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Carotenoids of yeasts isolated from the Brazilian ecosystem

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Abstract

The carotenoid composition of pigmented yeasts isolated in Brazil was studied. The yeasts were cultured in yeast malt broth at 200 rpm, 25 °C, for 5 days, without illumination. Open column, thin layer and high performance liquid chromatography were used to separate, identify and quantify the carotenoids. The major pigments found in these yeasts were torulene and β-carotene. β-Carotene predominated in *Rhodotorula graminis*-125, *Rhodotorula glutinis* and *Sporobolomyces roseus*, while torulene was the principal carotenoid in *Rhodotorula mucilaginosa*. The yeast *R. glutinis* had the highest total carotenoid production (881 μg/l), followed by *R. graminis* (594 μg/l), *Rhodotorula mucilaginosa*-137 (590 μg/l), *Rhodotorula mucilaginosa*-108 (562 μg/l) and *Rhodotorula mucilaginosa*-135 (545 μg/l). *Rhodotorula minuta* and *S. roseus* had the lowest carotenoid contents (168 and 237 μg/l, respectively). In μg/g of dry cells, *R. glutinis* had a total carotenoid concentration of 132 μg/g.

Keywords: Carotenoid; Torulene; Yeast; Fermentation; HPLC

1. Introduction

Carotenoids are among the most important constituents in food. As natural colourants, conferring yellow to red colour, they have decisive influence on the acceptability of many foods. Nutritionally, some carotenoids are precursors of vitamin A. In terms of human health, carotenoids are among the bioactive phytochemicals credited with the reduced risk for degenerative diseases such as cancer, cardiovascular diseases, macular degeneration and cataract (Astorg, 1997; Gaziano & Hennekens, 1993; Krinsky & Johnson, 2005; Olson, 1999). Thus, aside from promoting carotenoid-rich foods, sources of carotenoids for food and feed additives and supplements are being sought.

Traditionally, carotenoids have been marketed as dried powder or extracts from plants, such as annatto, paprika and saffron. Natural colourants from plant sources, however, suffer from diminishing or unstable supply of raw materials, subject to climatic conditions, as well as varying colourant level and quality of the final product. The international market for carotenoids has been met mainly by synthetic carotenoids with structures identical to those of natural carotenoids, but there is growing demand for natural sources.

Microbial carotenoids have been studied and their potential recognized over the years (Nelis & de Leenheer, 1991). Commercial application has, however, been limited. In recent years there has been a resurgence of interest in the use of microbial sources of carotenoids.

The main reason for the interest in using microorganisms to produce compounds that can otherwise be isolated from plants and animals or synthesized is the ease of increasing production by environmental and genetic manipulation. The commercial utilization of microorganisms with biotechnological potential to produce carotenoids is presently limited by the high cost of production. However, the cost of carotenoid production by fermentation can be minimized by the use of inexpensive industrial by-products as nutrient sources (Aksu & Eren, 2005).

β-Carotene from the microalga *Dunaliella salina* is being produced in commercial scale by Australia, Israel and the

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United States (Dufossé et al., 2005). Small plants are also operating in Chile, Mexico, Cuba, Iran and Taiwan. Recently, commercial production of astaxanthin from another microalga, *Haematococcus pluvialis*, has started by the United States, Japan and India.

Carotenoids are also produced by yeasts, bacteria and fungi. Red yeasts, which accumulate carotenoids, belong to the basidiomycetous classes, such as *Rhodotorula*, *Rhodosporidium* and *Sporobolomyces*. The major pigments are β-carotene, γ-carotene, torulene and torularhodin (Simpson, Chichester, & Phaff, 1971).

Brazil has a diverse and unique ecosystem that can provide yeasts with biotechnological potential for carotenoids. The present study was aimed at finding yeasts from the Brazilian environment, capable of producing carotenoids of possible commercial importance.

2. Materials and methods

2.1. Yeast strains

Strains of *Rhodotorula glutinis* and *Rhodotorula minuta*, both isolated from persimmon leaves, and *Sporobolomyces roseus*, isolated from the soil under the persimmon tree, were obtained from the Fundação André Tosello (Campinas, São Paulo, Brazil). In addition, *Rhodotorula mucilaginosa*-12 from tomato sauce as a contaminant, *Rhodotorula mucilaginosa*-108 from soil of the University of Campinas; *Rhodotorula graminis*-125 from sugar cane leaves, and *Rhodotorula mucilaginosa*-135 and *Rhodotorula mucilaginosa*-137 from the soil of Holambra (São Paulo, Brazil) were also isolated and investigated. Identification of these strains was described in detail by Maldonade, Scamparini, and Rodriguez-Amaya (2007), based on the comparative analysis of distinguishing morphological, physiological and metabolic characteristics of the strains studied. The cultures were maintained by monthly transfers to YM (yeast extract, 3 g/l; malt extract, 3 g/l; peptone, 5 g/l; glucose, 10 g/l) agar slants and stored at 4 °C. These yeasts were selected from 242 wild strains because they presented more intense colouration. Yeasts are focalized in our studies because they are less likely to pose food safety problems than bacteria and fungi. Some yeasts are already permitted for use in food and feed.

2.2. Culture conditions and harvesting procedure

Each starter culture was prepared by inoculating one loop of a slant culture into 50 ml of YM broth (Difco, Detroit, Michigan) in a 150 ml Erlenmeyer flask, incubating at 25 °C, 200 rpm for 72 h in a shaker. Fermentation experiments were carried out in 500 ml Erlenmeyer flasks containing 200 ml of YM broth. Each flask was inoculated with starter culture (5%, v/v) and incubated at 25 °C for 5 days at 200 rpm, without illumination. Each strain was cultivated three times (three fermentation trials) under the same conditions.

After cultivation, the cells were harvested by centrifugation at 16,000*g* for 20 min, washed twice with deonized water and centrifuged again. The dry mass was gravimetrically determined by drying centrifuged and washed samples at 105 °C to a constant weight.

2.3. Carotenoid analysis

The carotenoid content was determined according to procedures described by Rodriguez-Amaya (1999). Wet cells, collected by centrifugation, were macerated with Hyflosupercel with a mortar and pestle and exhaustively extracted with acetone. The carotenoids were transferred to petroleum ether in a separatory funnel by the addition of water and washed free of acetone. The pigment solution was dried with anhydrous sodium sulphate and quantified spectrophotometrically (Davies, 1976). Prior to open column chromatography, the solution was concentrated in a rotary evaporator (<40 °C).

Chromatography on a MgO:Hyflosupercel (1:2) open column was carried out with the following mobile phases: 1%, 2%, 5% ethyl ether in petroleum ether; 2%, 5%, 8%, 10% to 100% of acetone in petroleum ether; and 2%, 5%, 10% to 50% of water in acetone. The eluates in acetone–petroleum ether were washed with water and those in acetone–water were transferred to petroleum ether. All eluates were concentrated for further chromatography.

For high performance liquid chromatography (HPLC), the extract was concentrated in a rotary evaporator, dried under nitrogen, and redissolved in 1 ml acetone with the aid of sonication. An aliquot of 10 μl was then injected into the liquid chromatograph. A Varian liquid chromatograph (model 9010) (Varian Inc., Palo Alto, CA) equipped with a UV–vis photodiode detector (model 994) (Waters Corporation, Milford, MA) was used. The column was Vydac 218 TP54, C18, 5 μm, 4.6 mm × 250 mm (The Separations Group, Hesperia, CA). The mobile phase for *R. glutinis* was tetrahydrofuran:H_2O:methanol in a linear gradient from 15:4:81 (v/v/v) to 35:0:65 (v/v/v) in 40 min, maintaining this proportion until the end of the run. The mobile phase for *R. mucilaginosa* strains was tetrahydrofuran:H_2O:methanol from 15:4:81 (v/v/v) to 30:0:70 in a linear gradient in 40 min, maintaining this proportion until the end of the run. The flow rate was 0.5 ml/min.

The carotenoids were identified with the combined use of several parameters: UV–vis absorption spectra, position on the column, thin layer chromatography (TLC) (silica) R_F values, HPLC retention times and for the xanthophylls, specific group chemical reactions. The mobile phase for TLC was 5% methanol in toluene. The chemical reactions carried out were acetylation with acetic anhydride, methylation with acidified methanol, iodine-catalyzed isomerization, reduction with sodium borohydride, and epoxide tests (Eugster, 1995; Rodriguez-Amaya, 1999). The concentrations were determined spectrophotometrically (Davies, 1976), based on the maximum absorbance, of the carotenoid fractions obtained from the open column.

3. Results and discussion

The results obtained after 5 days of cultivation of the yeasts in YM medium broth are presented in Table 1. The production of biomass varied among the yeasts. *R. mucilaginosa*-137 had the highest biomass yield (8.6 g/l), followed by *R. graminis*-125 (8.5 g/l) and *R. mucilaginosa*-108 (8.3 g/l), while *S. roseus* had the lowest biomass yield (3.3 g/l). The yields obtained in the present study for *R. mucilaginosa* are higher than that (4.2 g/l) reported by Aksu and Eren (2005). For *R. glutinis* our result (6.5 g/l) falls within the range (from 5.2 to 9.3 g/l) obtained by Buzzini and Martini (1999) but lower than those (from 9.0 to 13 g/l) of Bhosale and Gadre (2001).

The pH of the medium for all the yeasts decreased from the initial YM pH of 6.8. The media of *R. minuta* and *S. roseus* had high residual sugar, indicating that the carbon source had not been well consumed.

Nine carotenoids were identified in the Brazilian yeasts: phytofluene (7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene), β-carotene (β,β-carotene), β-zeacarotene (7′,8′-dihydro-β,ψ-carotene), γ-carotene (β,ψ-carotene), neurosporene (7,8-dihydro-ψ,ψ-carotene), hydroxy-torulene, torulene (3′,4′-didehydro-β,ψ-carotene), echinenone (β,β-caroten-4-one), torularhodin (3′,4′-didehydro-β,ψ-caroten-16′-oic acid).

The first fraction obtained from the MgO:Hyflosupercel open column, a colourless zone before the first coloured band, showed the visible absorption spectrum typical of phytofluene, with well-defined peaks at 331, 347 and 367 nm in petroleum ether. It was found in appreciable amount only in *R. minuta*.

Fraction 2, orange in colour, was eluted from the column with 2–6% ethyl ether in petroleum ether. It showed the characteristic absorption curve of β-carotene, with λ_{max} at 447 and 475 nm and a shoulder at 423 nm in petroleum ether, and co-eluted with authentic β-carotene in TLC and HPLC. The absence of substituents was shown in the TLC plate developed with 5% methanol in toluene, where it ran with the solvent front. Exposition of the TLC plate to HCl gas confirmed the absence of epoxide groups, the orange colour of the spot not changing to blue or green.

The following band (fraction 3), eluted with 6–10% ethyl ether in petroleum ether, was identified as β-zeacarotene. The R_F value of 0.96 on the TLC plate indicated its carotene nature and the spectrum with λ_{max} at 403, 424 and 450 nm in petroleum ether was consistent with a carotenoid of nine conjugated double bonds, eight in the polyene chain and one in a β-ring.

Changing the mobile phase to 1–10% acetone in petroleum ether, another band (fraction 4) was separated on the MgO:Hyflosupercel column, which showed a visible absorption spectrum typical of γ-carotene. This bright-orange monocyclic carotenoid had an adsorption spectrum with λ_{max} at 435, 458 and 490 nm in petroleum ether, commensurate with a carotenoid having 11 conjugated double bonds, 10 in the polyene chain and one in the ring. The R_F on the silica plate of 0.97 indicated the absence of substituents.

Fraction 5, yellow-orange in colour, was eluted with 30–80% acetone in petroleum ether. The visible spectrum had well defined peaks at 412, 436 and 465 nm in petroleum ether, typical of neurosporene with nine conjugated double bonds in the polyene chain. The R_F of 0.96 was consistent with a carotene.

Torulene, fraction 6 (Fig. 1), was eluted with 2–6% of acetone in water. The reddish-pink torulene absorbed maximally at 460, 484 and 518 nm in petroleum ether. In all strains studied, torulene appeared as a mixture of (all-*E*) and (*Z*)-torulene, with peaks at 458, 483 and 515, with the (all-*E*)-isomer predominating. As a carotene it ran with the solvent front in the silica TLC ($R_F = 0.98$). In the MgO:Hyflosupercel column for the carotenoids of *R. mucilaginosa*-137 and *R. mucilaginosa*-108, two (*Z*) fractions were separated from the (all-*E*)-isomer. The first was a carotene, (*Z*)-torulene, with an R_F of 0.98. The second was tentatively identified as (*Z*)-hydroxy-torulene with maximum absorbance at 456, 481 and 513 nm with an R_F of 0.61. The location of the hydroxyl group was not determined.

The next band (fraction 7) on the column was eluted with 6–10% of acetone in water, was tentatively identified as echinenone, a keto carotenoid with one conjugated carbonyl group. Its spectrum had an unsymmetrical broad peak at 456 nm and a shoulder at 476 nm in petroleum ether. Since its concentration in all the strains analyzed was very low, reduction with sodium borohydride could not be carried out. The R_F was estimated to be about 0.83.

Table 1
Fermentation parameters obtained after 5 days of cultivation in YM medium broth

Yeast strain	Dry biomass (g/l)	Final pH of medium	Residual sugar (g/l)
Rhodotorula mucilaginosa-135	7.2 ± 0.16	6.1 ± 0.05	0.2 ± 0.05
Rhodotorula mucilaginosa-137	8.6 ± 0.20	6.3 ± 0.07	0.3 ± 0.03
Rhodotorula graminis-125	8.5 ± 0.31	6.4 ± 0.07	0.3 ± 0.04
Rhodotorula mucilaginosa-108	8.3 ± 0.14	6.4 ± 0.04	0.2 ± 0.03
Rhodotorula mucilaginosa-12	6.8 ± 0.17	5.7 ± 0.07	0.5 ± 0.08
Rhodotorula glutinis	6.7 ± 0.12	6.5 ± 0.08	0.2 ± 0.06
Rhodotorula minuta	5.1 ± 0.15	5.1 ± 0.06	3.6 ± 0.27
Sporobolomyces sp.	3.3 ± 0.10	5.8 ± 0.07	4.2 ± 0.19

Means and standard deviations of triplicate samples.

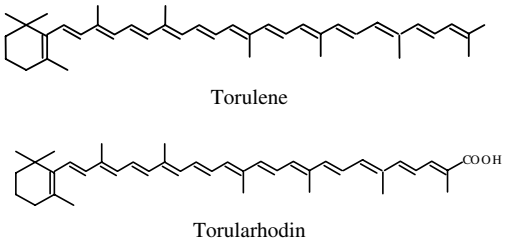

Fig. 1. Typical carotenoids in yeasts.

Torularhodin, fraction 8 (Fig. 1), a carboxylic acid carotenoid, was eluted with 25% of acetone in water. This pink pigment absorbed maximally at (465), 492 and 523 nm in petroleum ether and had an R_F of 0.48–0.50. After acetylation, the R_F increased to 0.92.

The HPLC chromatograms of the carotenoids of Brazilian yeasts are shown in Figs. 2 and 3. The visible spectra obtained by the photodiode array detector corroborated those obtained spectrophotometrically of fractions isolated by the open column (MgO:Hyflosupersel). Two (*Z*)-torulenes separated well from the (all-*E*)-form and the spectrum obtained by the photodiode array detector showed the cis-peak at about 380 nm clearly.

Practically, the same carotenoids were found in the Brazilian yeast studied, but considerable quantitative differences were seen among the strains (Table 2). *R. mucilaginosa*-135, *R. mucilaginosa*-137, *R. mucilaginosa*-108, *R. mucilaginosa*-12 had similar profiles, having torulene as principal carotenoid and β-carotene as the second major carotenoid (Fig. 2). On the other hand, β-carotene predominated in *R. glutinis*, *R. graminis*-125 and *S. roseus* (Fig. 3), with torulene as the second major carotenoid. *R. minuta* had the most different pattern, the main carotenoid being β-zeacarotene followed by phytofluene.

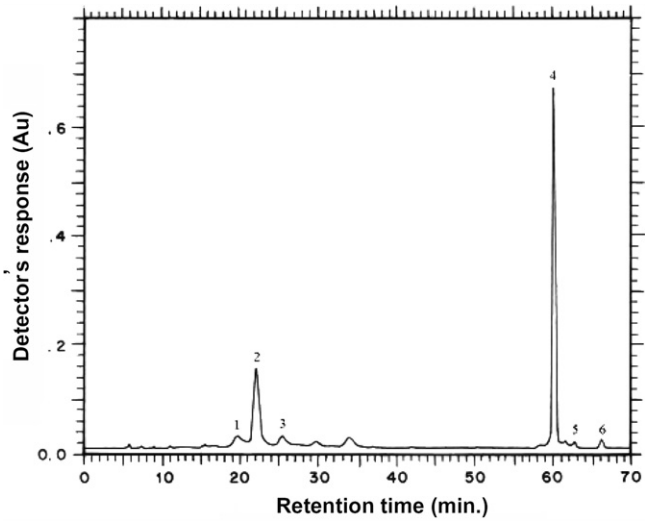

Fig. 2. Typical HPLC chromatogram of the carotenoids from *R. mucilaginosa*-137, *R. mucilaginosa*-135, *R. mucilaginosa*-108 and *R. mucilaginosa*-12 strains. HPLC conditions are described in the text. Detection was at 450 nm. Peak identification: (1) torularhodin, (2) β-carotene, (3) γ-carotene, (4) (all-*E*)-torulene, (5) (*Z*)-torulene and (6) (*Z*)-torulene.

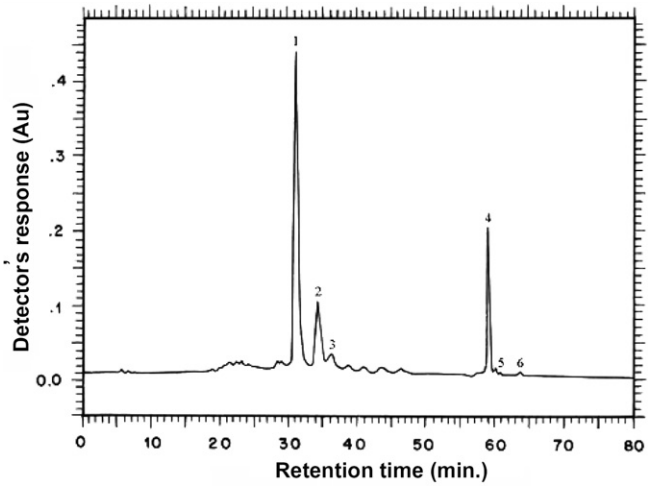

Fig. 3. Typical HPLC chromatogram of the carotenoids from *R. glutinis*. HPLC conditions are described in the text. Detection was at 450 nm. Peak identification: (1) β-carotene, (2) γ-carotene, (3) unidentified, (4) (all-*E*)-torulene, (5) (*Z*)-torulene and (6) (*Z*)-torulene.

Torularhodin was the third carotenoid in the *R. mucilaginosa* strains, but was not detected in *R. graminis*-125 and *R. minuta*. It ranked fourth in *R. glutinis* and *S. roseus*, in which the third carotenoid was γ-carotene. According to Frengova, Simova, and Beshkova (1995), the torularhodin content produced by *R. glutinis* vary significantly, from 56.0% to 78.3% of the total carotenoids, depending on the culture conditions.

Phytofluene, the colourless carotenoid, was found only in *R. minuta*. Phytofluene, neurosporene, β-zeacarotene and γ-carotene are known intermediates in the biosynthesis of β-carotene and torulene.

In the present work, the percentages of the carotenoids found in *R. glutinis* were: torularhodin (2%), torulene (30%), β-carotene (43%), γ-carotene (16%), β-zeacarotene (4%) and neurosporene (5%). These data agree with those obtained by Simpson, Nakayama, and Chichester (1964), who also identified β-carotene, torulene and torularhodin as major carotenoids of *Rhodotorula* sp. and *Rhodosporidium* sp., grown on a basal medium (Difco). Subsequently, Simpson et al. (1971) determined the carotenoids in *R. glutinis* after 12 days of fermentation as: torulene (28%), torularhodin (24%), β-carotene (25%), γ-carotene (13%), β-zeacarotene (1%) and neurosporene (1%). On the other hand, Frengova, Simova, Pavlova, Beshkova, and Grigrova (1994) reported that torularhodin was the major carotenoid of *R. glutinis*, reaching a maximum concentration of 182 μg/g dry cells on the sixth day of cultivation in whey ultrafiltrate medium, followed by β-carotene (44 μg/g). Torulene content reached 23 μg/g dry cells on the fifth day and then decreased. Torularhodin was also the major carotenoid found in *R. glutinis* (60–79%) by Buzzini and Martini (1999) in a medium containing concentrated grape as carbohydrate source.

Table 2
Carotenoid production (μg/l) of Brazilian yeasts

Carotenoid	*R. mucilaginosa*-135	*R. mucilaginosa*-137	*R. graminis*-125	*R. mucilaginosa*-108	*R. mucilaginosa*-12	*R. glutinis*	*R. minuta*	*Sporobolomyces*
Phytofluene	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)	56 (11 ± 0.5)	– (–)
Neurosporene	5 (0.7 ± 0.1)	15 (2.0 ± 0.7)	– (–)	– (–)	Tr (Tr)	46 (7.0 ± 0.4)	18 (3.5 ± 1.1)	– (–)
β-Zeacarotene	Tr (Tr)	13 (1.7 ± 0.4)	21 (2.2 ± 0.6)	12 (1.5 ± 0.3)	Tr (Tr)	30 (5.0 ± 0.6)	71 (14 ± 0.9)	18 (5.0 ± 0.2)
Echinenone	7 (1.0 ± 0.2)	Tr (Tr)	Tr (Tr)	19 (2.1 ± 0.2)	Tr (Tr)	Tr (Tr)	Tr (Tr)	Tr (Tr)
γ-Carotene	26 (3.7 ± 0.4)	47 (4.5 ± 0.7)	84 (9.8 ± 0.5)	30 (3.5 ± 0.7)	Tr (Tr)	142 (21 ± 0.6)	Tr (Tr)	20 (6.0 ± 0.5)
β-Carotene	95 (13 ± 0.3)	139 (16 ± 0.2)	303 (36 ± 0.2)	129 (16 ± 0.1)	105 (16 ± 0.4)	382 (57 ± 0.2)	23 (4.5 ± 0.2)	118 (36 ± 0.1)
Torulene	365 (51 ± 0.3)	352 (42 ± 0.2)	186 (22 ± 0.4)	307 (38 ± 0.3)	372 (54 ± 0.4)	261 (39 ± 0.2)	– (–)	71 (22 ± 0.2)
Torularhodin	47 (6.6 ± 0.7)	24 (2.8 ± 0.6)	– (–)	65 (7.0 ± 0.6)	10 (2.0 ± 0.8)	20 (3.0 ± 0.5)	– (–)	10 (3.0 ± 0.8)
Total	545 (76 ± 0.4)	590 (69 ± 0.5)	594 (70 ± 0.5)	562 (68 ± 0.6)	487 (72 ± 0.6)	881 (132 ± 0.2)	168 (33 ± 0.4)	237 (72 ± 0.6)

Tr, traces; –, not detected. Values in parentheses are in μg/g dry cell. Means and standard deviations of triplicate samples.

The total carotenoid concentration (sum of the individual carotenoid concentrations) in μg/l of fermentation medium is shown in Table 2. *R. glutinis* had the highest yield of total carotenoid (881 μg/l), followed by *R. graminis*-125 (594 μg/l), *R. mucilaginosa*-137 (590 μg/l), *R. mucilaginosa*-108 (562 μg/l), *R. mucilaginosa*-135 (545 μg/l), and *R. mucilaginosa*-12 (487 μg/). *S. roseus* and *R. minuta* had lower carotenoid contents (237 and 168 μg/l, respectively).

In terms of the dry cell (Table 2), the carotenoid production values were: *R. glutinis*, 132 μg/g; *R. mucilaginosa*-135, 76 μg/g; *S. roseus*, 72 μg/g; *R. mucilaginosa*-12, 72 μg/g; *R. graminis*-125, 70 μg/g; *R. mucilaginosa*-137, 69 μg/g; *R. mucilaginosa*-108, 68 μg/g; *R. minuta*, 33 μg/g. Although *R. glutinis* remained to be the strain with highest carotenoid content and *R. minuta* the lowest, the order of the other strains was different because of differences in the biomass produced.

Perrier, Dubreucq, and Gayzy (1995) investigated 13 strains of *Rhodotorula* from a culture collection, grown on yeast nitrogen base medium supplemented with glucose, and obtained total carotenoid in μg/g dry weight of 70 for *R. glutinis*, 95 for *R. graminis*, 40 for *R. minuta* and 100 for *R. mucilaginosa*.

Of the major carotenoids found in the yeasts investigated, torulene, the major carotenoid of *R. mucilaginosa*, is an interesting carotenoid for commercialisation. Having 13 double bonds, it has a nice reddish colour, in contrast to β-carotene, which has a yellow to orange colour, depending on the concentration. Probably because it has not been found in food, possible effect of torulene on human health has not been studied. Structurally, however, this compound fulfills the requirement for provitamin A, i.e. a β-ring without substituents and a lateral polyene chain of 11 carbons. Since the antioxidant property has been associated with the conjugated double bond system, the efficiency being greater with a higher number of double bonds (Foote, Chang, & Denny, 1970; Terão, 1989), this carotenoid should also be an efficient antioxidant. In fact, torularhodin, the carboxylated derivative of torulene (Fig. 1), was found *in vitro* studies, to be more potent in quenching singlet oxygen and scavenging peroxyl radicals than β-carotene (Sakaki, Nakanishi, Tada, Miki, & Komemushi, 2001; Sakaki, Nochide, Komemushi, & Miki, 2002). β-carotene is the principal carotenoid found in *R. glutinis*, but aside from being marketed as nature identical synthetic carotenoid, commercial natural source of this carotenoid is already available, particularly the microalga *Dunaliella salina*. Thus, our current research is focusing on optimizing culture conditions to increase carotenoid production, reduce production costs by using low-cost culture media (i.e. utilizing industrials wastes), increasing the efficiency of extraction and directing the biosynthetic pathway to torulene.

4. Conclusions

The qualitative and quantitative composition of carotenoids in pigmented yeasts depends on the genus and the culture conditions. β-Carotene predominated in *R. graminis*-125, *R. glutinis* and *S. roseus*, while torulene was the major carotenoid in *R. mucilaginosa*.

Per volume of medium, the yeast *R. glutinis* had the highest carotenoid production, followed by *R. graminis*, *R. mucilaginosa*-137 and *R. mucilaginosa*-135. *R. minuta* and *S. roseus* had the lowest carotenoid contents.

The yeasts investigated show potential as microbial sources for producing carotenoids by fermentation.

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