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Textile-dye polluted waters as a source for selecting chromate-reducing yeasts through Cr(VI)-enriched microcosms

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ABSTRACT

Chromate-resistant microorganisms able to reduce toxic Cr(VI) into insoluble Cr(III) are seen as promising candidates for alleviating Cr(VI)-contamination. In this study, chromate-reducing yeasts could be isolated from a textile-dye effluent and associated biofilm by using microcosm methodology with periodical 1 mM Cr(VI)-pulses. Viable cell count seemed to reveal a progressive tolerance increase to Cr(VI). However, fungal colony numbers decreased after 108 h of cultivation most likely as a consequence of the accumulated toxic effects of metal during enrichment. From 49 different Cr(VI)-tolerant fungal morphotypes isolated under selective conditions, 12 yeasts showed resistance up to 50 mM and 6 filamentous fungi up to 2 mM. These highly tolerant yeasts could be subsequently grouped into 8 OTUs (Operational Taxonomic Units) according to the ITS1-NL4 RFLP (Restriction Fragment Length Polymorphism) analysis. From them, microsatellite amplification, sequencing and Cr(VI)-removal ability allowed to select two representative isolates. A polyphasic approach including morphological, physiological/biochemical characterization and molecular taxonomy analysis allowed to identify these isolates as *Cyberlindnera jadinii* M9 (previously *Pichia jadinii*) and *Wickerhamomyces anomalus* M10 (previously *Pichia anomala*). *Cy. jadinii* M9 and *W. anomalus* M10 were grown in YNB' medium plus 1 mM Cr(VI) at 25 °C and pH 5.0, causing complete chromium removal before reaching 48 h of cultivation. Flame Atomic Absorption Spectroscopy (FAAS) assays suggested that Cr(VI) withdrawal was coupled to Cr(III) appearance. Electron microscopy studies indicated absence of precipitates on the cell wall region or microprecipitates into the cellular cytoplasm. These complementary results revealed that biospeciation to Cr(III) would the main detoxification mechanism in selected chromate-resistant yeasts, which could be thus envisaged as promising tools for future biological treatment of Cr(VI) pollution.

1. Introduction

Diverse industrial activities like leather tanning, stainless-steel production, chrome plating and polishing, chromate manufacturing, pigment production, wood-preserving facilities and petroleum refineries extensively use hexavalent chromium in the form of chromate or dichromate (Sultan and Hasnain, 2005; Thacker et al., 2006). These actions finally lead to the heavy pollution of soils, ground and surface waters, as well as atmosphere. Alarmingly, more than 170,000 tons of chromium waste is annually discharged to the environment (Kamaludeen et al., 2003).

Cr(VI) compounds are recognized as extremely toxic to living organisms, causing allergies, irritations and respiratory track disorders (Barceloux, 1999; Poljsak et al., 2010). Along with the strong Cr(VI) oxidizing nature, its intracellular reduction to Cr(III) gives rise to following reactions with nucleic acids and other cell components in biological systems with consequent mutagenic and carcinogenic effects (Barceloux, 1999; Camargo et al., 2003; Poljsak et al., 2010). Therefore, biospeciation of Cr(VI) to Cr(III) in chromium-polluted sites has been regarded as a potentially useful process for the remediation of Cr(VI)-contaminated environments (Camargo et al., 2003; Sultan and Hasnain, 2005).

On the other hand, Cr(III) at low concentrations constitutes an essential micronutrient for many higher organisms, being related to carbohydrate metabolism, synthesis and conformational stability of proteins and nucleic acids (Debski et al., 2004; Krishna and Philip, 2005; Poljsak et al., 2010). From the toxicological point of view, Cr(III) is relatively insoluble in water, being comparatively less toxic

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than Cr(VI) (Cervantes et al., 2001). Accordingly, Cr(VI) has been described as 100 times more toxic and 1000 times more mutagenic than Cr(III) (Morales-Barrera and Cristiani-Urbina, 2006).

Biological treatment of heavy metal-containing wastewater by using microorganisms has become one of the most active research fields in recent years (Drogui et al., 2005; Cheung and Gu, 2007). Compared to conventional chemical treatments (e.g. chemical reduction plus precipitation, ion exchange, adsorption on activated coal, etc.), biological management exhibits many advantages, which include (i) low operation cost, (ii) steady performance, and (iii) easy recovery of some valuable metals. Biological systems employing processes such as bioreduction, bioaccumulation or biosorption with living cells have been extensively examined on their chromium removal abilities (Krishna and Philip, 2005; Sultan and Hasnain, 2005; Villegas et al., 2008; Poljsak et al., 2011).

Several microorganisms have the exceptional ability to adapt to and colonize noxious metal polluted environments which would be inhabitable by other higher organisms. Among eukaryotic microorganisms, *Candida utilis*, *Schizosaccharomyces pombe* and *Candida intermedia* have been found to be effective in accumulating aggressive Cr-compounds (Păs et al., 2004; Poljsak et al., 2010), whilst others (*Candida maltosa*, *Candida* sp., *Lecythophora* sp. NGV1, *Candida* sp. NGV9, *Aureobasidium pullulans* VR-8) have developed the ability to bioconvert them into stable, non-toxic and bioavailable forms (Ramírez-Ramírez et al., 2004; Juvera-Espinosa et al., 2006; Cheung and Gu, 2007; Villegas et al., 2008; Poljsak et al., 2010). It has been already elucidated that Cr(VI) is assimilated *via* non-specific anion carriers which, under normal growth conditions, are involved in sulfate and phosphate anions uptake (Poljsak et al., 2010). In the case of some chromium resistant genotypes, the ability to survive this heavy metal in contaminated wastewaters would be related to decreased Cr(VI) uptake or the ability to reduce it (Poljsak et al., 2010). These findings have been the basis to propose heavy metal-resistant varieties of selected yeast species as promising candidates for bioremediation of chromate contaminated waters (Poljsak et al., 2011).

It has been previously cited in the literature that heavy metals can be found together with dyestuffs in real textile industrial effluents (Aksu et al., 2007). Based on these observations, this report describes the isolation, selection and identification of Cr(VI)-tolerant yeasts from a textile dye effluent-receiving site (Tucumán, Argentina). Involved chromate removal mechanisms were also preliminary investigated in order to evaluate their potential use for future environmental clean-up proposals.

2. Materials and methods

2.1. Enrichment and isolation of chromium-resistant microorganisms

Chromium-tolerant eukaryotic microorganisms (yeasts and filamentous fungi) were isolated from liquid effluents and wall-adhered biofilm from textile-dye effluent channel drainage in the proximity of a textile factory (Famaillá, Tucumán, Argentina). These samples were randomly selected and collected in sterile plastic containers. Effluent physicochemical analysis was performed by SAT laboratory (*Sociedad Aguas del Tucumán*, Tucumán, Argentina), as soon as the samples arrived.

Sterile YNB' liquid culture medium (Fernández et al., 2009) was amended with 10% v/v liquid effluent in order to reach a final working volume of 100 ml. Culture medium (YNB') containing Yeast Nitrogen Base (Difco YNB w/o amino acids and ammonium sulfate, 10% v/v), sucrose (50 g l^{-1}), and $(NH_4)_2SO_4$ (0.6 g l^{-1}), was supplemented with 1 mM Cr(VI) plus antibiotics (tetracycline and chloramphenicol, 50 mg l^{-1} each). Microcosm cultures (in 500-ml Erlenmeyer flasks) were incubated at 25 °C with agitation (250 rpm) for 108 h. Biofilm samples were pretreated (at 25 °C, 200 rpm, 2 h) by suspending 1 g of sample in 20 ml of a solution containing (in g l^{-1}): glucose, 1; yeast extract, 0.5; and Tween 80, 0.01. This pretreated suspension was subsequently used for microcosm inoculation.

Periodic Cr(VI)-enrichments of cultures were performed by adding 5.2 mg-Cr(VI) pulses (in 1 ml) at 36 and 72 h of cultivation. At three different time intervals during microcosm enrichment (i.e. before the first Cr(VI) pulse: at 36 h; before the second pulse: at 72 h and, at the end of cultivation: 108 h), 0.1 ml-aliquots were withdrawn, serially diluted and plated onto 2% w/v agar plates, both with YNB' and YNB' plus 1 mM Cr(VI). Plates were incubated at 25 °C for 72 h, and daily monitored for growth development. Results were expressed as Colony Forming Units (CFU) per ml. Chromium resistant isolates representing different colony morphotypes were isolated, maintained on the same agar medium and stored at 4 °C.

A $K_2Cr_2O_7$ stock solution containing 5.2 mg Cr(VI) ml^{-1} (100 mM) in bidistilled water was used as the hexavalent chromium source for culture medium. A filter-sterilized Cr(VI)-stock solution was added to YNB' culture medium under sterile conditions.

2.2. Screening and selection experiments

The obtained isolates were grown at 25 °C on solid YNB' medium supplemented with increasing Cr(VI) concentrations (1–50 mM) and microbial growth was daily observed during 7 days. Fungal isolates showing the highest tolerance at this stage were selected for following assays. Highly Cr(VI)-tolerant isolates were subsequently grown in YNB' liquid medium amended with 1 mM Cr(VI). Cultures (10 ml in 100-ml Erlenmeyer flasks) were inoculated with either one colony or one mycelium-covered agar plug from yeast or filamentous fungal cultures, respectively, previously grown for 3–4 days at 25 °C on Czapek Malt (CZM) agar plates (in g l^{-1}: malt extract, 40; sucrose, 30; $NaNO_3$, 3; K_2HPO_4, 1; $MgSO_4$ $7H_2O$, 0.5; KCl, 0.5; $FeSO_4$ $7H_2O$, 0.5; agar 2% w/v, initial pH 4.5). Liquid cultures were incubated at 25 °C and 250 rpm for 96 h. At regular intervals (24 h), the culture was pelleted by centrifugation (10,500 × *g*, 15 min) and the supernatant was collected for Cr(VI) quantification by the *S*-diphenilcarbazide (DPC) method, as described below.

Selected isolates were then further evaluated in YNB' liquid medium (30 ml in 250-ml Erlenmeyer flasks) added with growing Cr(VI)-concentrations (1, 2, 4, 6 and 15 mM). Cultures were performed as described above but in this case, incubation was carried out for 72 h. Spent broth samples were centrifuged (10,500 × *g*, 15 min) and the supernatant was set aside for Cr(VI) quantification. To estimate biomass dry weight (BDW), the pellet was washed twice with distilled water, collected in 0.45-μm cellulose acetate membrane filters and then, dried at 105 °C until constant weight.

2.3. General molecular procedures

Yeast DNA extraction was performed by using the Yeast Lysis Buffer (YLB) method, as previously described (Pajot et al., 2008). An initial molecular characterization was carried out by amplification of polymorphic regions of genomic DNA using microsatellite primers $(GAC)_5$ and $(GTG)_5$, following the protocol described by Rodrigues and Fonseca (2003). Restriction Fragment Length Polymorphisms (RFLP) analysis was performed using ITS1-NL4 PCR-amplified fragments after digestion with *Hha*I, *Dde*I, *Hinf*II and *Hae*III restriction enzymes, according to the supplier's instructions.

Ribosomal DNA regions including the large subunit (LSU) 28S rDNA D1/D2 domain, 5.8S rDNA and internal transcribed spacers (ITS1-ITS2) were amplified and sequenced as previously described (Pajot et al., 2008). Sequences were edited with DNAMAN program

version 5.2.2 (Lynnon BioSoft, Vandreuil, QB, Canada). Dendogram was constructed on the basis of the retrieved validated sequences from GenBank, using the Neighbor-Joining method (Saitou and Nei, 1987). Sequence comparisons were performed using the basic local alignment search tool (BLAST) program within the GenBank database. The ClustalW computer program was used for alignment of multiple sequences (Thompson et al., 1994). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al., 2007).

2.4. Morphological and physiological characterization

Morphological examination as well as classical biochemical and physiological tests were performed in accordance to standard yeast identification protocols (Yarrow, 1998). Nitrate and nitrite assimilation tests were both assayed in the auxanographic and the standard liquid medium assays. Carbon and nitrogen sources utilization was examined on solid YNB' and Yeast Carbon Base (YCB) media (Difco), respectively, at 25 °C. Cultures were incubated up to 21 days, and daily examined. Medium pH was adjusted to 5.5, as required.

For microscopy purposes, cells were grown in Yeast Morphology (YM) broth for 3 days at 26 °C and 250 rpm. Hyphae formation was assayed in YM agar by the Dalmau plate technique (Warren and Hazen, 1995). In order to observe the influence of Cr(VI) in yeast cell morphology, 2-day-old cultures grown in the presence of Cr(VI) were compared with metal-deprived control cultures. These samples were examined by transmission electron microscopy (TEM, Zeiss EM 109, Oberkochen, Germany). All the samples were treated according to standard protocols.

2.5. Kinetics of Cr(VI) removal by selected yeast isolates

The initial Cr(VI) concentration in YNB' medium was set at 1 mM, at pH 5.0. Inocula were prepared by transferring one colony of a 3-day-old Czapek Malt (CZM) agar culture to 50 ml CZM liquid medium (in a 250-ml Erlenmeyer flask), followed by cultivation under shaking conditions (25 °C, 250 rpm) during 24 h (Fernández et al., 2010). These inocula were used to seed (10%, v/v) YNB' liquid medium. Cultures were performed in 1000-ml Erlenmeyer flasks with a working volume of 200 ml at 250 rpm, and 25 °C for 96 h. From each sampling time, two culture broth aliquots were centrifuged (10,500 × *g*, 15 min). From one set of aliquots, supernatants were kept for extracellular chromium determination (total Cr). The remaining pelleted cells were washed twice with distilled water and preserved at −20 °C until intracellular chromium determination. With the other set of samples, supernatants were analyzed for residual Cr(VI) by DPC (see below), pH, reducing sugars (3,5-dinitrosalicylic acid (DNSA) method; Miller, 1959), and ammonium (indophenol blue method; Koroleff, 1976) consumption. BDW was determined as previously described.

2.6. Analytical determination of Cr(VI), Cr(III) and total chromium

Remaining Cr(VI) in cell-free supernatants was quantified by using a miniaturized version (Fernández et al., 2009) of the method reported by Urone (1955). This method was based on the colorimetric reaction between Cr(VI) and *S*-diphenylcarbazide (DPC) in acid solution, which generates a pink–violet color. The reaction is very sensitive and the absorbance is measured at 540 nm using a spectrophotometer.

Total chromium concentration was measured in washed pelleted biomass and cell-free supernatant samples after digestion with drops of 65% (w/v) HNO_3, followed by warming up on a hot plate until the suspension was clarified. After cooling down, original sample volume was made up by adding distilled water. The resulting solutions were analyzed for atomic Cr by Flame Atomic Absorption Spectroscopy (FAAS). As a control, an aliquot of uninoculated culture medium was similarly digested with HNO_3 and analyzed according the same procedure.

The difference calculated between total chromium and Cr(VI) concentrations was assumed as Cr(III), as already postulated (Villegas et al., 2008).

All the experiments were carried out in triplicate and average values with the corresponding standard deviations are herein reported.

3. Results

3.1. Isolation of Cr(VI)-tolerant microorganisms

The effluent spectrophotometric analysis showed chromium levels lower than 0.010 mg l^{-1} (Table 1), a low value considering previous reports about polluted waters in Argentina (Romero et al., 1997; Ronco et al., 2001). In order to select Cr(VI)-resistant fungal strains, microcosm methodology with periodical Cr(VI)-pulses was applied for sample enrichment. Results of viable cell count (CFU) seemed to indicate that the microbial population developed a progressive tolerance to the metal presence, until a certain limit. After the second pulse of Cr(VI), the total count of fungal microorganisms showed an increment when plated on YNB' medium (from 3.7×10^3 to 1.4×10^5 CFU ml^{-1} and, from 2.8×10^3 to 3.0×10^4 CFU ml^{-1}, when the sample came from the effluent or biofilm, respectively). However, at 108 h of cultivation, accumulated toxic effects of metal at

Table 1
Physicochemical analysis of textile-dye industry effluent.

PARAMETERS	Units	Effluent
Total alcalinity	mg l^{-1}	370
Conductivity	μS cm^{-1}	1010
BDO	mg l^{-1}	630
COD	mg l^{-1}	1700
Total hardness	mg l^{-1}	1160
Color		Greenish blue
Consumed oxygen	mg l^{-1}	190
Dissolved oxygen	mg l^{-1}	0
pH		7.2
Turbidity	UNT	58
Total residues	mg l^{-1}	895
Fixed solids	mg l^{-1}	605
Volatile solids	mg l^{-1}	290
Suspended solids	mg l^{-1}	200
Fixed suspended solids	mg l^{-1}	80
Volatile suspended solids	mg l^{-1}	120
Settleable solids (2 h)	mg l^{-1}	3
Aluminum	mg l^{-1}	0.098
Ammonia	mg l^{-1}	0.49
Arsenic	mg l^{-1}	<0.020
Cadmium	mg l^{-1}	<0.003
Calcium	mg l^{-1}	28
Cyanide	mg l^{-1}	<0.020
Chlorides	mg l^{-1}	70
Copper	mg l^{-1}	<0.2
Chromium	mg l^{-1}	<0.010
Magnesium	mg l	21.8
Manganese	mg l^{-1}	<0.020
Mercury	mg l^{-1}	<0.001
Nitrates	mg l^{-1}	4
Nitrites	mg l^{-1}	0.02
Lead	mg l^{-1}	<0.015
Sulfate	mg l^{-1}	100
Sulfides	mg l^{-1}	<1
Phosphates	mg l	0.462
Iron	mg l	0.38
Fluorine	mg l^{-1}	0.204

high concentrations and/or culture age, led to a decrease in the number of viable fungal colonies (4.5×10^4 and 1.0×10^4 CFU ml^{-1} for effluent and biofilm samples, respectively). When plated on YNB' plus 1 mM Cr(VI), it was also observed an increase before the second pulse, although the registered CFU numbers were lower than those obtained without the metal selection pressure (at the end of the cultivation, 2.6×10^2 and 6.8×10^2 CFU ml^{-1} in the case of effluent or biofilm, respectively).

From Cr(VI)-resistant fungal specimens able to maintain this phenotype when repeatedly subcultured in 1 mM-Cr(VI)-amended medium, representatives of each different colony morphotype were taken as particular isolates, and arbitrarily named. Among 49 pure fungal isolates, yeasts represented the minority. However, they exhibited the highest tolerance when further analyzed for their capacity to grow on solid YNB' medium containing from 0 to 50 mM Cr(VI). A total of 18 fungal isolates (12 yeasts and 6 filamentous fungi) showed the highest tolerance to Cr(VI) (up to 50 mM Cr(VI) for yeasts and up to 2 mM Cr(VI) for filamentous fungi). Those fungal microorganisms selected from this initial screening step were further evaluated in liquid YNB' medium containing 1 mM Cr(VI). At 24 h, yeasts showed the best performance as compared to the filamentous fungal isolates, and were thus accordingly selected.

In order to continue with the yeast selection process, molecular biology methods were employed. PCR-amplified products (ITS1-NL4) obtained from selected yeasts genomic DNA, were digested with restriction enzymes (*Dde*I, *Hha*I, *Hinf*I and *Hae*III). Restriction profiles analysis allowed clustering the 12 analyzed yeasts into 8 Operational Taxonomic Units (OTUs). One representative from each OTU was selected for the following analysis. Variability of the selected isolates could be further screened by PCR amplification with primers for microsatellite analysis, as mentioned in the Materials and methods section.

This latter molecular fingerprinting tool permitted the reorganization of selected yeast isolates into 4 groups. According to this technique, some of the evaluated isolates exhibited identical amplification profiles either with one or another of the used primers. In a previous work, Andrade et al. (2006) established that these primers would allow the differentiation of yeasts of different species, but would not reveal significant differences at an intraspecific level. According to that concept, the number of representative isolates could be restricted discarding those that might belong to the same species (Fig. 1). Finally, isolates M9, M10, M12 and M17 were chosen as representatives of each group and their subsequent molecular identification was performed.

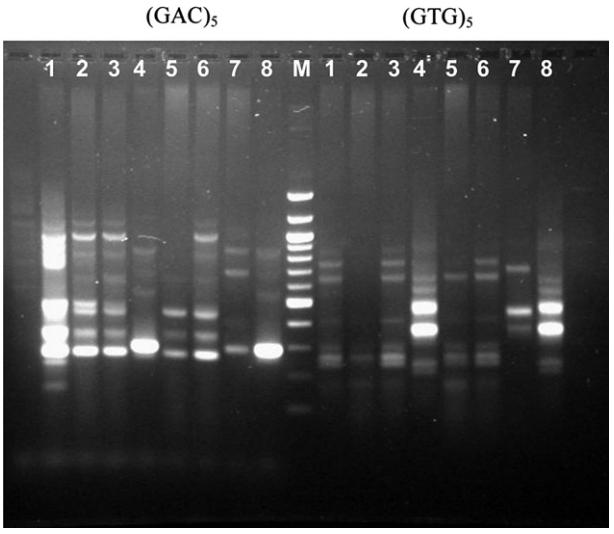

Fig. 1. Microsatellite amplification patterns in 2% agarose gel electrophoresis obtained with primers $(GAC)_5$ in the left and $(GTG)_5$ to the right. Lanes, M: 1-Kb molecular weight marker, yeast isolates 1: M3, 2: M7, 3: M9, 4: M10, 5: M12, 6: M15, 7: M17 and 8: M18.

3.2. Characterization and identification of selected isolates

Sequence analysis of ITS1-NL4 PCR-amplified fragments of microsatellite-based selected yeasts revealed that isolates M9 and M12 showed 99% similarities with its closest relative *Cyberlindnera jadinii* NRRL Y-1542^T (GenBank accession number: EF550309, previously *Pichia jadinii*; Kurtzman et al., 2008), whilst M10 and M17 showed 99% similarities with *Wickerhamomyces anomalus* NRRL Y-366^T (GenBank accession number: EF550341, previously *Pichia anomala*; Kurtzman et al., 2008). In a subsequent step, considering the sequence identity between isolate M9 with M12, and M10 with M17, one representative of each group was selected according to its best 1 mM Cr(VI) removal rate in liquid medium (data not shown). According to this further selection criterion, *Cy. jadinii* M9 (GenBank accession number FJ865435) and *W. anomalus* M10 (GenBank accession number FJ865436), whose phylogenetic relationships are depicted in Fig. 2, were chosen for subsequent assays.

Analysis of physiological and biochemical properties is essential for yeasts global characterization, and results also valuable for the diagnosis and identification of specific groups potentially interesting from a biotechnological perspective. Therefore, to complete the polyphasic approach, molecular identification was complemented through the evaluation of *Cy. jadinii* M9 and *W. anomalus* M10 growth responses on standard carbon and nitrogen compounds, the assimilation profile of a non-conventional aliphatic compound (hexadecane) as well as other fermentation features (Table 2). At 25 °C, glucose and sucrose were readily fermented within 48 h, while galactose, maltose and trehalose were not fermented at all even after 2 weeks of incubation, for both strains (Table 2). *Cy. jadinii* M9 and *W. anomalus* M10 showed different responses for D-glucosamine, L-rhamnose, erythritol and D-sorbitol (Table 2). Further physiological and biochemical features are also displayed in Table 3.

Yeast colonies moderately grew on YM agar, reaching a diameter close to 0.5 cm with defined margins and without surface irregularities after 3–4 days of incubation at 25 °C. Texture of colonies was buttery in the case of *Cy. jadinii* M9 whilst creamy and velvety for *W. anomalus* M10. In both cases, colony color was white to white-beige, with a slightly yellowish reverse, and exhibited a sweet aromatic smell when older. Microscopic characteristics were in agreement with the standard description (Kurtzman, 1998): ovate budding and spheroidal-elongated single cells, along with short chains or branched septate pseudohypae. Yeasts micromorphology during growth in YM broth is also displayed in Fig. 3.

3.3. Time course of Cr(VI) biospeciation to Cr(III)

The ability of selected strains to reduce the initial 1 mM Cr(VI) concentration in culture medium through its bioconversion to Cr(III) was subsequently evaluated. Reduction of Cr(VI) began during the first hours of cultivation for both yeast isolates (Fig. 4), and Cr(VI) reduction rate became highest during the exponential growth phase. Most of soluble Cr(VI) was removed from culture broth after 24 h of growth for *W. anomalus* M10, while in the case of *Cy. jadinii* M9 it sharply decreased to 80% at this time, being completely reduced (and not detected even as traces) at 48 h of culture.

It was also herein noted that Cr(VI) titers at time zero, according to DPC, were lower than those obtained by FAAS assuming that total chromium was Cr(VI) (Fig. 4). The mentioned difference in initial Cr(VI) concentration was nearly the same for both strains.

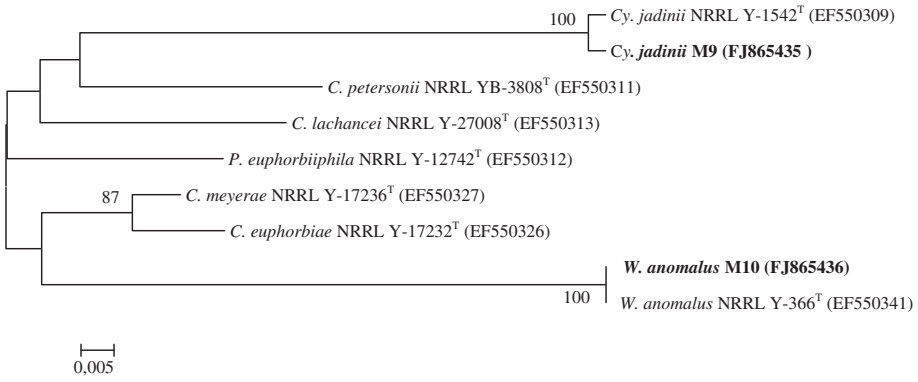

Fig. 2. Evolutionary relationships of M9 and M10 yeast isolates based on the 28S rDNA D1/D2 domain, 5.8S rDNA; ITS1 and ITS2 rDNA sequences. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.32445281 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 575 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

Spontaneous Cr(VI) reduction did not however occurred in the uninoculated culture medium even after 120 h of incubation, indicating the absence of unspecific Cr(VI) reduction by medium components (YNB' medium) (Fernández et al., 2010).

To evaluate total chromium content in cell-free supernatants and to check if cell bioaccumulation was taking place (in the pelleted cell fraction), a total chromium determination by FAAS was carried out. As depicted in Fig. 4, total chromium concentration remained practically constant throughout cultivation; most of the total initial chromium was always present in solution and a very little amount was kept in the cell fraction.

Specific chromium bioaccumulation (mg of Cr accumulated per g of BDW) declined with the biomass increase as cultivation progressed. Values obtained between 0 and 120 h of culture ranged from 1.196 to 0.786 mg Cr g^{-1} BDW for *Cy. jadinii* M9, and from 0.817 to 0.260 mg Cr g^{-1} BDW for *W. anomalus* M10. In the case of *Cy. jadinii* M9 this value remained stable from 48 h of culture, while in *W. anomalus* M10 this occurred after 72 h, events most likely related to the beginning of the stationary growth phase (Fig. 4A,B).

Growth, pH and ammonium consumption kinetics were similar for both yeasts, though *W. anomalus* M10 reached higher BDW values (8.7 g l^{-1}) than *Cy. jadinii* M9 (5.6 g l^{-1}). Nevertheless, sucrose consumption profiles were different. Whilst *Cy. jadinii* M9 spent almost 100% sucrose, *W. anomalus* M10, the highest biomass producer, consumed lower amounts of C-source (~45%).

Finally, no cell-associated heavy metal particles were evident in the Cr(VI)-grown yeast samples when compared to metal-deprived yeast controls, as analyzed by transmission electron microscopy (TEM) (data not presented). These results represented further evidence on the reduction removal nature of the Cr(VI) detoxification process performed by selected yeasts.

4. Discussion

Analyzing microcosm experiments, it would be reasonable to presume that the increase in viable cell counts with subsequent chromium pulses was based on a progressive cell tolerance development in the presence of this heavy metal. However, the reduction of cell viability after 108 h of cultivation might derive from the accumulated chromium toxic effects during the enrichment process and/or culture age. The lower counts recorded when Cr(VI)-enriched-microcosm samples were plated on YNB' medium plus Cr(VI) simply evidenced the selective nature of this medium. Nevertheless, it would be worthwhile to mention that the number of viable microorganisms able to grow on Cr(VI)-amended medium (~0.6% for effluent isolates; ~7% for biofilm isolates) was significantly higher than values previously reported (<0.1%) (Baldi et al., 1990).

The finding of higher counts of Cr-resistant viable specimens from biofilm samples was not surprising. Usually, the high exopolymer content of biofilms results beneficial for both entrapping dispersed solids and biosorbing dissolved metals. This microenvironment with high pH or high CO_2 may favor metal precipitation. Besides, microbial interaction can promote or allow the survival of sensitive strains (Malik, 2004). The progressively higher tolerance to metal concentrations might be related to the constitutive synthesis of metallothionein or other metal binding proteins or else changes in the genetic makeup (Malik, 2004).

A microorganism is considered cultivable or not, depending on the sample nature and the culture conditions (Müller et al., 2002). Microcosm methodology with periodical Cr(VI) pulses could lead to an increased number of isolated microorganisms able to tolerate or resist high chromium concentrations and at high growth rates. Interestingly, higher tolerance to Cr(VI) in yeasts isolated from natural sources than in laboratory collection strains was already highlighted (Ksheminska et al., 2003). Anyway, we could not associate the effluent Cr(VI) concentration with the presence of strains able to reduce high Cr(VI) concentrations. Thus, Cr(VI)-resistance strategies could be a shared feature among different strains in the microbial community.

Typical levels of Cr pollution have been reported in the order of 25–150 μg l^{-1} (Kaszycki et al., 2004). Markedly different values of Cr(VI) concentration, such as 0.03 and 60 μg l^{-1}, might be found in textile industry wastewaters (Juvera-Espinosa et al., 2006). Literature refers total Cr contents like 0.1–0.5 μg l^{-1} in seawater, 0.3–0.6 μg l^{-1} in non-polluted river and surface water, 5–50 μg l^{-1} in polluted river water, and up to 200 μg l^{-1} in severely polluted water systems. For the case of drinking water, Cr(VI) is regulated under the 50 μg l^{-1} maximum contaminant level (MCL) (Bobrowski et al., 2004). Despite the low chromium concentrations found in the herein described samples (10 μg l^{-1}, Table 1), this fact did not impede the isolation of Cr-tolerant strains as representatives of a small fraction of the community that could be cultivated under these restrictive culture conditions. In accordance, Polti et al. (2007) also reported isolation of chromium-resistant actinomycetes from polluted sites where this heavy metal was not abundant.

Table 2
Physiological properties of selected isolates.

	P. jadinii[a]	*Cy. jadinii* M9	*P. anomala*[a]	*W. anomalus* M10
Assimilation of carbon compounds				
D-Glucose	+	+	+	+
D-Galactose	−	−	V	−
L-Sorbose	−	−	−	−
D-Glucosamine	−	−	−	+
D-Ribose	−	−	V	−
D-Xylose	+	+	V	+
L-Arabinose	−	−	V	−
D-Arabinose	−	−	−	−, w
L-Rhamnose	−	−	−	+
Sucrose	+	+	+	+
Maltose	+	+	+	+
D-Trehalose	+	+	+	+
α-Methyl-D-glucoside	+	+	+	+
Cellobiose	+	+	+	+
Salicin	+	+	+	+
Melibiose	−	−	−	−
Lactose	−	−	−	−
Raffinose	+	+	+	+
Melezitose	+	+	+	+
Inulin	+/w	−	−	−
Soluble starch	−	+	+	+
Glycerol	+	+	+	+
Erythritol	−	−	+	+
Ribitol	−	−	V	−
D-Glucitol	+	−	+	−
D-Mannitol	+	+	+	+
myo-Inositol	−	−	−	−
D-Gluconate	+	+	V	+
DL-Lactate	+	+	+	+
Succinate	+	+	+	+
Citrate	+	+	+	+
Methanol	−	−	−	−
Ethanol	+	+	+	+
D-sorbitol	DNA	+	DNA	−
Fermentation				
D-Glucose	+	+	+	+
D-Galactose	−	−	V	−
Maltose	−	−	V	−
Sacarose	+	+	+	+
D-Trehalose	−	−	−	−
Assimilation of nitrogen compounds				
Ammonium sulfate	DNA	+	DNA	+
Nitrate	+	+	+	+
Nitrite	DNA	−, w	DNA	+
Assimilation of aliphatic compound				
n-Hexadecane	−	−	−	−

[a] (Data from Kurtzman, 1998; +: growth within 7 days; −: no growth after 21 days; w: weak growth response, V: variable growth; DNA: data not available).

Studies on the tolerance and interaction of yeasts with chromium have been usually restricted to strains of single, systematically selected groups (see Ksheminska et al., 2005 and the references therein). To the best of our knowledge, strains of *Cy. jadinii* and *W. anomalus* have not been previously reported for their ability to resist and reduce Cr(VI). Hence, we propose these yeast selected isolates (M9 and M10, respectively) as novel biotechnologically useful Cr(VI)-reducing yeasts.

Table 3
Miscellaneous physiological properties of selected isolates.

	P. jadinii[a]	*Cy. jadinii* M9	*P. anomala*[a]	*W. anomalus* M10
Vitamin-free	+	+	+	+
0.1% cycloheximide	DNA	−	DNA	−
0.01% cycloheximide	DNA	−	DNA	−
Urea hydrolisis	DNA	−	DNA	−
DBB test	−	−	−	−
19 °C growth	+	+	+	+
25 °C growth	+	+	+	+
30 °C growth	+	+	+	+
37 °C growth	+	+	V	+
50% glucose	DNA	−	DNA	−
10% NaCl + 5% glucosa	−	+/w	V	+/w

[a] (Data from Kurtzman, 1998; +: growth within 7 days; −: no growth after 21 days; w: weak growth response, V: variable growth; DNA: data not available).

Microbial ability to reduce Cr(VI) has been thought as a cost-effective and eco-friendly alternative since several years ago (Tseng and Bielefeldt, 2002; Ramírez-Ramírez et al., 2004; Juvera-Espinosa et al., 2006). It should be highlighted that the selective conditions herein used allowed isolating highly Cr(VI)-tolerant fungal morphotypes, up to 50 mM for yeasts and up to 2 mM in filamentous fungi. Meanwhile, most of the microbial isolates usually reported exhibited Cr(VI)-reducing activities within the range of 0.1–0.5 mM (Ksheminska et al., 2003; Juvera-Espinosa et al., 2006). Moreover, different morphological changes have been associated in response to heavy metals, both in filamentous fungi and yeasts, e.g. in fruiting structures, shape, surface or size and intracellular architecture (Muter et al., 2001; Silóniz et al., 2002; Ksheminska et al., 2005). However, no heavy metal deposition into the cells or morphological alterations were herein evident in Cr(VI)-treated yeasts, either under optical or electronic microscopy evaluation.

One large group of cell types have the ability to respond to undesirable changes in the environment when stress-generating agents such as Cr(VI) are present. Responses involve sensing and signal transduction, adaptation of gene expression profile, different metabolic activities and other features. Organisms have devised two main strategies to counteract this metal stressor, which may usually include either the restriction of metal-ion entry into the cell or its sequestration, both mechanisms leading to a reduction of the free ion levels in the cytosol. Tolerance against Cr(VI) of a given strain will therefore be changed if the responsible genes for Cr(VI) uptake, GSH metabolism or intracellular peroxide and superoxide production are altered, whereby the cellular redox system will be overbalanced (Czakó-Ver et al., 1999; Gazdag et al., 2003; Poljsak et al., 2010).

Considering that Cr(III) is most likely the only stable and soluble chromium state formed by Cr(VI) reduction, it could be inferred that the remaining chromium herein quantified by FAAS in supernatant was Cr(III). Its presence in supernatant would be unsurprising since under physiological conditions Cr(III) is positively charged and hence, cell membranes are impermeable to it. Then, binding to an organic compound would be required to pass across the plasmatic membrane (Raspor et al., 2000; Păs et al., 2004). Ramírez-Ramírez et al. (2004) also described a *C. maltosa* strain able to chemically reduce Cr(VI) to Cr(III) and similar speculations were proposed by Villegas et al. (2008) about the Cr(VI)-resistance in *Lecythophora* sp. NGV1, *Candida* sp. NGV-9 and *Aureobasidium pullulans* VR-8. Cr(VI) reduction ability was also reported in different *Candida* species (Muter et al., 2001; Juvera-Espinosa et al., 2006).

The high initial Cr(III) values herein observed at the beginning of cultivation for both strains (Fig. 4), would hardly represent an immediate reduction of Cr(VI) to Cr(III). Probably, these values resulted from the underestimation of Cr(VI) by DPC at time zero, since Cr(III) values were calculated from the difference between total chromium (by FAAS) and Cr(VI) (by DPC). On the other hand, the metal species added to culture medium was Cr(VI) and its immediate biotic reduction would be unfeasible, whilst the abiotic reduction was experimentally discarded. This difference may be related to the inoculum chemical composition, which might mask the Cr(VI) real value. The mentioned difference in initial Cr(VI) was almost the same for both strains, supporting the inoculum chemical composition influence on Cr(VI)-determination by DPC as the most probable interference.

The observation that total chromium remained almost constant in solution while Cr(VI) was progressively removed reinforced the idea of Cr(VI) reduction (Morales-Barrera et al., 2006). Biospeciation to Cr(III) would be the most probable interpretation

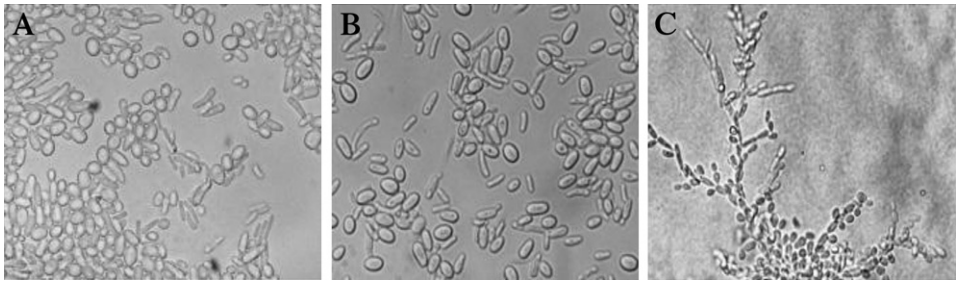

Fig. 3. Optical micrographs (100 × magnification) corresponding to the unicellular growth of *Cy. jadinii* M9 (A), *W. anomalus* M10 (B) and filamentous growth (pseudohyphae) by microculture technique (C), on YM-agar.

assumed from this study, as it represents a more stable, less toxic and less mobile oxidation state, whilst the adsorptive removal of Cr(VI) as well as its removal by culture medium components could be neglected.

With regard to other cultivation parameters during Cr(VI) removal, sugar requirement as energy source or electron donor has been also postulated by Villegas et al. (2008), who found that *Lecythophora* sp. NGV-1, *Candida* sp. NGV-9 and *A. pullulans* VR-8 only reduced chromate if glucose or another suitable electron donor was present. In the present work, substantial sucrose consumption accompanied the Cr(VI) removal process. Orozco et al. (2007) also reported higher Cr(VI) removal ability in lactose-supplemented activated sludges than in systems without this sugar.

Scarce information is currently available concerning filamentous fungi and yeasts with Cr(VI) bioremediation capacity. Considering that among the toxic heavy metals encountered in dye-containing effluents hexavalent chromium can be frequently found (Aksu et al., 2007), the ability of selected yeasts to tolerate a combination of textile dye plus Cr(VI) during enrichment microcosms would be not unexpected. Yeasts are widespread in Nature and play different roles with major economic and social significance in human culture (Silóniz et al., 2002). Results from this work give evidence of the significant potential of selected yeasts for chromium bioremediation, resulting talented candidates for alleviating this polluting metal from environment.

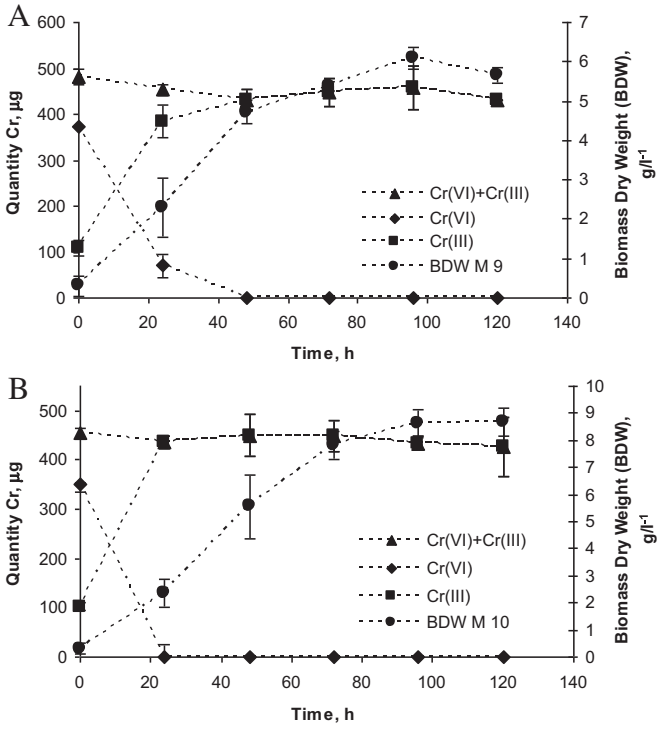

Fig. 4. Growth curves and chromium biospeciation profiles during *Cy. jadinii* M9 (A) and *W. anomalus* M10 (B) cultures in YNB' medium plus 1 mM Cr(VI) at 25 °C and 250 rpm. Heavy metal quantification included Cr(VI) and total chromium (per 10 ml of sample). Not visible standard deviation bars indicate they do not exceed symbol size.

5. Conclusions

The finding of outstanding Cr(VI)-bioremediating yeasts such as *Cy. jadinii* M9 and *W. anomalus* M10 from a low chromium-content polluted site revealed that resistant specimens could be not exclusively related to the pollutant concentration in the environment.

Remarkable Cr(VI) biospeciation capability without deleterious effects on growth was revealed as the main detoxification mechanism in selected yeasts. Particular highlights of these results involve the irreversibility of this process, with a reduced risk of metal release back to the environment, and the possibility to design on this basis biological strategies to alleviate Cr(VI)-pollution by means of cost-effective green technology.

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