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Relationship among carotenoid production, copper bioremediation and oxidative stress in *Rhodotorula mucilaginosa* RCL-11

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ABSTRACT

Rhodotorula mucilaginosa RCL-11, a pigmented yeast isolated from a filter plant of a copper mine in the province of Tucumán, Argentina, supports high concentrations of the heavy metal Cu(II). Copper overload augmented carotenoid biosynthesis in this yeast, modifying at the same time the relative proportion of the pigments produced. Inhibition of the synthesis pathway with diphenylamine suggests an inverse relationship between carotenoid and copper biosorption by *R. mucilaginosa* RCL-11. The increased activity of superoxide dismutase and catalase measured under inhibition of carotenoid biosynthesis could explain these observations. Exposure to H_2O_2, a second oxidative stress agent, alone or in combination with Cu(II) also modified the carotenoid content, both qualitatively and quantitatively. The change in the relative proportion of the carotenoids torularhodin, torulene and beta-carotena, as well as the detection of gamma-carotene in the presence of H_2O_2 and Cu(II) allows to hypothesize that the carotenoids produced by *R. mucilaginosa* RCL-11 plays different roles in the oxidative stress response of this yeast.

1. Introduction

As the result of partial reduction of oxygen, aerobic metabolism entails the production of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl radicals ($HO^\bullet$). ROS are potent oxidants that can damage all cellular components, including DNA, lipids and proteins [1]. Both enzymatic and non-enzymatic systems are involved in the cell defense against these damaging oxidants [2]. Key components in the enzymatic defense are the superoxide dismutases (SOD), which eliminate the superoxide radical, and the catalases (CAT), responsible for the H_2O_2 remotion [3]. Together with enzymatic antioxidant mechanisms, low molecular weight compounds also interact with oxidizing species detoxifying them. The most significant non-enzymatic antioxidants involved in direct scavenging of ROS or recycling of oxidized compounds are ascorbate, glutathione, alfa-tocopherol and carotenoids [4].

Carotenoids are one of the most important low molecular weight antioxidants. These compounds form a group of more than 600 molecules found in most life forms fulfilling diverse functions, from their original evolutionary role as photosynthetic or light-quenching pigments to antioxidants and precursors of vitamin A. The most significant part of the carotenoid molecule are the conjugated double bonds that determines their color and biological action [5]. A number of microorganisms, including bacteria, algae, molds and yeasts, produce a broad range of carotenoids, including gamma- and beta-carotene, thorulene, lycopene and astaxanthin [6]. Yeasts of the genera *Rhodotorula*, *Rhodosporidium*, *Sporobolomyces* and *Phaffia* have been described to possess a remarkable ability to produce carotenoids when they are grown under unfavorable conditions, including UV radiation, temperature, solvents and heavy metals [7,8].

Metal pollutants are generated through a wide range of industrial activities and continue to be released into the environment at harmful quantities. Toxic levels of the biologically essential metal copper are often associated with certain mineral ores and industrial or agricultural discharges [9]. Organisms routinely exposed to this metal in their ecological niches need to develop resistance systems, since heavy metals increase the ROS level. For instance, *Saccharomyces cerevisiae* cells exposed to lead increase the intracellular levels of ROS and display morphological changes related to programmed cell death by apoptosis [10]. Microbial cells possess efficient adaptation mechanisms to survive in these adverse environments. An example of this adaptation mechanism is the biosynthesis of carotenoids in yeasts exposed to metals [11].

Rhodotorula mucilaginosa RCL-11, a copper-tolerant yeast that belongs to the phylum Basidiomycota, was isolated from filter plant

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of a copper mine located in the NorthWest of Argentina [12]. This microorganism is capable of accumulating up to 44% of the copper from a medium supplemented with 0.5 mM $CuSO_4$ as a Cu(II) source. Under these conditions, accumulation of dark grains in the cytoplasm of the yeast revealed by electron microscopy has been related to the decrease in the extracellular concentration of copper [13].

Similar to other *Rhodotorula* species, *R. mucilaginosa* RCL-11 produces carotenoid pigments, including alpha-, beta- and gamma-carotene, torulene and torularhodin [14]. Despite the large amount of information available on carotenoids production by this species, relationship between the ability of copper uptake and carotenoids content has not yet been studied. In the present work the evidence presented shows that carotenoid biosynthesis is inversely related to the copper bioaccumulation by *R. mucilaginosa* RCL-11. In addition, this heavy metal is involved in the triggering of an oxidative stress response in which carotenoid pigments and ROS detoxifying enzymes play a role in this yeast.

2. Materials and methods

2.1. Microorganism and culture conditions

R. mucilaginosa RCL-11 was isolated from wastewater sediment collected from a mine plant copper filter in the province of Tucumán, Argentina. Complete identification was previously carried out through molecular and physiological characterization [12]. Strain is available upon request to the corresponding author. Cultures were prepared in 250 ml Erlenmeyer flasks containing 50 ml YNB-glucose (Difco), buffered with 50 mM Tris-succinate (pH 5) inoculated at a final concentration of 10^7 cells ml^{-1} with an active overnight pre-inoculum. Incubation was performed at 30 °C on a rotary shaker at 250 rpm during 72 h. Carotenoid inhibition was achieved by adding diphenylamine (DPA) (Sigma), a specific carotenogenesis inhibitor [4], to the culture medium at a final concentration of 50 μM before inoculation. Growth was monitored by optical density at 600 nm.

2.2. Determination of copper uptake

To analyze the influence of Cu(II) on *R. mucilaginosa* physiology, before inoculation culture media where supplemented with 0.5 mM $CuSO_4$ as Cu(II) source. Total copper ion concentration in the supernatant was measured by flame atomic absorption spectrophotometry with AAnalyst 400 Atomic Absorption Spetrometer (PerkinElmer). At regular times, samples from cultures where withdrawn and supernatants obtained by centrifugation at 300 × *g* for 5 min were filtered and diluted before analysis. Copper concentrations were determined by comparison with a standard curve of $CuSO_4$. Relative copper removal was defined as the extracellular heavy metal concentration divided per A_{600} in order to normalize the biosorption to the yeast growth, and expressed as mM $A_{600}{}^{-1}$.

2.3. Oxidative stress culture conditions

Four cultures of *R. mucilaginosa* RCL-11 were prepared as described above and two of them were supplemented with 0.5 mM of $CuSO_4$. After 6 h of culturing at 30 °C, one supplemented flask and one non-supplemented flask were further supplemented with hydrogen peroxide at a final concentration of 5 mM and cultivation was continued as described above. Absorbance at 600 nm was determinate at 24 h and 48 h. After 48 h, cultures were centrifuged at 300 × *g* for 5 min and carotenoids were extracted as described below.

2.4. Extraction and quantification of carotenoids

At early stationary growth phase, *R. mucilaginosa* cultures were centrifugated for 10 min at 500 × *g* and cell pellets were stored at −20 °C until further analysis. Pigments were extracted with DMSO–acetone according to Sedmak [15] with slight modification. Briefly, cells were suspended in DMSO and incubated for 30 min at 55 °C. After centrifugation and collection of the organic phase, pellets were extracted twice with acetone at room temperature. Pellets containing substantial residual pigmentation were finally extracted with DMSO as described above and pooled organic phases were shaked with petroleum ether supplemented with saturated NaCl solution. To extract polar compounds distilled water was added to the petroleum ether phase containing the carotenoids. Petroleum ether was evaporated and the dried extracts were resuspended in 1 ml of acetone. Equal biomass quantities determined by wet weight measurements were processed in order to standardize the carotenoid concentration. Carotenoid absorption profiles were determined by scanning the absorbances between 350 and 600 nm using a Beckman DU 640 spectrophotometer. Total carotenoid quantification was performed spectrophotometrycally at 490 nm using the extinction coefficient of torulene ($\varepsilon_{1\%1\,cm}$ 2680 l mol^{-1} cm^{-1} according to Simpson [16]).

2.5. Identification of carotenoids by RP-HPLC

Organic extracts prepared as described above where analyzed by reverse-phase HPLC in order to identify the carotenoids produced by *R. mucilaginosa* RCL-11 essentially as described previously [15,17]. RP-HPLC analyses of the carotenoid extracts were performed using a Waters e2695 HPLC equipment with a online diode-array detector (Waters Corporation). A Phenomenex Gemini C_{18} column (150 mm × 4.6 mm, 3 μm particle size) maintained at 35 °C was used with an integrated Phenomenex Security Guard C_{18} pre-column. The mobile phase consisted of a linear gradient of acetone from 70 to 100% in 20 min eluted at 0.6 ml min^{-1} and finally held at 100% for 25 min. Carotenoids were identified by their retention times and by comparison of the spectral features with those of pure compounds or with reported data.

2.6. Determination of SOD and CAT activities

Cells from cultures prepared as described above were harvested after 24 h of cultivation and washed twice with distilled water for analysis of SOD and CAT activities. Cells were disrupted in 50 mM Tris–HCl buffer, pH 8.0, using glass beads and vortexing for 15 min. Cell-free extracts were centrifuged for 5 min at 300 x *g*. Total protein content of extract was determined by the Bradford method (BioRad) using bovine serum albumin as a standard.

SOD activities in protein extracts were analyzed in natives Tris–glycine 10% polyacrylamide gels as described previously [18]. Equal amounts of proteins were separated by electrophoresis and gels were stained for SOD activity with the tetrazolium salt 3-[4,5-dimethyltiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and phenazine methosulfate under light exposition. Densitometry of native gels was carried out on a GelDoc 2000 analyzer (Bio-Rad) and band densities were determined using the Quantity One software (Bio-Rad). For comparison purposes, SOD activity in protein extracts from control cultures was considered as the 100%.

Determination of CAT activities was performed according to Jakubowski [19]. Briefly, the decrease in absorbance at 240 nm was monitored with a Beckman DU 640 spectrophotometer at 25 °C when protein extract was added in to 60 mM sodium phosphate buffer pH 7.4 containing 22 mM H_2O_2. One unit of enzyme activity (U) was defined as the amount of enzyme that converted 1 μmol of substrate per min at 25 °C. Specific activity was expressed as U per mg of protein.

2.7. Statistic analysis

All data are expressed as means ± standard deviation of at least triplicate determinations of independent cultures. Data were subjected to analysis of variance. When significant differences were found, Fischer LSD test was used to express differences among treatments.

3. Results

3.1. Modification of carotenoid biosynthesis by Cu(II) overload

As mentioned above, supplementation of culture media with Cu(II) as $CuSO_4$ produced a remarkable change in pigmentation intensity of *R. mucilagiona* RCL-11 (data not shown). Addition of 0.5 mM $CuSO_4$ to the culture medium produced a twice increase in carotenoid synthesis changing from 1.7 mg l^{-1} in control culture medium to 3.3 mg l^{-1} under copper supplementation (Fig. 1A). In order to analyze the role played by Cu(II) in pigment modification, its production were characterized under carotenoid inhibition and heavy metal supplementation. Diphenylamine (DPA) is a compound largely utilized to interfere the carotenoid synthesis in both eukaryotic and prokaryotic microorganisms. Diphenylamine (DPA), blocks the sequence of de-saturation reactions by inhibiting phytoene synthase, leading to an accumulation of phytoene together with other, normally absent, saturated carotenoids [20,21]. As expected, 50 μM DPA produced almost the complete inhibition of carotenoid synthesis by *R. mucilaginosa* RCL-11 decreasing the pigment concentration to 0.26 mg l^{-1}. The cultivation of cells in presence of both copper and DPA remained carotenoid synthesis bellow 1 mg l^{-1}. However, compared to non-supplemented growth medium these treatments seemed to modify not only quantitatively but also qualitatively the carotenoids present in *R. mucilaginosa* RCL-11 cells, as shown in Fig. 1B. Absorption scanning of organic extracts after copper overload presented the highest

Table 1
Composition of carotenoids produced by *R. mucilaginosa* RCL-11 as determined by RP-HPLC coupled to an online diode-array detector under control conditions, in presence of 0.5 mM $CuSO_4$, 5 mM H_2O_2 and a combination of both stressors.

	Retention time (min)	mg l^{-1} (%)[a]			
		Control	+$CuSO_4$	+H_2O_2	+$CuSO_4$ + H_2O_2
Torularhodin	~18.5	2.24 (58.33)	5.37 (57.37)	5.22 (28.89)	4.03 (29.27)
Torulene	~20.8	0.82 (21.35)	2.33 (24.89)	8.21 (45.59)	5.60 (40.67)
Gamma-carotene	~21.2		0.85 (9.08)	2.24 (12.44)	2.34 (16.99)
Beta-carotene	~21.8	0.77 (20.21)	0.81 (8.65)	2.34 (12.99)	1.80 (16.99)
Total		3.8 (100)	9.36 (100)	18.01 (100)	13.77 (100)

[a] Percentage of the different pigments related to the total concentration of carotenoids.

absorbance at 460 nm. Noteworthy, when *R. mucilaginosa* RCL-11 yeasts were grown in culture medium with DPA and Cu(II), a differential spectrum was observed. Compared with the control medium, a shift in the maximum absorption was determined, changing from 460 nm to 450 nm, which suggests a modification in the carotenoid profiles that could be taking place under this latter condition (Fig. 1B). To test this hypothesis, organic extracts were chromatographied by RP-HPLC coupled with a diode-array detector and carotenoids presents in each sample were identified and quantified. Analysis revealed that *R. mucilaginosa* RCL-11 strain expressed three major carotenoids identified as torularhodin, torulene and beta-carotene. Under the assayed condition, gamma-carotene was not detected. Cu(II) exposure produced an increase in the three compounds (Table 1) as well as the appearance of gamma-carotene, with a retention time between torularhodin and beta-carotene. Relative proportion of torularhodin contents remained almost constant (58.33 and 57.33%, in control and copper supplemented media, respectively), with an increase of more than 15% in torulene percentage: 21.35% in control medium and 24.89% under copper supplementation. Although the concentration of beta-carotene presents in organic extracts changed from 0.78 mg l^{-1} to 0.81 mg l^{-1}, its proportion decreased to less than 10%. This observation could be attributed to the appearance of gamma-carotene in the analyzed samples obtained from copper-overloaded cultures, as discussed below. Interestingly, higher values of total carotenoid concentrations were obtained with the utilization of RP-HPLC, compared to the spectrophotometer determinations. This observation could be attributed to a better sensitivity of the former method.

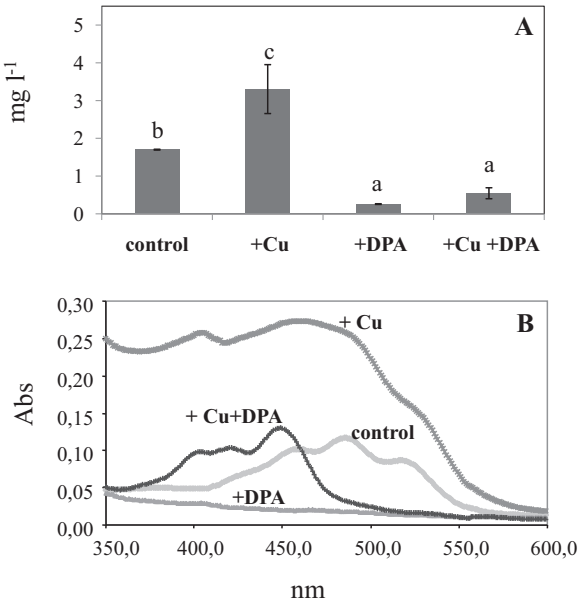

Fig. 1. Biosynthesis of carotenoids in *R. mucilaginosa* RCL-11 under Cu(II) stress and carotenoid inhibition. (A) Total carotenoid pigments obtained from cells grown for 48 h in culture medium supplemented or not with 0.5 mM $CuSO_4$ as Cu(II) source, and with or without addition of 50 μM DPA were measured at 490 nm. (B) Absorption scanning was evaluated by absorption spectrophotometry between 350 and 600 nm. Same letters mean no significant differences; different letters mean significant differences as determined by a Fischer LSD test ($p < 0.05$).

3.2. Effect of inhibition of the carotenoids biosynthesis on R. mucilaginosa RCL-11 growth and Cu(II) removal

Pigment biosynthesis was inhibited with diphenylamine and growth and bioaccumulation of the heavy metal were evaluated. As depicted in Fig. 2A, 50 μM of DPA in the culture media affected the growth of *R. mucilaginosa*: after 48 h of incubation A_{600} were 17.2 ± 1.0 and 14.7 ± 0.3 in control and DPA-supplemented cultures, respectively. It is possible that DPA has a toxic effect per se, or the lack of carotenoids caused by the DPA addition affects yeast growth [21]. Addition of this heavy metal had a detrimental effect on the growth of *R. mucilaginosa* RCL-11. $CuSO_4$ at a concentration of 0.5 mM decreased the cell yield, as can be observed in the $A_{600} = 6.7 \pm 1.7$ after 48 h (Fig. 2A). Interestingly, supplementation with 50 μM of DPA maximized the effect of copper addition: biomass determined as the A_{600} was about 50% higher ($A_{600} = 3.6 \pm 1.1$) when carotenoid biosynthesis was not inhibited in the culture medium with $CuSO_4$ the remaining Cu(II) concentration in the culture medium after incubation of strain with or without carotenoids inhibiting agent was quantified by flame atomic absorption spectrophotometry. After 48 h of incubation, extracellular $CuSO_4$ concentrations in both conditions were virtually the same, being decreased between 0.18 and 0.15 mM. These values represented a 35 and 40% decrease with respect to that at the beginning of the experience. However, when cell densities measured as the absorbance at 600 nm were considered ($A_{600} = 6.7 \pm 1.7$ and 3.6 ± 1.1 in copper and copper plus DPA supplemented media, respectively), an increase in the relative removal of Cu(II) could be determined when carotenoid biosynthesis was inhibited with 50 μM DPA. Relative copper removal increased from 0.053 to 0.083 mM A_{600}^{-1} with DPA addition suggesting that under the assayed conditions the interference of carotenoid biosynthesis promoted the heavy metal remotion.

3.3. Enzymatic antioxidant response under inhibition of carotenoids biosynthesis

Previous reports showed that exposure to heavy metals triggered the overexpression of SOD and CAT in *R. mucilaginosa* RCL-11, enzymes directly involved in responses to oxidative stresses [13]. In order to find out whether a relationship could exist between carotenoid production, copper removal and the antioxidant response, superoxide dismutase and catalase activity were assayed in extracts of yeast cells exposed to 0.5 mM $CuSO_4$ and 50 μM DPA. An increase in activity of both antioxidant enzymes was observed in response to the Cu(II) overload. Catalase

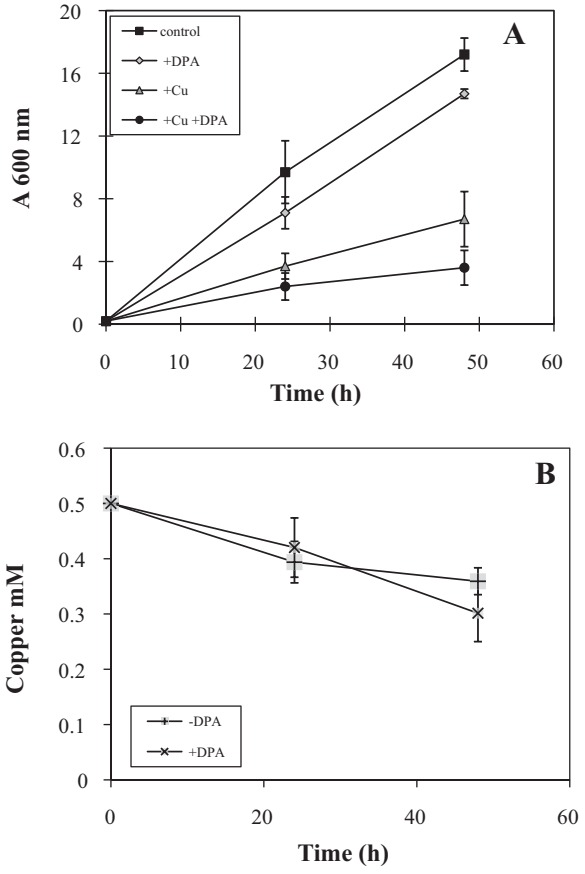

Fig. 2. Effect of inhibition of carotenoids biosynthesis and Cu(II) overload on *R. mucilaginosa* RCL-11 growth and Cu(II) uptake. (A) Growth of *R. mucilaginosa* RCL-11 cultures was monitored measuring the absorbance at 600 nm after 24 and 48 h in control culture medium (■) or under 50 μM DPA (◊), 0.5 mM Cu(II) (△) or a combination of 0.5 mM Cu(II) and 50 μM DPA (●) addition. (B) Decrease in copper ion concentration by *R. mucilaginosa* RCL-11 cells was measured after 24 and 48 h in culture media supplemented with 0.5 mM $CuSO_4$ (X), or a combination of 0.5 mM $CuSO_4$ and 50 μM DPA (+). Data are expressed as the means ± standard deviation from four independent experiments.

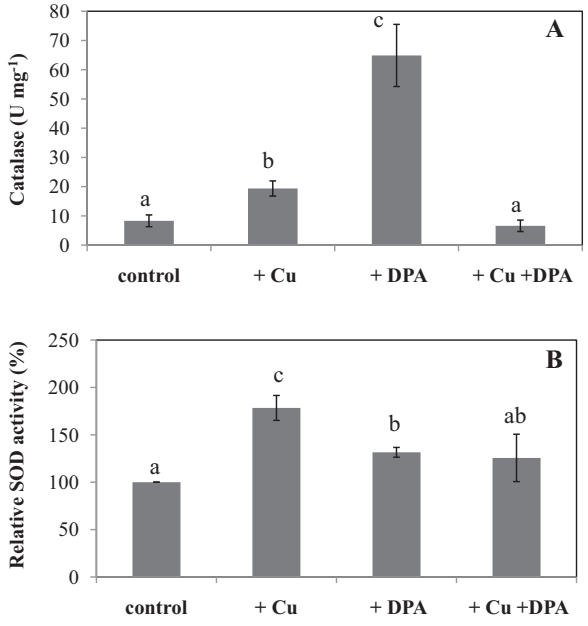

Fig. 3. Catalase and superoxide dismutase activity in *R. mucilaginosa* RCL-11 in the presence and absence of 0.5 mM Cu(II) and/or 50 μM DPA. (A) Catalase activity was measured in crude cell-free extracts as described in Section 2.6. (B) Crude cell-free extracts were loaded onto native gels for quantification of SOD activity. Same letters mean no significant differences; different letters mean significant differences as determined by a Fischer LSD test ($p < 0.05$).

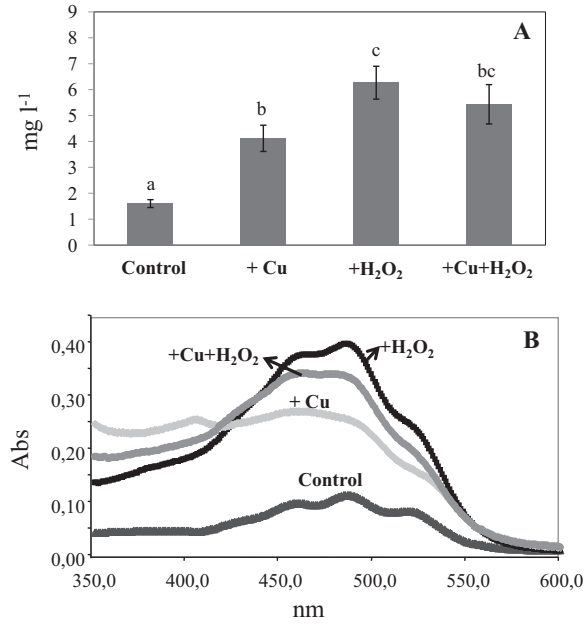

Fig. 4. Effect of oxidative stress by H_2O_2 addition on carotenoids biosynthesis. (A) Carotenoid synthesis by *R. mucilaginosa* RCL-11 cells grown during 48 h in medium supplemented or not with $CuSO_4$, 5 mM H_2O_2 or combination of both was measured at 490 nm. (B) Absorption scanning was evaluated by absorption spectrophotometry between 350 and 600 nm. Same letters mean no significant differences; different letters mean significant differences as determined by a Fischer LSD test ($p < 0.05$).

activity increased from 8.29 ± 2.0 U mg^{-1} in the control condition, to 19.35 ± 2.5 U mg^{-1} in the growth medium with 0.5 mM $CuSO_4$. When the production of carotenoid pigments was inhibited with 50 μM DPA, the highest CAT activity could be measured, attaining a value of 64.88 ± 10.6 U mg^{-1} (Fig. 3A). Unexpectedly, simultaneous addition to *R. mucilaginosa* cultures of copper and DPA inhibitory agent showed no significant difference compared to the control treatment, in terms of catalase activity. On the other hand, and in comparison to non-treated cells, SOD activity was 78.3 ± 13.0% higher under supplementation with copper (Fig. 3B). DPA in the culture media produced a significant rise in superoxide dismutase activity of 131.5 ± 5.1%. Similar to the behavior determined for CAT activity, simultaneous addition of DPA and Cu(II) produced just a non significant increase in SOD activity compared to the control.

3.4. Effect of a second oxidative stress on pigment production by R. mucilaginosa RCL-11

The results presented above suggested a relationship among copper exposure, carotenoid biosynthesis and oxidative response of *R. mucilaginosa* RCL-11. To analyze whether a second oxidative stress factor could modify the effect caused by copper on *R. mucilaginosa* RCL-11, the response of the yeast cells to the presence of 0.5 mM Cu(II) and 5.0 mM H_2O_2 was assayed. Fig. 4A shows the stimulating effect of Cu(II), H_2O_2 and a combination of both on carotenoid synthesis. Addition of 5 mM H_2O_2 to *R. mucilaginosa* RCL-11 cultures induced the highest concentration of carotenoids, increasing from 1.8 mg l^{-1} in control condition to 6.2 mg l^{-1} (Fig. 1A; for comparison purposes, values obtained under copper exposure are also included). In contrast, when both copper and hydrogen peroxide were present in the culture media, *R. mucilaginosa* RCL-11 produced carotenoids at a concentration of

5.45 mg l^{-1}, between the values obtained in the control and the copper exposed cultures. Similar to what was previously described for the copper treatment, addition of 5 mM H_2O_2 alone or in combination with 0.5 mM $CuSO_4$ not only modified quantitatively the carotenoid pigments but also qualitatively. A characteristic spectrum was observed for H_2O_2 treatment with three absorption peaks at 460, 480 and 525 nm, being de second one the most abundant (Fig. 4B). The combination of copper and H_2O_2 in culture media showed a spectrum similar to that determined under the hydrogen peroxide treatment but the intensity of the absorbance peaks were lower (Fig. 4B). Quantification of carotenoid pigments by HPLC showed that torularhodin concentrations under H_2O_2 and Cu(II) stresses were similar (i.e., 5.22 and 5.37 mg l^{-1}, respectively), and slightly lower when both stressors were present (4.03 mg ml^{-1}) (Table 1). The increase determined from 0.82 to 8.21 mg l^{-1} in torulene concentration when cultures where stressed with hydrogen peroxide was attenuated to 5.6 mg l^{-1} when copper was also present. A similar behavior could be determined for beta-carotene concentration, which changed from 0.77 under control conditions to 2.34 and 1.8 mg l^{-1}, when H_2O_2 and $CuSO_4$ plus H_2O_2 where added, respectively. Similar to what was previously described, gamma-carotene normally undetectable in control conditions, presented comparable values in cultures with H_2O_2 alone (2.24 mg l^{-1}) or in combination with Cu(II) (2.34 mg l^{-1}). Noteworthy, as depicted in Table 1, the percentages of torularhodin and torulene seemed to be inverted in comparison to the control conditions when hydrogen peroxide alone was present. When the proportion of beta-carotene was analyzed and compared to the values obtained from the control medium, this pigment showed the highest percentage variability among the different assayed conditions. Similar to what was previously mentioned, higher values of total carotenoid concentrations were obtained with the utilization of RP-HPLC, compared to the spectrophotometer determinations.

3.5. Effect of oxidative stress on the growth of R. mucilaginosa RCL-11

The growth of yeast cells exposed to both stressors was evaluated, considering that copper exposure alone or in combination with hydrogen peroxide changed the composition of carotenoids produced by *R. mucilaginosa* RCL-11. In addition, these pigments have been largely related to resistance to oxidative stress in different microorganims. Growth were differentially modified by Cu(II) or H_2O_2 exposure. As shown in Fig. 5, hydrogen peroxide alone or in combination with copper promoted a longer lag phase, compared to the control medium. In contrast, after 24 h of aerobic incubation without inducing any oxidative stress response, cell densities measured as the A_{600} attained a value of 11.9 ± 1.9. When only the heavy metal was utilized as a stressor, intermediate values of $A_{600} = 4.6 \pm 0.8$ could be measured suggesting that under the assayed conditions 0.5 mM H_2O_2 could exert a stronger influence on the growth of *R. mucilaginosa* RCL-11. Yeast cells exposed to H_2O_2 started to grow after 24 h, attaining cell densities after 48 h comparable to copper addition alone (A_{600} 8.7 ± 0.6), and higher than the values determined when Cu(II) stressor was also present (A_{600} 6.4 ± 0.5). However, these values were remarkable lower than 16.1 ± 0.9, the cell densities of control cultures after 48 h of incubation.

4. Discussion

R. mucilaginosa RCL-11 possesses a remarkable capacity for copper uptake through sequestration and extrusion of cytoplasmic copper ions [13]. This characteristic is of high relevance for the bioremediation of heavy metals from contaminated environments.

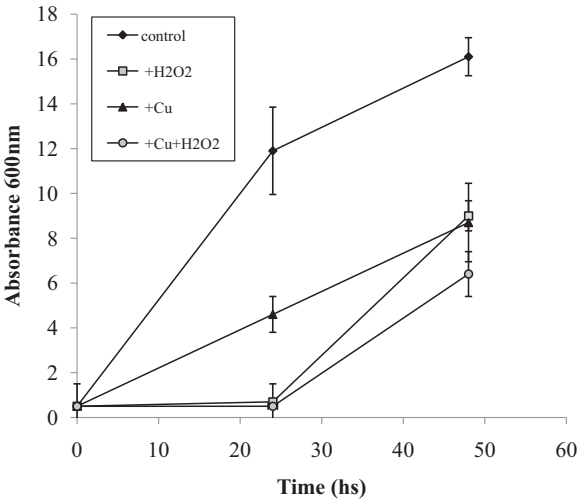

Fig. 5. Effect of $CuSO_4$ and H_2O_2 on the growth of *R. mucilaginosa* RCL-11. Yeast growth was evaluated by absorbance at 600 nm after 24 and 48 h in presence of 0.5 mM $CuSO_4$, 5 mM H_2O_2, and combination of H_2O_2 and $CuSO_4$.

In this microorganism, the over-expression of proteins involved in stress response is directly related to the resistance to this heavy metal, as revealed the analysis of the proteome of the cells cultured in media supplemented with 0.5 mM $CuSO_4$ [22]. Similar to other colored species, the characteristic pigmentation of the colonies is due to the production of carotenoids. Torularhodin, torulene and beta-carotene are the main pigments produced in *Rodothorula* species [17]. Libkind and Van Brook [14] demonstrated a carotenoid proportion of 83.4% for torularhodin, 10.8% for beta-carotene and 5.7% for torulene in *R. mucilaginosa* CRUB 006. Likewise, torularhodin, the most oxidized pigment, was the principal carotenoid produced in *R. mucilaginosa* RCL-11 under control conditions, followed by torulene and beta-carotene (Table 1). As other secondary metabolites, carotenoid biosynthesis is influenced by diverse factors such as light, temperature, aeration, solvents and divalent cations (i.e., Ba, Fe, Mg, Ca, Zn and Co) [23]. Buzzini [24] reported that trace elements exert a selective influence on the carotenoid profile in *R. graminis* DBVPG 7021: Al(III) and Zn(II) had a stimulatory effect on beta-carotene and gamma-carotene synthesis, while Zn(II) and Mn(II) had a inhibitory effect on torulene and torularhodin production. Bhosale and Gadre [11] observed that divalent cations, including Zn(II), Co(II), Fe(II) and Cu(II) had a stimulatory effect on volumetric production and cellular accumulation of carotenoids in *Rhodotorula glutinis*. In the present report, evidence is presented showing that also the heavy metal copper can increase the concentration of these pigments in *R. mucilaginosa* RCL-11 (Fig. 1). A similar behavior has been observed when *Phaffia rhodozyma* NRRL Y-10922 was cultured in defined media supplemented with $CuSO_4$ at concentrations below 3.5 μM [25]. In contrast to *R. mucilaginosa* RCL-11, which doubles its carotenoid content, higher concentrations of Cu(II) decreased the pigment production of NRRL Y-10922 strain. It has been postulated that divalent cations can activate or inhibit specific carotenogenic enzymes (i.e., specific desaturases involved in carotenoid biosynthesis) altering the quantity of the pigments the cell produces [6]. As described above, heavy metal addition not only changed the concentration but also the relative profile of the carotenoids synthesized by *R. mucilaginosa* RCL-11. It is then plausible that the same biochemical changes could take place in *R. mucilaginosa* RCL-11 when the cells are exposed to high concentrations of Cu(II). Carotenoids are important molecules for the pharmaceutical, chemical and food industries. The mutagenesis of pigmented yeast has been proposed to obtain over-producing strains [6]. However, the modification of

the culture conditions, i.e., the addition of heavy metals such as copper, could be a promising way to increase the yield of these valuable compounds by microorganisms for which molecular techniques have not been developed.

As mentioned above, it has been postulated that the main resistance mechanism to copper overload of *R. mucilaginosa* RCL-11 is the sequestration of the heavy metal. However, the analysis of the influence of carotenoids on the bioremediation of copper showed an inverse relationship between pigment production and copper bioremediation. The increase from 0.053 to 0.083 mM $A_{600}{}^{-1}$ in the relative copper removal after DPA addition, representing a raise of 55% in the cell capability to bioremediate, suggests that the cell response to copper exposure is more complex, as discussed below. In addition, this finding is of biotechnological importance since it suggests that a non-pigmented mutant of *R. mucilaginosa* RCL-11 could be more effective to concentrate, remove and recover metals from streams and could enhance the efficiency of wastewater treatment processes. A similar approach has been achieved for the biosorption of Cr (VI) by *R. mucilaginosa* UCM Y-1776, which highly support the hypothesis presented in this work [26]. Production of carotenoids could be part of a physiological response triggered to avoid, at least in part, the intracellular accumulation of heavy metals. When DPA is added, carotenoids are not produced and more copper is accumulated; when culture media are not supplemented with DPA, more pigments are produced and copper is also accumulated, although to a lesser extent.

A link between carotenoids and reactive oxygen species (ROS) has previously been established [6]. For instance, these pigments have a demonstrated role in the prevention of oxidative injury due to ROS exposure in pigmented heterotrophic yeasts [27]. The maximum catalase activity in the absence of carotenoid synthesis (see Fig. 3A) shows that *R. mucilaginosa* RCL-11 responded to the lack of pigments induced by DPA, at least in part, activating other antioxidant mechanisms such as catalase and superoxide dismutase activity. Yan et al. [28] showed that introduction of the beta-carotene gene into yeasts with a deficiency in cytosolic catalase activity increased cell resistance to H_2O_2 stress. The authors suggested that carotenoids would substitute catalase to protect the yeast from H_2O_2-induced oxidative damage [28]. An increase in catalase activity in *R. mucilaginosa* RCL-11 could be explained by the same mechanisms, suggesting that increased catalase activity can substitute carotenoids in protecting the yeast from the oxidative damage caused by the heavy metal copper. Superoxide dismutase activity seems to exhibit a similar behavior under treatment with carotenoid inhibitor agent. It has been suggested that the absence of CuZnSOD in pigmented yeasts is complemented by the presence of carotenoproteins that act as an extra mitochondrial antioxidant [4].

The connection between the carotenoid biosynthesis and the oxidative stress response of *R. mucilaginosa* RCL-11 triggered by the heavy metal exposure is highlighted by the modification of the carotenoid profile when the yeast cells are cultured with copper and hydrogen peroxide. Liu and Wu [29] suggested that carotenoid biosynthesis in *Xanthophyllomyces dendrorhous* can be stimulated by H_2O_2 as an antioxidative response. As described above, copper and H_2O_2 not only increased the concentration of pigments in *R. mucilaginosa* RCL-11 cells, but also modified the relative proportions of the carotenoids. When copper was the only stressor utilized, the main change in the pigment profile was represented by a decrease in the relative proportion of beta-carotene and the presence of gamma-carotene, which was undetectable in the control culture condition with the analytical techniques employed (Table 1). Gamma-carotene is the precursor molecule in the biosynthesis of the other pigments produced by *R. mucilaginosa* [6]. It is plausible that the detection of this common precursor is directly related to the increase in the carotenoid biosynthesis. In concordance, the examination of the relative pigment proportions shows that under Cu(II) exposure, the relative percentage of torulene and the appearance of gamma-carotene are favored in detriment of beta-carotene. When yeast cultures were supplemented with H_2O_2 alone, the main changes determined were related to the total carotenoid quantities, the detection of the precursor gamma-carotene, and inverted percentages between torularhodin and torulene (see Fig. 1 and Table 1). It is therefore possible that this stressor not only induces changes in the quantities of the carotenoids, but also in a specific carotenoid response. In accord with that, the addition of both Cu(II) and H_2O_2 produced modifications comparable to those observed when the hydrogen peroxide was the only stressor (see Table 1). Most likely, after 48 h of incubation H_2O_2 had decomposed from the culture media leaving Cu(II) alone exerting its oxidative effect on yeast cells. *R. mucilaginosa* RCL-11 growth under the different assayed conditions is in concordance with this hypothesis (see Fig. 5). The extended lag phase always determined in culture media supplemented with hydrogen peroxide could be attributed to this oxidative agent. After 24 h of incubation, cultures with H_2O_2 tended to grow as in the control medium; at the same time cultures stressed with $CuSO_4$ and H_2O_2 start an exponential phase comparable to that observed when the heavy metal was alone (see Fig. 5).

It is probable that the described changes determined in the carotenoid production of *R. mucilaginosa* RCL-11 are related to specific responses to different stress conditions. For instance, evidence has been presented showing that torularhodin has a central role in the photoprotection of *R. mucilaginosa* and *S. cerevisiae* [27,30]. In contrast, *R. glutinis* responded to oxidative stress after addition of ROS-generating reagent H_2O_2 by switching its carotenoid biosynthesis from beta-carotene to torularhodin, suggesting that an increase in the production of torularhodin reduced the susceptibility to injury induced by ROS in this species [31]. Beyond its role as a precursor of torularhodin, the specific biological function of torulene has remained more elusive. Saelices et al. [32] demonstrated that light, through the WC-1/WC-2 phoreceptor system, is involved in the cleavage of torulene to torularhodin mediated by the *cao-2* gene product of *Neurospora crassa*. Those findings clearly show that high levels of specific carotenoids are required under certain environmental conditions. Detailed studies are required to evaluate the function of the torulene carotenoids accumulation, and the concomitant decrease in the proportion of torularhodin by *R. mucilaginosa* RCL-11 when hydrogen peroxide is present, independently of the presence or not of the heavy metal copper.

The ability of pigmented yeasts to respond to stress factors through the overproduction of significant metabolites is of biotechnological importance in both bioremediation and carotenoids production at industrial level. To the best of our knowledge, this report is the first to link the carotenoid contents with the ability to bioremediate environments contaminated with Cu(II) by *R. mucilaginosa*.

Further studies are currently in progress in order to maximize the capability for bioremediation of heavy metals by *R. mucilaginosa* RCL-11 through the modification of the stress response.

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