Journal of Basic Microbiology 2009, *49*, 1–9

Research Paper

Responses of *Candida fukuyamaensis* RCL-3 and *Rhodotorula mucilaginosa* RCL-11 to copper stress

Liliana B. Villegas[1], María J. Amoroso[1,2] and Lucía I. C. de Figueroa[1,3]

[1] PROIMI – CONICET, Av. Belgrano y Pje. Caseros, Tucumán, Argentina
[2] Microbiología General, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Argentina
[3] Microbiología Superior, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Argentina

The effect of high Cu(II) concentrations on superoxide dismutase (SOD) and catalase (CAT) activity in *Candida fukuyamaensis* RCL-3 and *Rhodotorula mucilaginosa* RCL-11, previously isolated from a copper filter at a mine plant in Argentina, was studied. Addition of 0.1, 0.2 and 0.5 mM Cu(II) to the culture medium increased total SOD and CAT activity in both strains. Native polyacrylamide gel electrophoresis revealed two bands with SOD activity for *C. fukuyamaensis* RCL-3 and only one for *R. mucilaginosa* RCL-11; the three bands corresponded to MnSOD.

Intracellular accumulation of copper and morphological changes was observed using electron microscopy. Dark bodies examined with transmission electron microscopy (TEM) after 48 h of incubation probably corresponded to copper deposits. The number of dark bodies in *R. mucilaginosa* RCL-11 grew with increasing incubation time, whereas in *C. fukuyamaensis* RCL-3 the amount decreased. Scanning electron micrographs (SEM) of *C. fukuyamaensis* RCL-3 did not reveal any differences compared with the control, but *R. mucilaginosa* RCL-11 cells were bigger than control ones. TEM confirmed absence of compartmentalization mechanisms in Cu(II) detoxification since electron-dense bodies were mainly found in the cytoplasm.

Keywords: Copper(II) uptake / Yeasts / Catalase (CAT) activity / Superoxide dismutase (SOD) activity

Received: July 03, 2008; accepted: January 07, 2009

DOI 10.1002/jobm.200800218

Introduction

Copper is an essential component of several important enzymes, based on its ability to undergo redox transitions between Cu(I) and Cu(II). The use of *Saccharomyces cerevisiae* as a model organism has served as a powerful system for the study of copper homeostasis, and has enabled identification of copper transport proteins in other fungi and higher eukaryotes such as plants and mammals [1, 2]. It is known that extracellular copper in the form of Cu(II) is reduced to Cu(I) by metalloreductases, and transported across the plasma membrane through high and low-affinity copper transporters [3, 4]. After crossing the plasma membrane, cells induce biosynthesis of small soluble proteins known as "copper-chaperones", which efficiently bind copper atoms and have the ability to distribute the ions to specific intracellular destinations [5].

At the same time this characteristic of cells to use copper is a threat to the organism when the metal is present at high concentrations, since it catalyzes the production of reactive oxygen species (ROS) via the Fenton-reaction [6, 2]. At high concentrations, Cu(II) is toxic and this toxicity is exploited in agriculture as commercial fungicides and algicides [7].

Yeasts, like other aerobic organisms, are continuously exposed to ROS formed as by-products during normal cellular metabolism. These forms of oxygen are highly damaging to cellular constituents, like DNA, lipids and proteins [8]. However, ROS are detoxified via superoxide dismutases (SODs), which dismutate the superoxide in two steps to O_2 and H_2O_2. Subsequently,

Correspondence: Lucía I.C. de Figueroa, PROIMI – Biotecnología – CONICET, Av. Belgrano y Pje. Caseros T4001MVB, Tucumán, Argentina, Liliana B. Villegas, PROIMI – Biotecnología – CONICET. Av. Belgrano y Pje. Caseros T4001MVB, Tucumán, Argentina
E-mail: proimiunt@arnet.com.ar, villegas@proimi.org.ar
Phone: +54 381 434488
Fax: +54 381 4344887

the H_2O_2 is detoxified by catalases (CATs). Both enzymes create the first and the most important line of antioxidant defense [9].

Under physiological conditions, endogenous cellular antioxidant defense systems are adequate to maintain ROS at basal, harmless levels, and repair damages. However, under stress conditions, the levels of ROS exceed the antioxidant capacity of the cells, and consequently the cells face oxidative stress. This unbalanced situation can result from: (i) a decrease in antioxidants, due to depletion of such defenses; (ii) an increased production of ROS, or both [10].

In a previous work, two yeasts with multiple heavy metal tolerance, including Cu(II), were isolated from a copper filter at a mine plant in the province of Tucumán, Argentina. The strains were identified as *Candida* sp. (GenBank AY743221) and *Rhodotorula mucilaginosa* (GenBank AY437842) by sequencing the D1/D2 region of the 26S rDNA gene. The growth rate of both yeasts decreased with increasing Cu(II) concentration, and the strains were also able to absorb this metal ion from the culture medium. However, the copper accumulation profiles of both strains were different: whereas the metal was released by *Candida* sp. RCL-3, *R. mucilaginosa* RCL-11 did not show this characteristic [11]. Based on these facts, the aim of the current study was to determine oxidative stress caused by elevated Cu(II) concentrations by measuring SOD and CAT activities and to study the passage of copper ions into yeast cells and the effect on their morphology.

Materials and methods

Microorganisms

Candida sp. RCL-3 and *Rhodotorula mucilaginosa* RCL-11 were previously isolated and identified by Villegas *et al.* [11]. Complete identification of these yeasts was carried out using conventional techniques, which were based on morphological, physiological and biochemical criteria described by Yarrow [12].

Enzyme assaying

Cells were incubated in YNB-glucose (Difco) medium, which was buffered with 50 mM Tris-succinate (pH 5.5) and supplemented with 0.1, 0.2 and 0.5 mM Cu(II). Flasks were inoculated and incubated during 24, 48 and 72 h as previously described by Villegas *et al.* [11]. Cells were harvested by centrifugation and washed twice with 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA. Cell homogenates were prepared by using glass beads in phosphate suspension in 2 ml Eppendorf tubes (0.5 ml beads and 1 ml cell suspension). Ten pulses of 1 min each on a vortex mixer were used, with 1 min intervals on ice between pulses. The homogenates were centrifuged at 10,000 × *g* during 20 min at 4 °C, supernatants were recovered and used as enzyme source. Total protein content in the supernatants was determined by the Bradford method [13] using bovine serum albumin as reference protein.

SOD activity

SOD activity was determined according to a modified method by Beauchamp and Fridovich [14]. This assay is based on the competition between SOD and an indicator molecule, nitro blue tretrazolium (NBT), for superoxide ions produced by a photochemical reaction in the presence of riboflavin.

Native polyacrylamide gel electrophoresis (PAGE) was performed according to Sambrook *et al.* [15], loading each lane with 10 μg total protein. One μg of MnSOD from *Escherichia coli* and 1 μg of CuZnSOD from bovine serum were included as reference enzymes. Electrophoresis was performed at 100 V for 3 h. The gel was incubated for 15 min in 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA, followed by immersion in 1 mg ml^{-1} NBT for 15 min in the dark. Finally the gel was incubated for 15 min at room temperature in 50 mM potassium phosphate buffer (pH 7.8) containing 0.03 mg ml^{-1} riboflavin and 5 mg ml^{-1} methionine under gentle shaking and also in the absence of light. Next, the gel was exposed to light for 20–30 min. Areas with SOD activity remained clear whereas areas without the enzyme turned violet due to the formation of formazan. To discriminate between CuZnSOD and MnSOD, samples were incubated with 10 mM H_2O_2 for 30 min before being loaded on the gel, as CuZnSOD is sensitive to H_2O_2 [16].

To quantify total SOD activity 0.01 ml of sample was mixed in the dark with 1.5 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.4 ml of NTB solution (1 mg ml^{-1}), 1 ml of riboflavin solution (0.03 mg ml^{-1}) and methionine (5 mg ml^{-1}). The mixtures were exposed to light for 20 min, at such intensity that absorbance at 550 nm in the absence of sample increased at a rate of 0.05 min^{-1}. The reading obtained with 0.01 ml of distilled water, under the same assay conditions, was defined as 100% NBT reduction. One unit (U) of SOD was defined as the amount of enzyme that caused 50% inhibition of NBT reduction to blue formazan under the test conditions.

CAT activity

Catalase activity was measured spectrophotometrically at room temperature following the decrease in absorp-

tion at 240 nm of a solution containing 50 mM potassium phosphate buffer (pH 7.5), 0.5 mM EDTA, 10 mM H_2O_2 and 0.01 ml sample [9]. One unit of CAT was defined as the amount of H_2O_2 in mM consumed per minute. Results of total SOD and CAT activity are expressed as U per mg of total proteins.

Statistical analyses

Spectrophotmetric results obtained in the present work were expressed as mean value of at least triplicate determinations of independent cultures. Statistical significance of differences among values was assessed by using the Student's t-test and ANOVA. A probably level of $P < 0.05$ was used throughout this study.

Transmission Electron Microscopy (TEM)

Cells were incubated with 0.5 mM Cu(II) during 24, 48 and 72 h as described above. Growth of both yeasts for 24 h in the absence of Cu(II), was included as control.

Cells were centrifuged at 8,000 g during 10 min, washed twice with sterile distilled water and fixed overnight with 3.16% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Then, cells were post-fixed with 2% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 12 h. Samples were subsequently dehydrated with ethanol series and absolute acetone and cells embedded in Spurr resins. Ultra thin sections were stained with uranyl acetate and lead citrate and mounted on copper grids [17]. Observations were made with a Zeiss EM 109 transmission electron microscope.

Scanning Electron Microscopy (SEM)

SEM was carried out with cells of both strains cultured for 48 h with or without 0.5 mM Cu(II). Samples were fixed and post-fixed with glutaraldehyde and osmium tetroxide, respectively, and dehydrated as mentioned above. Afterwards they were coated with gold and yeast cell properties were examined with a Jeol 35 CF scanning electron microscope.

Results

According to rDNA sequence analysis of the D1/D2 domain of 26S rDNA previously published by Villegas *et al.* [11] and conventional yeast identification assays carried out in this work, *Candida* sp. RCL-3 was characterized as *Candida fukuyamaensis.* This result was mainly based on its ability to assimilate D-arabinose, mannitol and starch and ferment glucose, trehalose, sucrose, raffinose and galactose. Accordingly the strain was named as *C. fukuyamaensis* RCL-3. Results with *R. mucilaginosa* RCL-11 were coincident with the assay for *R. mucilaginosa* described by Yarrow [12].

SOD and CAT activities

Endogenous catalase and superoxide dismutase activities were measured in cell-free extracts of *C. fukuyamaensis* RCL-3 and *R. mucilaginosa* RCL-11 to determine oxidative stress levels when Cu(II) was added to the culture medium.

Fig. 1 shows electrophoresis profiles of SOD activity. The results were obtained using cell-free extracts of both yeasts grown with 0.5 mM Cu(II) and without (control) during 72 h. Whereas a single band with SOD activity appeared in *R. mucilaginosa* RCL-11 (Fig. 1A, lanes 1 and 2), *C. fukuyamaensis* RCL-3 exhibited two

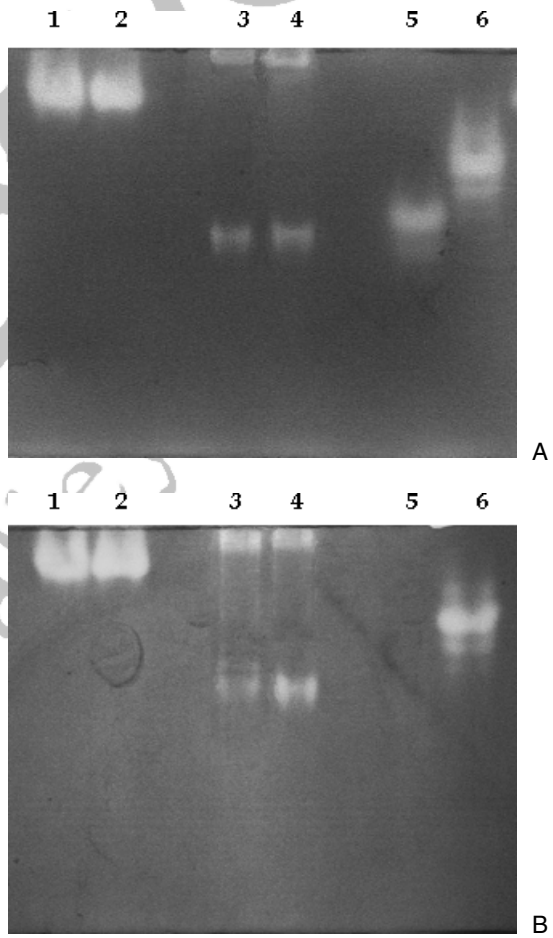

Figure 1. Activity staining for SOD in cell free extracts in 10% native PAGE.
A: Cell extracts incubated in the presence of 0.5 mM Cu(II) during 72 h and SOD standards; B: The same cell extracts as in A, but after treatment with 10 mM H_2O_2. Lane 1: *R. mucilaginosa* RCL-11 control cells; lane 2: *R. mucilaginosa* RCL-11 incubated with 0.5 mM Cu(II); lane 3: *C. fukuyamaensis* RCL-3 control cells; lane 4: *C. fukuyamaensis* RCL-3 incubated with 0.5 mM Cu(II); lane 5: CuZnSOD from bovine; lane 6: MnSOD from *E. coli.*

bands (Fig. 1A, lanes 3 and 4). All bands presented different electrophoresis patterns compared to reference SODs (Fig. 1A, lanes 5 and 6). Fig. 1B shows the results of yeast cells of both cell-free extracts and reference SODs after treatment with H_2O_2. Neither the samples nor the standard MnSOD were affected by the process. In contrast, reference CuZnSOD was inhibited by H_2O_2.

Total SOD activity was also measured and showed an unimportant increase with increasing incubation time of *C. fukuyamaensis* RCL-3 and *R. mucilaginosa* RCL-11. However, in the presence of an initial Cu(II) concentration of 0.5 mM SOD activity was more than twice compared to the control (Fig. 2A and B).

The behavior of CAT activity was similar to that of total SOD activity described above, but the two yeasts differed. When *C. fukuyamaensis* RCL-3 was grown in the absence of Cu(II) it presented 8, 10 and 17 times more CAT activity than *R. mucilaginosa* RCL-11 after 24, 48 and 72 h, respectively (Fig. 3A and B). Furthermore, when *C. fukuyamaensis* RCL-3 was incubated with 0.5 mM Cu(II), CAT activity was only 1.5 times more than the control after 72 h of incubation (Fig. 3A), while CAT activity in *R. mucilaginosa* RCL-11 was 15 times more than its control after the same incubation time (Fig. 3B).

Intracellular copper localization

To determine the intracellular distribution of the metal, transmission electron microscopy (TEM) was carried out.

Cell images of *C. fukuyamaensis* RCL-3 and *R. mucilaginosa* RCL-11 obtained after 24, 48 and 72 h of incubation in the presence of 0.5 mM Cu(II) were compared with control cells (Figs. 4 and 5). *C. fukuyamaensis* RCL-3 showed a typical ascomycete wall with a thin and electro-dense external layer and clear internal zone (Fig. 4A), while *R. mucilaginosa* RCL-11 displayed a thin-

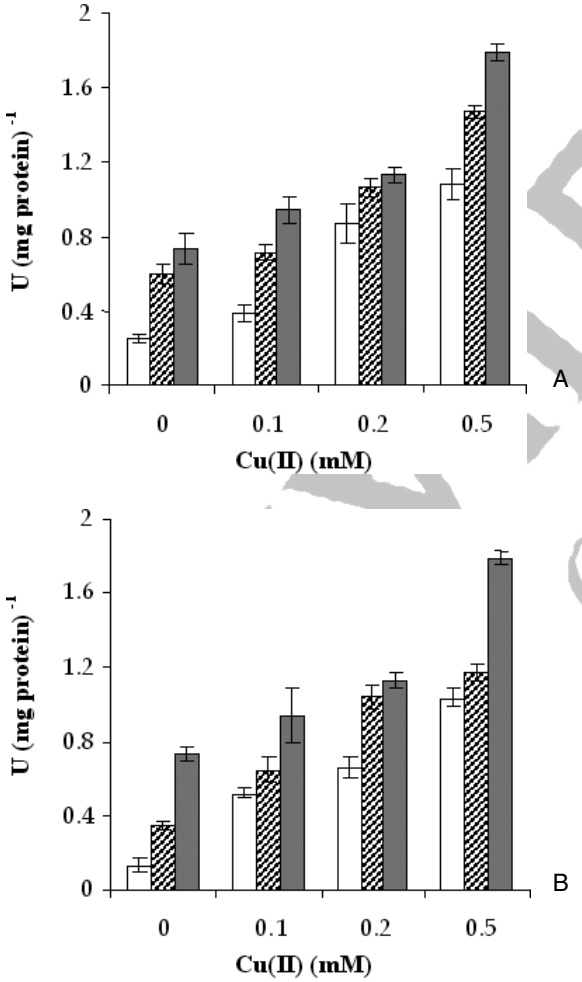

Figure 2. Total SOD activity in cell free extracts of *C. fukuyamaensis* RCL-3 (A) and *R. mucilaginosa* RCL-11 (B) grown with 0.1, 0.2 and 0.5 mM Cu(II) and without Cu(II) after (□) 24, (▨) 48 and (■) 72 h of incubation.

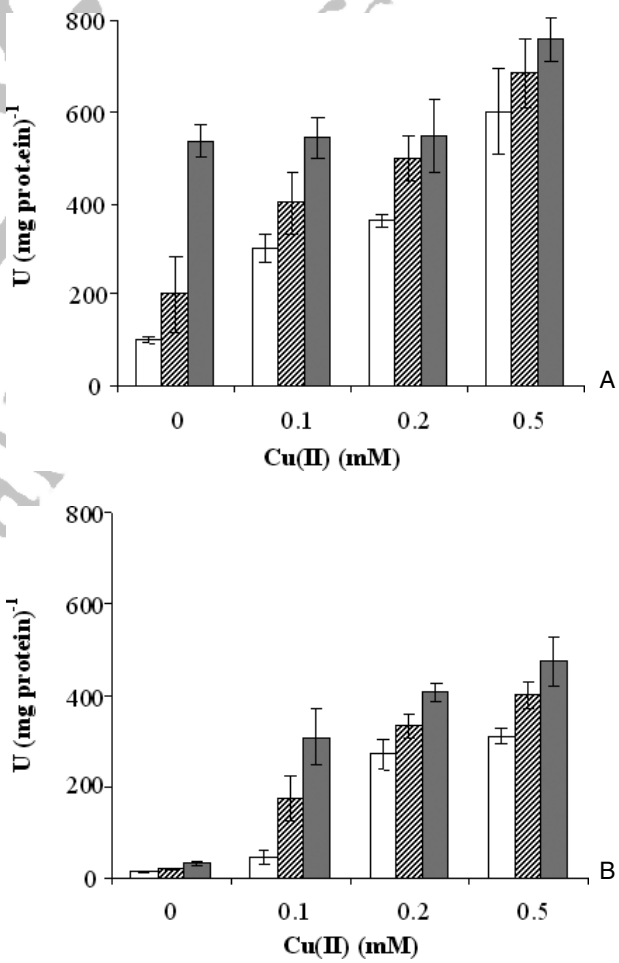

Figure 3. CAT activity in cell free extracts of *C. fukuyamaensis* RCL-3 (A) and *R. mucilaginosa* RCL-11 (B) grown with 0.1, 0.2 and 0.5 mM Cu(II) and without Cu(II) after (□) 24, (▨) 48 and (■) 72 h of incubation.

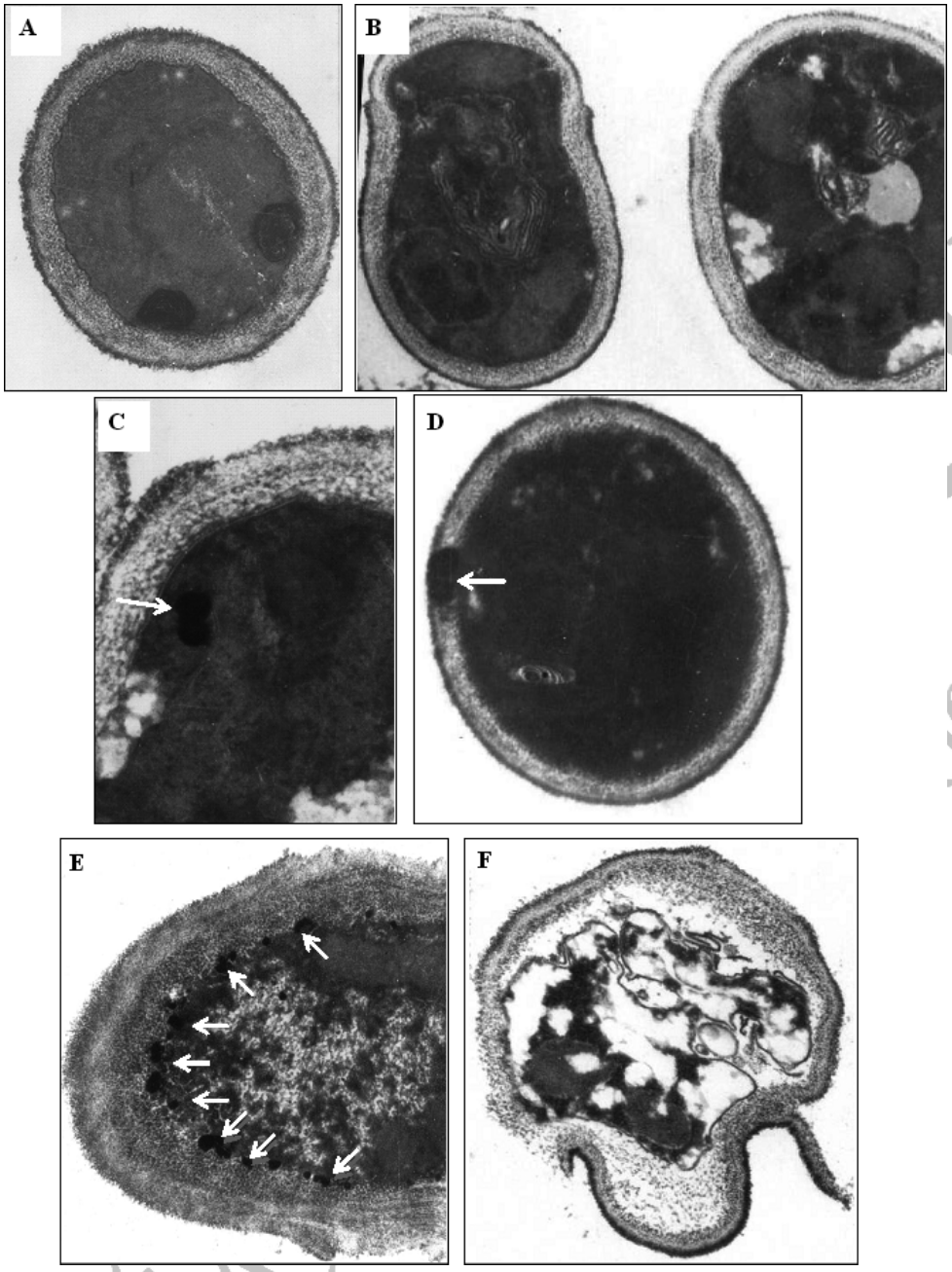

Figure 4. TEM micrographs of *C. fukuyamaensis* RCL-3 grown in the absence (A) and the presence of 0.5 mM Cu(II) (B to F). B: 24 h; C and D: 48 h; E and F: 72 h of incubation. Arrows indicate electron-dense bodies.
B, E and F: 33,300X; A and D: 50,080X and C: 82,640X.

ner wall with higher electro density (Fig. 5A). This characteristic is typical of basidiomycetes, that form multi-layers, which alternate between clear and electron-dense [18].

Figs. 4 and 5 also reveal the sequential effects of 0.5 mM Cu(II) on cellular structures and possible copper location after different incubation times. After 24 h of growth both yeasts showed evident cytoplasmic disorders due to clear and electron-dense zones, indicating cytoplasmic lysis (Figs. 4B and 5B). Moreover, *C. fukuyamaensis* RCL-3 showed highly developed cell membrane structures and thinner regions in the cell wall (Fig. 4B). *R. mucilaginosa* RCL-11 presented areas of cytoplasmic lysis mainly located next to the cell wall (Fig. 5B). After 48 h of growth, the two yeasts showed similar characteristics to those after 24 h of growth and the presence

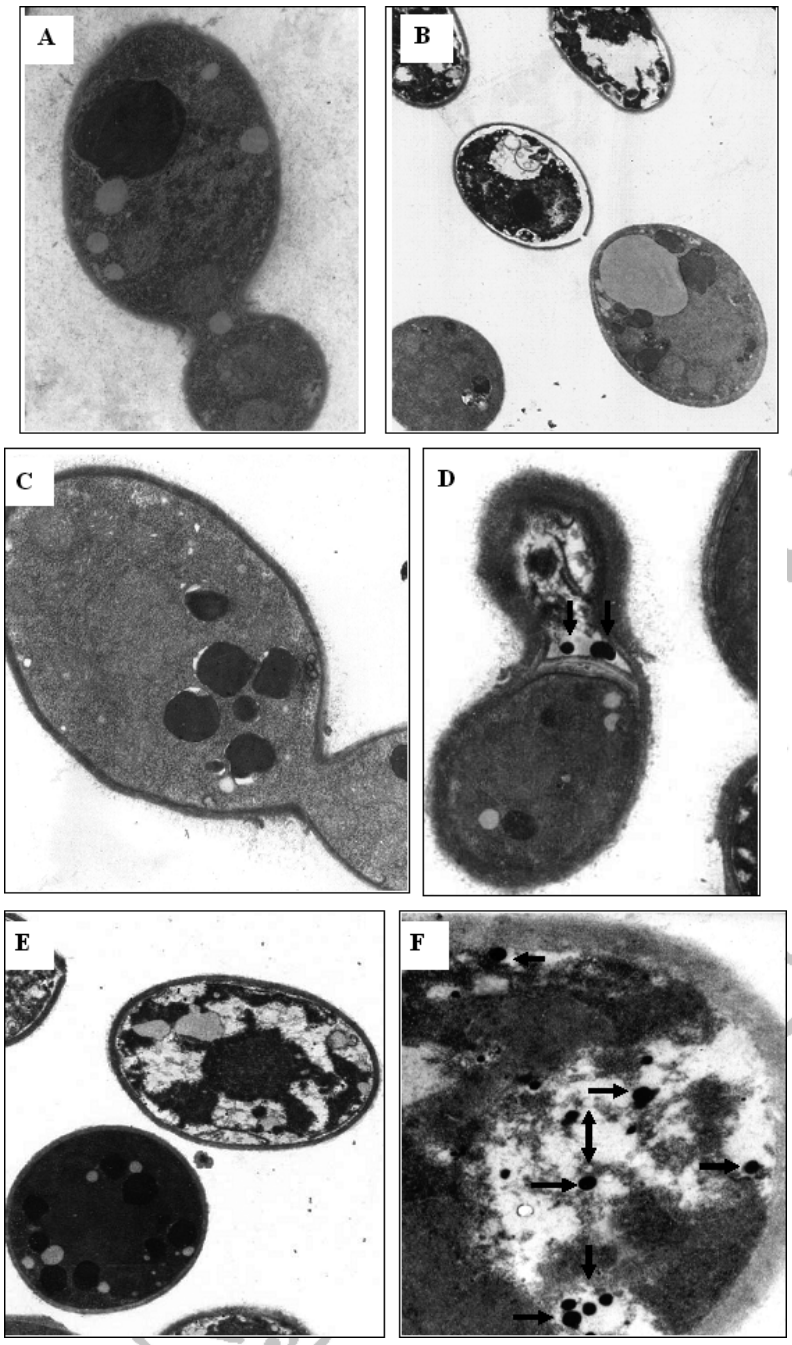

Figure 5. TEM micrographs of *R. mucilaginosa* RCL-11 grown in the absence (A) and the presence of 0.5 mM Cu(II) (B to F). B: 24 h; C and D: 48 h; Eand F: 72 h of incubation. Arrows indicate electron-dense bodies.
A: 18,700X; B, C, D and E: 33,300X; F: 82,640X.

of dark bodies. Whereas *C. fukuyamaensis* RCL-3 showed these bodies in the cytoplasm and cell wall (Fig. 4C and D), *R. mucilaginosa* RCL-11 displayed small bodies within membranous structures that may correspond to peroxisomes and larger bodies in the cytoplasm next to the cell wall (Fig. 5C and D). After 72 h, few *C. fukuyamaensis* RCL-3 cells presented dark bodies, which were mainly located next to the cell wall (Fig. 4E). Most of the cells showed loss of their typical shape, with clearer cytoplasm and loosening of the cell wall as shown in Fig. 4F. On the other hand, *R. mucilaginosa* RCL-11 showed many dark bodies in the cytoplasm with a characteristic morphology similar to the control (Fig. 5E and F).

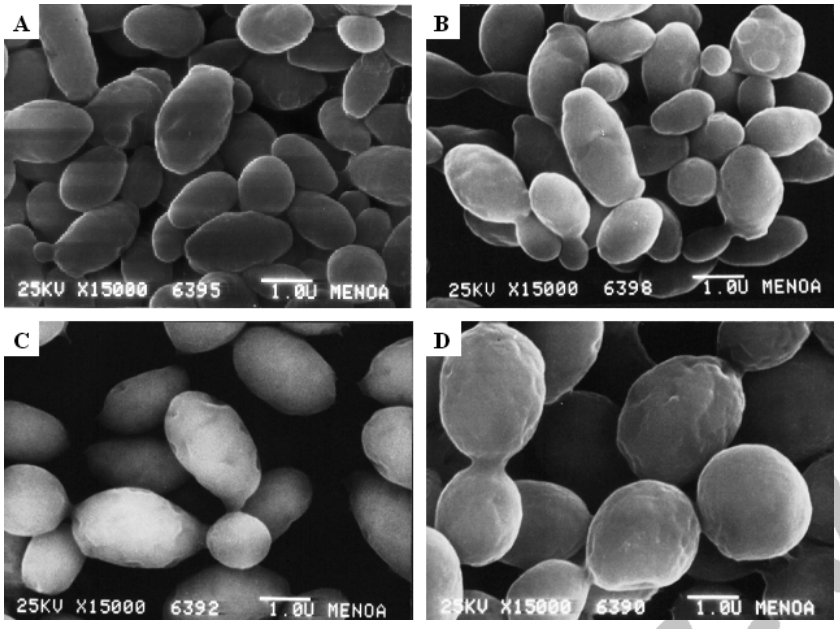

Figure 6. SEM micrographs of *C. fukuyamaensis* RCL-3 grown in the absence (A) and presence (B) of 0.5 mM Cu(II) and *R. mucilaginosa* RCL-11 grown without (C) and with (D) 0.5 mM Cu(II) after 48 h of incubation.

Size and shape properties of yeast cells incubated with and without 0.5 mM Cu(II) were compared by Scanning Electron Microscopy (SEM). Surprisingly, while *C. fukuyamaensisi* RCL-3 did not reveal any differences when compared with the controls (Fig. 6A and B), *R. mucilaginosa* RCL-11 cells grown in the presence of 0.5 mM Cu(II) (Fig. 6D) were bigger (2.6×2 μm approximately) than control cells, which presented a size of 2.3×1.3 μm (Fig. 6C).

Discussion

To determine the oxidative stress level produced by different Cu(II) concentrations on *C. fukuyamaensisi* RCL-3 and *R. mucilaginosa* RCL-11, activity of endogenous CAT and SOD was measured. An increase in CAT and SOD activity in both yeasts correlated with Cu(II) concentrations probably occurs in response to high ROS levels. Consequently, presence of 0.1, 0.2 and 0.5 mM Cu(II) produced high oxidative stress levels. Abe *et al.* [19] studied the SOD activity in a deep-sea and copper-tolerant yeast, *Cryptococcus* sp. N6. Total SOD activity was 25 and 100 times higher when this strain was grown with 1 and 10 mM Cu(II), respectively, compared with growth in the absence of endogenous copper ions. In native polyacrylamide gel electrophoresis, *Cryptococcus* sp. N6 showed a single band with SOD activity. CAT activity was only 1.4 and 1.1 times superior compared to growth without copper (control). The results of the SOD activity from *R. mucilaginosa* RCL-11 are in agreement with results by Abe *et al.* [19]. CAT activity in *Cryptococcus* sp. N6 was similar to that obtained with *C. fukuyamaensis* RCL-3 in the presence of 0.5 mM Cu(II) after 72 h of incubation but not with *R. mucilaginosa* RCL-11. However, Fujs *et al.* [20] found different results with *C. intermedia* as a model organism for the study of oxidative stress response to Cu(II). CAT activity decreased 30% when the cells were treated with Cu(II) but total SOD activity was not significantly altered.

It is well known that yeasts contain two intracellular SODs: MnSOD and CuZnSOD. CuZnSOD is predominantly located in the cytoplasm and it can act as an antioxidant and a copper chaperone [21]. Consequently, induction of this enzyme due to Cu(II) presence should be possible as was reported by Ito *et al.* [22], who worked with *Yarrowia lipolytica* grown in the presence of 2 mM Cu(II) in complex medium. On the other hand, MnSOD is generally found in the mitochondria matrix but an unusual MnSOD was found in *C. albicans* cytoplasm. This microorganism does not possess an amino-terminal peptide enabling mitochondrial import [23]. These authors postulated that the switch from CuZnSOD to cytoplasmic MnSOD expression reflects the requirement for extra protection against ROS and copper toxicity, a mechanism not found in *S. cerevisiae*. In the present work, the bands from *C. fukuyamaensis* RCL-3 and *R. mucilaginosa* RCL-11 were not inhibited by H_2O_2 treatment, and Cu(II) stimulated MnSOD but not CuZnSOD activity.

Moore *et al*. [24] indicated the absence of CuZnSOD in pigmented yeasts and they attributed this to presence of carotenoid pigments that act as antioxidants. However, Hernández-Saavedra [25] demonstrated the existence of CuZnSOD in *Udeniomybes puniceus*, a pigmented yeast, and activity increased with Cu(II) presence. The same authors also confirmed absence of CuZnSOD in *R. mucilaginosa* and *R. graminis*, which demonstrated a single band with SOD activity. These results would explain the absence of CuZnSOD activity in yeast extract from *R. mucilaginosa* RCL-11 and this enzyme was not detected in *C. fukuyamaensis* RCL-3 either. It is also possible that the main defense mechanism of *C. fukuyamaensis* RCL-3 against copper could be extrusion of cytoplasmic copper ions into the medium as was reported by Villegas *et al*. [11]. Fig. 4D shows dark bodies in the cell wall, they could be related with the copper extrusion. For this reason, expression of CuZnSOD would not be necessary. It is important to mention that the efflux of copper has also been reported for *C. albicans* and *Y. lipotytica* but not for *S. cerevisiae* [26, 22]. On the other hand, existence of two MnSOD enzymes postulated by Lamarre *et al*. [23] as mentioned above, explains the two bands with MnSOD activity present in *C. fukuyamaensis* RCL-3 and absence of CuZnSOD. This yeast could have a resistance mechanism which is similar to that of *C. albicans* and differs from *S. cerevisae.*

Regarding CAT activity, it is known that *S. cerevisiae* has two CATs: catalase A, located in peroxisomes, and catalase *T* in the cytoplasm [21]. *R. mucilaginosa* RCL-11, which has the capacity to keep copper inside the cell [11], presented higher CAT activity than *C. fukuyamaensis* RCL-3. This explains the presence of numerous peroxisomes when *R. mucilaginosa* RCL-11 was incubated in the presence of 0.5 mM Cu(II) (Fig. 5C).

Only few studies about morphology changes produced by copper have been conducted in yeasts. However, in the presence of uranium, *S. cerevisiae* presented deposits of this metal as fine needle-like crystals on the cell wall and membrane [27]. Studies carried out in a vanadate-tolerant yeast, *Hansenula polymorpha*, grown in the presence of vanadate, revealed changes in cellular ultra structure [28]. Electron micrographs of *P. guilliermondii* ATCC 201911 incubated with Cr(VI) revealed significant changes observing dark entities principally located in the vacuole [29].

Based on these previous studies, the electron-dense bodies found in *C. fukuyamaensis* RCL-3 and *R. mucilaginosa* RCL-11 after 48 h of growth in the presence of Cu(II) would correspond to intracellular copper deposits.

Ramsay and Gadd [30] determined the absence of a compartmentalization mechanism in copper detoxification by yeasts, and Silóniz *et al*. [31] observed in *P. guilliermondii* cells grown with Cu(II) and stained with a fluorochrome, that this metal ion could be localized intracellular at the beginning of the experiment, however after 48 h the cultured cells were completely stained, thus making it difficult to point out the location of the ion. These results are in agreement with the observations in *C. fukuyamaensis* RCL-3 and *R. mucilaginosa* RCL-11 of the current work since electron-dense bodies were mainly found in the cytoplasm.

Concluding remarks

Copper uptake reported by Villegas *et al*. [11] in *C. fukuyamaensis* RCL-3 and *R. mucilaginosa* RCL-11 and the dark bodies presence in the cytoplasm observed by electron microscopy could indicate that the resistance mechanism in these yeasts is through sequestration and extrusion of cytoplasmic copper ions rather than avoidance or compartmentalization.

The resistance mechanism, morphological changes and oxidative stress response depend as much on the yeast strain as on the growth conditions. The ability of metal processing by yeasts can be used to concentrate, remove and recover metals from streams and could enhance the efficiency of wastewater treatment processes. Further studies are necessary as different mechanisms of copper resistance may affect different bioremediation strategies.

Acknowledgements

This work was supported by grants from the Consejo de Investigaciones de la Universidad Nacional de Tucumán, CIUNT (Grants N° E/229 and D/220), Argentina. The authors acknowledge electron microscopy by Dr Winik (Universidad Nacional de Tucumán, Argentina) and technical assistance of Mr. Claudio Delfini and are also most grateful to Dr. Carlos Nieto for suggestions and critical reading of the manuscript.

References

[1] Clemens, S., Kim, E.J. Neumann, D. and Schroeder, J., 1999. Tolerance to toxic metals by a gene gamily of phytochelatin synthases from plants and yeast. EMBO J., **18**, 3325–3333.

[2] Puig, S. and Thiele, D.J., 2002. Molecular mechanisms of copper uptake and distribution. Curr. Opin. Chem. Biol., **6**, 171–180.

[3] Hassett, R. and Kosman, D.J., 1995. Evidence for Cu(II) reduction as a component of copper uptake by *Saccharomyces cerevisiae*. J. Biol. Chem., **270**, 128–134.

[4] Koch, A.K., Peña, M.M. and Thiele, D.J., 1997. Copper-binding motifs in catalysis, transport, detoxification and signaling. Chem. Biol., **4**, 549–560.

[5] Rees, E.M. and Thiele, D.J., 2004. From aging to virulence: forging connections through the study of copper homeostasis in eukaryotic microorganisms. Curr. Opin. Microbiol., **7**, 175–184.

[6] Halliwell, B. and Gutteridge, J.M., 1990. The antioxidants of human extracellular fluids (review). Arch Biochem Biophys., **280**, 1–8.

[7] Barceloux, D.G., 1999. Copper. Clin. Toxicol., **37**, 217–230.

[8] Imlay, J.A., 2003. Pathways of oxidative damage. Annu. Rev. Microbiol., **57**, 395–418.

[9] Lushchak, L. and Gospodaryov, D., 2005. Catalases protect cellular proteins form oxidative modification in *Saccharomyces cerevisiae*. Cell Biol. Int., **29**, 187–192.

[10] Costa, V. and Moradas-Ferreira, P., 2001. Oxidative stress and signal transduction in *Saccharomyces cerevisiae*: insights into ageing, apoptosis and diseases (review). Mol, Aspects Med., **22**, 217–46.

[11] Villegas, L.B., Amoroso, M.J. and Figueroa, L.I.C., 2005. Copper tolerant yeasts isolated from polluted area of Argentina. J. Basic Microbiol., **45**, 381–391.

[12] Yarrow, D., 1998. Methods for the isolation, maintenance and identification of yeasts. In: The Yeasts, a Taxonomic Study, 4th edn. (C.P. Kurtzman and J.W. Fell, eds.), pp. 77–100. Elsevier Science BV, Amsterdam.

[13] Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., **72**, 248–254.

[14] Beauchamp, C. and Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem., **44**, 276–287.

[15] Sambrook, J., Maniatis, T. and Fritsch, E.F., 1989. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory Press.

[16] Liochev, S.I. and Fridovich, I., 2002. Copper, zinc superoxide dismutase and H_2O_2. J. Biol. Chem., **277**, 34674–34678.

[17] Venable, J.R. and Coggeshall, R., 1965. A simplified lead-citrate stain for use in electron microscopy. J. Cell. Biol., **25**, 407–408.

[18] Moore, R.T., 1998. Cytology and ultrastructure of yeasts and yeastslike fungi. In: The Yeasts, a Taxonomic Study, 4th edn. (C.P. Kurtzman and J.W. Fell, eds.), pp. 33–44. Elsevier Science BV, Amsterdam.

[19] Abe, F., Miura, T., Nagahma, T., Inoue, A., Usami, R. and Horokoshi, K., 2001. Isolation highly copper-tolerant yeast, *Cryptococcus* sp., from the Japan Trench and the induction of superoxide dismutase activity by Cu^{2+}. Biotechnol. Lett., **23**, 2027–2034.

[20] Fujs, S., Gazdag, Z., Poljsak, B., Stibilj, V., Milacic, R., Pesti, M., Raspor, P. and Batic, M., 2005. The oxidative stress response of the yeast *Candida intermedia* to copper, zinc and selenium exposure. J. Basic Microbiol., **45**, 125–135.

[21] Jamieson, D.J., 1998. Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. Yeast, **14**, 1511–1527.

[22] Ito, H., Inouhe, M., Tohoyama, H. and Joho, M., 2007. Characteristics of copper tolerance in *Yarrowia lipolytica*. Biometals, **20**, 773–80.

[23] Lamarre, C., LeMay, J.M., Deslauriers, N. and Bourbonnais, Y., 2001. *Candida albicans* expresses an unusual cytoplasmic manganese-containing suproxide dismutase (SOD3 gene product) upon the entry during the stationary phase. J. Biol. Chem., **276**, 43784–43791.

[24] Moore, M.M., Breedveld, M.W. and Autor A.P. 1989. The role of carotenoids in preventing oxidative damage in the pigmented yeast *Rhodotorula mucilaginosa.* Arch. Biochem. Biophys., **270**, 419–431.

[25] Hernández-Saavedra, N.Y., 2003. Cu, Zn superoxide simutase in *Rhodotorula* and *Udeniomyces* spp. Isolated from sea water: cloning and sequencing the encoding region. Yeast, **20**, 479–492.

[26] Weissman, Z., Berdicevsky, I., Cavari, B.-Z. and Kornitzer, D., 2000. The high copper tolerance of *Candida albicans* is mediated by a P-type ATPase. Proc. Natl. Acad. Sci. USA, **97**, 3520–3525.

[27] Volesky, B. and May-Phillips, H.A., 1995. Biosorption of heavy metals by *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol., **42**, 797–806.

[28] Mannazzu, I., Guerra, E., Strabbioli, R., Pediconi, D. and Fatichenti, F., 1998. The vanadate-tolerant yeast *Hansenula polymorpha* undergoes cellular reorganization during growth in, and recovery from, the presence of vanadate. Microbiology, **144**, 2589–2597.

[29] Ksheminska, H., Fedorovych, D, Babyak, L., Yanovych, D., Kaszycki, P. and Koloczek H., 2005. Chromium(III) and (VI) tolerance and bioaccumulation in yeast: a survey of cellular chromium content in selected strains of representative genera. Process Biochem., **40**, 1565–1572.

[30] Ramsay, L.M. and Gadd, G.M., 1997. Mutants of *Saccharomyces cerevisiae* defective in vacuolar function confirm a role for the vacuole in toxic metal ion detoxification. FEMS Microbiol. Lett., **152**, 293–298.

[31] Silóniz, M., Balsolobre, L., Alba, C., Valderrama, M. and Peinado, J., 2002. Feasibility of copper uptake by the yeast *Pichia guilliermondii* isolated form sewage sludge. Res. Microbiol., **153**, 173–180.

