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Heavy metal capture by autochthonous yeasts from a volcanic influenced environment of Patagonia

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Heavy metals at elevated concentrations are a major threat to agricultural and human health. Typically, human activities tend to release these metals to the environment in aqueous solutions, generating high levels of pollution due to the mobility of the heavy metals. The aim of the present work was to assess heavy metal tolerance in yeasts isolated from Río Agrio – Lake Caviahue volcanic acidic aquatic environment and to evaluate the capacity of selected strains to capture metals in acidic culture media conditions. The ability of three yeast species, *Cryptococcus agrionensis*, *Cryptococcus* sp. 2, and *Coniochaeta fodinicola*, to tolerate and capture metals in live cultures has been evaluated. These three yeast species showed high tolerance to low pH and elevated concentrations of metals, thus implying their autochthonous status. Minimal inhibitory concentration (MIC) for growth obtained for these isolates showed elevated tolerance to the six heavy metals evaluated and were significantly higher than those registered for other microorganisms. *C. agrionensis* was able to capture 15.80 mg (g biomass)$^{-1}$ of Cu^{2+} (MIC: 0.22 g L^{-1}), *Cryptococcus* sp. 2 was able to capture 36.25 and 65.28 mg (g biomass)$^{-1}$ of Ni^{2+} and Zn^{2+}, respectively (MIC: 0.56 and 1.68, respectively), and *C. fodinicola* was able to capture 67.11 mg (g biomass)$^{-1}$ of Zn^{2+} (MIC: 3.75). This work reported the ability of yeasts to capture metals in acidic conditions for the first time. We hope that it represents the step-stone for future researches in the ability and metabolism of yeasts form acidic aquatic environment related to metal tolerance and capture.

Additional supporting information may be found in the online version of this article at the publisher's web-site.

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Introduction

In recent years, social attention has been drawn to environmental pollution due to the higher level of environmental awareness as well as concern of toxicological effects of pollutants on human health [1]. Heavy metals are found in nature as minerals, which are scarcely available for living organisms. Because of this, microorganisms have evolved in order to require very low concentrations of metals (mainly in order of milligrams per liter in a nutritive culture medium) for metabolism. Human activities (industrial and mining activities mainly) release high amounts of toxic heavy metals into the environment, and their presence in water bodies and soils have great impact in the biodiversity of both, micro- and macro-organisms [2–4]. Other important fact is that metals gain mobility when pH decreases. Acids in the environment tend to leachate toxic heavy metals, increasing their mobility and bioavailability, which is of particular concern in acid mine drainages.

Since metals cannot be metabolically degraded; the only way of reducing metal availability in the

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environment is by removing them [4]. A major problem for bioremediation technologies to be applied is that heavy metal discharges are usually acidic (e.g., acid mine drainages, industrial effluents). It is a fact that remediation technologies in acidic environments are difficult. For physicochemical remediation technologies, the main objective is to neutralize pH by adding alkali substance as sodium and potassium hydroxide. But this strategy has many disadvantages as promoting chemical changes in the environment, unspecific precipitation of ions and the possibility of further contamination by byproducts and toxic sludges [5–9].

Bioremediation presents some interesting advantages. Firstly, almost all microorganism cells are able to capture metal ions in cell surface through binding them to negatively-charged molecules such as polycarbonates, proteins, or other cell wall components, a process known as biosorption [6, 10]. Secondly, many microorganisms can actively interact with metal ions using metabolic pathways and enzymes [11]. Bioremediation in acidic environments is limited by pH. Many works have dealt manly with metal biosorption in acidic medium [12–20]. Although biosorption is one strategy of bioremediation technologies, is of vital importance since the sorption of the metal ions to the functional groups of the cell surface is the main step of most bioremediation strategies. In this area, some works have reached encouraging results when dealing with low pH. For example the work carried out by Bankar et al. [21] in which the yeasts *Yarrowia lipolytica* was able to capture Cr^{6+} at pH 1. Also, many authors have suggested that microorganisms inhabiting extreme environments would be more suitable for bioremediation porpoises [20, 22–31].

The survey of microorganisms that inhabit acidic environments represents an important opportunity to overcome this limitation. The physiological versatility displayed by yeasts and its presence in acidic aquatic environments, grants them a decisive position in the development of this technology. Since yeasts are complex eukaryotic microorganisms that can thrive in extreme environments, much attention has been focused on them in the past years [19, 26, 32–34].

In the present work, we describe the capabilities of yeast species isolated from the Río Agrio – Lake Caviahue (RAC) acidic aquatic environment, a volcanic origin environment, to tolerate metals and determine the minimal inhibitory concentration for growth for Cd^{2+}, Cu^{2+}, Co^{2+}; Li^{+}, Ni^{2+}, and Zn^{2+}. We focus on three species with relevant characteristics to the environment and we also describe their ability to capture different metal ions in acidic conditions which are usually present in many industrial and mine waste effluents.

Materials and methods

Yeast strains and isolation medium

Pure cultures of 165 isolates corresponding to 33 yeasts species were studied. Of these, 91 strains were obtained and identified as described by Russo et al. [19]. The remaining 74 were isolated using two selective approaches: Río Agrio – Lake Caviahue (RAC) water selection (36 isolates) and metal enriched medium (38 isolates).

For the RAC water selection, 45 ml water samples from three distinctive sampling sites were sterilized throughout filtration by using 0.22 μm nitrocellulose Millipore filters (Millipore, Bedfore, MA) and collected into sterile 250 ml Erlenmeyer flasks. Water used for this experiment belonged to the source of Upper Río Agrio (URAs; pH 1.24), the Upper Río Agrio before it enters in the Lake Caviahue (URA; pH 2.80), and the Lake Caviahue surface water (LC; pH 3.00). Before inoculation, all sterile water samples were added with 5 ml 10-fold concentrate Malt Yeast Peptone (MYP) broth ($g\,L^{-1}$: malt extract 70.0; yeast extract 5.0; peptone-soytone 25.0) as nutrient source.

For inocula filters, homogenized 1200 ml of URA water was speared in three equal volumes of 400 ml and filtered with 0.45 μm nitrocellulose Millipore® filter by means of a Nalgene® device and each filter were used to inoculate Erlenmeyer flasks. This procedure ensures that all Erlenmeyer flasks were inoculated with the same inoculum, hence any difference in the variety of isolated yeasts depended exclusively on the effect of the physicochemical conditions of URAs, URA, and LC water. Incubation took place at 20 °C with orbital shaking (150 rpm) for 120 h. One hundred microliter samples were spread into solid MYP3 medium (see Supplementary Information for culture medium preparation). Plates were incubated for 72 h at 20 °C and 96 h at 4 °C. Single colony morphotypes were isolated and purified for further identification.

For metal enriched medium, homogenized 1200 ml of water from the URA was speared in three equal volumes of 400 ml and filtered as mentioned before. Filters were used to inoculate Erlenmeyer flasks containing MYP3 medium added with volumes of concentrated metal solutions in order to achieve defined metal concentrations. Metal used for this experiments are depicted in Table 1. Erlenmeyer flasks were added with the lower metal concentration and incubated at 20 °C with orbital shaking (140 rpm) for 120 h. One hundred microliter samples were spread into solid MYP3 medium. Plates were then incubated as mentioned before. An additional 100 μl sample was used to inoculate an Erlenmeyer flask

Table 1. Heavy metals and ion concentration used for metal selection culture medium and agar diffusion assay.

		Selective medium		Agar diffusion assay	
Metal salt	Metal ion	1° step ($g\,L^{-1}$)	2° step ($g\,L^{-1}$)	Ion concentration ($g\,L^{-1}$)[a]	Ion concentration ($g\,L^{-1}$)[b]
$CdSO_4 \cdot H_2O$	Cd^{2+}	0.06	0.77	6.0	7.0
$CoSO_4 \cdot 7H_2O$	Co^{2+}	0.06	0.94	4.0	5.0
$CuSO_4 \cdot 5H_2O$	Cu^{2+}	0.04	0.20	1.5	2.0
$FeSO_4 \cdot 7H_2O$	Fe^{2+}	N/A	N/A	N/A	2.0
$Li_2SO_4 \cdot H_2O$	Li^{+}	0.65	3.12	6.5	9.0
$NiSO_4 \cdot 6H_2O$	Ni^{2+}	0.40	0.56	0.9	1.5
$ZnSO_4 \cdot 7H_2O$	Zn^{2+}	0.80	3.80	7.0	7.5

N/A, not assayed.
[a]Concentration used for all strains except *Cryptococcus* sp. 2.
[b]Concentration used for *Cryptococcus* sp. 2.

with fresh culture medium with the higher metal concentration. Incubation was carried out in the same conditions as above mentioned for 120 h and 100 μl samples were spread in solid MYP3 medium and incubated as above mentioned. All experiments were carried out in triplicate.

Physiological and molecular yeast identification

Morphological characteristics and physiological tests were performed according to Yarrow [35]. DNA was extracted from yeast biomass according to Libkind et al. [36]. The mini/microsatellite primed-PCR fingerprinting technique (MSP-PCR) was used according to Libkind et al. [36] using the minisatellite primer M13 and the synthetic microsatellite oligonucleotide $(GTG)_5$. For sequence analysis, DNA was amplified using NL1 and NL4 primers. Cycle sequencing of the region D1/D2 at the 5′ end of the 26 S rRNA gene domain was performed following Libkind et al. [36]. The sequences obtained were aligned with those included in the GenBank database with the Basic Local Alignment Search Tool (BLAST at http://www.ncbi.nlm.nih.gov) [37].

Qualitative and quantitative screening of heavy metal resistance

Tolerance was qualitative assessed in an agar diffusion assay [32, 38] for 165 strains. Briefly, solid yeasts nitrogen base added with 1% glucose (YNBG) plates were prepared. A central ditch (length/width/depth) 70/5/3 mm was made with a sterile spatula. Yeast cultures were inoculated in lines extending from the ditch in perpendicular angle. *Xanthophyllomyces dendrorhous* strain CRUB 1511 was included for comparative purposes. Once strains were inoculated, the ditch was filled with 900 μl of a concentrated metal solution (Table 1). For *Cryptococcus* sp. 2, assays were performed on MYP3 medium with higher metal concentration. Instead of using Hassen et al. [38] inhibition criterion, an arbitrary distance parameter was proposed in order to determine tolerance levels upon tested metal concentrations. Strains were qualified in three levels depending on the distance at which colony grew from the ditch: strongly tolerant (between 0 and 2.5 mm), tolerant (between 2.5 and 5 mm), and sensitive (more than 5 mm).

Minimal inhibitory concentration (MIC) for growth was measured for seven of the most tolerant strains in microtiter plates [39, 40] containing YNBG broth. Strains selected were *Cryprococcus agrionensis* CRUB 1310^T, *Cryptococcus* sp. 2 CRUB 1564, *Cryptococcus* sp. 6 CRUB 1638, *Coniochaeta fodinicola* CRUB 1361, *Rhodotorula taiwanensis* CRUB 1414, *Rhodotorula mucilaginosa* CRUB 1441, and *Rhodosporidium toruloides* CRUB 1401. Microtiter plate containing known metal concentration (Table 2) in Yeast Nitrogen Base (Difco) and glucose ($20\,g\,L^{-1}$) were inoculate with 1 μl of a cell suspension with an optical density of 0.9 at 620 ηm (OD_{620}). Microtiter plates were placed in an automated microtiter plate reader (Bioscreen C MBR, GrowthCurves) and incubated at 22 °C with continuous shaking. Yeasts growth was evaluated by OD measuring in a range of 450–620 ηm, every 2 h for a period of 120 h. Growth was considered positive when OD measures were equal or superior to 0.5. Positive control (culture medium without metals) and negative

Table 2. Metal ion concentrations used for MIC assays.

	Metal ion					
	Cd^{2+}	Co^{2+}	Cu^{2+}	Li^{+}	Ni^{2+}	Zn^{2+}
Concentration ($g\,L^{-1}$)	3.10	0.94	1.72	6.25	1.13	7.50
	1.55	0.47	0.86	3.13	0.56	3.75
	0.78	0.24	0.43	1.56	0.28	1.88
	0.39	0.12	0.22	0.78	0.14	0.94
	0.19	0.06	0.11	0.39	0.07	0.47

control (culture medium with metal and non-inoculum) were added in all cases. All experiences were performed in triplicate for each strain.

Selection of metal concentration and metal capture assays

In order to assess the best metal concentration for capture assays, *C. agrionensis* CRUB 1310^T, *Cryptococcus* sp. 2 CRUB 1564, and *C. fodinicola* CRUB 1361 strains were cultured in test tubes containing 5 ml YNBG broth with different metal concentrations (Supporting Information Table S1). Metal concentration tested for this assays were based on MIC concentration determined previously. The metals used for these experiments were Cd^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+}. The selection of these metals was based on their presence in the Río Agrio – Lake Caviahue environment and the potential environmental toxicity [19, 34]. Culture medium were inoculated with 100 µl of a cell suspension ($OD_{620} = 0.5$) and incubated for 120 h in an Innova 4000 Shaker (New Brunswick) at 20 °C with orbital shaking (160 rpm). Growth was considered positive if OD_{620} values were higher than 1.0.

Metal capture assays were performed in YNBG broth in Erlenmeyer flasks. Culture medium was prepared with 9 ml 10-fold concentrated YNBG, and 76.5 ml of sterile distilled water with pH 2.5 (adjusted with 5 N sulphuric acid added after sterilization). Flasks were inoculated with 4.5 ml of a cell suspension ($OD_{620} = 0.5$) and incubated at 20 °C with orbital shaking (160 rpm) for 72 h or until the culture reached an OD_{620} value of 1.0. Once growth was achieved, a metal solution with known concentration was added and, immediately afterwards, 5, 2, and 0.2 ml samples were taken. The 5 ml samples were used to determine the exact metal concentration in the flask at the beginning of the experiment (named T_0). The 2 ml samples were collected to measure dry weight and pH, and 0.2 ml samples were collected to measure OD. After metal additions flasks were incubated as detailed before for 72 h and new 5, 2, and 0.2 ml samples were collected.

Metal content was measured in cell-free supernatants, obtained by centrifugation of the 5 ml samples at 1500*g* for 15 min and recovery of the liquid phase in new pretreated tubes (washed with nitric acid 0.1 N, then washed 10 times with sterile deionized water and dried at 70 °C for 72 h). Supernatants were stored at 4 °C until atomic absorption spectroscopy was analyzed with a Shimadzu AA6500 (Shimadzu Co., Kyoto, Japan). The final dry cell biomass was obtained by centrifugation at 1500*g* for 5 min and drying at 105 °C until constant weight was established. pH of supernatant was measured with a pH-meter.

Results

Yeasts isolation and identification using selective strategies

When water from the environment was used 36 yeast strains were isolated, 27 of them being isolated with water from upper Río Agrio (URA), 8 with water from Lake Caviahue (LC), and only 1 with water from upper Río Agrio source (URAs). The 36 yeast strains were grouped by conventional and molecular techniques in 12 groups. A total of 12 species were identified, 74% of which corresponded to basidiomycetous yeasts (Supporting Information Table S2). A clear effect of the water of the environment in the variety of the isolated yeasts was observed, since all culture medium were inoculated with the same inoculum.

When metals were added to the culture medium 38 isolates were obtained and assorted into five species: *C. agrionensis* (Cd^{2+} 0.06 g L^{-1}; Co^{2+} 0.06 g L^{-1}, and Cu^{2+} 0.04 g L^{-1}), *Cryptococcus* sp. 6 (Cu^{2+} 0.20 g L^{-1}), *C. huempii* (Li^{+} 0.65 g L^{-1}), *C. fodinicola* (Co^{2+} 0.94 g L^{-1}, Li^{+} 3.12 g L^{-1}, Ni^{2+} 0.40 and 0.56 g L^{-1}, and Zn^{2+} 0.80 and 3.80 g L^{-1}), and *R. taiwanensis* (Cd^{2+} 0.06 g L^{-1}; Co^{2+} 0.06 g L^{-1}, and Cu^{2+} 0.04 and 0.20 g L^{-1}).

Yeasts tolerance to metals in solid culture medium and MIC results

For qualitative assays, of the 33 yeasts species tested, 6 species showed a remarkable tolerance to most of the assayed metals (Supporting Information Table S1). *Cryptococcus* sp. 2 strains were the most tolerant since they have no inhibition against any metal. *Cryptococcus* sp. 6 also showed a high tolerance followed by *C. agrionensis*, with some variability between strains, but the majority of them were highly tolerant to metal contact, except Co^{2+}. *C. fodinicola* strains showed more consistent results, except for Co^{2+} and Zn^{2+}. *R. mucilaginosa* and *R. taiwanensis* showed similar results. *Rhodosporidium t.* showed the lowest tolerance levels. The most tolerant strains of each species (*C. agrionensis* CRUB 1310^T, *Cryptococcus* sp. 2 CRUB 1564, *Cryptococcus* sp. 6 CRUB 1638, *C. fodinicola* CRUB 1631, *R. mucilaginosa* CRUB 1441, *R. taiwanensis* CRUB 1414, and *R. toruloides* CRUB 1401) were selected for MIC assays.

Minimal inhibitory concentration (MIC) for growth obtained is depicted in Table 3. All strains showed higher MIC values in comparison with those obtained by other researchers. Another interesting point worth highlighting is that MIC values were significantly lower than inhibitory metal concentrations obtained in solid culture medium.

Table 3. Qualitative tolerance and MIC ($g\,L^{-1}$) for most tolerant yeast.

Species	Strain	Cd^{2+}		Co^{2+}		Cu^{2+}		Li^{+}		Ni^{2+}		Zn^{2+}	
C. agrionensis	CRUB 1310T	++	**0.78**	++	0.47	++	0.22	++	**>6.25**	+	0.07	++	**>7.50**
Cryptococcus sp. 2	CRUB 1564	++	0.19	++	**>0.94**	++	0.22	++	3.13	++	**0.56**	++	1.88
Cryptococcus sp. 6	CRUB 1638	++	0.19	++	<0.06	++	0.22	++	1.56	++	0.07	++	<0.47
C. fodinicola	CRUB 1631	–	<0.19	++	**>0.94**	++	**>1.72**	++	3.13	++	**0.56**	++	3.75
R. taiwanensis	CRUB 1414	++	**0.78**	++	0.24	++	0.11	++	1.56	+	**0.56**	+	1.88
R. mucilaginosa	CRUB 1441	++	**0.78**	++	0.12	++	0.11	++	1.56	++	<0.07	–	3.75
R. toruloides	CRUB 1401	++	**0.78**	++	**0.47**	++	<0.11	++	3.13	++	**0.56**	+	0.94
X. dendrorhous[a]	CRUB 1511	–	nd	–	nd	–	nd	+	nd	–	nd	–	nd
E. coli[b]		nd	0.06	nd	0.06	nd	0.06	nd	nd	nd	0.06	nd	0.10
P. anomala[c]	CCY 38-1-30/31	nd	0.56	nd	nd	nd	0.32	nd	nd	nd	0.12	nd	1.31
Sp. salmonicolor[c]	CCY 19-4-6/9	nd	0.22	nd	nd	nd	0.13	nd	nd	nd	0.06	nd	0.07

nd, not determined.
Highest MIC values are highlighted in bold.
[a]*X. dendrorhous* was assayed by triplicate for each metal ion.
[b]Adapted from Nies [23].
[c]Adapted from Vadkertiová and Sláviková [51].

Because of their high tolerance and MIC values obtained, *C. agrionensis* CRUB 1310^{T}, *Cryptococcus* sp. 2 CRUB 1564, and *C. fodinicola* CRUB 1631 were selected for further analysis on their capability to capture metals in acidic culture medium.

Metal capture assays in acidified YNBG medium

After 120 h of incubation *C. agrionensis* CRUB 1310^{T} was able to grow only with Cu^{2+} at $0.22\,g\,L^{-1}$. *Cyptococcus* sp. 2 CRUB 1564 was able to grow with Cd^{2+} ($0.19\,g\,L^{-1}$), Cu^{2+} ($0.22\,g\,L^{-1}$); Ni^{2+} ($0.15\,g\,L^{-1}$), and Zn^{2+} ($6.50\,g\,L^{-1}$). *C. fodinicola* was able to grow with Cu^{2+} ($1.72\,g\,L^{-1}$), Ni^{2+} ($0.15\,g\,L^{-1}$), and Zn^{2+} ($6.50\,g\,L^{-1}$). The results obtained in this assay were used as guidelines for the capture assays.

All strains were able to capture soluble metal ions in the acidified culture medium. The pH of the culture medium remained at 2.5 throughout the assay, thus yeasts metabolisms did not change this condition. Since no metal precipitation was observed in the culture medium at all time, any decrease in the concentration of the metals could only be explained by capture (both sorption and/or uptake) mediated by yeast biomass. *C. agrionensis* CRUB 1310^{T} was able to capture 15.80 mg Cu^{2+} $(g\,biomass)^{-1}$ (Fig. 1). *Cryptococcus* sp. 2 CRUB 1564 was able to capture 36.25 and 65.28 mg $(g\,biomass)^{-1}$ of Ni^{2+} and Zn^{2+}, respectively. Despite the ability to grow in culture medium supplemented with Cd^{2+}, this strain was unable to capture this metal, hence being able to tolerate it but not interact with it. *C. fodinicola* CRUB 1631 was able to capture only Zn^{2+} up to 67.11 mg $(g\,biomass)^{-1}$, but was unable to capture neither Cu^{2+} nor Ni^{2+}. The capability of these yeasts to tolerate metals is a clear mechanism against toxicity.

Discussion

The low ascomycetous percentage obtained here was higher in comparison to previous data from the Río Agrio – Lake Caviahue (RAC) environment [19], but was similar to the results obtained by Gadanho et al. [41]. Furthermore, they registered higher number of ascomycetous yeasts when culture medium amended with water of the environment was used. Still, yeasts flora of RAC environment is dominated by basidiomycetous species, which are more nutritionally versatile than ascomycetous species [42].

The effect caused by the water of the environment was also observed by Gadanho et al. [41]. It is not surprising that the higher diversity of yeasts was found when the inoculum was exposed to water from Upper Río Agrio

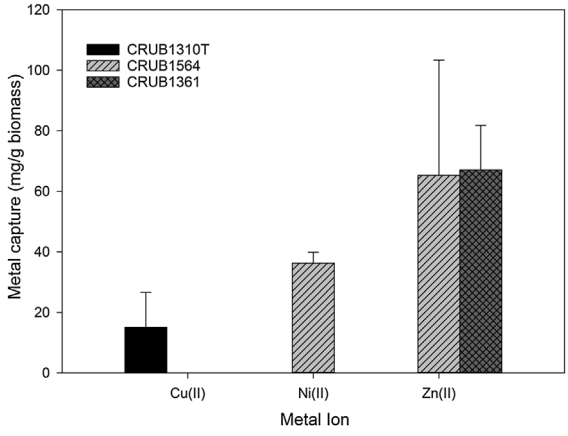

Figure 1. Metal capture (in mg $[g\,biomass]^{-1}$) for three yeast strains in acidified (pH 3) YNBG culture medium. Mean values and SD are represented.

(URA), since the filtered yeasts trapped in the filter were obtained from the URA and also most adapted species were previously isolated in that sampling site [19, 20].

Pure metals added to the culture medium exert higher selective effect in comparison to water from the environment. Although water from the environment has a mixture of metals, their concentration are not as high as those used in this experiment [34] and it also may have other compounds that could enhance yeast growth (as nutrients, vitamins, or amino acid). Nevertheless, the aim of this assay was to obtain metal-tolerant yeast strains and the low diversity obtained was initially promising for the isolation of putative metal-tolerant yeasts species. In concordance with other researches [4, 38, 43–45] observations, cadmium exerted the highest toxicity.

When using the metal enrichment technique, some yeast species were isolated when high metal concentration was applied, but not when low metal concentration was applied. This was the case of *Cryptococcus* sp. 6, which was isolated culture medium added with $0.20\,g\,L^{-1}$ copper but not in culture medium added with $0.04\,g\,L^{-1}$ of the same metal ion. This can be explained by the similar morphology between this species and *C. agrionensis*, which was isolated from the lower copper concentration. It is very likely that *Cryptococcus* sp. 6 was always present, but less abundant in comparison with *C. agrionensis*. Also, in the case of *C. fodinicola*, this species was isolated only in the higher concentrations of cobalt ($0.94\,g\,L^{-1}$) and lithium ($3.12\,g\,L^{-1}$). This species grows slowly, and after 120 h of incubation it remains as punctual colonies, making it difficult to isolate. Nevertheless, since it grows in the higher concentrations of metals, the species seems to be highly tolerant to them.

Regarding to metal toxicity, interestingly, almost all studied strains were highly tolerant to Cu^{2+}, Li^{+}, and Zn^{2+}, indicating a possible adaptation to the presence of these particular metal ions. Comparing tolerance to metals, yeast species could be ordered as follows (from most to least tolerant): *Cryptococcus* sp. 6 > *Cryptococcus* sp. 2 > *C. agrionensis* > *C. fodinicola* > *R. mucilaginosa* ≥ *R. taiwanensis* >> *R. toruloides*.

When exposed to Cu^{2+}, *C. agrionensis* colonies acquired brownish pigmentation, different from the pale pink color expected for this species [46]. This change of color was extremely suggestive, since similar changes were observed when Cu^{2+} was included by metallothineins into cytoplasm in other yeasts species [47]. This implies that *C. agrionensis* might be able to interact with Cu^{2+} ions, capturing them in the biomass. Further studies should be carried put in order to elucidate the precise interaction between this yeast species and copper.

Differences observed in yeasts tolerance between solid and liquid culture media can be easily explained since contact between cells and metals is higher in liquid, rather than in solid media. Similar results were observed by Villegas et al. [32], where the difference in tolerated concentrations reached about 10-fold greater in solid media when comparing to liquid media. Minimal inhibitory concentration (MIC) for growth values obtained were in concordance to qualitative tolerance obtained previously, with the clear exception of *R. toruloides*, which was the less tolerant yeasts species when diffusion assays were employed. Cd^{2+} and Ni^{2+} were the most toxic metals tested, followed by Cu^{2+} and Co^{2+}, whereas Li^{+} and Zn^{2+} showed lower toxicity. *C. fodinicola* CRUB 1631 strain showed an outstandingly high tolerance to Cu^{2+}, higher than tested concentration. Similar results were obtained for *Cryptococcus* sp. 2 CRUB 1564 and *C. fodinicola* CRUB 1631 in Co^{2+}, and for *C. agrionensis* CRUB 1310^{T} in Li^{+} and Zn^{2+}.

This latter species proved to be particularly interesting given its phylogenetic relationship to three *Cryptococcus* species isolated from an extreme acidic aquatic environment of Portugal [33, 41, 46]. This phylogenetic relationship indicates that all four species might have evolved to thrive in such acidic environment. Also, *C. agrionensis* is strongly related to Río Agrio – Lake Caviahue environment, since almost all isolates belonged to the upper Río Agrio [19].

C. fodinicola was also isolated at S. Domingos mine, Portugal (named *Lecythophora* sp. MSD 302 and 270 strains, 99% similarity in D1/D2 region) and at Ranger Uranium Mine, Australia. These strains were isolated from the most extreme conditions in all Portuguese, Australian, and Argentinean acidic aquatic environments ([41, 48] and the present work). Strains isolated from RAC were only isolated at a pH 2.2 when metals were added to culture medium. This result shows concordance with those obtained by Vázquez-Campos et al. [48] whose isolates were obtained at low pH (1.7–1.8) and high metal concentration. Portuguese isolates proved to have extremely high tolerance to Cu^{2+}, being able to tolerate up to $24.8\,g\,L^{-1}$ (Dr. Gadanho pers. com.). Similar trends were also observed in *Cryptococcus musci* isolated from leaves in heavy metal polluted environments [49], which was able to tolerate up to $13.075\,g\,L^{-1}$ of cadmium. These strategies of high tolerance to metals might be directly related to an evolutionary response of the microorganisms in order to protect it from the toxic agent, thus preventing it to enter in the cytoplasm of the cell. Nevertheless, studding its capacity to capture this particular heavy metal is highly interesting.

Cryptococcus sp. 2 seems to have evolved to survive only in these acidic environments, since the growth of this yeast is strongly reduced when culture medium pH rise above 3. This same species was also isolated from S. Domingos mines in Achada do Gamo sampling site [41], showing the same remarkable characteristics. The physiological characteristics of this particular species represent a clear proof that *Cryptococcus* sp. 2 is an autochthonous yeast species of acidic aquatic environments and the first acidophilic yeast isolated. Also, the growth of this yeasts species seems to be augmented by the presence of Fe^{2+} (unpublished data). The high tolerance to metals displayed by all strains of *Cryptococcus* sp. 2 suggested us an evolved metabolism to deal with metals.

All three species showed remarkable physiological characteristics. Many authors suggested that microorganisms thriving in extreme environments are excellent candidates for biotechnological applications [20, 22–31]. Physiological adaptation to acidic conditions grants them great potential in bioremediation of metals. Autochthonous yeasts from RAC acidic aquatic system must have evolved to tolerate the extreme conditions of this environment, including the presence of metals. The results obtained this far for these species indicate a great potential for bioremediation purposes using live biomass.

The main restriction to the possible application of bioremediation as in situ technology is the dependence of this process with pH. Many works have dealt with this problem, concluding that pH should be adjusted between 4 and 7 in order to be able to apply bioremediation strategies without metal precipitation (due to alkalinity) or re-mobilization (due to acidity) [6, 12, 18, 21]. This represents a major problem since the main source of metals in aquatic environments proceeds from acidic origins (e.g., acid rock drainages, acid mine drainages, metal industries, geothermal waters, etc.). In this work, three yeast species able to capture metal in acidic conditions (pH = 2.5) are described. The potential application of these yeasts in bioremediation processes at acidic conditions is promising.

An important factor to be noticed is that all yeast strains herein tested were highly tolerant to metals. Live biomass was chosen for these experiments since metabolic processes involving bioremediation are more versatile than simple adsorption to cell surface. But this poses some difficulties. One of the main problems is the tolerance of the yeasts to metal concentrations as they are found in the polluted environments to be treated. If metal concentration exerts levels of toxicity that alters yeast's growth, they will not be able to capture metals by active metabolism. Thus tolerant strains should be selected. But many authors found that tolerant microorganisms are less able to capture metals since they may evolve means to isolate from the toxic compounds [12, 44]. This could be the case of Cd^{2+}, since all yeast strains tested in this work were able to tolerate this metal, but none was able to capture it. Cd^{2+} is a particularly complex metal since it has no biological known function and is highly toxic to microorganisms. Cd^{2+} associated metabolic pathways are well known, mainly in *Saccharomyces cerevisiae*, thus tolerance can be well explained [50]. On the other hand, researchers proved that adapted yeasts could be more effective in capturing metals. de Silóniz et al. [25] proved that a strain of *Pichia guilliermondii* adapted to Cu^{2+} was able to capture more Cu^{2+} than the non-adapted strain. It seems that metal toxicity/necessity should be taken into consideration when choosing a tolerant strain for certain metals.

It is worth noting that in our work, metal capture was not necessarily related to metal tolerance obtained previously. For example, even though *C. fodinicola* CRUB1631 was the most tolerant to Cu^{2+} (more than 1.72 g L^{-1}), *C. agrionensis* CRUB1310^T was able to capture this metal having a lower metal tolerance (0.22 g L^{-1}). This result seems to be in accordance to other works previously cited which indicates that tolerance is opposite to capture ability.

The results obtained this far show evidences that autochthonous acidic aquatic environment yeasts can be proposed for bioremediation techniques. Eccles [5] stated that the selection of bioremediation strategies instead of classical physicochemical remediation should be based on stronger foundations than costs alone. Further studies should be carried out with these yeast species in order to be able to assess the applicable potential of them in bioremediation processes.

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Conflict of interest

All authors declare no conflicts of interest.

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