Biometals (2008) 21:591–600
DOI 10.1007/s10534-008-9145-8

Chromate removal by yeasts isolated from sediments of a tanning factory and a mine site in Argentina

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Received: 30 May 2007 / Accepted: 17 May 2008 / Published online: 5 June 2008

Abstract Twenty-one yeast-like microorganisms were isolated from tannery effluents and from a nickel–copper mine in Argentina. They were tested for their Cu(II), Ni(II), Cd(II) and Cr(VI) tolerance in qualitative assays on solid medium. Three isolates were selected for their multiple tolerance to the different heavy metals and highest tolerance to Cr(VI). According to morphological and physiological analysis and 26S rDNA D1/D2 domain sequences the isolates were characterized as: *Lecythophora* sp. NGV-1, *Candida* sp. NGV-9 and *Aureobasidium pullulans* VR-8. Resistance of the three strains to high Cr(VI) concentrations and their ability to remove Cr(VI) were assessed using YNB-glucose medium supplemented with 0.5 and 1 mM Cr(VI). Chromate removal activity was estimated by measuring remaining Cr(VI) concentration in the supernatant using the colorimetric 1,5-diphenylcarbazide method and total chromium was determined by flame atomic absorption spectroscopy. The results indicate that the initial Cr(VI) concentration negatively influenced growth and the specific growth rate but stimulated the metabolic activity of the three strains; resistance to Cr(VI) by these strains was mainly due to reduction of Cr(VI) rather than chromium bioaccumulation. This study showed the potential ability of these strains as tools for bioremediation of Cr(VI) from contaminated sites.

Keywords Bioremediation · Cr(VI) removal · Cr(VI) tolerant yeasts

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Introduction

Contamination by chromium compounds is caused by a variety of industrial applications eventually leading to heavy pollution of soil, surface water and atmosphere. Major sources of chromium contamination include the metal finishing industry, petroleum refineries, iron and steel industries, textile dyeing and leather tanning. In nature the most common and stable oxidation states of this element are Cr(VI) and Cr(III) (Barceloux 1999; Iyer et al. 2004). Cr(III) is an essential trace element well known for its particular role in the maintenance of normal carbohydrate

metabolism in mammals and yeasts (Debski et al. 2004). It has also been suggested that this ion is involved in the tertiary structure of proteins and the conformation of cell RNA and DNA (Gulan Zetic et al. 2001; Zayed and Terry 2003). However, at high concentrations, Cr(III) showed negative effects on cellular structures. In contrast, Cr(VI) is always toxic to living organisms causing allergies, eczema, irritations and respiratory track disorders, and it is a strong mutagenic and carcinogenic agent. This toxic action is due to the fact that Cr(VI) complexes can easily penetrate cellular membranes and undergo immediate reduction reactions leading to the formation of various reactive intermediates. These intermediates are harmful to cell organelles, proteins and nucleic acids (Raspor et al. 2000; Cervantes et al. 2001; Plaper et al. 2002). In vivo Cr(VI) toxicity is 1,000-fold more cytotoxic than Cr(III) (Biedermann and Landolph 1990; Barceloux 1999). Apart from its toxicity, Cr(VI) is highly soluble and thus mobile and biologically available in the ecosystems. In contrast, Cr(III) has a high affinity for organic compounds resulting in the formation of complexes that precipitate as amorphous hydroxides (Palmer and Wittbrodt 1991).

Argentina is an important producer of leather in the world. Chromium sulphate [Cr(III)] is used as a tanning agent and transformed into Cr(VI), which results in severe contamination in the environs of tanneries. Cr(VI) removal from water bodies and wastewater is necessary to avoid environmental pollution. Traditional technologies such as chemical reduction and subsequent precipitation, ion exchange or reverse osmosis are expensive and only limited removal is achieved (Silóniz et al. 2002). As a result, new techniques are required to reduce Cr(VI) concentrations to acceptable environmental levels at low cost. This problem has inspired studies on metal decontamination using microorganisms and biological methods as an alternative to conventional methods due to their eco-friendly nature.

Cr(VI) removal or uptake by yeasts is gaining much attention since these eukaryotic microorganisms have proved to be useful in biotechnological processes (Kaszycki et al. 2004; Pas et al. 2004; Ramírez-Ramírez et al. 2004; Ksheminska et al. 2005). The aim of this work was to describe procedures for the isolation and selection of Cr(VI) tolerant yeasts and to study their ability to remove chromate from a synthetic culture medium.

Materials and methods

Sampling and isolation

Sediment samples were aseptically collected from wastewater sediment from tannery ponds, located in Nonogasta, La Rioja province, Argentina, and sediment from a nickel–copper mine located in Virorco, San Luis province, Argentina. Samples of 20–30 g were randomly selected, transported in sterile plastic bags, kept at 4°C until use and processed as described previously by Villegas et al. (2005). Micromorphology and colonial features were examined and reproductive forms were tested in different media to determine whether the isolates belonged to the same group.

Pure cultures were maintained on YEPD agar slants containing (in g l^{-1}): glucose, 20; yeast extract, 10; peptone, 10; and agar, 15. Slants were incubated at 30°C for 24–48 h, subsequently stored at 4°C and sub-cultured at regular intervals.

Qualitative screening on solid medium for heavy metal tolerance

Heavy metal tolerance of the isolates was tested using the agar diffusion method. Heavy metal solutions were poured into ditches according to Villegas et al. (2004). In order to avoid possible interactions of heavy metal compounds with complex components of the medium, yeast nitrogen base (YNB) without amino acids was used as culture medium. YNB-glucose agar contained (in g l^{-1}): yeast nitrogen base without amino acids (DIFCO), 6.7; glucose, 20; and agar, 15. Experiments were performed separately with the following heavy metals: Cu(II) as $CuSO_4$, Ni(II) as $NiSO_4$, Cd(II) as $CdCl_2$ and Cr(VI) as $K_2Cr_2O_7$ at different concentrations (1–10 mM). All heavy metal solutions were prepared in bi-distilled water and filter-sterilized. Microbial growth was used as a qualitative parameter of heavy metal tolerance. Microorganisms that grew up to the ditch were considered tolerant to the concentration analysed, while no growth was considered sensitive. The growth inhibition halo around the ditch gave an estimate of the degree of tolerance in cases in which no complete tolerance was observed. Sterile distilled water was used as control and *Saccharomyces cerevisiae* ATCC 32051 was used as reference strain.

Semi-quantitative assays of Cr(VI) tolerance

Sensitivity towards Cr(VI) was tested by agar diffusion assays using wells filled with 50 µl of sterile Cr(VI) solutions at different concentrations (10–100 mM of Cr(VI) as $K_2Cr_2O_7$) according to the method described by Villegas et al. (2004). In order to avoid residual growth, cells were starved in sterile distilled water during 4 h prior to inoculation. The diameter of the growth inhibition was measured after incubation at 30°C during 72 h. An inhibition zone larger than 10 mm in diameter was arbitrarily considered as a metal-sensitive response (i.e. the lawn yeast was sensitive), whereas inhibition zones of tolerant yeasts were 10 mm or smaller.

A water-saturated well was used as control and *S. cerevisiae* ATCC 32051 as reference strain.

Characterization of selected isolates

Selected isolates were characterized by traditional physiological and biochemical criteria described by Yarrow (1998) and *S. cerevisiae* ATCC 32051 was included as reference strain in all tests. Molecular characterization, DNA sequence analysis of the D1/D2 region of the 26S rRNA gene, using NL1 (GCATATCAATAAGCGGAGGAAAAG) and NL4 (GGTCCGTGTTTCAAGACGG) universal primers were also carried out (Kurtzman and Robnett 1998).

Total genomic DNA was extracted according to the physical method described by Sampaio et al. (2001). This technique is based on cellular lysis using glass beads and high temperatures favouring the rupture of the cell wall. PCR amplifications of 26S rDNA were performed in 25 µl reaction volumes using an automated GeneAmp® PCR System 9700 Thermal Cycler (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). PCR products were run on 1.5% (w/v) agarose gel electrophoresis, stained with ethidium bromide and then visualized using a Gel Doc 2000 Image Analyzer (Bio-Rad Laboratories, Hercules, CA, USA) (Sambrook et al. 1989). Nucleotide sequences were obtained with an ABI Prism 3730 Automated Sequencer. The 26S rDNA D1/D2 domain sequences obtained from the selected isolates were edited with CROMAS version 1.55. Comparison of these sequences with partial 26S rDNA sequences published in the GenBank was performed using the BLAST tool from the National Center for Biotechnology Information (NCBI) (http//:www.ncbi.nlm.nih.gov) to search similar sequences in public databases.

Quantitative Cr(VI) tolerance analysis

Quantitative (CrVI) tolerance assays were performed in 500 ml Erlenmeyer flasks containing 250 ml YNB-glucose medium separately supplemented with 0.5 and 1 mM Cr(VI). Flasks were inoculated at a final concentration of 0.01–0.02 g biomass l^{-1} (1×10^6 CFU ml^{-1}) with an active overnight pre-inoculum and incubated at 30°C on a rotary shaker at 250 rpm until a stationary phase was reached (80–100 h). The pH was buffered at 5–5.5 with 50 mM Tris–succinate (Ross and Parkin 1989). Media inoculated with each of the selected strains without Cr(VI) solution and non-inoculated medium with Cr(VI) were included as controls and incubated under identical conditions. Samples of 5 ml were taken every 4 h and centrifuged at 7,000*g* for 10 min; supernatants were kept at 4°C for residual glucose and chromium concentration analyses. Sediments were washed twice with bi-distilled water. Dry weight was determined using aluminium foil cups dried to constant weight at 80°C.

Specific growth rates (μ, h^{-1}) were calculated from the slope of the linear regression of the natural logarithm of culture biomass versus time.

Residual glucose was determined by the dinitrosalicylic acid (DNS) method described by Miller (1959) and modified as follows: 500 µl of supernatant and 750 µl of 1% DNS (dissolved in 6% NaOH) were mixed and incubated for 10 min in a boiling water bath and subsequently absorbance was recorded at 590 nm. Readings were interpolated from a standard curve prepared with a series of glucose dilutions (0–1 g l^{-1}).

Analytical chromium determinations

Chromate removal was estimated by measuring remaining Cr(VI) concentration in the supernatant during the assay. Cr(VI) was quantified by the colorimetric (540 nm) 1,5-diphenylcarbazide method (APHA 1992). Total chromium concentration in the supernatant (extracellular) and in the hot nitric acid digested cell pellets (intracellular) was determined by flame atomic absorption spectroscopy (FAAS). Total chromium in sediment samples was measured by

FAAS after hot digestion of the samples with nitric acid.

Statistical analyses

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

Results and discussion

Sampling and isolation

Total chromium concentrations were between 0.750 and 1.500 for sediment samples from the tannery ponds and between 0.008 and 0.010 mg Cr g^{-1} for nickel–copper mine sediments. The high chromium concentration in the tannery samples could be due to anthropogenic activities in the zone, as chromium compounds are used as tanning agents.

After a series of dilution and plating assays, 13 different pure isolates with colonies and cellular morphology typical to yeasts were obtained from tannery ponds and 8 from sediment samples from the nickel–copper mine. The isolates from tannery samples were labelled NGV (Nonogasta) and from mine samples VR (Virorco) followed by a serial number.

Qualitative screening for heavy metal tolerance on solid medium

Along with the reference strain the ability of the 21 isolates to tolerate four heavy metals was studied first. This assay was carried out to find out if the isolates were capable of tolerating one or more of the heavy metals studied. Figure 1 shows the percentage of the isolates that were able to grow abundantly. As can be observed, Cd(II) was the most toxic of the four heavy metals for all isolates followed by Cr(VI), Ni(II) and Cu(II) for isolates from the nickel–copper mine. Similar results were obtained with yeasts isolated from sewage sludge (Balsalobre et al. 2003) and with a yeast isolated from wastewater sediment samples collected

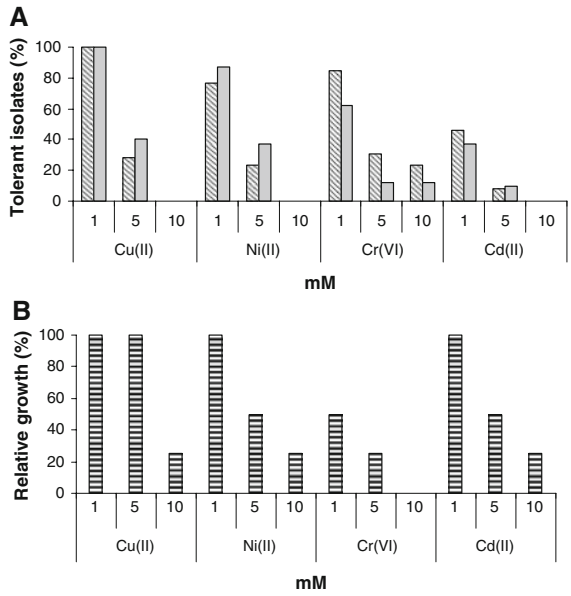

Fig. 1 Qualitative tolerance of isolates to four heavy metals, Cd(II), Cr(VI), Ni(II) and Cu(II), on solid medium: (**a**) isolates obtained from tannery ponds (▨) and from a nickel–copper mine (■). (**b**) Relative growth of *S. cerevisiae* ATCC 32051

from a copper filter at a mine plant, located in the province of Tucumán, Argentina (Villegas et al. 2005). In contrast, the isolates obtained from the tannery ponds showed highest tolerance to Cr(VI), followed by Cu(II) and Ni(II). The reference strain, *S. cerevisiae* ATCC 32051, displayed abundant growth in the presence of 1 and 5 mM Cu(II) and 1 mM Ni(II) and Cd(II) and moderate growth in the presence of 5 mM Ni(II), Cd(II) and 1 mM Cr(VI). No growth was observed at the highest concentrations (Fig. 1b). Based on abundant growth at 10 mM Cr(VI) (highly Cr(VI) tolerant) and moderate growth in the presence of the other heavy metals tested, NGV-1, NGV-9 and VR-8 were selected for further Cr(VI) tolerance assaying.

Semi-quantitative analysis of Cr(VI) tolerance

This assay is useful for comparing Cr(VI) tolerance between the selected isolates and reference strain. The increase in metal concentration with plate diffusion experiments resulted in a marked inhibition of growth. The results showed that *S. cerevisiae* ATCC 32051 was unable to grow in the presence of Cr(VI) whereas the tolerance order for the isolates

was as follows: VR-8 > NGV-1 > NGV-9 with a tolerance of 60, 50 and 30 mM Cr(VI), respectively.

Qualitative and semi-quantitative Cr(VI) tolerance assays show that the reference strain, *S. cerevisiae* ATCC 32051, was less tolerant than the isolates obtained from sediment samples. These results are coincident with those obtained by other authors, who demonstrated that yeasts isolated from sites contaminated with heavy metals were more tolerant than collection strains with similar characteristics (Ksheminska et al. 2003; Ramírez-Ramírez et al. 2004; López-Archilla et al. 2004; Srivatava and Thakur 2006). These observations suggest that natural strains develop mechanisms that allow them to adapt to stress conditions in the habitats, where they were isolated.

Characterization of selected isolates

Comparison of 26S rDNA D1/D2 domain sequences obtained from NGV-1 (GenBank DQ523176) with entries in the GenBank sequence database revealed 93.11% and 92.36% homology with *Lecythophora decumbens* CBS 153.43 (GenBank AF35397) and *L. hoffmanii* CBS 245.38 (GenBank AF353599) respectively. Macroscopic and microscopic examination of NGV-1 showed a typical *Lecythophora* morphology as previously described by Weber et al. (2002): relatively slow colonial growth with colonies initially mucoid and cream coloured becoming more leathery, pink or salmon. In young cultures cells were yeast-like and narrow hyphae separated by a septum appeared with age. Conventional and molecular tests were insufficient for identification at species level and consequently NGV-1 was assumed to correspond to the *Lecythophora* genus and named *Lecythophora* sp. NGV-1. The nearest species of NGV-9 (GenBank DQ523174) was *Pichia spartinae* CBS 6059 (GenBank U45764) with 98.5% homology. Macroscopic and microscopic characteristics of NGV-9 agreed with the description of *P. spartinae*: butyrous and white colonies with spheroidal cells found as single cells, in short chains or pseudohyphae and a thin ring was formed on the surface of assimilation media. However, the diagnosis differed from the standard description of *P. spartinae* by Kurtzman and Fell (1998) regarding the ability to assimilate L-sorbose and soluble starch and ferment sucrose after 15 days of incubation. Ascospore formation, a characteristic of this genus, was not observed. Therefore, NGV-9 was called *Candida* sp. NGV-9. Finally, VR-8 (GenBank DQ523175) showed 100% homology with *Aureobasidium pullulans* CBS 621.80 (GenBank AF361049) sequence. Traditional morphological and physiological criteria described by Kurtzman and Fell (1998) were also coincident with *A. pullulans* and consequently it was called *A. pullulans* VR-8.

These yeast and yeasts-like genera are normally found in terrestrial habitats, in the earth or on plant surfaces. Many authors have isolated these types of yeast from environments contaminated with heavy metals, e.g. *Candida* strains were isolated from tannery and industrial waste (Baldi et al. 1990; Pepi and Baldi 1992; Dönmez and Aksu 2001; Ramírez-Ramírez et al. 2004), *Lecythophora* were isolated from an environment with low pH and high heavy metal concentrations, demonstrating their ability to tolerate and grow in such extreme habitats (López-Archilla et al. 2004), whereas *A. pullulans* was isolated from a site contaminated with Pb, Cd and Zn (Bewley 1980) and from stainless steel industry effluents (Santos and Linardi 2001).

Quantitative Cr(VI) tolerance analysis

Growth inhibition of yeasts by Cr(VI) was also assessed quantitatively, analyzing the following parameters: biomass production, specific growth rate and glucose consumption. Growth in the presence of 0.5 or 1 mM Cr(VI) was compared with growth without Cr(VI) (control).

Candida sp. NGV-9 was affected most by Cr(VI) presence. At 1 mM Cr(VI) growth was poor: it reached only 10% of control biomass after 22 h and diminished afterwards (Fig. 2b$_1$). This would indicate death or cellular lyses, so this Cr(VI) concentration was considered lethal for this yeast. *A. pullulans* VR-8 growth was least affected at 0.5 mM Cr(VI) (17% less biomass than the control) but in the presence of 1 mM Cr(VI) after 100 h of incubation, growth inhibition was similar to that obtained with *Lecythophora* sp. NGV-1 after 80 h of incubation (Fig. 2a$_1$ and c$_1$, respectively).

As can be observed in Table 1, the specific growth rate (μ) decreased in relation to the control: 40% and 50% for *Lecythophora* sp. NGV-1, 50% and 60% for *Candida* sp. NGV-9 and 36% and 70% for *A. pullulans* VR-8 with increasing Cr(VI) concentration.

In contrast, specific glucose consumption rose with increasing incubation time and Cr(VI) concentration. While *Lecythophora* sp. NGV-1 consumed 14% and 21% more glucose than its control after 40 and 60 h of incubation in the presence of 0.5 and 1 mM Cr(VI) respectively (Fig. $2a_2$), glucose consumption by *Candida* sp. NGV-9 surpassed the control in 25% after 60 h of incubation at 0.5 mM Cr(VI) (Fig. $2b_2$). Moreover, specific glucose consumption by *A. pullulans* VR-8 increased 13% and 33% at 0.5 and 1 mM Cr(VI) respectively in relation to its control. Both values were reached after 20 h of incubation and then remained constant (Fig. $2c_2$).

These results indicate that the initial Cr(VI) concentration in the culture medium negatively influenced growth and the specific growth rate but stimulated glucose consumption in the three strains.

Similar growth inhibition was reported by Dursun et al. (2003) who worked with *Aspergillus niger* in the presence of 1 mM Cr(VI) but its μ, with respect to its control, was more affected by Cr(VI) than the μ observed in the current study. Moreover, Ksheminska et al. (2005) studied Cr(III) and Cr(VI) tolerance in 51 naturally occurring wild type yeast strains belonging to various systematic groups. The results indicated profound differences within the yeast

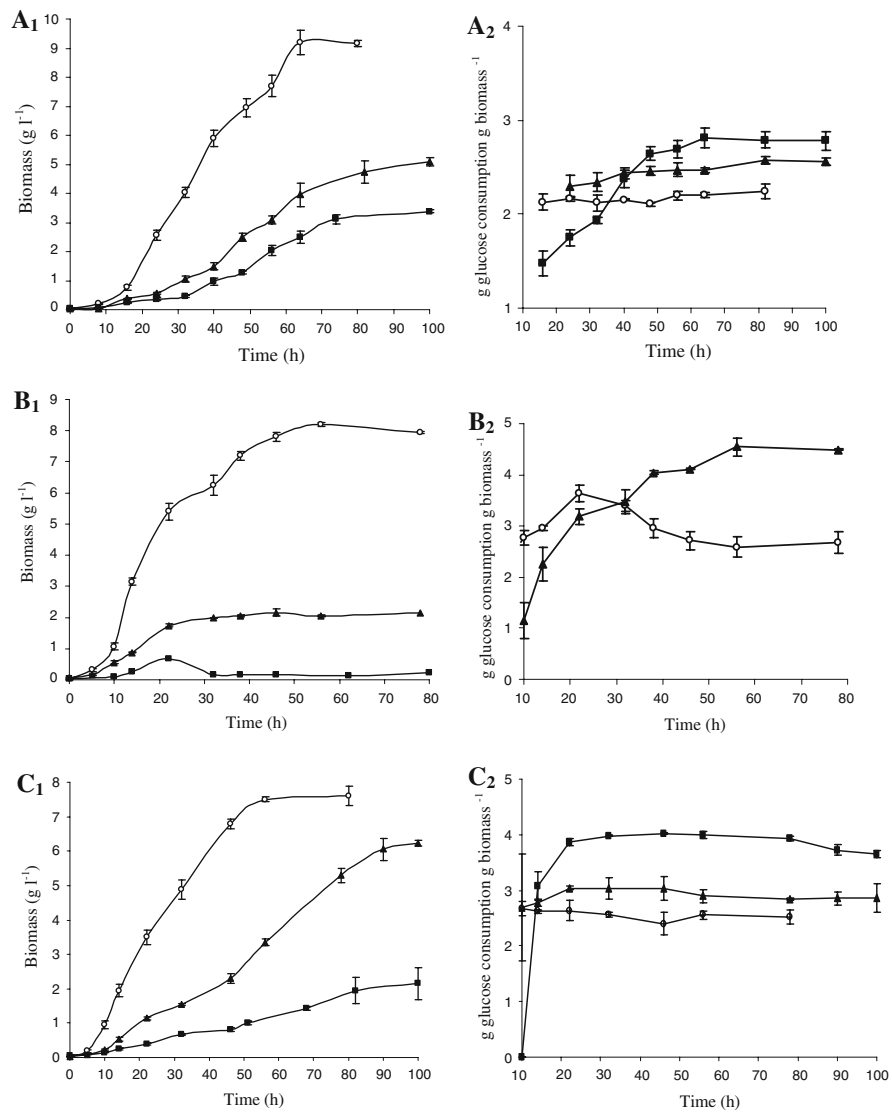

Fig. 2 Effect of different Cr(VI) concentrations on cell growth (**a_1**; **b_1** and **c_1**) and on specific glucose consumption in relation to biomass expressed as g glucose g biomass^{-1} (**a_2**; **b_2** and **c_2**) by: (**a**) *Lecythophora* sp. NGV-1; (**b**) *Candida* sp. NGV-9 and (**c**) *A. pullulans* VR-8. (○) control; (▲) 0.5; (■) 1 mM Cr(VI)

Table 1 Effect of Cr(VI) concentration on the specific growth rates of *Lecythophora* sp. NGV-1, *Candida* sp. NGV-9 and *A. pullulans* VR-8

Strain	Specific growth rates (μ h^{-1})		
	Cr(VI) concentration (mM)		
	0	0.5	1
Lecythophora sp. NGV-1	0.10 ± 0.06	0.063 ± 0.005	0.052 ± 0.008
Candida sp. NGV-9	0.30 ± 0.04	0.15 ± 0.02	0.11 ± 0.01
A. pullulans VR-8	0.31 ± 0.02	0.20 ± 0.02	0.073 ± 0.005

groups studied and this diversity was not only found among different genera but even between strains belonging to the same taxonomic group.

The results described by the authors mentioned above were similar to the current study, even though they used complex media. Laxman and More (2002) studied Cr(VI) toxicity in *Streptomyces griseus* and found that tolerance was higher in complex than in semi-synthetic medium indicating that the organic compounds of the first reduced Cr(VI) and/or inhibited its availability. In the present work YNB-glucose medium was used, which is poorer than semi-synthetic medium regarding organic compounds (glucose, peptone, yeast extract, NH_4NO_3, $MgSO_4$ and $CaCl_2$). At the same Cr(VI) concentration, *Lecythophora* sp. NGV-1 and *A. pullulans* VR-8 displayed growth inhibition values slightly lower than *S. griseus*.

Determination of chromium concentration

Cr(VI) resistance mechanisms are not well known in yeasts. Earlier studies in some species of *Candida* and *Rhodosporidium* showed that chromate resistance was related to reduced ion uptake and not to biological reduction of Cr(VI) to Cr(III) (Baldi et al. 1990; Pepi and Baldi 1992; Krauter et al. 1996). Nevertheless, more recent studies with some yeast and bacteria genera showed that Cr(VI) reduction to Cr(III) and chromium bioaccumulation are related to chromate resistance (Muter et al. 2002; Srinath et al. 2002; Ramírez-Ramírez et al. 2004; Liu et al. 2006; Tang et al. 2006; Cabrera et al. 2007). Some authors only obtained chromium accumulation with yeast or filamentous fungus strains. They found that Cr(VI) bioaccumulation depended on the physiological state of the cells, the density of the biomass, incubation time and the kind of strain used (Kaszycki et al. 2004; Ksheminska et al. 2005; Srivastava and Thakur 2006).

To determine yeast ability to remove or absorb chromium, total intra and extracellular chromium, as well as supernatant Cr(VI) were measured. At 0.5 and 1 mM Cr(VI) *Lecythophora* sp. NGV-1 diminished 90% and 55% of Cr(VI) respectively, while *Candida* sp. NGV-9 reduced 40% and only 10% Cr(VI), (Fig. 3a_1, b_1). No changes in total extra and intracellular chromium were observed by FAAS. *A. pullulans* VR-8 grown at 0.5 mM Cr(VI) removed 100% of the Cr(VI) after 45 h of incubation and 68% of total chromium after 100 h of incubation. When this strain was incubated at 1 mM Cr(VI), 60% of Cr(VI) and only 15% of total chromium were eradicated (Fig. 3c_1). This 15% was found in the pellet: 0.24 ± 0.03 and 0.37 ± 0.03 mM in the presence of 0.5 and 1 mM Cr(VI) respectively.

No spontaneous Cr(VI) reduction occurred in non-inoculated medium after 100 h of incubation indicating that the components of the medium do not reduce Cr(VI) levels.

It is important to mention that *A. pullulans* VR-8 produced exopolysaccharides (EPS) which could not be separated from the pellets by centrifugation techniques. The residual chromium could be adsorbed by EPS and/or inside the cells. Breierová et al. (2002) confirmed that some yeasts synthesize extracellular polymers with the ability to adsorb important amounts of Cd(II) to protect themselves. Studies with an *Enterobacter cloaceae* strain demonstrated that total chromium accumulation in cells was constant and independent of Cr(VI) concentrations. However production of EPS and their capacity to chelate chromium rose with increasing Cr(VI) concentrations in the culture medium (Iyer et al. 2005). Studies with yeasts reported that the amount of bound heavy metals depended on the composition and amount of EPS produced by the different strains (Mikes et al. 2005).

The results in the present work indicate that Cr(VI) resistance by *Lecythophora* sp. NGV1, *Candida* sp. NGV-9 and *A. pullulans* VR-8 was mainly due to Cr(VI) removal rather than chromium bioaccumulation.

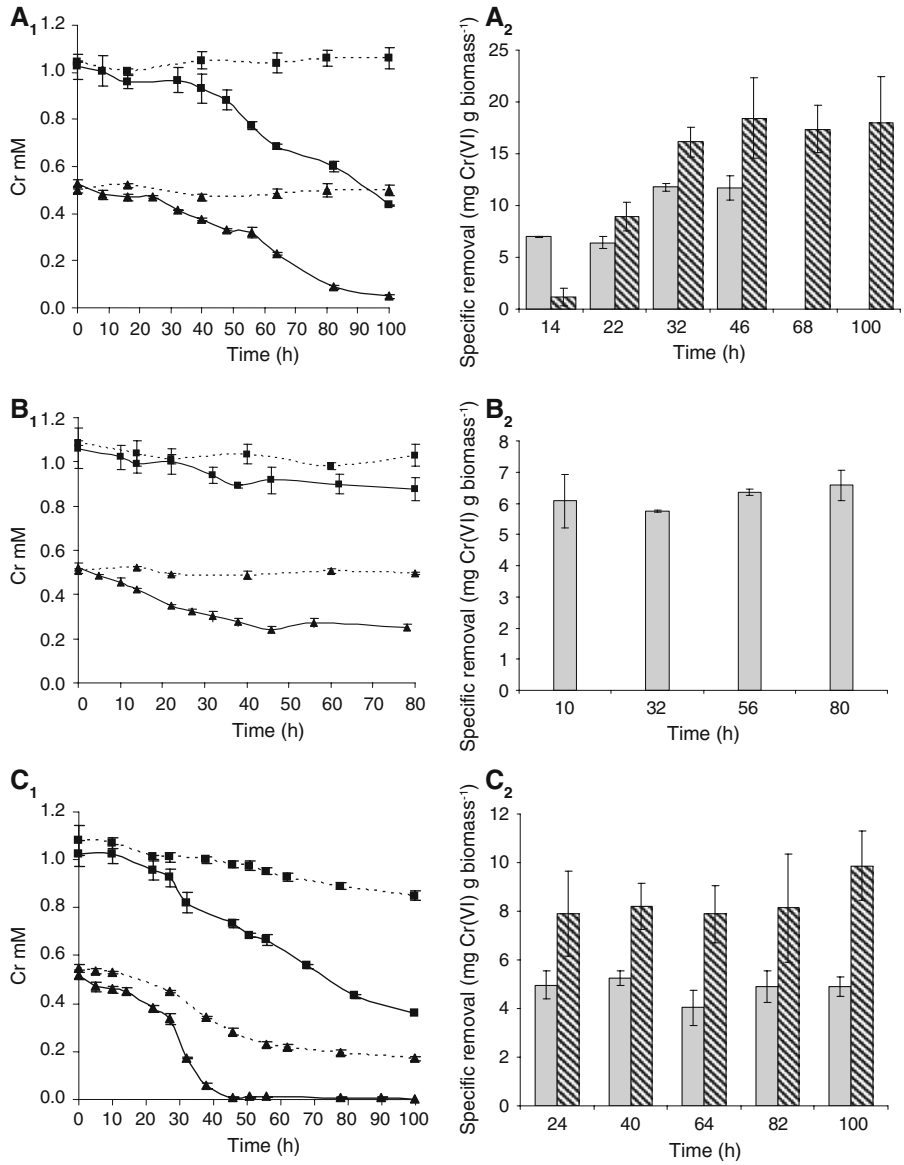

Fig. 3 (**a**$_1$; **b**$_1$ and **c**$_1$) extracellular Cr(VI) concentration (–) and total Chromium (- - -) kinetics in the presence of 0.5 (▲) and 1 mM Cr(VI) (■); (**a**$_2$; **b**$_2$ and **c**$_2$) specific Cr(VI) removal (mg Cr(VI) g biomass^{-1}) at 0.5 (▒) and 1 mM Cr(VI) (▨) by (**a**) *Lecythophora* sp. NGV-1; (**b**) *Candida* sp. NGV-9 and (**c**) *A. pullulans* VR-8

In order to compare the ability of the three strains to remove Cr(VI), specific chromate removal was determined (defined as mg of Cr(VI) removal per gram of biomass). The results showed that Cr(VI) removal increased with the initial Cr(VI) concentration in the medium: *Lecythophora* sp. NGV-1 reached maximum elimination after 24 h of incubation independently of the Cr(VI) concentration (Fig. 3a$_2$). *Candida* sp. NGV-9 presented maximum specific removal after 10 h of incubation in the presence of 0.5 mM Cr(VI) (Fig. 3b$_2$). This result was similar to that with *Lecythophora* sp. NGV-1 although this strain showed higher growth than *Candida* sp. NGV-9 at the same Cr(VI) concentration (Fig. 2b$_1$). While both strains presented constant values during the assay, specific Cr(VI) removal by *A. pullulans* VR-8 increased with elapsing incubation time and this strain showed the highest values:

12.5 ± 0.5 and 17.3 ± 1 mg Cr(VI) g biomass^{-1} after 32 and 46 h of incubation in the presence of 0.5 and 1 mM Cr(VI) respectively (Fig. 3c_2).

Specific glucose consumption augmented with increasing specific Cr(VI) removal, whereas growth and μ concomitantly decreased with increasing initial Cr(VI) in the culture medium, an effect most evident in *A. pullulans* VR-8. Probably, these strains required glucose as energy source or electron donor to remove Cr(VI). These results are in agreement with those reported by Wang and Xiao (1995) and Laxman and More (2002), who found that *Pseudomonas fluorescens* and *S. griseus* cells only reduced chromate if glucose or another suitable electron donor was present. Orozco et al. (2007) also reported that active sludges with lactose were more capable of removing Cr(VI) than systems without this sugar.

It is known that Cr(VI) uses sulphate and phosphate transport routes to penetrate the cellular membrane due to its structural similarity to these anions. However, Cr(III) is positively charged under physiological conditions and hence the cell membranes are impermeable to this metal. Therefore, Cr(III) requires binding to an organic compound to be able to pass the plasmatic membrane (Raspor et al. 2000; Pas et al. 2004). Considering that Cr(III) is the only stable and soluble chromium component formed by Cr(VI) reduction, it could be inferred that the chromium remaining in the supernatant in this work was Cr(III). The incapacity by *Lecythophora* sp. NGV-1 and *Candida* sp. NGV-9 to accumulate chromium, could be explained by the low bioavailability of Cr(III) ions in the medium or by hindered transport across the yeast cell membranes under the incubation conditions, as well as the presence or absence of certain components of the culture medium. Although, *A. pullulans* VR-8 diminished total supernatant chromium, it was impossible to determine if chromium had accumulated in the cells or if it was adsorbed by EPS. Further studies on Cr(VI) mechanisms in these strains are necessary.

The biological effects of chromium depend on its oxidation state: Cr(VI) is water soluble and highly toxic to most organisms whereas Cr(III) is less water soluble and relatively innocuous. Thus, reduction of Cr(VI) to Cr(III) represents a valuable strategy to control chromium dispersion and it is a potentially useful mechanism to detoxify pollution. Yeasts are a very diverse group of eukaryotic microorganisms, and Cr(VI) resistance mechanisms can differ considerably among species. Further research will be necessary in order to establish the potential and benefits of strains isolated from the environment in the development of new and economic biotechnological processes. Such processes aim at diminution of Cr(VI) from contaminated environments and treatment of industrial effluents with dangerous concentrations of this heavy metal.

Acknowledgements This work was supported by grants from CIUNT and CONICET, Argentina. Technical assistance of Mr. Claudio Delfini and FAAS facilities provided by Dr. Orlando Villegas (Universidad Nacional de San Luis, Argentina) are gratefully acknowledged.

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