International Journal of Food Science +Technology

Original article

Carotenoid profile produced by *Bacillus licheniformis* Rt4M10 isolated from grapevines grown in high altitude and their antioxidant activity

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(Received 29 March 2018; Accepted in revised form 13 June 2018)

Summary *Bacillus licheniformis* (BL) strains are used for industrial production of exoenzymes, antibiotics and secondary metabolites, and also serve as probiotics. Some strains sequester heavy metals, promoting plant growth or inducing plant systemic resistance against stresses. However, investigations on the biological functions and applicability of carotenoids produced by BL had been less studied. In this study, the carotenoid profile produced by BL Rt4M10 was characterised during the accumulation period of culture and in response to diphenylamine (DPA), inhibitor of carotenoid biosynthesis. The antioxidant scavenging capacities of the carotenoid extracts against oxidative reactions inside the microbial cell and *in vitro* were also evaluated. Eighteen different carotenoids were analysed; their profile was modified along bacterial growth. DPA decreased carotenoid production by 70%, as well as the cells' survival in hydrogen peroxide and the antioxidant capacity, thus demonstrating the role of carotenoids from Rt4M10 in oxidative stress.

Keywords Antioxidant activity, *Bacillus licheniformis*, carotenoids, pigments.

Introduction

Bacillus licheniformis (BL) is a Gram (+) bacterium known for the synthesis of a wide range of medicinal, agricultural, pharmaceutical and industrial products. In the last years, it has been extensively used for large-scale industrial production of exoenzymes, antibiotics and chemicals (Elshaghabee *et al.*, 2017; Wu *et al.*, 2018). BL strains are categorised by the World Health Organization as a GRAS organism (Generally Recognized As Safe), being active against many pathogens and free of toxins. *B. licheniformis* is also used as probiotic (Elshaghabee *et al.*, 2017). The scientific evidence shows their benefits on gastrointestinal disorders and inflammation of animals and humans; as well, it has protective effects in the pediatric patient undergoing radiotherapy (Du *et al.*, 2018). However, despite the various benefit-promoting effects of BL, the importance of carotenoid production has been understudied.

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Carotenoids are widespread naturally occurring yellow, orange and red pigments produced by higher plants, algae and fungi (Mercadante & Egeland, 2004). Generally, they are tetraterpenes (C40); however, in bacteria, a diverse range of carotenoids with C30, C40 and C50 backbones can be found (Sandmann, 2014). The industrial demand for carotenoids has been increased because of their potential applications. Due to their useful biological activities as antioxidants, protectors against ultraviolet (UV) radiation and natural food colorants, carotenoids are commercially used (Shegokar & Mitri, 2012). Humans and animals are unable to synthesise *de novo* these compound and thus rely on their diet to supply them. Few carotenoids can be commercially produced via chemical synthesis, and their use is prohibited in some cosmetic and food industries or is strictly regulated (Jaswir *et al.*, 2011). Currently, commercial production of carotenoids is mostly carried out by extraction from plant or algae tissues (Ishida *et al.*, 2009). However, microbial production also has great potential in terms of efficiency,

doi:10.1111/ijfs.13879

production and innocuous environmentally friendly methods (Sandmann, 2014; Mata-Gómez *et al*., 2014).

Inhibitors are frequently used to elucidate carotenoid biosynthesis. Diphenylamine (DPA) is an inhibitor of phytoene desaturase a key enzyme in carotenoid biosynthetic pathway (Sandmann, 2014).

The aim of this study was the evaluation of carotenoid pattern produced by BLRt4M10 (Rt4M10). The effects of DPA and incubation time on carotenogenesis were analysed, and their effect on the antioxidant capacity of compounds produced by Rt4M10 was evaluated.

Materials and methods

Reagents and standards

Diphenylamine (DPA, Tetrahedron Laboratories Pvt. Ltd. in Thane, Maharashtra, India) was used. Methanol (MeOH), ethanol (EtOH) and acetone (JT Baker Chemical Co., Phillipsburg, New Jersey, USA) analytical grade were used for sample preparation and carotenoid extractions; tert-Butyl methyl ether (TBME), 2,6-di-tert-butyl-4-methylphenol (BHT), triethylamine (TEA) (Sigma-Aldrich Co., St. Louis, MO, USA), acetonitrile, ethyl acetate (AcEt) and MeOH (Merck, Darmstadt, Germany) were used as solvent carriers in HPLC analysis. The authentic standards β-apocaroten-8-al, zeaxanthin, β-carotene, violaxanthin, neoxanthin and lutein were obtained from Sigma-Aldrich Co. Trolox reagent (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), $NaH_2PO_4{\cdot}12H_2O$, $Na_2HPO_4{\cdot}12H_2O$, fluorescein and 2,20-azobis-2-methylpropionamidine dihydrochloride (AAPH) were purchased from Sigma-Aldrich (Steinheim, Germany). μM β-cyclodextrin (CD) was provided by Ferromet S.A. (Buenos Aires, Argentina).

Microorganism and culture conditions

Rt4M10 previously isolated from roots of *V. vinifera* by Salomon *et al*. (2014) was used in this work (GenBank accession number KF717083.1). One colony of Rt4M10 was precultured on Luria Broth Base (Miller's LB Broth Base Invitrogen, Buenos Aires, Argentina) medium in an orbital shaker (Boeco PSU-10i, Germany) at 32 °C and 135 r.p.m. Then, 500 μL of this preinoculum was cultured in 500-mL flasks with 125 mL of LB. The growth was monitored by spectrophotometric absorbance at 600 nm (OD_{600}, biomass production) in UV-Vis spectrophotometer Cary 50 (Varian Inc., Mulgrave, Australia) and by colony forming units (CFU) mL^{-1} determination at every 12 h until the stationary phase reached as previously described (Cohen *et al*., 2008). The carotenoid pattern was characterised in response to DPA. DPA was dissolved in EtOH; the solution was filtered through a 0.22-μm membrane filter. When the bacteria culture achieves an OD_{600} nm = 0.3, the filtrated DPA was added to the medium with minimal effects on cell growth. Rt4M10 with 100 μM of DPA (Rt4M10 + DPA) and without DPA (Rt4M10−DPA) was cultivated aerobically from 96 to 168 h at 30 °C and 135 r.p.m. in an orbital shaker in darkness. Culture and carotenoids' extraction were carried out on ice under darkness or dim light to avoid pigments photooxidation. Cells were harvested by centrifugation (Hermle Z 326 K centrifuge, Hermle Labortechnik GmBH, Germany) at 8000 ***g*** 4 °C for 5 min. The upper layer was discarded, and the extracts (residual cell) were stored at −80 °C until extraction.

Carotenoid extraction

Residual cells were extracted with 3 mL MeOH: acetone for (7:3, v/v) 16 h at 4 °C in darkness. Cell suspensions were sonicated in ultrasonic ice water bath (Cleason 1106, Buenos Aires, Argentina) at 200 W, 4 °C for 10 min. Then, the extract was centrifuged three times (7500 ***g***, 5 min, 4 °C). The upper organic phase was collected, pooled and concentrated under reduced pressure in a rotary evaporator according to Salvadores (2012). Concentrated samples (CS) were stored at −80 °C.

Chromatographic analysis

Prior to chromatographic analysis, the extracts obtained from Rt4M10 cultures were resuspended in MeOH:AcEt (4:1) and BHT (0.1% w/v), filtered through a polytetrafluoroethylene membrane (0.2 μm; Herts, UK) and centrifuged (2 min, 13 000 ***g***). Then, the final extracts were transferred to inserts in amber vials and 2 μL of internal standard (β-apocaroten-8-al 200 ng μL^{-1}) was added. Afterwards, samples were filtered through a 0.45 μm pore size nylon membrane filters (Whatman®, GE Healthcare Life Sciences, Switzerland) and then 20 μL was injected in a Perkin-Elmer series 200 high-performance liquid chromatograph equipped with a photodiode array detector (HPLC-DAD; PerkinElmer, Shelton, CT). An YMC30 column (250 × 4.6 mm; particle size 5 μm) from YMC Europe (Schermbeck, Germany) was used for separation of the compounds at 25 °C (according to Aschoff *et al*., 2015 with modifications). A gradient of binary solvents consisting of 3% ddH2O in MeOH containing 0.05 M ammonium acetate (Solvent A) and 100% TBME (Solvent B), and 0.1% (w/v) TEA was used. A flow rate of 1 mL min^{-1} elution was done according to the following programme: isocratic 20% B for 20 min followed by a linear gradient from 20 to 50% B in 4 min, isocratic 50% B for 4 min, then a

Table 1 Carotenoids separated on HPLC and their spectral characteristics by DAD

Compound tentative identification	K′[a]	α[b]	Spectral characteristics (nm)[c]	% III/II[d]	Q-ratio[e]
Lutein epoxide	1.97	1.61	417 444 468	20	
Neochrome	3.16	1.56	401 425 450	75	
Luteoxanthin	4.94	1.50	400 424 450	90	
Unknown I	7.43	1.14	444 468 498	71	
Plectaniaxanthin	8.44	1.07	450 480 508	20	
Phytoene	9.02	1.00	276 286 297	10	
13-Z-bacterioruberin	9.04	1.19	386 463 488 520	38	0.75
all-Z-cryptoxanthin	10.77	1.01	400 422 448 476	10	0.52
9-Z-violaxanthin (diester)	10.88	1.03	394 416 438 467	97	0.34
2-hydroxytorularhodin	11.16	1.01	389 468 495 528	49	0.13
Torularhodin	11.25	1.03	470 495 528	45	
9-Z-bacterioruberin	11.61	1.02	386 464 487 520	46	0.25
Z-torulene	11.79	1.02	386 461 485 517	33	0.72
Bacterioruberin 1 glycoside	12.05	1.02	389 465 492 527	39	0.14
E-torulene	12.24	1.02	387 463 489 520	69	0.25
Bacterioruberin 2-glycoside	12.43	1.06	387 465 492 523	45	0.19
Spheroidenone	13.23	1.01	458 483 516	77	
Spirilloxanthin	13.40	1.05	388 468 493 526	49	0.12
Unknown II	14.04	1.01	457 484 514	47	
C_{50}-β-carotene (Z)	14.21	1.01	387 465 498 522	45	0.15
Unknown III (isomer 1)	14.31	1.08	458 485 517	48	
Unknown III (isomer 2)	15.50	1.01	455 486 517	80	
β-apocaroten-8-al (IS)	7.05		462		

[a]Retention factor.
[b]Separation factor.
[c]Gradient movil phase of MeOH containing 3% H_2O and 0.05 mol L^{-1} ammonium acetate (solvent A) and 100% TBME containing 1% (w/v) TEA (solvent B).
[d]Ratio of the height of the longest wavelength absorption peak, designated III, and that of the middle absorption peak, designated II, taking the minimum between the two peaks as baseline.
[e]Quotient between the cis (Z) peak band and band II.

linear increase to 68% B in 2 min, isocratic at 68% B for 2 min, followed by a linear decrease to 20% B, with a final wash with 100% MeOH and re-equilibration for 15 min at the starting conditions. Photodiode array detection was performed from 190 to 700 nm, and the quantification was carried out by peak area measurements at 420 nm. Identification of carotenoids was achieved by matching retention times and comparing UV-visible fine spectra with authentic standard. Due to the lack of bacterial standards for some carotenoids, these compounds were tentatively identified by retention factor, the localisation of the maximum absorption wavelength (λ_{max}) and degree of spectral vibrational fine structure (Table 1) according to various researchers (Weber *et al.*, 2007; Mandelli *et al.*, 2012; Steiger *et al.*, 2012; Aschoff *et al.*, 2015; Yang *et al.*, 2015). Standard curves for the quantification of carotenoids were acquired by plotting amount (ng) against area obtained by triplicate according to the peak area ratios with β-apo-8′-carotenal.

Antioxidant activity (AOA) inside the microbial cell

Assessment of sensitivity to H_2O_2 was carried out as the procedure described by Carbonneau *et al.* (1989) with modifications. Briefly, RT4M10 + DPA and RT4M10−DPA were cultivated before the end of the stationary phase as previously described and diluted with isotonic solution to a final concentration of 10^8 CFU mL^{-1}. Samples of 1 mL were prepared and placed in a water-ice bath.

Accurate quantities of 30% H_2O_2 solution were added to the cell suspension to obtain H_2O_2 concentration 0, 25, 50, 75 and 100 mM. Then, cells were incubated with H_2O_2 in darkness at 0 °C and plated gradually over a period of 1 and 3 h. Colonies were kept at 30 °C and counted after 4–5 days. In each experiment, controls incubated without H_2O_2 at 0 °C in darkness were used; under these conditions, cell increment was not significant and therefore was not taken into account in survival calculation.

AOA *in vitro*

Hydrophilic oxygen radical absorbance capacity (ORAC) assay

The ORAC of hydrophilic compounds ($ORAC_H$) was determined according to Berli *et al.* (2012) with modifications. The residues of carotenoid extraction were re-extracted with 10 mL of acetone/water/acetic acid, (70:29.5:0.5, v/v/v). After adding solvent, the tube was vortexed 30 s followed by sonication at 37 °C 5 min and then kept at room temperature for 10 min with occasional shaking. After centrifugation at 4600 r.p.m. for 15 min, the supernatant was removed and transferred to a volumetric flask and diluted to 25 mL.

Fifty microlitres aliquots of suitable diluted sample in NaH_2PO_4-Na_2HPO_4 buffer (75 mM, pH 7.0) and Trolox standards prepared in KH_2PO_4-K_2HPO_4 buffer (pH 7.0) at different concentration (0–50 μM) were added to a 96-well plate. Then, 100 μL of fluorescein solution (20 nM) and the mixture incubated at 37 °C for 7 min before the addition of 50 μL of 140 mM peroxyl radical generator AAPH. Fluorescence was monitored using 485 nm excitation and 538 nm emissions at 1-min intervals for 90 min on a microplate fluorometer (Fluoroskan Ascent FL, Thermo Fisher Scientific Inc, Wilmington, DE). The decay area of the fluorescence under the curve during 90 min was calculated, and the ORAC was expressed as μmol of Trolox equivalents per L of samples (μM TE), respectively.

Lipophilic ORAC assay

The ORAC of lipophilic compound ($ORAC_L$) was determined according to Prior *et al.* (2003) with modification. For $ORAC_L$ assay, the dried extracts detailed in carotenoid extraction section were dissolved in 250 μL of acetone and then diluted with 750 μL of a 7% CD solution acetone: H_2O (1:1 v/v). For further dilution, as a blank and to dissolve Trolox standards for the lipophilic assay a 7% CD solution was used. Also, it was used. For CS+DPA and CS−DPA lipophilic analysis, 50 μL of 7% CD solution was added to the 96-well microplate. The fluorescein solution (80 mM) and AAPH (530 nM) were added in the same way as that for the hydrophilic assay. Readings were initiated immediately with the same experimental conditions of $ORAC_H$ assay.

Statistical analysis

Statistical analysis was carried out with InfoStat version 2013 (Grupo InfoStat, FCA, UNC, Argentina). The results were tested for homogeneity of variance using Cochran's test and analysed by one-way analysis of variance (ANOVA) and Tukey's multiple range tests (TMRT). Significant differences were considered at probability of $P \leq 0.05$, and replica numbers of reported data are specified in each figure or table legend.

Results and discussion

Culture growth and effect of DPA on carotenoid accumulation

Cultures Rt4M10 grown in LB medium showed an exponential phase from 10 to 48 h, then the stationary phase took place from 72 to 120 h, followed by the decay phase (Fig. 1a). In the experimental conditions used, DPA incorporated 12 h after the culture growth, depressed carotenoid production (Fig. 1b), and decreased culture growth from 24 h onwards, as it has been previously reported in cell suspension cultures (Moliné *et al.*, 2010). However, the size of plated colonies with DPA (+DPA) was comparable to those without the depressor of carotenoid biosynthesis (−DPA). As well, CFU in both treatments were similar from 24 to 72 h. Notwithstanding, RT4M10+DPA grew slower than Rt4M10-DPA. The later reached the maximum value of CFU at 48 h and maintained it approximately until 120 h, then decayed but always with the highest values (Fig. 1a). As expected, +DPA inhibited carotenoid biosynthesis by about 70% (Fig. 1b) at the different times (24, 48, 72, 96 and 168 h). Therefore, the colour of the colonies was different, an orange pellet in Rt4M10-DPA and a pale yellow pellet in +DPA (Figure S1).

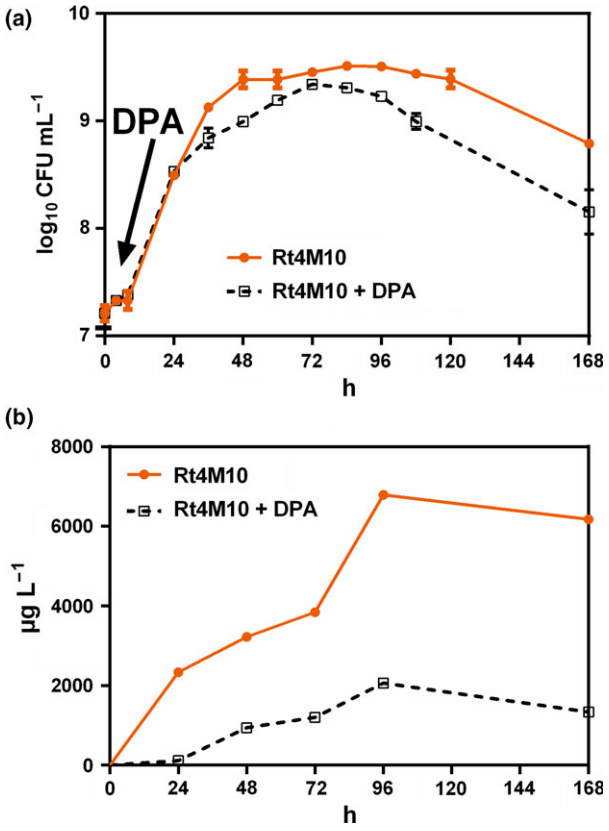

Figure 1 a) Effect of DPA on the cell growth (log10 CFU mL^{-1}) and b) total carotenoids (sum of identified/tentatively identified carotenoids expressed in μg L^{-1}), produced by Rt4M10. The strain was cultivated for 168 h at 32 °C as described in Materials and Methods. Closed circle: strain Rt4M10 grown without DPA (Rt4M10−DPA); open square: strain Rt4M10 grown with 100 μM DPA (Rt4M10+DPA). Each point represents an average of three independent experiments with standard error.

There are non-pigmented *Bacillus* species while others are able to produce carotenoid pigments that render them yellow (Duc *et al.*, 2006), yellow-orange and red (Khaneja *et al.*, 2010) in colour. In *B. firmus* strains, an alternative C30 biosynthetic pathway has been reported (Steiger *et al.*, 2012; Pérez-Fons & Fraser, 2012). At the moment, however, there is scarce information about carotenoids produced by BL strains. Only one strain of BL isolated from seagrass has been informed as producing the carotenoid pigment diadinoxanthin (C40), although in the Prokaryotic Carotenoid Database (ProCarDB), no carotenoid has been reported for this strain (Nupur *et al.*, 2016).

Profile and quantification of carotenoids by HPLC-DAD

The chromatograms obtained in the analysis of carotenoids along the different phases of Rt4M10 culture

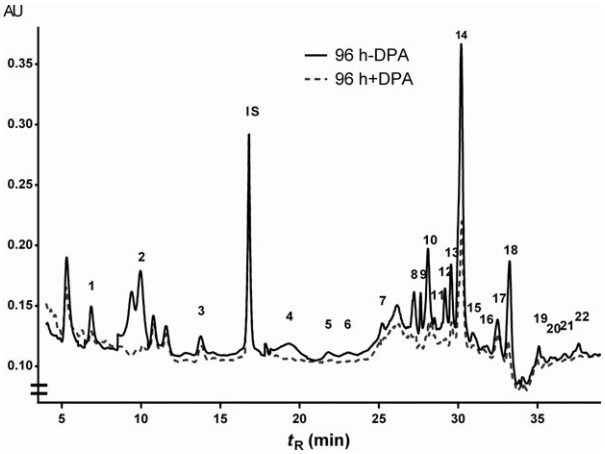

Figure 2 HPLC-DAD chromatogram with a C30 column of the carotenoids extracted from Rt4M10 cultivated for 96 h at 32 °C without DPA (CS−DPA, continuous line), and with DPA μmol 100 L^{-1} (CS+DPA, dotted line), as described in Materials and Methods. Peak identification: 1, lutein epoxide; 2, neochrome; 3, luteoxanthin; IS, (internal standard) β-apocaroten-8-al; 4, unknown I; 5, plectaniaxanthin; 6, phytoene; 7, 13-Z-bacterioruberin; 8, all-Z-cryptoxanthin; 9 9-Z-violaxanthin (diester); 10, 2-hydroxytorularhodin; 11, torularhodin; 12, 9-Z-bacterioruberin; 13, Z-torulene; 14, bacterioruberin monoglycoside; 15, E-torulene; 16, bacterioruberin diglycoside; 17, spheroidenone; 18, spirilloxanthin; 19, unknown II; 20, C50-β-carotene (Z); 21, unknown III; 22, unknown IV. Spectral characteristics (Table 1). AU, arbitraries units; TR, retention time.

growth showed well-separated peaks (α, Table 1). HPLC-DAD analysis of CS−DPA revealed 22 distinctive peaks (Fig. 2) with defined spectral characteristics (Table 1). The carotenoids tentatively identified were (peak 1–22): lutein epoxide, neochrome, luteoxanthin, β-apocaroten-8-al (IS, internal standard), unknown I, plectaniaxanthin, phytoene, 13-Z-bacterioruberin, all-Z-cryptoxanthin, 9-Z-violaxanthin (diester), 2-hydroxytorularhodin, torularhodin, 9-Z-bacterioruberin, Z-torulene, bacterioruberin monoglycoside, E-torulene, bacterioruberin diglycoside, spheroidenone, spirilloxanthin, unknown II, C50-β-carotene (Z), unknown III and unknown IV. Therefore, eighteen carotenoids were tentatively identified in CS−DPA cultures, but only eight were detected in the presence of DPA. In both treatments, the content of carotenoids was changed along the time (24, 48, 72, 96, 168 h), quantitatively and qualitatively (Table 2). In Fig. 2, the chromatographic profiles of the carotenoids produced in CS+DPA and CS−DPA are shown. As it can be seen in Table 2 and Figure S2, the maximum concentration of carotenoids in both treatments was found at the end of the stationary phase (6779 μg L^{-1} and 2062 μg L^{-1}, respectively, Fig. 1b). Therefore, it may be assumed that these secondary metabolites are produced by bacteria acclimated to adverse conditions, as RT4M10 has been isolated from soil and roots of vineyards at high altitude (1450 m a.s.l.) exposed to high UV-B radiation (26.45 μW cm^{-2}) (Salomon *et al*., 2014). At 96 h, a 70% reduction of the carotenoids was found in DPA treatment, probably by inhibition of the phytoene desaturase. In CS−DPA treatment at 96 h, C40-carotenoids (45.92%), C50-carotenoids (47.77%) and minor unknown compounds (6.31%) were detected. Among them, the most abundant was the group of C50 (bacterioruberin, BRu). The isomer 13-Z-BRu at 96 h (44 μg L^{-1}) was identified, while the 9-Z-BRu isomer was found in all the phases of bacterial growth, in concentration of 107–362 μg L^{-1}. Also, conjugated forms of BRu were found, as monoglycoside (BRu monoglc) and diglycoside (BRu diglc), reaching a maximum between 24 to 168 h. On the other hand, in CS+DPA treatment, BRu monoglc was determined but in lower levels, except at 72 h where no significant difference with CS−DPA was found. In CS+DPA, BRu diglc was determined at the end of the bacterial growth (96–168 h) and in low concentrations (50 and 10 μg L^{-1}, respectively). Also, C50-β-carotene was detected but at lower concentration respect to the other C50. These results are in agreement with reportes in other bacteria and archaea (Mercadante & Egeland, 2004; Calegari-Santos *et al*., 2016). Glycosylated carotenoids, like BRu monoglc and BRu diglc, increase bacterial membrane rigidity and decrease water permeability (Falb *et al*., 2008). BRu also protects the halophile microorganism from DNA damaging agents such as ionising radiation, UV radiation and hydrogen peroxide (Singh & Gabani, 2011), probably due to its ability to act as free radical scavenger and quenching singlet oxygen (Mandelli *et al*., 2012). Z-Torulene (TRu) was identified in both treatments during different intervals of time, except at 24 h. The E-TRu form was detected only in CS−DPA, at stationary phase (72–168 h). Also, the carboxylated derivative of TRu, Torularhodin (TRo), was determined in CS−DPA at the beginning of the bacterial growth (24 and 48 h; 329 μg L^{-1} and 341 μg L^{-1}, respectively). In contrast, in CS+DPA treatment, TRo was found only at 48 h (92 μg L^{-1}), perhaps as a consequence of growth delay due to DPA application. The hydroxyl derivate (2-hydroxy-TRo) was also found in both treatments. Whereas, in the absence of DPA, 2-hydroxy-TRo was identified along the bacterial growth, from 24 to 96 h (978 μg L^{-1}, maximum level), decreasing at 168 h (454 μg L^{-1}); with DPA, it was quantified in low concentration and at 96 h (62 μg L^{-1}) and 168 h (26 μg L^{-1}). All this compounds depend on the culture growth and the inhibitor. A correlation of TRo and 2-hydroxy-TRo with DPA was found, since the first compounds appeared at exponential phase, while 2-hydroxy-TRo did it at the end of the stationary phase, perhaps due to its higher oxidative state. Zoz *et al.*

Table 2 Concentration (μg L^{-1})[b] of carotenoids at 24, 48, 72, 96 and 168 h in CS−DPA and CS+DPA on a C_{30} HPLC column and their spectral characteristics following DAD

Compound[a]	24 h[c]	24 h+DPA[d]	48 h[c]	48 h+ DPA[d]	72 h[c]	72 h+ DPA[d]	96 h[c]	96 h+ DPA[d]	168 h[c]	168 h+ DPA[d]
Lutein epoxide	nd	nd	nd	nd	205.0	nd	178.6	nd	170.5	nd
Neochrome	nd	nd	nd	nd	nd	nd	165.65	nd	158.3	nd
Luteoxanthin	121.2	nd	134.4	115.8	152.5	125.0	156.6	88.3	160.3	86.2
Unknown I	8.24	nd	13.7	nd	23.1	nd	53.6	nd	nd	nd
Plectaniaxanthin	nd	nd	nd	nd	nd	nd	10.2	nd	nd	nd
Phytoene	nd	nd	6.4	nd	13.7	nd	15.7	nd	nd	nd
13-Z-bacterioruberin	nd	nd	nd	nd	nd	nd	44.1	nd	nd	nd
all-Z-cryptoxanthin	nd	nd	10.3	7.3	D	nd	42.9	nd	nd	nd
9-Z-violaxanthin	nd	nd	104.1	nd	137.9	nd	251.6	nd	237.8	nd
2-hydroxytorularhodin	427.1	nd	422.5	nd	342.1	nd	978.4	61.5	453.9	26.5
Torularhodin	329.4	nd	340.7	92.0	D	nd	D	nd	nd	nd
9-Z-bacterioruberin	283.2	nd	226.1	nd	107.2	nd	362.3	nd	295.2	nd
Z-torulene	nd	nd	170.3	nd	256.0	124.0	315.4	207.1	198.6	101.8
Bacterioruberin glycoside	958.7	117.7	1053	605.3	1095	1076	1530	1176	1682	904.0
E-torulene	nd	nd	nd	nd	77.2	nd	82.5	nd	60.1	nd
Bacterioruberin diglycoside	98.0	nd	108.3	nd	156.3	nd	329.6	50.2	405.6	10.0
Spheroidenone	112.1	nd	149.9	nd	207.4	nd	451.0	nd	462.3	nd
Spirilloxanthin	nd	nd	485.2	nd	859.6	nd	1044	481.6	998.1	208.0
Unknown II	nd	nd	nd	nd	Nd	nd	158.6	nd	178.1	nd
C_{50}-β-carotene (Z)	nd	nd	nd	nd	145.7	nd	246.0	nd	308.1	nd
Unknown III	nd	nd	nd	nd	62.6	nd	149.5	nd	162.9	nd
Unknown III	nd	nd	nd	nd	nd	nd	225.3	nd	245.3	nd

[a]Tentative identification (see Table 1).

[b]Average concentration (relative to internal standard) of three replicates with %DRS (per cent relative standard deviation) <25%.

[c]24, 48,72, 96 and 168 h.

[d]24, 48,72, 96 and 168+ inhibitor DPA.

nd compound no detected, D compound detected (concentration below the quantification limit of analytical method).

(2015) suggested the possible use of TRu and TRo as components of cosmetics and food. Studies of toxicity conducted on rats demonstrated that β-carotene, TRo and TRu produced by *R. glutinis* yeasts can be used as innocuous food additives (Latha & Jeevaratanm, 2012). Also, TRu and TRo could be able to inhibit growth of prostate cancer (Du *et al.*, 2016). On the other hand, phytoene was quantified in low concentration in CS−DPA, but it was undetectable in CS+DPA, suggesting that phytoene acts as precursor being immediately metabolised to others carotenoids. Phytoene is a common precursor of C40 and C50 carotenoid compounds.

The following xanthophylls were determined in the group of C40 without DPA: luteoxanthin and spheroidenone starting at 24 h; all-Z-cryptoxanthin, 9-Z-violaxanthin (diester) and spirilloxanthin at 48 h; lutein epoxide starting at 72 h and neochrome and plectaniaxanthin at 96 h, respectively. While luteoxanthin was identified in all the growth phases of Rt4M10 (from 24 to 168 h), in the treatment with DPA the production started at 48 h and declined at 96 h. The all-Z-cryptoxanthin was also found in CS+DPA only at 48 h. All together the results suggest that the enzymatic activity and the biosynthetic pathways involved in the carotenoid production are less affected by DPA. It is important to note that both all-Z-cryptoxanthin and 9-Z-violaxanthin are precursors of ABA in plants. It has been also found in a previous work that Rt4M10 produced ABA in chemically defined medium (Salomon *et al.*, 2014), so this finding supports that all-Z-cryptoxanthin and (9)-Z-violaxanthin, like in plants, may be intermediates of ABA pathway in the bacterium.

Spheroidenone and spirilloxanthin was observed to have scavenger function (Šlouf *et al.*, 2012). Finally, in the extracts without DPA, four unknown carotene-like compounds were found, unknown I–IV.

Antioxidant activity AOA inside the microbial cell

Correlation between total carotenoid concentration and sensitivity to H_2O_2 of the Rt4M10 cultures was assessed. No difference was observed in Rt4M10−DPA when they were suspended in increasing H_2O_2 concentrations (0 to 100 mM) by 1 h. When it was maintained in the same concentrations H_2O_2 by 3 h, the CFU mL^{-1} decreased only in H_2O_2 100 mM. However, the survival rate of Rt4M10 + DPA was significantly decreased by treatment with H_2O_2 (50–100 mol L^{-1}) for 1 h and CFU diminished drastically after 3 h, revealing

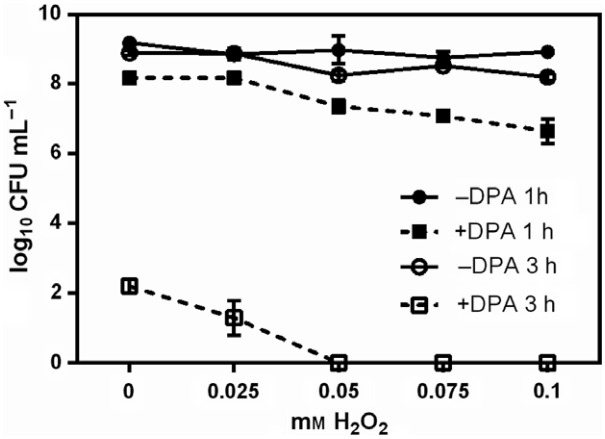

Figure 3 Bacterial survival ($\log_{10}$ UFC mL^{-1}) of Rt4M10 without DPA (Rt4M10−DPA) and with 100 μM DPA (Rt4M10+DPA) exposed to H_2O_2 0, 25, 50, 75 and 100 mM by 1 and 3 h.

differences between the treatments with DPA and without DPA (Fig. 3). Therefore, carotenoids protected Rt4M10 cells against oxidant activity of H_2O_2 demonstrating their role as antioxidants. The efficiency of the carotenoids as scavengers of reactive oxygen species determined by other authors (Miller *et al.*, 1996; Chisté *et al.*, 2011) is in agreement with our results.

AOA *in vitro*

The ORAC method was performed for quantifying the antioxidant capacity of the total extracted compounds of CS+DPA and CS−DPA at 48 and 168 h. In CS−DPA, the AOA data obtained from 48 and 168 h through the $ORAC_H$ assay was 2036 ± 288 and 1251 ± 18 μM TE, respectively. While the AOA data for CS+DPA were 96 ± 14 and 4.6 ± 2.2 μM TE at those two times proposed (Fig. 4a). In CS−DPA, the $ORAC_L$ assay values were 4369 ± 20 and 2408 ± 119 μM TE at 48 and 168 h, respectively. In CS+DPA, the $ORAC_L$ assay results were 1350 ± 189 and 1807 ± 531 μM TE at 48 and 168 h (Fig. 4b). These results showed that hydrophilic extract of CS−DPA treatment presents higher ORAC values compared with CS+DPA (between 95% and 98% at 48 and 168 h, respectively). In lipophilic extracts of CS−DPA, a similar behaviour was observed (60% and 25%, respectively). These results show that lipophilic and hydrophilic extracts of CS−DPA possess antioxidant capacity against peroxyl radical. Therefore, the high antioxidant capacities ($ORAC_H$ and $ORAC_L$) of *in vitro* CS−DPA were evidenced by the production of carotenoids by Rt4M10. This result explains that DPA is able to inhibit the synthesis of carotenoids, but as it was incorporated in the half of the exponential phase

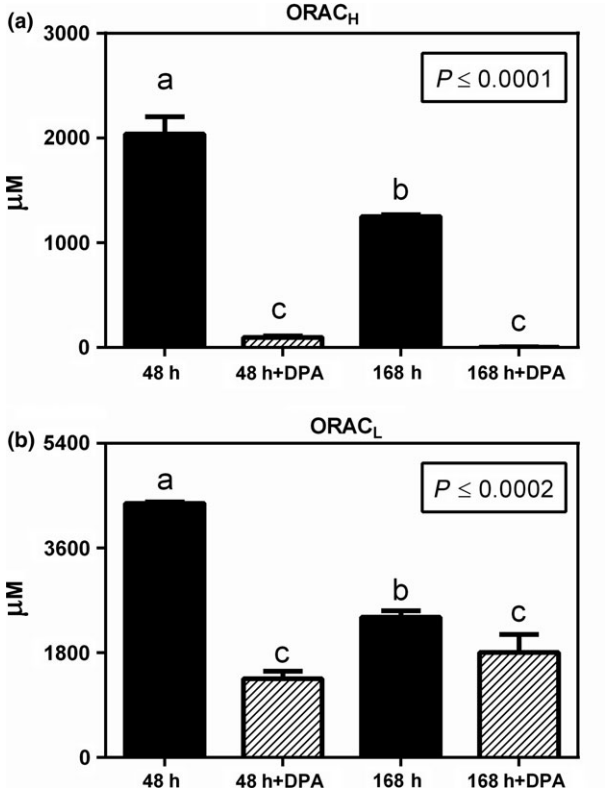

Figure 4 a) Hydrophilic (ORACH) and b) lipophilic (ORACL) antioxidant capacity expressed as μmol of Trolox equivalents per L (μM TE) of concentrated samples without DPA (CS−DPA) and with 100 μM DPA (CS+DPA) extracted from Rt4M10 at 48 and 168 h.

(12 h), the bacteria have the possibility to synthesise carotenoid compounds although in minor concentration.

Conclusions

In this study, the characterisation of eighteen carotenoids was performed by HPLC-DAD in extracts from Rt4M10. The highest carotenoid production occurred at stationary phase. These findings suggest two biosynthetic branching of carotenoid pathways in the experimental conditions used, in which phytoene is the precursor of C40 and C50-carotenoids, once the stationary phase is established in Rt4M10 culture. However, further studies on identification of carotenoids produced by Rt4M10 are required by techniques as mass spectrometry, nuclear magnetic resonance and/or Raman spectroscopy. Thus, the AOA inside the microbial cell and *in vitro* demonstrated in this work may be the basis for future investigations for carotenoid synthesis as candidates for biological active compounds in cosmetics, pharmaceutical and foods industries. These results help us to understand another mechanism

present in BL that increases their biotechnological potential.

Acknowledgment

This study was financed by Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET, PIP 11220130100185CO to A. C.), Instituto Nacional de Tecnología Agropecuaria (INTA, project PRET MZASJ-121101) to V. J. and Universidad Nacional de Cuyo (SECyT-UNCuyo to R. B., A. C., A. A. and P. P.). A. C., R. B. and P. P. are career members of CONICET; V. J. is researcher of INTA. E. D has a fellowship of Consejo Interuniversitario Nacional.

Conflict of interest

The authors declare no conflict of interests.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article:

Figure S1. Cellular pellet produced by Rt4M10 at 168 h grown without DPA (Rt4M10−DPA, left) and with DPA (Rt4M10+DPA, right). The strain was cultivated for 168 h at 32 °C as described in Materials and Methods.

Figure S2. Relative levels of carotenoids extracted from Rt4M10, concentrates sample without DPA (CS−DPA) and CS+100 μM DPA (CS+DPA) at 24, 48, 72, 96 and 168 h.