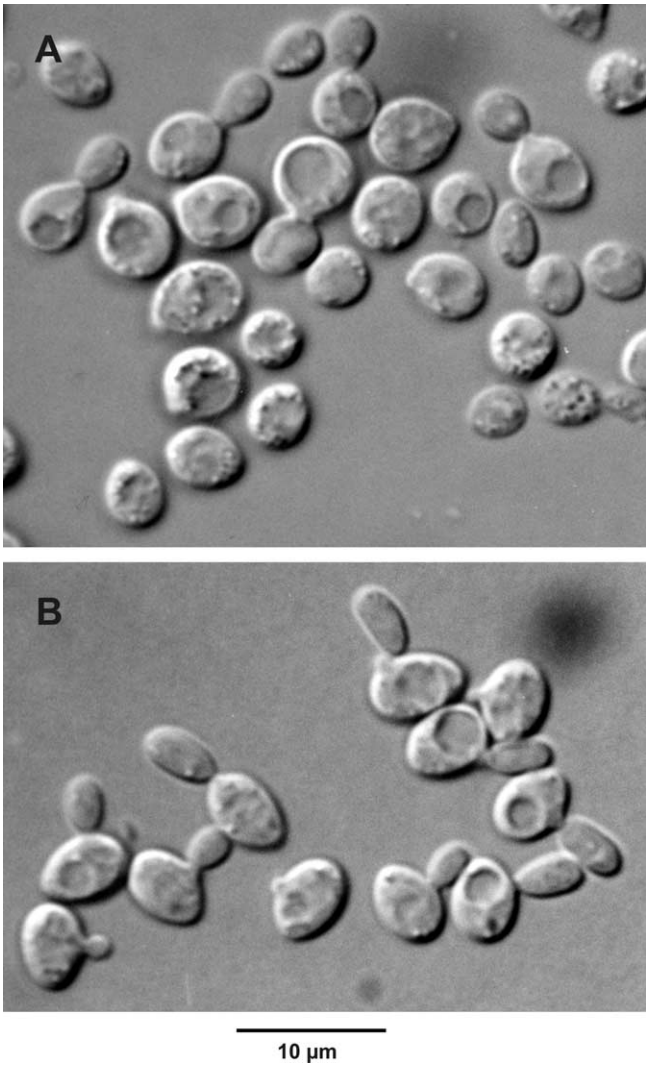

Fig. 2. Microscopic features of *Dioszegia* CAR034. (A) Vegetatively budding cells on ME agar with 20% sucrose after 7 days. (B) Production of conidia on cornmeal agar after 48 h.

Table 2
The closest matches for the ITS (GenBank accession DQ003332), 18S (DQ0033300) and 28S (DQ003331) ribosomal DNA sequences of strain CAR034 producing pigment **1**

ITS (complete)	18S (5′ partial)	28S (5′ partial)
1. *Dioszegia takashimae* AY562159 (99.6%)	*Dioszegia zsoltii* AF385444 (99.9%)	*Dioszegia takashimae* AY562150 (99.8%)
2. *Dioszegia catarinonii* AY562154 (97.1%)	*Dioszegia hungarica* AB032638 (99.5%)	*Dioszegia zsoltii* AY562152 (99.7%)
3. *Dioszegia zsoltii* AY562164 (96.7%)	*Dioszegia crocea* D31648 (99.4%)	*Dioszegia catarinonii* AY562143 (99.0%)

from a slight projection (Fig. 2A). More elongated asymmetric cells interpreted as conidia (Fig. 2B), 4–5 × 2–3 μm, were commonly produced within 24 h especially on weaker media (cornmeal agar and 0.2% ME agar). Sexual reproductive structures or chlamydospores were not observed on any of the agar media used.

Identification of CAR034 was attempted by analysing its complete ITS and 5′ partial 18S and 28S rDNA sequences, details of which are summarized in Table 2. Including primers, the lengths of amplificates were 541 nt (ITS), 821 nt (18S) and 655 nt (28S). The yeast *Dioszegia takashimae* was the closest match in GenBank/EMBL searches with ITS as well as 28S rDNA amplificates; 18S rDNA data for this species were not available in these data bases. Unfortunately, no valid species description of *D. takashimae* has been published as yet, so that we were unable to verify our identification by reference to the type description. However, other *Dioszegia* spp. were the next closest hits in our sequence searches (Table 2), confirming the assignment of CAR034 to the genus *Dioszegia* in its narrow boundaries defined by phylogenetic analyses (see Takashima et al., 2001; Bai et al., 2002; Scorzetti et al., 2002).

The carbon and nitrogen utilization spectrum of CAR034 was as follows: D-glucose (+), D-galactose (+), fructose (+), mannose (+), L-sorbose (delayed), cellobiose (+), lactose (−), maltose (+), melibiose (+), sucrose (+), α,α-trehalose (+), melezitose (+), raffinose (+), inulin (−), starch (−), D-arabinose (delayed), L-arabinose (+), L-rhamnose (+), D-ribose (+), D-xylose (+), erythritol (−), galactitol (+), D-glucitol (−), glycerol (−), *myo*-inositol (−), D-mannitol (+), ribitol (weak, delayed), methanol (−), ethanol (−), citric acid (−), D-galacturonic acid (delayed), D-glucuronic acid (+), D-gluconic acid (+), DL-lactic acid (+), succinic acid (+), α-D-methyl glucoside (delayed), salicin (weak, delayed), $(NH_4)_2SO_4$ (weak), KNO_3 (weak), $NaNO_2$ (−), L-lysine (+), ethylamine · HCl (delayed), cadaverine · 2HCl (delayed). These data corresponded well with the spectra reported by Takashima et al. (2001) and Bai et al. (2002) for several of the *Dioszegia* spp. listed in Table 2.

2.3. Chemotaxonomic implications

Pigment **1** was originally described from fruit-bodies of *Sarcoscypha* (formerly *Plectania*) *coccinea* (Fr.) Lamb. where it occurred mainly as the 1′,2′-diester and 2′-ester with unsaturated fatty acids (Arpin and Liaaen-Jensen, 1967; Vacheron et al., 1969), and it has also been reported from a few other ascomycetes (see Gill and Steglich, 1987). However, to our knowledge this pigment has been found in only two basidiomycetes, occurring in its free form in both cases. These are the red yeasts *Cryptococcus laurentii* (Kuff.) Skinner var. *flavescens* Lodder et Kreger-van Rij strain CBS 4256 (Bae et al., 1971) and *Rhodotorula aurantiaca* (Saito) Lodder strain CBS 317 (Liu et al., 1973). Although the former has now been separated from *C. laurentii* as *C. flavescens*, it is closely related to *C. laurentii* (Takashima et al., 2003) which belongs to the *Bulleromyces* lineage of the Tremellales (Hymenomycetes). In contrast, *R. aurantiaca* has been placed in the *Erythrobasidium* lineage of the Urediniomycetes (Fell et al., 2000; Nakase, 2000; Takashima et al., 2001). The *Dioszegia* clade of the *Cryptococcus luteolus* lineage, although belonging to the Hymenomycetes, is taxonomically far removed from *C. laurentii* (Gácser et al., 2001; Bai et al., 2002; Scorzetti et al., 2002), and the present report therefore identifies a third phylogenetic grouping of red yeasts, *Dioszegia*, as producers of pigment **1**. This suggestion is confirmed by the presence of **1** in a second red yeast isolate belonging to *Dioszegia*, and obtained from a leaf of *Clematis* sp. collected at the same sampling site (Davoli, P., Weber, R.W.S., unpublished). Further, **1** was the dominant pigment in both our *Dioszegia* strains whereas *C. flavescens* (Bae et al., 1971) as well as *R. aurantiaca* (Liu et al., 1973) contained a diversity of carotenoids, including 2-hydroxyplectaniaxanthin (Liu et al., 1973) which was absent from both our *Dioszegia* isolates.

2.4. Biosynthesis of *1*

When (−)-nicotine, a well-known inhibitor of carotenoid cyclization (Goodwin, 1980; Bouvier et al., 1997), was added at different concentrations to well-aerated shaken-flask cultures of *Dioszegia* CAR034 in YES medium, the concentration of **1** decreased, and lycopene (**2**) accumulated stoichiometrically (Fig. 3). No other pigments were observed except for a basal level of γ-carotene (β,ψ-carotene, **3**) which remained constant at all nicotine concentrations tested. This hints at an early formation of the β-ionone ring and suggests lycopene as the acyclic precursor of monocyclic carotenoids in *Dioszegia*, supporting the classical pathway from **2** via **3** and torulene (3′,4′-didehydro-β,ψ-carotene, **4**) (Simpson et al., 1964; Arpin and Liaaen-Jensen, 1967; Goodwin, 1980; see Fig. 4). An effect similar to that of nicotine has been reported for CPTA [2-(4-chlorophenylthio)triethylamine], resulting in lycopene accumulation at the expense of β-carotene, torulene and torularhodin in *Rhodotorula* spp. (Hayman et al., 1974).

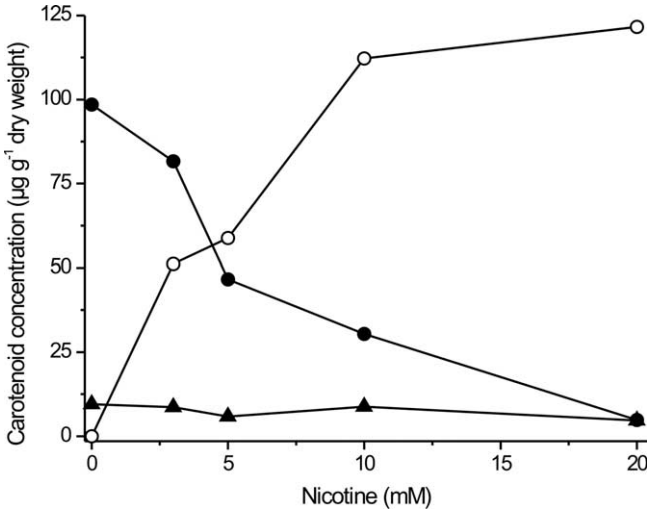

Fig. 3. The concentrations of **1** (●), **2** (○) and **3** (▲) in shaken-flask cultures of *Dioszegia* CAR034 incubated in the presence of increasing concentrations of nicotine. Results are shown as average of three independent replicates.

The absence of hydroxylated acyclic carotenoids in our cultures of *Dioszegia* CAR034 supplemented with nicotine would suggest that the introduction of a tertiary hydroxy group at C-1′ is also inhibited, as described in the literature for the bacterium *Rhodomicrobium vannielii* Duchow and Douglas (Singh et al., 1973; Britton et al., 1977). This is consistent with the hypothesis that hydration at C-1/C-1′ and cyclization of acyclic carotenoids share a common reaction mechanism (Britton et al., 1975, 1977). Therefore, addition of water across the C-1′,2′ double bond and subsequent hydroxylation at C-2′ must occur as late events in the bioynthesis of **1**, which represents the end product of carotenogenesis in *Dioszegia*. The proposed biosynthetic pathway for formation of **1** is summarized in Fig. 4.

2.5. Production of *1* under oxidative stress

When grown in YES medium in shaken-flask culture, the carotenoid spectrum of *Dioszegia* CAR034 was influenced by the degree of aeration. In standard flasks, **3** was the dominant pigment, with only low quantities of **1** present, whereas the reverse was found in the well-aerated baffled flasks (Fig. 5). Biosynthesis of **1** was therefore stimulated by aeration conditions equivalent to those favouring biosynthesis of torularhodin over β-carotene in *Sporobolomyces roseus* (Davoli et al., 2004), and of astaxanthin over β-carotene in *Phaffia rhodozyma* (Weber and Davoli, 2003). The latter switch was described by Johnson and Lewis (1979) as requiring an oxygen dissolution rate

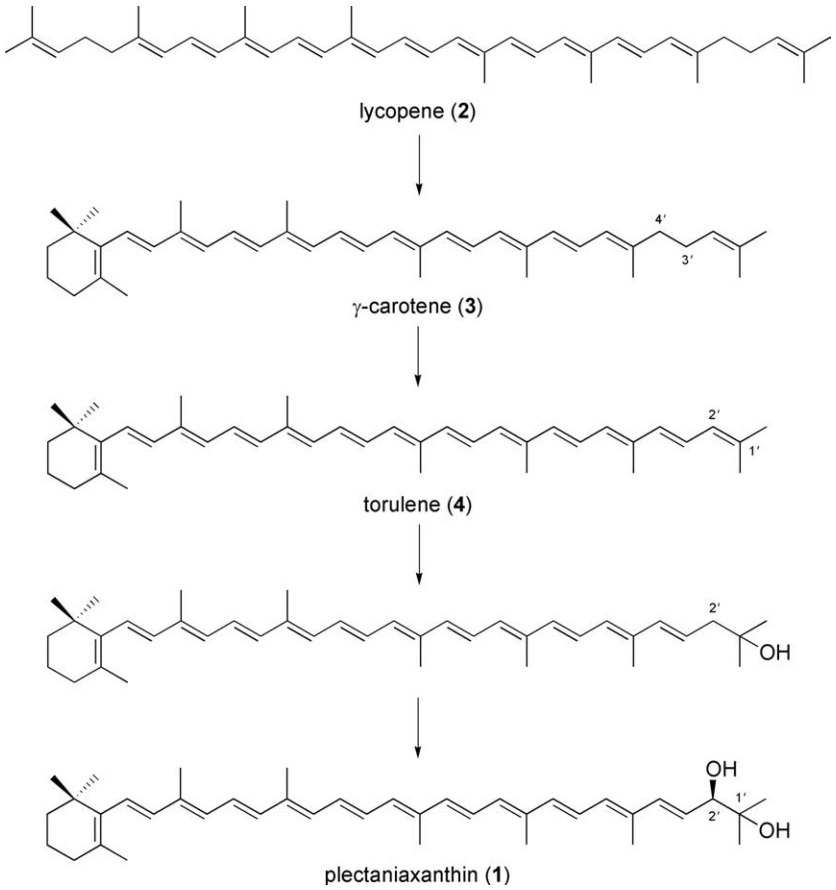

Fig. 4. Proposed biosynthetic pathway of **1** in *Dioszegia*.

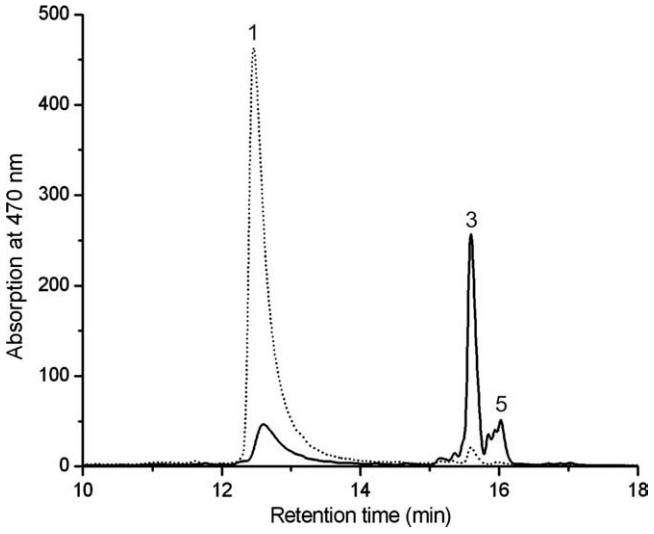

Fig. 5. HPLC chromatograms of crude extracts obtained from *Dioszegia* CAR034 grown for 5 days at 21 °C in standard flasks (solid line) and indented flasks (dotted line). Extracts were obtained from the same volume of culture. Peaks due to plectaniaxanthin (**1**), γ-carotene (**3**) and an unidentified minor carotenoid (**5**) are indicated.

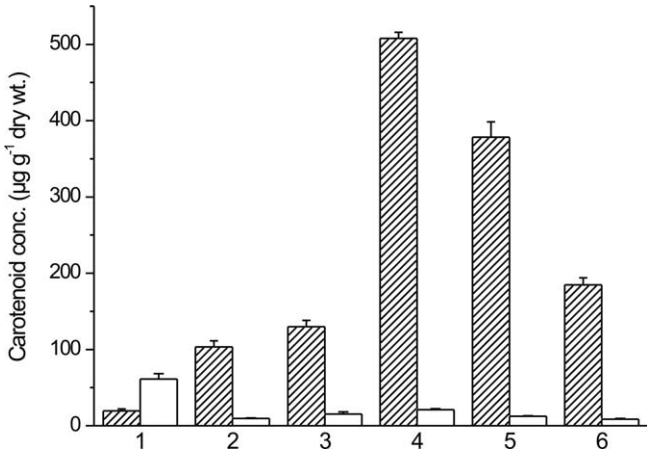

Fig. 6. Concentration of **1** (hatched bars) and **3** (empty bars) in cells of *Dioszegia* CAR034 grown in YES medium in indented (2–6) or standard (1) conical flasks. Carotenoid production was examined after incubation for 5 days at 21 °C (1 and 2), 10 days at 4 °C (3), 5 days at 21 °C with 2% ethanol (4), 5 days at 21 °C with 100 μM duroquinone (5), and 5 days at 21 °C with 5 mM H_2O_2 (6). Results are shown as average ± SD of three independent replicates.

exceeding 30 mmol O_2 l^{-1} h^{-1}. In fermentor cultures of CAR034, the switch from **3** to **1** occurred at an aeration rate of at least 0.25 l air (i.e., 120 mmol O_2) l^{-1} culture h^{-1}.

In addition to **1** and **3** which were the two major pigments of *Dioszegia* CAR034, cells grown at reduced aeration also contained traces of one or more unidentified apolar pigments **5** (Fig. 5). Liquid cultures showed only a modest increase in biomass in baffled flasks (8.1 g dry weight l^{-1} after 5 days) as compared to standard flasks (6.2 g l^{-1}). The quantification of **1** and **3** in cells of *Dioszegia* CAR034 under different culture conditions is summarized in Fig. 6. Incubation in baffled flasks at 4 °C for 10 days resulted in an equivalent biomass to that at 21 °C for 5 days, and the pigment concentrations and composition were also similar.

The occurrence of the single pigment **1** at substantial concentrations (>100 μg g^{-1} dry weight) in *Dioszegia* CAR034 at high aeration called for further experiments to address its potential physiological role as an antioxidant. Several agents have been used to induce oxidative stress, including duroquinone which is a generator of intracellular superoxide radicals (Rossi et al., 1986), and H_2O_2 which decomposes in the presence of Fe^{2+} ions to give hydroxyl radicals. Another inducer of oxidative stress is ethanol which is likely to have a complex effect on carotenoid biosynthesis. Firstly, a direct stimulation of HMG-CoA reductase activity by ethanol has been observed in *Phaffia* and other yeasts, leading to an enhanced biosynthesis of isoprenoids including carotenoids (Gu et al., 1997). The role of mevalonic acid as a key intermediate in carotenoid biosynthesis has also been suggested on the basis of biochemical studies (see Sandmann and Misawa, 2002). Secondly, the first degradation product of ethanol, acetaldehyde, can be further metabolized in a number of ways, including oxidation by aldehyde oxidase to give acetic acid and the superoxide radical (Jones, 1989). This pathway has also been suggested for *Phaffia* (Gu et al., 1997).

Suitable concentrations of the above three inducers of oxidative stress were determined in preliminary experiments, whereby maximum stimulation of production of **1** in shaken baffled flasks was observed when 5 mM H_2O_2, 100 μM duroquinone, or 2% ethanol were added to freshly inoculated cultures of CAR034. Growth was strongly retarded or altogether absent when higher concentrations of these substances (10 mM, 200 μM and 5%, respectively) were used (Fig. 7; see also Schroeder and Johnson, 1993). Their presence had little effect on the production of **3**, whereas concentrations of **1** were enhanced 1.8-fold with 5 mM H_2O_2, 3.1-fold and 3.7-fold with 50 or 100 μM duroquinone (respectively), and 4.9-fold with 2% ethanol (Fig. 6). In all these experiments the cell biomass showed only minor variations (6.2–7.7 g dry weight l^{-1} culture).

2.6. Ecophysiological considerations

Although a substantial body of literature has been published on putative antioxidant roles of oxidized carotenoids in a range of red yeasts, and the stimulation of their biosynthesis under conditions of oxidative stress, the present report is the first one to implicate pigment **1** in this context. Further, our producing organisms, *Dioszegia* spp., have not previously been described to react to oxidative stress in such a way. Two examples have received the particular attention of previous workers. In *Phaffia*, β-carotene is the predominant pigment at low aeration, whereas most kinds of oxidative stress induce its oxidation towards astaxanthin (Schroeder and Johnson, 1993). Secondly, in red yeasts belonging to the genera *Rhodotorula* and *Sporobolomyces* (Urediniomycetes), the enhanced biosynthesis of

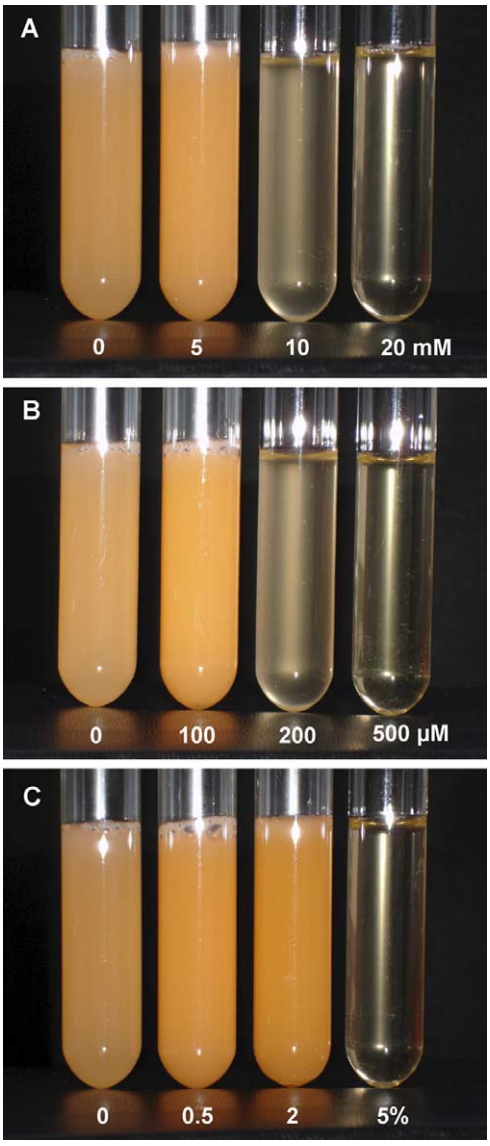

Fig. 7. Determination of threshold concentrations of inducers of oxidative stress. Samples of 5 days old shaken-flask cultures of CAR034 augmented with inhibitor concentrations of H_2O_2 (A), duroquinone (B) and ethanol (C) as indicated were filled into test tubes to compare the pigmentation of yeast cells in suspension.

carotenoids under oxidative stress leads towards the monocyclic pigments torulene and torularhodin, with β-carotene levels remaining relatively static (Sakaki et al., 2002; Davoli et al., 2004). This latter biosynthetic route probably shares its early steps with that leading to **1**, torulene being the branch-point (Fig. 4; see Sandmann and Misawa, 2002). The shift towards xanthophylls at oxidative stress makes sense in the light of the suggestion by Martin et al. (1999) that β-carotene is an effective antioxidant only at relatively low oxygen tension whereas it may in fact act as a pro-oxidant, i.e., react with triplet oxygen, at high oxygen concentrations (see also Krinsky and Yeum, 2003; Lowe et al., 2003).

Light is a powerful stimulant of singlet oxygen formation in its own right as well as in concert with photosensitizing substances, and illumination by white and UV light has been shown to enhance carotenoid biosynthesis in a range of yeasts (Schroeder and Johnson, 1995a,b; Sakaki et al., 2000; Bhosale, 2004). Although firm ecological data are lacking, UV irradiation of leaf surfaces has been shown to inhibit the growth of non-pigmented yeasts slightly more strongly than that of either red yeasts or 'black yeasts', i.e., species with heavy deposits of the sun-screen pigment melanin in their cell walls (Ayres et al., 1996). In this context, we note that *Dioszegia* CAR034 was isolated from leaves grown in a habitat exposed to high solar irraditation.

Unfortunately, few comparative data are available to indicate the antioxidant properties of different carotenoids in living organisms, and none seem to have been published for **1** as yet. However, torularhodin, which is structurally similar in being monocyclic but carrying a carboxy group at its linear end, has been shown to be an effective peroxyl radical scavenger in the red yeast *Rhodotorula glutinis* (Sakaki et al., 2001). It is a fair working hypothesis that pigment **1** fulfils a similar role in *Dioszegia*, since it is accumulated at much higher intracellular concentrations than have been reported for torularhodin under oxidative stress (Sakaki et al., 2000, 2002; Davoli et al., 2004). Further experiments are in progress to test this hypothesis. Another area worthy of detailed examination concerns the unravelling of molecular events involved in perception and transduction of the oxidative stress signal, and the identity of the enzymes being up-regulated during enhanced synthesis of pigment **1**. HMG-CoA reductase is likely to play a pivotal role in regulating carotenoid biosynthesis, as indicated by the strong stimulation of production of **1** by ethanol observed in the current study, and also general biochemical considerations of carotenoid biosynthesis in other fungi (Sandmann and Misawa, 2002).

3. Experimental

3.1. Isolation, cultivation and identification of CAR034

Fresh leaves of *Crataegus* sp. were collected at Old Winchester Hill (Hants., UK) on 6 October 2002. Epiphytic yeasts were isolated by shaking individual leaves in 25 ml aliquots of sterile distilled water, followed by spreading 0.1 ml samples on 2% malt extract (ME) agar augmented with 1 mg l^{-1} benomyl (Riedel-de Haën, Seelze, Germany). Strain CAR034 is being maintained as lyophilized cells and on ME agar at 4 °C in the Culture Collection, Department of Biotechnology, University of Kaiserslautern. For microscopic examination, this yeast was grown for 7 or 28 days at 21 °C on YM agar (Difco), yeast-glucose (YG) agar containing 4 g yeast extract, 10 g malt extract and 4 g glucose l^{-1}, 0.2% and 2% ME agar, 2% ME agar with 20% sucrose, cornmeal agar (Difco), yeast extract-sucrose (YES) agar containing 20 g sucrose, 4 g yeast extract, 1 g KH_2PO_4 and 0.5 g $MgSO_4 \cdot 7H_2O$ $litre^{-1}$ tap water (Davoli et al., 2004), and 0.5% ribitol agar (Kucsera et al., 1998). Identification was performed by analysis of selected

ribosomal DNA sequences as described in detail by Schwarz et al. (2004). DNA was extracted by means of the phenol–chloroform method (Sacks et al., 1995) from yeast cells grown in liquid YES medium. The entire ITS1–5.8S rDNA–ITS2 region was amplified using the primer pair ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG) and ITS4 (5′-TCCTCCGCTTATTGATATGC); the 5′ segment of the 18S rDNA gene with the primers NS1 (5′-GTAGTCATATGCTTGTCTC) and SR4 (5′-AAACCAACAAAATAGAA); and the 5′ region of the 28S rDNA gene with the primers LR0R (5′-GTACCCGCTGAACTTAAGC) and LR3 (5′-CCGTGTTTCAAGACGGG) (Vilgalys and Hester, 1990; White et al., 1990; http://www.biology.duke.edu/fungi/mycolab). Database searches were carried out using the FASTA function in the GCG Wisconsin Package. C and N utilization spectra were determined as described by Yarrow (1998), growing the yeast in 1.5 ml volumes in shaken 24-well plates.

For studies on the physiology of pigment production, CAR034 was grown in 500 ml conical flasks with ('baffled flasks') or without a single indentation, containing 100 ml liquid YES medium. Cotton wool bungs were made loosely in order to provide maximum aeration in the indented flasks. Incubation was on an orbital shaker (120 rpm, 5 cm amplitude) at 21 or 4 °C in dim room light. Following preliminary experiments to determine threshold concentrations, the effect on carotenoid synthesis was quantified by cultivation in the presence of various substances known to influence carotenoid production in other red yeasts. These included 5 mM H_2O_2, 50 and 100 μM duroquinone (Sigma–Aldrich), 2% (v/v) ethanol, and 5–20 mM (−)-nicotine (Carl Roth).

For purification of **1**, cultivation of CAR034 was carried out in a 20-l stirred (120 rpm) and aerated (0.4 l air l^{-1} culture min^{-1}) fermentor (C6; Biolafitte) containing 15 l YES medium. Incubation was for 6 days at 21 °C.

3.2. Extraction and purification of carotenoids

Carotenoids were obtained as described in detail by Davoli et al. (2004), by centrifugation of yeast cells, freezing of the pellet at −20 °C, thawing, incubation in DMSO for 12 h, and repeated extraction of the yeast pellet with acetone. The coloured solvent fractions were pooled and extracted by phase separation with light petroleum, and the apolar phase was concentrated under reduced pressure to yield an oily crude extract.

In order to eliminate sterols and other lipids from the crude extract (1.35 g), this was redissolved in 20 vol. acetone and incubated overnight at −80 °C. The colourless precipitate was separated by filtration through Whatman No. 5 paper. This procedure was repeated 3 times, yielding 50 mg carotenoid-enriched extract. Final purification was performed by silica gel chromatography (Kieselgel 60; 63–200 μm particle size; Merck), with **3** (0.3 mg) eluting in pure cyclohexane, and **1** (20 mg) eluting in cyclohexane/ethyl acetate (1:1, v/v).

3.3. Identification and quantification of carotenoids

HPLC analyses of crude extracts and purified carotenoid preparations were carried out in a Hewlett-Packard HP1090 Series II liquid chromatograph fitted with a LiChrospher 100 RP-18 column (5 μm particle size; 250 × 4 mm; Merck). The flow rate was 1 ml min^{-1}, and the injection volume was 10 μl. The gradient was from 70% to 100% acetone in 15 min (see Steel and Keller, 2000; Davoli et al., 2004). Preliminary identification of carotenoids was by their retention time and UV/visible absorption spectra (300–600 nm) in comparison with commercial standards of β-carotene and lycopene (**2**) or our own purified standards of torulene (**4**), γ-carotene (**3**) and plectaniaxanthin (**1**). Quantification of **1** and **2** was achieved by reference to calibration curves established with pure standards, and of **3** as described by Davoli and Weber (2002b). All quantification data were obtained from triplicate cultures.

The identification of carotenoids in crude extracts was confirmed by mass spectra recorded with a Hewlett-Packard Series 1100LC-MSD liquid chromatograph-mass spectrometer in the atmospheric pressure-chemical ionization (APCI) mode (Davoli and Weber, 2002b), but using a water/acetone gradient as described above; both positive (PI) and negative ionization (NI) modes were used. Purified **1** was identified on the basis of its MS fragmentation, UV/visible spectra in methanol and *n*-hexane using a Perkin–Elmer Lambda 16 spectrometer, IR spectrum in KBr obtained with an IFS-48 FT-IR spectrometer (Bruker, Karlsruhe, Germany), and by means of NMR spectroscopy. ^{1}H and ^{13}C NMR spectra of **1** were recorded in $CDCl_3$ solution with a Bruker Avance 400 spectrometer operating at 400.13 and 100.61 MHz, respectively. ^{13}C resonances were measured in the inverse-detection mode with a spectral resolution of ±0.5 ppm. The following homonuclear and heteronuclear two-dimensional NMR techniques were utilized to aid in the assignment of ^{1}H and ^{13}C signals: gHMQC, gCOSY, gTOCSY and *J*-resolved spectroscopy. In particular, a ^{1}H–^{13}C gHMQC experiment was used for the assignment of protonated carbon resonances, setting the evolution delay to maximize one-bond correlations.

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