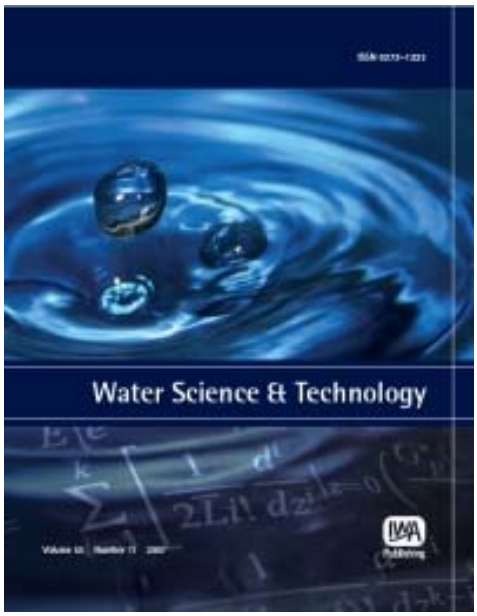

Cu(II) removal by *Rhodotorula mucilaginosa* RCL-11 in sequential batch cultures

Liliana B. Villegas, María J. Amoroso, Lucía I. C. de Figueroa and Faustino Siñeriz

ABSTRACT

The present study explored the ability of the yeast *Rhodotorula mucilaginosa* RCL-11 to adapt to increasing Cu(II) concentrations, measuring oxidative stress through superoxide dismutase and catalase activity in two parallel sequential batch assays. One assay was performed in Erlenmeyer flasks without aeration and a second in a fermentor in which the dissolved oxygen was maintained at 30% saturation. Both assays were carried out by increasing Cu(II) concentrations in five sequential steps: 0; 0.1; 0.2; 0.5 and 1 mM. Each assay was incubated at 30°C, 250 rpm and pH 5.5. While growth parameters of *R. mucilaginosa* RCL-11 decreased 90–95% with increasing Cu(II) concentration in the culture medium, the oxidative stress level increased from 30 to 55% in both assays. Cells grown under controlled oxygen conditions showed 30% more copper bioaccumulation and 10% glucose consumption when compared with cells grown without aeration. SOD activity was higher under controlled than whitout areation, whereas CAT activity was similar under both test conditions. Cu(II) bioaccumulation by *R. mucilaginosa* RCL-11 and a possible increase in this capacity by adaptation of the strain under controlled aeration represent a potential valuable tool for treatment of effluents or water bioremediation with high copper contents.

Key words | Copper(II) uptake, oxidative stress, *Rhodotorula mucilaginosa*, sequential batch

Liliana B. Villegas
María J. Amoroso
Lucía I. C. de Figueroa (corresponding author)
Faustino Siñeriz
PROIMI—CONICET,
Av. Belgrano y Pje. Caseros. T4001MVB,
Tucumán,
Argentina
E-mail: *lbvilleg@gmail.com*;
mjamoroso@ciudad.com.ar;
proimiunt@arnet.com.ar;
fsineriz@arnet.com.ar

María J. Amoroso
Microbiología General, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán,
Tucumán,
Argentina

Lucía I. C. de Figueroa (corresponding author)
Faustino Siñeriz
Microbiología Superior, Facultad de Bioquímica, Química y Farmacia,
Universidad Nacional de Tucumán,
Tucumán,
Argentina

INTRODUCTION

Copper is an essential element for all organisms because it either forms part of the structure or it is a cofactor of many enzymes. These enzymes are involved in an array of biological processes required for normal growth, development and maintenance of the cells. However, at higher concentrations copper becomes toxic for all organisms mainly because of its interaction with cellular iron stores and by an increase in the production of reactive oxygen species (ROS) via the Fenton-reaction (Puig & Thiele 2002; Gaetke & Chow 2003).

Cu(II) is known to be one of the most widespread heavy metal contaminants in the environment (Dönmez & Aksu 2001). Copper enters aquatic systems primarily through mining and industrial activities. These systems are often the source of drinking water (Salomons 1995; Audry *et al.* 2004). The impact of copper on aquatic systems and its distribution in food chains is a serious threat to animals and humans, resulting in a world-wide environmental problem.

Removal of heavy metals using common physicochemical processes are generally limited and very expensive (Eccles 1999). These processes include oxidation and reduction, chemical precipitation, filtration, electrochemical treatment, evaporation, ion-exchange and reverse osmosis. Poor selectivity, high reagent requirements and unpredictable metal ion removal are some other

doi: 10.2166/wst.2009.457

disadvantages associated with these techniques. Furthermore, reagents used for metal desorption are pollutants themselves, resulting in toxic sludge and secondary environmental pollution. Therefore, development of cost-effective alternatives such as bioremediation with microbial biomass has become of interest over the past decade (Silóniz *et al.* 2002; Malik 2004).

Many microorganisms have developed a variety of mechanisms to remove heavy metals from wastewater involving a variety of different mechanisms such as adsorption to cell surfaces, transport into the cell, intracellular accumulation or reduction to non-toxic or less toxic species (Gadd 2000; Lloyd 2003; Malik 2004).

In a previous publication, a copper-tolerant yeast was isolated from a polluted area in Argentina and identified as *Rhodotorula mucilaginosa* RCL-11. The microorganism was capable of accumulating up to 50% of the copper from a medium containing 0.5 mM Cu(II) (Villegas *et al.* 2005). After 48 h of growth in the presence of Cu(II), electron microscopy showed dark grains in the cytoplasm of *R. mucilaginosa* RCL-11. The number of dark bodies in the cells increased with increasing incubation time. Scanning electron micrographs revealed that *R. mucilaginosa* RCL-11 cells grown in the presence of Cu(II) were larger than those grown in the absence of the heavy metal (Villegas *et al.* 2009).

It is well known that 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, including DNA, lipids and proteins (Imlay 2003). ROS molecules are detoxified via superoxide dismutases (SODs) and catalases (CATs) and these enzymes represent the first and most important line of antioxidant defense (Lushchak & Gospodaryov 2005). To determine the oxidative stress level in *R. mucilaginosa* RCL-11 produced by different Cu(II) concentrations, endogenous CAT and SOD activity was measured (Villegas *et al.* 2009). The authors found an increase in both activities, which was mainly related to the high Cu(II) concentration in the culture medium, as the yeast had to cope with high ROS levels.

More studies with this strain will allow to evaluate the usefulness of it for using in bioremediation or in the treatment of water contaminated with copper.

MATERIALS AND METHODS

Strain, medium and culture conditions

Rhodotorula mucilaginosa RCL-11 was isolated by Villegas *et al.* (2005) from a copper filter in a mineral processing plant, located in the province of Tucumán, Argentina. Cells were incubated in yeast nitrogen base (YNB) without amino acids (Difco) with 20 g l^{-1} glucose as sole carbon source.

Two parallel sequential batch assays were carried out to assess copper removal by *R. mucilaginosa* RCL-11. One assay was performed in 500 ml Erlenmeyer flasks containing 250 ml of culture medium and the oxygen concentration in this assay was unregulated. The second assay was run in a 1.5 l stirred fermentor (LH Fermentation Inc., Emeryville, CA, USA) with a working volume of 1 l of the same culture medium. Dissolved oxygen was regulated and maintained at 30% saturation by supplying air automatically via a proportional-integrative-derivative (PID) controller. Both assays were performed at 30°C and 250 rpm. The medium was buffered with 50 mM Tris-succinate to maintain a constant pH of 5.5.

The flask and fermentor were inoculated with an active overnight pre-inoculum to contain a final concentration of 10^7 CFU ml^{-1}.

For Cu(II) assaying, a 100 mM $CuSO_4$ solution was added to the cultures to attain desired final Cu(II) concentrations. Sequential batch cultures were obtained by increasing Cu(II) concentrations through five sequential steps: 0; 0.1; 0.2; 0.5 and 1 mM. A first batch of both assays was carried out in the absence of Cu(II) for 24 h (control). A second batch was started after discarding most of the culture and replacing it with an equal amount of the same culture medium supplemented with 0.1 mM Cu(II); incubation time was 24 h. The third to fifth batch were performed similarly, but with 0.2, 0.5 and 1 mM of Cu(II) during 30, 50 and 100 h, respectively. The initial microbial concentration for each batch was adjusted to 10^7 CFU ml^{-1} with cells from the previous batch culture.

Growth parameters

Samples were taken every 6 hours and CFU ml^{-1} were determined by serially diluting fractions of the culture

and plating 100 μl of each dilution onto YEPD-agar: 10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 15 g l^{-1} glucose and 15 g l^{-1} agar.

Samples were also centrifuged at 10,000 *g* for 15 min and cells were washed twice with bi-distilled water. Dry weight was determined using aluminum foil cups dried to constant weight at 80°C. Supernatants were stored at 4°C for analysis of residual glucose and copper.

Residual glucose was determined by the Dinitrosalicylic acid (DNS) method described by Miller (1959). Specific growth rates (μ; h^{-1}) were calculated as the slope of the regression line of the natural logarithm of culture biomass versus time.

Copper determination

Total copper concentrations of supernatant samples were measured by atomic absorption spectrophotometry. For determination of intracellular metal concentrations, cells were harvested by centrifugation and washed twice with bi-distilled water. Subsequently, 100 μl of concentrated nitric acid were added to the cell pellet and the mixture was boiled until cells were completely mineralized. The original sample volume (10 ml) was adjusted with bi-distilled water.

Enzyme assaying

Cells were harvested by centrifugation at 10,000 *g* for 15 min at the end of each assay and washed twice with 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA. Cell extracts were prepared by agitating 1 ml of wet pellet with the amount equivalent to a volume of 0.5 ml of glass beads on a vortex mixer. Ten pulses of 1 min each were applied, with 1 min intervals on ice between pulses. Cell debris was removed by centrifugation at 10,000 *g* during 20 min at 4°C. The supernatants were retained and used as enzyme source. Total supernatant protein content was determined with the method by Bradford (1976) using bovine serum albumin as reference protein.

SOD enzyme activity

SOD activity was determined according to the method by Beauchamp & Fridovich (1971), modified by us. This assay is based on the competition between SOD and an indicator molecule, nitro blue tetrazolium (NBT), for superoxide produced by a photochemical reaction in the presence of riboflavin.

Native polyacrylamide gel electrophoresis (PAGE) was performed according to Sambrook *et al.* (1989), loading 10 μg of total protein in each lane. Electrophoresis was performed at 100 V for 3 h. After electrophoresis, 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 solution for 15 min in the dark. Subsequently, the gel was incubated in 50 mM potassium phosphate buffer (pH 7.8) containing 0.03 mg ml^{-1} riboflavin and 5 mg ml^{-1} methionine for 15 min at room temperature under gentle shaking in the dark. Then the gel was exposed to light for 20–30 min. Areas with SOD activity remained colourless after the gel turned violet as a result of formazan formation.

To quantify total SOD activity, 0.01 ml of sample was mixed in the dark with 1 ml of 50 mM potassium phosphate EDTA buffer (pH 7.8; containing 0.1 mM EDTA), 0.4 ml of NTB solution (1 mg ml^{-1}), 1 ml of riboflavin (0.03 mg ml^{-1}) and 0.5 ml of methionine (5 mg ml^{-1}). The mixtures were exposed to light for 20 min. Readings with 0.01 ml of water under the same conditions were considered a reference of 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. To discriminate between CuZnSOD and MnSOD, samples were incubated with 10 mM H2O2 for 30 min before being loaded onto the gel, as CuZnSOD is sensitive to H_2O_2 (Liochev & Fridovich 2002).

CAT enzyme activity

Catalase activity was measured spectrophotometrically at room temperature following the decrease in absorption 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 of cell free extract (Lushchak & Gospodaryov 2005). One unit of CAT was defined as H_2O_2 consumption (mM) per minute.

Results of total SOD and CAT activity are expressed as U per mg of total protein.

Statistical analyses

All results are expressed as mean values of at least triplicate determinations of independent cultures. The statistical significance of differences among values was assessed by using the Student's *t*-test and ANOVA. A probability level of $P < 0.05$ was used throughout this study.

RESULTS AND DISCUSSION

Growth parameters

Rhodotorula mucilaginosa RCL-11 growth curves in Erlenmeyer and fermentor sequential batch modes at increasing Cu(II) concentrations are shown in Figure 1. Both assays allowed to study the effect of adaptation to increasing Cu(II) concentrations and compare the effect of Cu(II) under controlled (fermentor) and without aeration conditions (Erlenmeyer flasks).

In the absence of copper (control), no significant growth differences were observed between controlled and without aeration (Figure 1A). When the yeast was incubated in the presence of 0.1 mM Cu(II) the lag phases under controlled and without aeration were similar, but growth under the two conditions differed significantly from controls. The final cell concentration in the fermentor ($3.90 \pm 0.35\,10^8$ CFU ml^{-1}) was similar to the control, whereas the cell concentration in flasks decreased with 40% in relation to the control (Figure 1B). Cells grown at 0.1 mM Cu(II) during 24 h were used as inoculum for the next culture (0.2 mM Cu(II)). The lag phase during controlled aeration at this concentration was longer and growth was slower than without aeration. CFU mL^{-1} decreased with 85% at without aeration compared to the control after 30 h and under controlled aeration growth declined with 90% without reaching the stationary phase (Figure 1C). When yeast cells grown at 0.2 mM Cu(II) were used as inoculum for the next culture containing 0.5 mM Cu(II), growth under controlled and without aeration showed similar results (Figure 1D). In the final subculture, 1 mM Cu(II) and using an inoculum from the 0.5 mM Cu(II) culture, the stationary phase for without aeration assay was reached after 40 h and under controlled aeration, the culture did not reach the stationary phase after 100 h (Figure 1E).

Specific growth rates (μ) for *R. mucilaginosa* RCL-11 at different Cu(II) concentrations are shown in Figure 2A. Rates declined with increasing copper concentrations, except for 0.1 mM Cu(II) under controlled aeration. Under these conditions μ was not affected compared to the control without Cu(II) addition. Specific glucose consumption increased up to mid exponential growth phase and from then it remained constant. Glucose consumption versus different Cu(II) concentrations is presented in Figure 2B. Under controlled aeration, glucose consumption increased with increasing Cu(II) concentrations until 0.5 mM, whereas this parameter declined under without aeration condition when the yeast was incubated at 0.1 mM Cu(II). Glucose consumption increased with increasing Cu(II) concentration in the culture medium using cells adapted to Cu(II) with a maximum consumption of 4.7 ± 0.3 g of glucose per g of biomass. At 1 mM of Cu(II) specific glucose consumption dropped with 35% in comparison to the control in flask experiments.

Several authors have reported that glucose or lactate used as electron donor affected the reduction rate of Cr(VI) (Laxman & More 2002; Villegas *et al.* 2008; Zhu *et al.* 2008). *R. mucilaginosa* RCL-11 probably requires glucose as energy source or electron donor for Cu(II) removal because its specific glucose consumption increased with increasing specific Cu(II) removal, even though its specific growth rate, μ, and the growth period decreased owing to the toxicity of the accumulated copper inside the cells.

Copper uptake

Bioaccumulation kinetics of copper by *R. mucilaginosa* RCL-11 was tested under both assay conditions. The increase in intracellular copper through time was correlated with the reduction of extracellular copper (Figure 3; for practical reasons only intracellular copper is given). In order to compare the capacity of the yeast to remove copper under both experimental conditions, specific bioaccumulation (q), defined as the amount of copper (mg) per gram of biomass, was also calculated (Figure 3). When the yeast was incubated in the presence of 0.1 mM Cu(II), copper

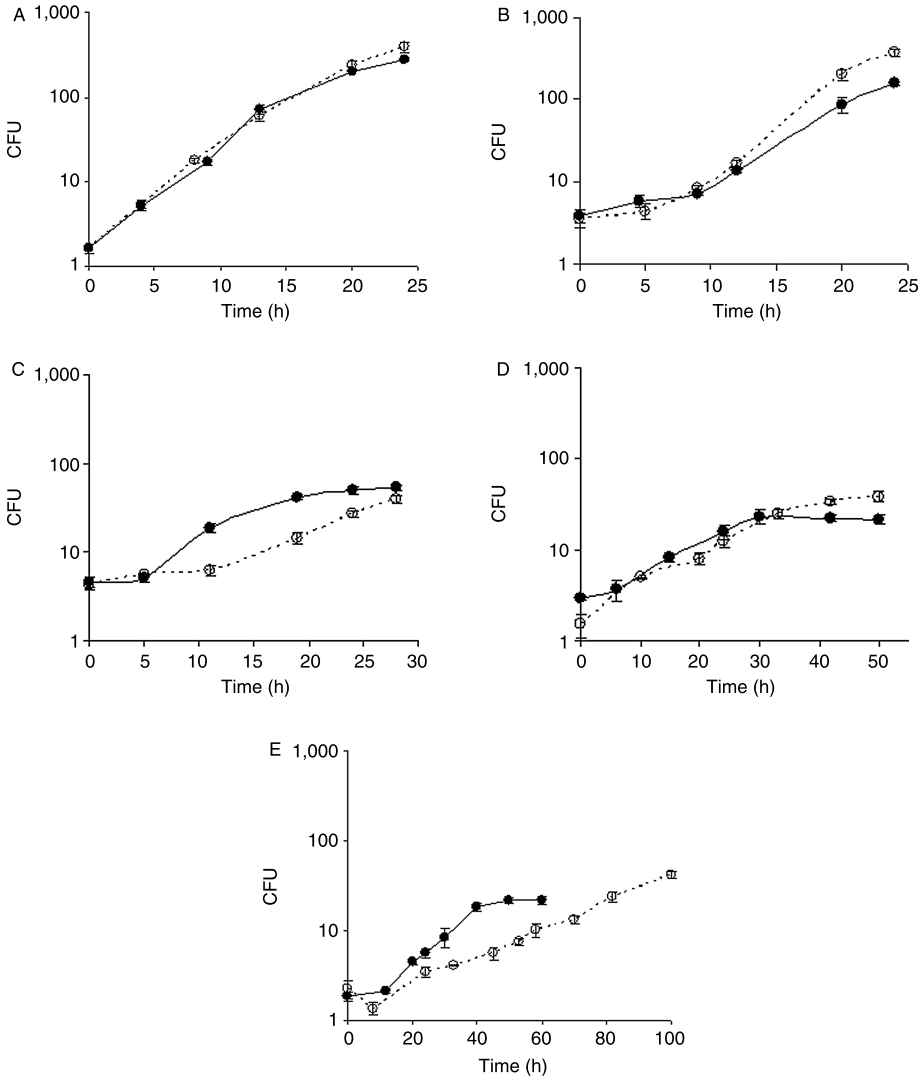

Figure 1 | Effect of serial increase of Cu(II) concentration on *R. mucilaginosa* RCL-11 growth. (A) Control (without Cu(II)); (B, C, D and E) 0.1 mM, 0.2 mM, 0.5 mM and 1 mM Cu(II), respectively. (- -○- -) controlled aeration (- -●- -) without aeration.

bioaccumulation reached 80% in the fermentor and 65% in flasks after 24 h of incubation but specific bioaccumulation decreased in both assays (Figure 3A). With pre-adapted cells in successive cultures with 0.2, 0.5 and 1 mM of Cu(II) in without aeration, bioaccumulation was 50, 25 and 30%, respectively. Maximum copper removal started during the exponential phase and remained constant thereafter. Under controlled aeration, copper removal was 50% compared to controls regardless of the initial Cu(II) concentration (Figure 3B–D). Specific bioaccumulation increased with increasing initial Cu(II) concentration in the culture medium through time until reaching maximum values and then remained constant. At an initial Cu(II) concentration of 1 mM maximum Cu(II) accumulation was 13 mg per g of biomass after 24 h under without aeration and 17 mg after 45 h under controlled aeration (Figure 3D). These results are coincident with the exponential growth phase. It is important to notice that Villegas *et al.* (2005) found that *R. mucilaginosa* RCL-11 lost its specific Cu(II) bioaccumulation capacity when this cation was added at increasing concentrations, and the strain could not grow at a Cu(II) concentration of 1 mM.

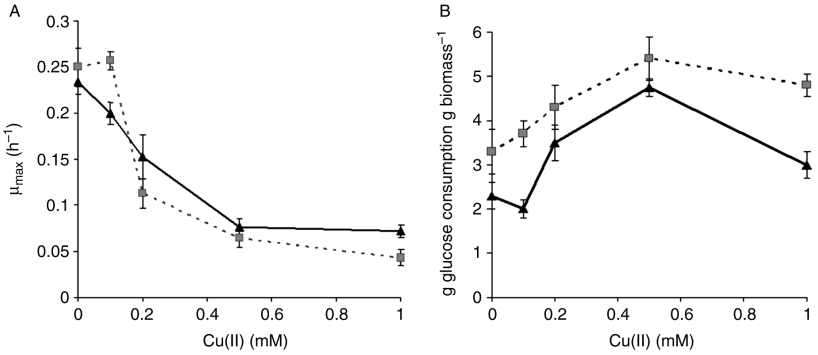

Figure 2 | Effect of different Cu(II) concentrations on (A) specific growth rate and (B) specific glucose consumption in relation to the biomass expressed as g glucose g biomass^{-1}. (----) controlled aeration (—) without aeration.

Adaptability of yeasts to high copper concentrations was previously reported by Dönmez & Aksu (1999, 2001), who showed that increasing Cu(II) concentrations in the growth medium caused growth inhibition of all kinds of yeasts. They also found that adapted cells accumulated Cu(II) more efficient than non-adapted cells. The level of Cu(II) accumulation depended on the metal concentration and yeast strain and best results were obtained with *Candida* sp.: 14.8 mg of copper per g biomass at 5 mM Cu(II). Our current results with *R. mucilaginosa* RCL-11 were slightly higher, representing more effective copper removal than Dönmez and Aksu obtained with *Candida* sp.

Silóniz *et al.* (2002) found that increasing concentrations of copper affected physiological parameters and bioaccumulation capacity of viable *Pichia guilliermondii*, isolated from sewage sludge in Spain. The growth rate of non-adapted cells of this yeast decreased with increasing copper concentrations, whereas Cu-induced cells of *P. guilliermondii* maintained constant biomass yield and growth rate, independently of the external copper concentration.

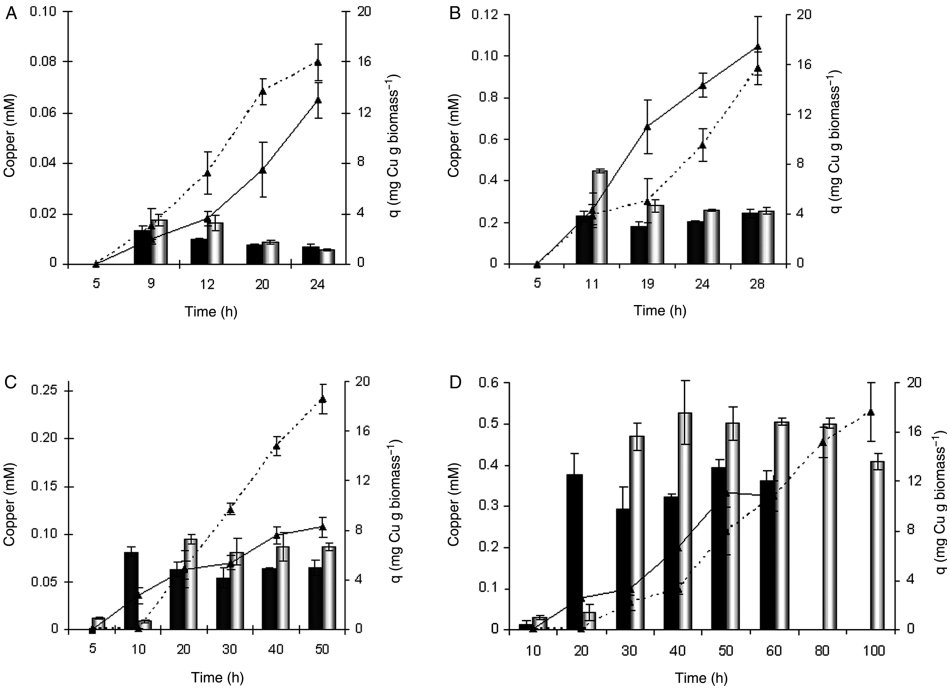

Figure 3 | Intracellular copper concentration of *R. mucilaginosa* RCL-11 cultures with controlled (----) and without (——) aeration. Specific bioaccumulation, *q* (mg Cu g biomass^{-1}), at different initial Cu(II) concentrations: (A) 0.1; (B) 0.2; (C) 0.5 mM and (D) 1 mM Cu(II). (□) controlled and (■) without aeration.

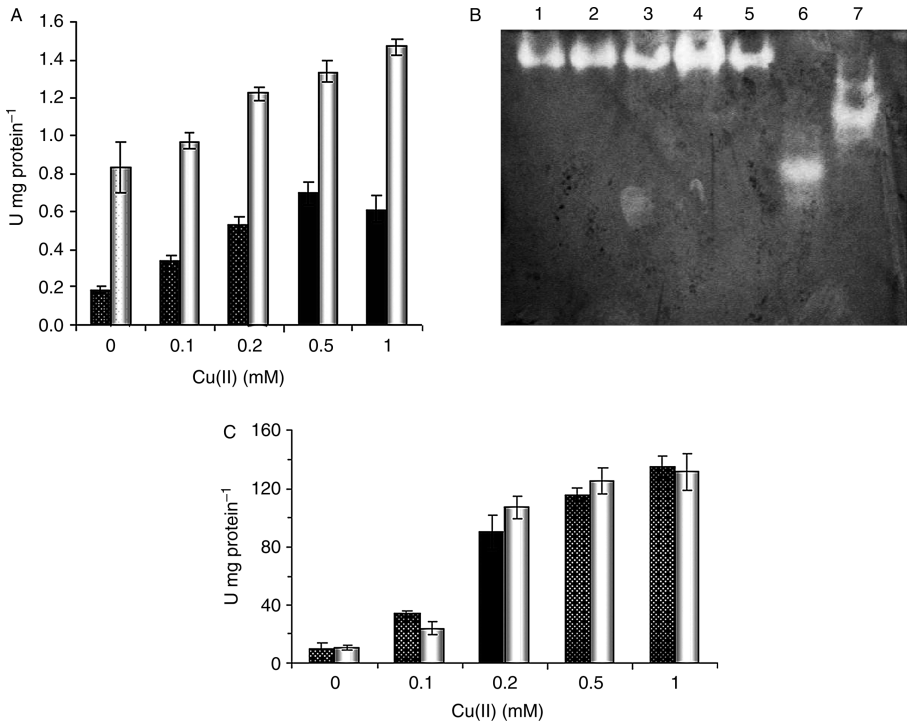

Figure 4 | (A) Total SOD activity in cell free extracts of *R. mucilaginosa* RCL-11 with (□) and without (■) controlled aeration. (B) Activity staining for SOD in cell free extracts (fermentor grown cell) in 10% native PAGE. Cell extracts were incubated in the presence of Cu(II) and SOD standards. lane 1, control cells; lane 2, cells incubated with 0.1 mM Cu(II); lane 3, cells incubated with 0.2 mM Cu(II); lane 4, cells incubated with 0.5 mM Cu(II); lane 5, cells incubated with 1 mM Cu(II); lane 6, bovine CuZnSOD and lane 7, *E. coli* MnSOD. (C) CAT activity in cell free extracts of *R. mucilaginosa* RCL-11 with (□) and without (■) controlled aeration.

SOD and CAT enzyme activity

Endogenous catalase and superoxide dismutase activity were measured in cell free extracts of *R. mucilaginosa* RCL-11 to evaluate the oxidative stress level when Cu(II) was added to the culture medium. Under both culture conditions, SOD activity increased with increasing Cu(II) concentration in the culture medium, but SOD activity in the fermentor was higher than in flasks (Figure 4A). The yeast showed a single band with SOD activity under both growth conditions. For practical purposes, the Figure 4B shows electrophoresis profiles of SOD activity for fermentor grown cells. Reference enzyme CuZnSOD was inhibited by H_2O_2 treatment, whereas SOD from *R. mucilaginosa* RCL-11 was not affected. On the other hand, CAT activity under controlled and without aeration was similar at the different Cu(II) concentrations assayed (Figure 4C).

A previous paper by Villegas *et al.* (2009) reports on SOD and CAT activity with non-adapted *R. mucilaginosa* RCL-11 cells grown at 0.2 and 0.5 mM of Cu(II). Total activity of both enzymes was higher than the results obtained with adapted cells in the current study.

It is well known that yeasts induce biosynthesis of small soluble proteins known as "copper-chaperones", which efficiently bind copper atoms in the cytoplasm. These proteins distribute the copper molecules to specific intracellular destinations (Rees & Thiele 2004). The decrease in SOD and CAT activity in the current study could be due to the fact that the cytoplasm of adapted cells has a larger number of proteins that sequester copper, which prevents the free ion to produce reactive oxygen species (ROS), thus decreasing oxidative stress. Consequently, adapted cells were able to grow at higher Cu(II) concentrations than non-adapted cells. The higher stress conditions under controlled aeration may be directly related to a greater ability of copper bioaccumulation.

R. mucilaginosa RCL-11 showed a single band of SOD activity, which was not inhibited by H_2O_2 treatment, suggesting that Cu(II) stimulated MnSOD but not CuZn-SOD activity (Figure 4B). This result confirms the absence

of CuZnSOD activity in yeast extract from *R. mucilaginosa* from non-adapted cells as reported by Hernández-Saavedra (2003) and Villegas *et al.* (2009).

CONCLUSIONS

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. Cu(II) bioaccumulation by *R. mucilaginosa* RCL-11 and a possible increase in this capacity by adaptation of the strain to copper under controlled aeration as observed in this study, suggests the usefulness of this strain for bioremediation or treatment of effluents containing high copper levels to avoid the water contamination or the bioremediation of contaminated water by this heavy metal. Further studies are necessary to develop a right bioremediation strategy.

ACKNOWLEDGEMENTS

This work was supported by grants from CIUNT and CONICET, Argentina. The authors also gratefully acknowledge Dr. Orlando Villegas (Universidad Nacional de San Luis, Argentina) for providing FAAS facilities and Mr Claudio Delfini for his valuable collaboration with the experimental design and technical assistance.

REFERENCES

Audry, S., Schäfer, J., Blane, G. & Jouanneau, J. M. 2004 Fifty year sedimentary record of heavy metal pollution (Cd, Zn, Cu, Pb) in the Lot River reservoirs (France). *Environ. Pollut.* **132**, 413–426.

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

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.

Dönmez, G. & Aksu, Z. 1999 The effect of copper(II) ions on the growth and bioaccumulation properties of some yeasts. *Process Biochem.* **35**, 135–142.

Dönmez, G. & Aksu, Z. 2001 Bioaccumulation of copper(II) and nickel(II) by the non-adapted and adapted growing *Candida* sp. *Water Res.* **35**, 1425–1434.

Eccles, H. 1999 Treatment of metal-contaminated wastes: why select a biological process? *Biotopic* **17**, 462–465.

Gadd, G. M. 2000 Bioremedial potential of microbial mechanisms of metal mobilization and immobilization. *Curr. Opin. Biotechnol.* **11**, 271–279.

Gaetke, L. M. & Chow, C. K. 2003 Copper toxicity, oxidative stress, and antioxidant nutrients. *Toxicology* **189**, 147–163.

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

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

Laxman, R. & More, S. 2002 Reduction of hexavalent chromium by *Streptomyces griseus*. *Miner. Eng.* **15**, 831–837.

Liochev, S. I. & Fridovich, I. 2002 Copper, Zinc superoxide dismutase and H_2O_2. *J. Biol. Chem.* **277**, 34674–34678.

Lloyd, D. 2003 Effects of uncoupling of mitochondrial energy conservation on the ultradian clock-driven oscillations in *Saccharomyces cerevisiae* continuous culture. *Mitochondrion* **3**, 139–146.

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

Malik, A. 2004 Metal bioremediation through growing cells. *Environ. Int.* **30**, 261–278.

Miller, G. L. 1959 Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31**, 426–428.

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

Rees, E. M. & Thiele, D. J. 2004 From aging to virulence: forging connections through the study of copper homeostasis in eukaryotic microorganisms. *Curr. Opin. Microbiol.* **7**, 1–10.

Salomons, W. 1995 Environmental impact of metals derived from mining activities: processes, predictions, prevention. *J. Geochem. Exp.* **52**, 5–23.

Sambrook, J., Maniatis, T. & Fritsch, E. F. 1989 *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory Press.

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

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

Villegas, L., Fernández, P., Amoroso, M. J., Figueroa, L. I. C., *et al.* 2008 Chromate removal by yeasts isolated from sediments of a tanning factory and a mine site in Argentina. *Biometals* **21**, 591–600.

Villegas, L., Amoroso, M. J. & Figueroa, L. I. C. 2009 Responses of *Candida fukuyamaensis* RCL-3 and *Rhodotorula mucilaginosa* RCL-11 to copper stress. *J. Basic Microbiol.* In press.

Zhu, W., Chai, L., Ma, Z., Wang, Y., Xiao, H. & Zhao, K. 2008 Anaerobic reduction of hexavalent chromium by bacterial cells of *Achromobacter* sp., Strain Ch1. *Microbiol. Res.* **163**, 616–623.