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ORIGINAL PAPER

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Torularhodin and torulene are the major contributors to the carotenoid pool of marine *Rhodosporidium babjevae* (Golubev)

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Abstract A carotenoid-producing yeast strain, isolated from the sub-arctic, marine copepod *Calanus finmarchicus*, was identified as *Rhodosporidium babjevae* (Golubev) according to morphological and biochemical characteristics and phylogenetic inference from the small-subunit ribosomal RNA gene sequence. The total carotenoids content varied with cultivation conditions in the range 66–117 μg per g dry weight. The carotenoid pool, here determined for the first time, was dominated by torularhodin and torulene, which collectively constituted 75–91% of total carotenoids under various regimes of growth. β-Carotene varied in the range 5–23%. A high-peptone/low-yeast extract (weight ratio 38:1) marine growth medium favoured the production of torularhodin, the carotenoid at highest oxidation level, with an average of 63% of total carotenoids. In standard yeast medium (YM; ratio 1.7:1), torularhodin averaged 44%, with increased proportions of the carotenes, torulene and β-carotene. The anticipated metabolic precursor γ-carotene (β,ψ-carotene) constituted a minor fraction (≤ 8%) under all conditions of growth.

Keywords *Rhodosporidium* · Marine yeast · Carotenoids · β-carotene · Torulene · Torularhodin

Introduction

Several urediniomycetous yeasts synthesize the three carotenoids: β-carotene, torulene and torularhodin, as their principal pigments. These genera include the anamorphic *Rhodotorula* and *Sporobolomyces* spp. and their teliomorphic counterparts *Rhodosporidium* and *Sporidiobolus*. They typically display reddish coloration, however, with variations due to differences in absolute and relative amounts of the three main carotenoids or the presence of additional pigments [7, 17].

Recently, there has been considerable interest in these yeasts as putative sources of carotenoids with commercial potential. *Rhodotorula* spp., in particular, have been the subject of studies on optimization of growth yield and carotenoid production, both in the wild-type and carotenoid-overproducing mutant strains. When cultivated on cheap agricultural byproduct carbohydrates, this yeast has been considered an interesting alternative source of β-carotene [2, 5, 10, 23]. There are established markets for β-carotene within the food and feed supplements industry, related to its well-known functions as provitamin A and colorant. Additionally, the carotenoids produced by the *Rhodotorula* group of yeasts have attracted research interest related to their function as scavengers of reactive oxygen species. Besides extensive studies on β-carotene, torularhodin has also been investigated for its role as a scavenger of peroxyl radicals [19] and as a singlet oxygen quencher [20].

Rhodotorula and related genera seem ubiquitous, as they have been isolated from terrestrial, fresh-water and marine environments and from a broad selection of climatic zones. With very few exceptions, strains which have been subject to physiological studies and mutagenic treatments have been of terrestrial origin. Here, we report the isolation and carotenoid-producing potential of a marine isolate of *Rhodosporidium babjevae* (Golubev), isolated from a zooplankton species in sub-arctic coastal waters.

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Materials and methods

Sample collection, isolation and cultivation

Three bright red-pigmented yeast isolates of indistinguishable colour and cell morphology were obtained

from whole-animal homogenates of the marine copepod, *Calanus finmarchicus*, collected in Grøtsundet, Northern Norway. The cells were routinely kept on agar plates (MAP) with the following composition: 10 g l^{-1} Difco Marine broth 2216, 10 g l^{-1} Difco Bacto-peptone, 10 g l^{-1} glucose, 15 g l^{-1} NaCl, 15 g l^{-1} agar. Cells for analyses were cultured in conical flasks on a rotary shaker (200 rpm) up to 140 h. Broth with the above nutrient composition or standard yeast medium (YM) broth (5 g l^{-1} Bacto-peptone, 3 g l^{-1} Bacto yeast extract, 3 g l^{-1} malt extract, 10 g l^{-1}glucose) was used.

Identification of strain

One isolate, CG-200, was subjected to the phenotypic yeast identification scheme at the German Collection of Microorganisms and Cell Cultures (DSMZ) (P. Hoffmann, personal communication) and to phylogenetic inference based on sequencing of the partial small-subunit ribosomal RNA (ssu-rRNA) gene. After mechanical disintegration by a French pressure cell, DNA was extracted and purified by established methods (extraction with phenol:chloroform:isoamylalcohol (25:24:1) and ethanol precipitation) from a 50 µl volume of concentrated cell material. The major part of the ssu-rRNA gene was amplified by the polymerase chain reaction from 50 ng DNA extract, employing the forward primer CTGGTTGATYCTGCCAGTAG and the reverse primer CAGGTTCACCTACRGAAACC, according to the following protocol: 95°C for 5 min, 30 cycles (95°C for 30 s, 53°C for 30 s, 72°C for 90 s) and 72°C for 7 min. The nucleotide sequence of the complete PCR product was determined in an automatic sequencer by Big Dye Terminator technology (Perkin Elmer), according to the manufacturer's protocol. The forward primer GCCAGCAGCCGCGGTAA (517F) was employed as sequencing primer, in addition to the end primers given above. The sequence has been deposited at the EMBL Nucleotide Sequence Database with accession number AJ780965. Phylogenetic inference based on unambiguously aligned sequences was done by the neighbour-joining method, as implemented in the ClustalX program [24]. Sequences with the following GenBank accession numbers were included: AB016293, AB018403, AB021697, AB042787, AB073270, AB073271, AB073275, D66883, U77395, X60180, X83827.

Extraction and identification of pigments

Standard precautions for work with carotenoids were taken [3]. French-press disintegrated cell suspensions (30 ml) were extracted with acetone (4×100 ml). The solvent volume was reduced to 200 ml under reduced pressure and the carotenoids transferred to hexane. A polar, hypophasic carotenoid was separated from non-polar, epiphasic carotenes by extraction with 10% 1 M KOH in methanol (100 + 2×50 ml). After neutralization with 1 M HCl, the acidic carotenoid was extracted into ether. Contaminating lipids were removed from the polar fraction by precipitation at −20°C in acetone, followed by methylation with diazomethane [14] and chromatography on neutral preparative TLC plates (silica:$CaCO_3$ 2:1 with 20% acetone in hexane as mobile phase). The identity of the methyl ester was subsequently determined by VIS spectroscopy, mass spectroscopy (EIMS spectra recorded on a Finnigan 95 XL instrument) and HPLC co-chromatography with an authentic standard [Hewlett Packard series HP1050 with a diode array detector, Interchim Uptisphere 5 ODB 250×4.6 mm column, *n*-C_6H_{14}/acetone/methanol/1 M NH_4OAc as mobile phase: 0/0/80/20 (0 min), 0/30/70/0 (30 min), 20/50/30/0 (50 min); 1.25 ml min^{-1}].

The non-polar carotenes were separated into three zones by preparative TLC on alkaline plates [silica/kieselguhr/MgO/$Ca(OH)_2$ 24/16/12/9 with 3% ether in pentane as mobile phase]. The identity of the carotenes was determined by VIS- and mass spectra and co-chromatography tests with authentic standards. For further identification data, see [4, 15].

Carotenoid quantification

Total carotenoids were determined in quantitative acetone extracts (3×30 ml) of disintegrated cells from 200 ml cultures. The absorbance at λ_{max} (487 nm) was related to $E_{1\%,\ 1\ cm} = 3{,}200$, reflecting the major contribution by torulene to the overall absorbance at λ_{max} [4]. Cell dry weight was determined after drying for 24 h at 105°C.

Relative amounts of individual carotenoids in water-free acetone extracts were determined from absorbance at 487 nm wavelength in the HPLC diode array chromatograms.

Results and discussion

The strain CG-200 was identified as *R. babjevae* (Golubev) based on morphological and physiological characteristics (Table 1). Discriminatory criteria against close relatives within the phylogenetically defined "*Rhodotorula glutinis sensu stricto*" group, which also include *R. diobovatum*, *Rh. glutinis* and *Rh. graminis* [11], were non-growth on L-arabinose and growth on maltose and melezitose. Phylogenetic inference based on a 1,629 bp fragment of the ssu-rRNA gene, with start and end positions homologous to positions 62 and 1,669 of the *Saccharomyces cerevisiae* gene [1], also affiliated the strain most closely with the four above-mentioned species (Fig. 1). Earlier studies have demonstrated monophyly of these genera by sequence analyses of alternative rRNA regions, including the internal transcribed spacer regions and the D1/D2 region of the lsu-rRNA gene [11].

Table 1 Physiological characteristics of strain CG-200

Glucose	+
Galactose	+
Sorbose	+
Rhamnose	W[a]
Dulcit	–
Inositol	–
Mannitol	+
Sorbitol	+
Glycerol	+
Erythritol	–
D-Arabinose	W
L-Arabinose	–
Ribose	W
D-Xylose	+
L-Xylose	W
Adonitol	+
α-Methylglycoside	+
Salicin	+
Cellobiose	+
Maltose	+
Lactose	–
Melbiose	–
Sucrose	+
Trehalose	+
Inulin	–
Melezitose	+
Raffinose	+
Starch	–
Xylitol	–
Gluconate	W
2-Keto-gluconate	+
5-Keto-gluconate	–
N-acetylglucosamine	–

[a]Weak response

Rhodosporidium babjevae was originally isolated from terrestrial plant material [13]. However, strain GC-200 was obtained from a marine copepod species, confirming the presence in marine environments. Marine habitats have also been demonstrated by Gadanho et al. [12], who have isolated a number of strains from seawater samples.

The present strain showed moderately psychrotrophic character, with an optimum specific growth rate (0.38 h^{-1} in YM medium) in the range 24–27°C and no growth at 37°C. No indication of a distinct marine adaptation was evident, as growth was not influenced by the presence or absence of NaCl or the sea salts mixture of the MAP medium.

The total carotenoid content varied with growth conditions, from 66 to 117 μg per g dry cell weight, when cultured in standard conical flasks (Fig. 2). Levels as low as approximately 10 μg g^{-1} [21] and higher than 900 μg g^{-1} [6] have been reported for wild-type *Rhodotorula* and related genera, but the majority of studies indicate typical concentrations to be in the range 80–300 μg g^{-1} [8–10, 16, 22, 23]. However, variations in growth conditions have strongly influenced the overall production of carotenoids.

As shown in the present study, β-carotene, torulene and torularhodin constitute the main carotenoid complement of *R. babjevae*. γ-Carotene (β,ψ-carotene), first suggested as a metabolic branching point between the β-carotene and torulene/torularhodin biosynthetic pathways by Simpson et al. [22], was also shown to contribute 3–8% to the total carotenoids. Squina et al. [23] observed up to 14% of γ-carotene in *Rh. glutinis* and Davoli et al. [9] up to 16% in *Sporobolomyces roseus*.

The plausible biosynthetic route for the carotenoids discussed is shown (Fig. 3), with increasing oxidation levels from γ-carotene (β,ψ-carotene) to torulene and finally to torularhodin. The relative abundance of the three major carotenoids has been shown to be highly variable, and both marked differences between strains of the same species [6] and strong influences of cultivation conditions have been demonstrated previously. The present *R. babjevae* strain conforms to the prevailing pattern for *Rh. glutinis* strains, with a predominance of torulene and torularhodin under most cultivation conditions. Collectively, these two compounds tend to constitute more than half of the carotenoid complement in *Rh. glutinis*, except when cultured under oxygen stress [6, 7, 9, 10, 20, 23]. Up to sevenfold variation in total carotenoid content per unit biomass and a 29–80% range in percentage torularhodin in a strain of *Rh. rubra* (*Rh. mucilaginosa*) have been induced by simply

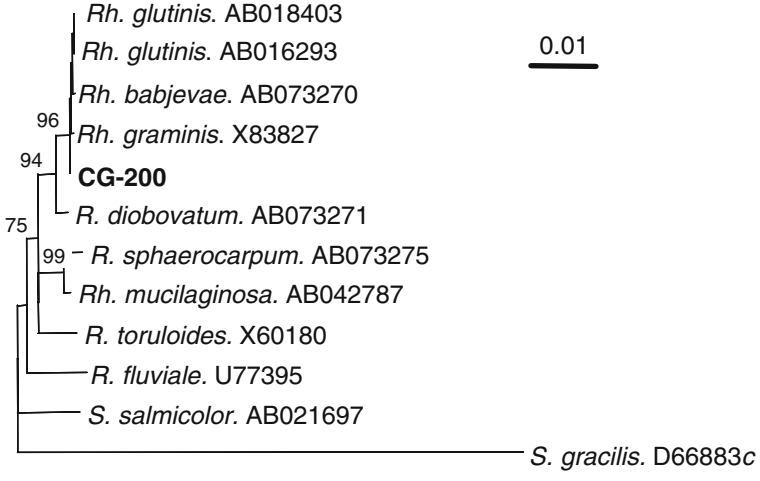

Fig. 1 Phylogenetic inference (neighbour-joining method) of *R. babjevae* and related species based on partial ssu-rRNA gene sequence. Bootstrap values >70% from 1,000 resamplings are presented. *Sporobolomyces gracilis* was used for rooting the tree

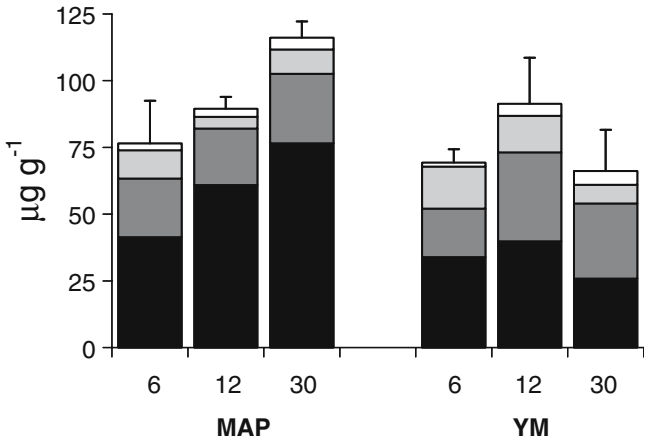

Fig. 2 Total contents and relative distribution of carotenoids in *R. babjevae* with different media and incubation temperatures (6, 12 and 30°C). *Black* torularhodin, *dark gray* torulene, *light gray* β-carotene, *white* γ-carotene. *Error bars* indicate standard deviations on total carotenoid content determinations (in triplicate)

manipulating the relative contents of yeast extract, peptone and salts in the growth media [23]. A less comprehensive approach in the present study, by employing two different growth media, confirmed an impact on the carotenoid composition of varying the same two organic nutrient sources. The differences were, however, less explicit, as the low-yeast extract (0.3 g l^{-1})/high-peptone (11.3 g l^{-1}) MAP medium resulted in torularhodin contents of 54–68% of total carotenoids at variable growth temperatures, as compared with 39–49% in conventional YM medium with ten times the yeast extract and half the peptone contents (Fig. 2). The higher percentages of β-carotene (15% in MAP, 23% in YM) were observed in cells cultured at the lowest temperature (6°C) in both media.

Both light [2, 20] and intensified oxygenations [8, 9, 18] have been shown to influence carotenoid production in the direction of a higher torularhodin fraction. In the present work, the only observed effect of incubation of liquid cultures in dark versus light was a minor delay in the onset of carotenoid accumulation. No significant shift towards a higher proportion of torularhodin was observed. In accordance with the results of Davoli et al. [9] on *Rh. glutinis*, intensified aeration by use of baffled cultivation flasks instead of conventional EM flasks resulted in higher total carotenoid contents (236 ± 14 µg per g dry weight), but basically the same distribution between carotenoid components (data not shown).

Previous studies have, to a major extent, focused on the potential for improvement of β-carotene synthesis through mutagenesis or growth medium optimization, as *Rhodotorula* and related genera are interesting alternative sources of this vitamin A precursor. With half a molecule of β-carotene intact, torulene and torularhodin potentially have around 50% provitamin A activity, as compared with β-carotene. Their antioxidant properties may be at least as beneficial in, for example, poultry feed supplements. Torularhodin has been demonstrated to be

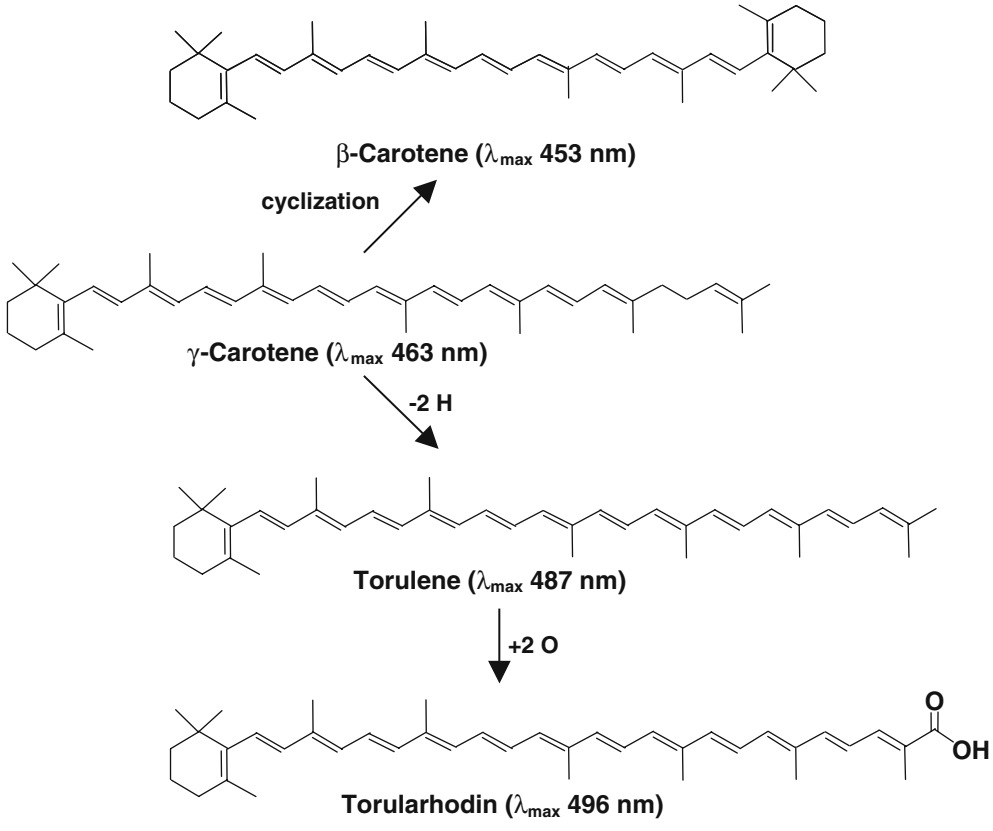

Fig. 3 Plausible biosynthetic pathways for the carotenoids in *R. babjevae* (Golubev). Absorption maxima refer to the middle, main maximum of the VIS spectrum in acetone

a more potent quencher of singlet oxygen than β-carotene, possibly due to its longer polyene chain [20]. Furthermore, an increased market potential as food and feed pigments has been pointed out for carotenoids with the shades of colour characteristic of torulene and torularhodin [7].

Presently, the *Rhodotorula* group of yeasts are the only known natural sources of these pigments, and therefore they are interesting objects for studying the regulation of the two pathways leading from γ-carotene (β,ψ-carotene) to the dicyclic β-carotene or to the more oxidized monocyclic carotenoids torulene and torularhodin (Fig. 3). So far, no plausible carotenol or carotenal intermediates for the oxidative transformation from torulene to torularhodin have been unequivocally identified from natural sources [4].

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