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Critical Influence of Culture Medium and Cr(III) Quantification Protocols on the Interpretation of Cr(VI) Bioremediation by Environmental Fungal Isolates

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Abstract The influence of culture medium composition on chromium(VI) quantification according to diphenylcarbazide (DPC) colorimetric determination was evaluated. Considering the eventual biospeciation of Cr(VI) as a mechanism of microbial bioremediation, the possibility to quantify Cr(III) in culture medium was also explored. Yeast nitrogen base (YNB) was identified as the least interferent culture medium for Cr(VI) quantification by DPC and it was applied to compare different strategies for Cr(III) oxidation. The most appropriate oxidation protocol consisted in the reaction with 80 mM KIO_4 at room temperature for 30 min prior to DPC. Parameters like basal culture medium (vitamins + salts + oligoelements), C and N source were systematically evaluated, either independently or in combination. Results demonstrated that C source was the most interferent culture medium component, being the use of sucrose preferable to glucose. A medium arbitrarily named as YNB′ (YNB without amino acids and ammonium sulfate plus 50 g L^{-1} sucrose and 0.6 g L^{-1} $(NH_4)_2SO_4$) was defined for Cr(VI)-amended fungal cultures. Kinetics of growth, Cr(VI) removal, and nutrient consumption for isolates *A. pullulans* VR-8, filamentous fungus PMF-1, and *Lecythophora* sp. NGV-1 were obtained. The order of Cr(VI) removal efficiency was as follows: *A. pullulans* VR-8 > *Lecythophora* sp. NGV-1 > filamentous fungus PMF-1, and a similar trend was observed for biomass yield and nutrients consumption. Studies on biospeciation by means of the selected Cr(III) oxidation protocol were unsuccessful, leading to Cr(VI) values much lower than expected. It revealed that this kind of protocols should be cautiously evaluated when studying microbial Cr(VI) bioremediation.

Keywords Cr(VI) · Yeast · Fungi · Culture medium · Cr(III) oxidation

1 Introduction

Chrome plating and polishing activities, inorganic chemical production, cooling tower and steel mill effluents, leather tanning, wood-preserving facilities, and petroleum refineries have been reported as the main source of chromium contamination. It represents a serious threat to water quality and the environment if no adequate treatments prior to disposal are implemented (Tseng and Bielefeldt 2002).

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Chromium(VI) has been designated a priority pollutant in many countries and by the United States Environmental Protection Agency (Ksheminska et al. 2003; Juvera-Espinosa et al. 2006). Its toxicity to biological systems is mainly based on the strong oxidizing potential which can lead to cellular damage. Also, carcinogenic and allergenic effects for mammals have been described, being 100 times more toxic and 1,000 times more mutagenic than Cr(III) (Morales-Barrera and Cristiani-Urbina 2006). On the other hand, Cr(III) is more stable and it constitutes a trace element for living organisms related to cell membrane stability and synthesis and stability of nucleic acids and proteins (Zetic et al. 2001). However, at high concentrations, it can complex with organic compounds interfering with metalloenzyme systems (Jamnik and Raspor 2003). Cr(VI) may also exert its toxicity as a redox active metal that can participate in Fenton reactions, being able to generate reactive oxygen species which cause cell oxidative stress (Jamnik and Raspor 2003).

The use of microorganisms for Cr(VI) bioremediation has been thought of as a cost-effective and ecofriendly alternative from several years ago, but research usually involved bacteria (Tseng and Bielefeldt 2002; Ramírez-Ramírez et al. 2004; Juvera-Espinosa et al. 2006). Up to 1990, no reports concerning Cr(VI)-resistant yeasts had appeared. Later on, diverse *Candida* species were related to chromium resistance and/or reduction (Baldi et al. 1990; Muter et al. 2001; Ramírez-Ramírez et al. 2004; Juvera-Espinosa et al. 2006; Guillén-Jiménez et al. 2008). Likewise, filamentous fungi have been revealed as promising candidates for Cr(VI) bioremediation in recent years (Morales-Barrera and Cristiani-Urbina 2008; Morales-Barrera et al. 2008).

Microorganisms have developed different strategies to resist heavy metal stress. Previous studies have demonstrated that yeasts are a very heterogeneous group in terms of chromium tolerance and accumulation and a variety of mechanisms have been evolved for metal uptake and metabolism (Ksheminska et al. 2005). In a diversity of yeast genera and strains, the interpretation of Cr(VI) removal has been correlated to mechanisms such as the bioaccumulation or the participation of certain protective/detoxifying metabolic activities such as riboflavin biosynthesis or metal-reducing activity (Ksheminska et al. 2005).

Both enzymatic and nonenzymatic mechanisms have been suggested for chromium reduction. In the first case, soluble enzymes able to reduce Cr(VI) to Cr(III) might be implicated. In the case of yeasts like *Candida* species, Cr(VI) reduction ability has been previously reported (Muter et al. 2001; Ramírez-Ramírez et al. 2004; Juvera-Espinosa et al. 2006). However, no enzymes concerning this activity have been isolated and characterized so far.

The use of CrO_4^- as a terminal electron acceptor has also been described (McLean and Beveridge 2001), but the strategy would be more likely in bacteria under anaerobic respiration conditions. In addition, nonspecific reduction of Cr(VI) by metabolic products, such as hydrogen sulfide, ascorbic acid, and thiols (glutathione, cysteine, and coenzyme M), under physiological conditions has also been suggested (Baldi et al. 1990). However, up to date, the complexity of yeast bioremediation still represents a challenging problem.

With respect to the quantification of Cr(VI) along cultivation, the commonly cited method consists on the colorimetric determination of remaining Cr(VI) in cell-free supernatants with *S*-diphenylcarbazide (DPC; Urone 1955). On the other hand, the Cr(VI) reduction to Cr(III) is usually indirectly estimated from total Cr measurements by atomic absorption spectroscopy (AAS; McLean and Beveridge 2001; Ramírez-Ramírez et al. 2004). Nevertheless, some works have reported different techniques consisting on the oxidation of Cr(III) to Cr(VI) by oxidants like $KMnO_4$ (Muter et al. 2001), hypobromite (Juvera-Espinosa et al. 2006; Morales-Barrera and Cristiani-Urbina 2008), or ammonium persulfate plus $AgNO_3$ (Bobrowski et al. 2004) and its subsequent quantification by DPC.

On all this context where the interpretation of Cr(VI) removal mechanisms appears somewhat troublesome per se, further interactions of this heavy metal with culture medium components cannot be underestimated. Chromium fate is highly dependent on pH and redox potential, as well as on the presence of different substances that may disturb the chemical equilibrium between chromium oxidation states, e.g., total organic carbon, ferrous iron, sulfide, and other inorganic compounds (Vitale et al. 1997; Zetic et al. 2001; Tseng and Bielefeldt 2002; Bobrowski et al. 2004; Guillén-Jiménez et al. 2008). Also, the presence of sugars may exert a significant influence since, despite not profusely documented, compounds like D-lactose have been already associated to the reduction of Cr(VI) to Cr(III) concomitantly with the formation of the aldonic acid (Roldán 2005).

Although the incidence of parameters like culture medium, pH, inoculum size, Cr valence and chemical nature, and cultivation mode on the efficiency of Cr(VI) removal by microbial cultures has been already stressed (Tseng and Bielefeldt 2002; Kaszycki et al. 2004; Ksheminska et al. 2005; Juvera-Espinosa et al. 2006; Morales-Barrera and Cristiani-Urbina 2006), no specific comments could be encountered concerning the interference of culture medium components on the Cr(VI) quantification. Likewise, no systematic trials have been previously described in order to quantify the biases obtained when oxidizing methods were intended for Cr(III) conversion to Cr(VI) prior to DPC determinations.

Therefore, one of the aims of this research was to weight the influence/interference of different components of culture medium on the Cr(VI) quantification as well as their incidence on yeast growth and metal removal. In addition, different strategies for Cr(III) oxidation and its subsequent quantification by DPC were systematically assessed.

2 Experimental

2.1 Culture Media for Chromium Quantification and Chromium Species Tested

Culture media preliminary tested for Cr(VI) quantification were: yeast nitrogen base (YNB without amino acids; DIFCO) with glucose (20 g L^{-1}) and $(NH_4)_2SO_4$ (5 g L^{-1}) as C and N source, respectively, pH 5.0; yeast extract peptone dextrose (YEPD); and MOPT (Fariña et al. 1998). Tested chromium species included Cr(VI) as $K_2Cr_2O_7$ and Cr(III) as $CrCl_3$, at 1 mM final concentration, and stock solutions (100 mM) were always prepared in bidistilled (bd) water.

2.2 Cr(III) Oxidation and Quantification Methods

The different Cr(III) oxidation methods tested are displayed in Table 1. For comparison purposes, $KMnO_4$ oxidation protocols were also tested according to APHA et al. (1975) and Muter et al. (2001). The possible interference of proteins on Cr(III) oxidation and subsequent Cr(VI) quantification was evaluated by means of the treatment with trichloroacetic acid (TCA) prior to 80 mM KIO_4 oxidation (protocol A3; Table 1). To do this, 500 µL of the sample was mixed with 500 µL 6% TCA. After vortexing, the mixture was left to stand for 10 min, centrifuged (10,300×*g*/10 min), and the supernatant was subjected to oxidation protocol A3 before Cr(VI) quantification by DPC, unless stated otherwise.

Also, in order to remove the eventual interference of metal ions other than Cr (APHA et al. 1975), a preliminary treatment of samples with cupferron was included after 80 mM KIO_4 oxidation (protocol A3; Table 1). The oxidized sample (2,400 µL) was treated

Table 1 Oxidation methods tested for Cr(III) conversion to Cr(VI) prior to DPC quantification

Protocol ID	Ammonium peroxidisulfate	KIO_4[a]
Reagents	100 µL concentrated H_2SO_4 + 100 µL 50% *w*/*v* ammonium peroxidisulfate + 1,800 µL sample (Panreac Química SA 2008)	(a) 1,900 µL sample + 100 µL 80 mM KIO_4
		(b) 1,800 µL sample + 100 µL concentrated H_2SO_4 + 100 µL 80 mM KIO_4
		(c) 1,900 µL sample + 100 µL supersaturated KIO_4
		(d) 1,800 µL sample + 100 µL concentrated H_2SO_4 + 100 µL supersaturated KIO_4
Conditions	Incubation at 100°C/2 h	Incubation at (1) 100°C/30 min, (2) RT/ON, (3) RT/30 min, (4) RT/30 min + adjust to pH 1–3 with 20% *w*/*v* NaOH
	Cool to RT	
	Add 1 mL bd H_2O	
	Adjust to pH 1–3 with 20% *w*/*v* NaOH	
	Make up to 5 mL with bd H_2O	Cool to RT, when necessary
		Make up to 5 mL with bd H_2O

[a] Periodate oxidation methods consisted in the combination of steps as follows: A1, A2, A3, B4, C3, and D4 where, for instance, A3 means oxidation step A + incubation step 3

RT room temperature, *bd* bidistilled, *ON* overnight

with 300 μL of an ice-cold cupferron solution (5 g in 95 mL of bd water). After mixing, it was allowed to stand in an ice bath for 1 min, and afterwards, three chloroform extractions were carried out (in 500-μL aliquots). After each phase separation, chloroform extracts were discarded and the final aqueous layer was carefully recovered and kept in a separate tube. The extracted sample was then boiled for 5 min, and after cooling to room temperature, DPC determination was performed as usual.

For the quantification of Cr(III) with ethylenediaminetetraacetic acid (EDTA), an aliquot of Cr(III) standard solution (0.26 g L^{-1}) was mixed with 200 μL of 0.8 N EDTA in a final reaction volume of 1 mL. After vortexing, optical density was measured at 540 nm and a calibration curve was constructed.

2.3 Maintenance and Cultivation of Yeast Strains

Fungal strains isolated from metal-containing environments (*Aureobasidium pullulans* VR-8 from a nickel–copper mine, San Luis, Argentina; *Lecythophora* sp. NGV-1 from tannery wastewater sediments, La Rioja, Argentina (Villegas et al. 2008); and the fungus PMF-1 isolated from a Cr-amended medium in the laboratory) were maintained by periodic subculturing on YNB as described above. Incubation was performed at 25°C for 3 days and plates were stored at 5°C.

For inoculum preparation, active cells from YNB plates were transferred to 25 mL of liquid YNB (filter sterilized) with the same composition as above in 125-mL Erlenmeyer flasks and incubated at 25°C and 250 rpm for 48 h. From these liquid cultures, a 5-mL aliquot was used for inoculation of 100 mL liquid YNB without amino acids and ammonium sulfate (basal YNB) plus 50 g L^{-1} sucrose and 0.6 g L^{-1} $(NH_4)_2SO_4$, hereafter identified as YNB′, in 500-mL Erlenmeyer flasks. A proper amount of a filter-sterilized Cr(VI) stock solution (100 mM, as $K_2Cr_2O_7$) was added to reach a 1-mM final concentration. Incubation was carried out at 25°C and 250 rpm.

2.4 Analytical Determinations

Aliquots of culture medium were periodically withdrawn, centrifuged at 10,300×*g*/25°C/15 min, and supernatants were subsequently analyzed for pH, Cr(VI) removal, reducing sugars, and ammonium consumption.

2.4.1 Cell Concentration

Biomass dry weight (BDW) was estimated after double washing of the pellets resulting from Section 2.4 with distilled water followed by drying at 85°C until constant weight.

2.4.2 Cr(VI) Quantification

Cr(VI) in solution was determined by colorimetric measurement of the pink/violet-colored complex formed after reaction with DPC in acid solution (APHA et al. 1975). A miniaturized protocol was developed for this purpose, as follows: to 50 μL of sample supernatant, 50 μL of 0.2 N H_2SO_4 was added and the volume was made up to 2 mL with bd water. After mixing with 40 μL of 5 mg DPC/mL acetone, the mixture was allowed to stand for 10 min and spectrophotometric determinations were performed at 540 nm (Beckman DU640) against a reagent blank. Chromium(VI) concentrations were proportional to their absorbance (up to 2.6 μg mL^{-1}) and were quantified by the use of an external $K_2Cr_2O_7$ standard with a seven-point calibration curve.

2.4.3 Reducing Sugars Concentration

They were quantified by using the dinitrosalicylic acid (DNSA) reagent (Miller 1959). A 500-μL aliquot of sample supernatant, diluted with distilled water if necessary to fit the measurement range, was mixed with 50 μL of 1 N HCl and heated in a boiling water bath for 10 min. Once cooled to room temperature, it was neutralized with 50 μL of 1 N NaOH and 750 μL DNSA reagent were added. The mixture was heated in a boiling water bath for 15 min and the reaction was stopped by freezing (10 min). After vortexing, absorbance was measured at 640 nm against a reagent blank. A six-point calibration curve was constructed with glucose as standard; linearity could be kept up to 1.0 g L^{-1}.

2.4.4 Ammonium Concentration

A microtechnique based on the Koroleff method was used for ammonium quantification (Koroleff 1976). Briefly, 1 mL of sample supernatant was mixed with 40 μL of phenol–alcohol solution (10% *w*/*v*), 40 μL of sodium nitroprussiate (0.5% *w*/*v*, aqueous), and

100 μL of freshly prepared oxidant reagent (100 mL alkaline complex [100 g sodium citrate + 5 g NaOH in 500 mL distilled water] + 25 mL commercial NaClO). After vortexing, the mixture was allowed to stand for 1 h in the dark and then optical density was measured at 630 nm. A seven-point calibration curve was constructed with $(NH_4)_2SO_4$ as standard; linearity could be kept up to 3.5 mg L^{-1}.

2.5 Statistical Analysis of Results

The statistical significance of differences among values was assessed by using the one-way analysis of variance test. Data were analyzed using the GraphPad InStat Instant Biostatistics package version 3.0.

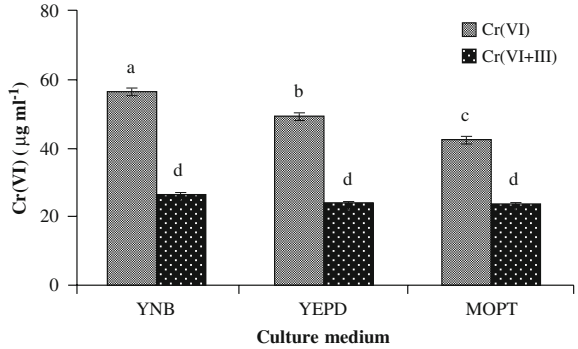

Fig. 1 Influence of culture medium on the Cr(VI) quantification by DPC. Media were supplemented with either 1 mM Cr(VI) or 1 mM Cr(VI+III) mixture. For details, see Section 2. *Bars with the same letter* indicate no significant differences. *a* vs. *b* ($P<0.01$); *a* vs. *c* ($P<0.001$); *b* vs. *c* ($P<0.01$)

3 Results and Discussion

3.1 Influence of Culture Medium and Cr(III) Oxidation Methods on the Cr(VI) Quantification

Different culture media containing an equal concentration of Cr(VI) (1 mM) or a mixture of equivalent amounts of Cr(VI) + Cr(III) (1 mM) were subjected to the conventional Cr(VI) quantification method by DPC. MOPT culture medium, previously designed for exopolysaccharide (EPS) production (Fariña et al. 1998), was included in this screening because, in one of the tested strains (*A. pullulans*), EPS production was suggested as one of the mechanisms for heavy metal removal (Suh and Kim 2000). According to the culture medium used, a progressive decrease of measurable Cr(VI) concentration was noted (Fig. 1) in the following order: YNB > YEPD > MOPT. As expected, in the case of chromium mixtures, only the hexavalent species could be quantified by DPC (Fig. 1).

When different Cr(III) oxidation methods were tested, a noticeable influence of culture medium was clearly evidenced (Figs. 2 and 3). Significant differences were also noted depending on the oxidation protocol applied, both for the same culture medium and the aqueous Cr mixture. Oxidation of Cr(III) in aqueous mixtures in the presence of Cr(VI) was not always efficient according to the protocol used (Figs. 2 and 3).

Some oxidation methods led to undetectable levels of Cr(VI) by DPC, either for aqueous Cr mixtures (80 mM KIO_4, protocol B4) or for YNB, YEPD, and MOPT culture media (ammonium peroxidisulfate protocol; Fig. 2). Concerning the interpretation of these results, it has been already reported that, while Cr(III) does not interfere with DPC spectrophotometric determination, Cr(III) oxidants may do it under certain reaction conditions (Bobrowski et al. 2004). In other cases, detectable Cr(VI) only achieved the levels corresponding to half of the added amount (i.e., ~0.5 mM), apparently indicating no Cr(III) oxidation (Figs. 2 and 3).

In an effort to improve the oxidation of Cr(III)-containing media with 80 mM KIO_4 under neutral conditions (protocol A3), some further trials including deproteinization with TCA and cupferron treatment were performed (Fig. 3). However, Cr(VI) values in culture media not only could not be enhanced but even became lower, particularly in the case of cupferron (Fig. 3), both for culture media and the aqueous Cr mixture.

For aqueous Cr mixtures, protocols that best worked were those involving 80 mM KIO_4 under neutral conditions (protocols A1–A3; Figs. 2 and 3). However, when these protocols were applied to culture media, Cr(VI) titers in YNB approached to half of the expected amount (i.e., ~0.5 mM), revealing the unsuccessful oxidation of Cr(III) under these conditions. Moreover, values were even lower for the other tested media (YEPD and MOPT; Figs. 2 and 3). Also, DPC-measured Cr(VI) values in culture media after oxidation with $KMnO_4$ (APHA et al. 1975; Muter et al. 2001) were quite lower than the ones obtained according to the herein selected protocol A3 (data not shown). Therefore, results indicated protocol A3 as one

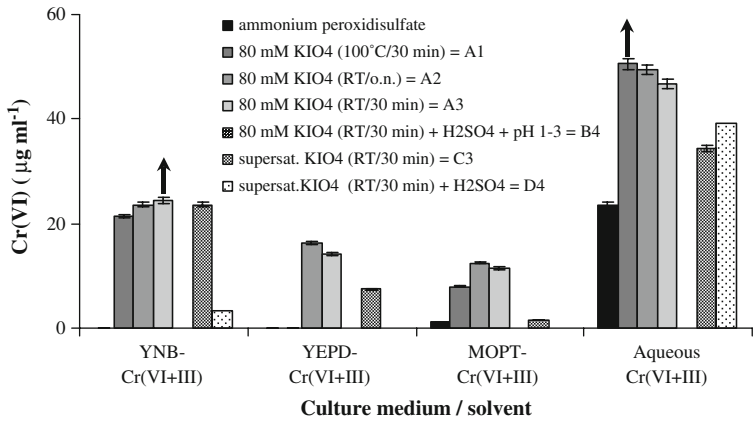

Fig. 2 Influence of culture medium on the Cr(III) oxidation according to different protocols. Media or bd water were amended with 1 mM Cr(VI+III) mixture prior to oxidation. For further details, see Table 1. Post oxidation Cr(VI) values were obtained by DPC quantification. Best values are denoted by an *upward arrow*

more likely susceptible to optimization and, accordingly, it was selected for subsequent experiments.

Based on all the preceding results, it could be concluded that the selection of culture medium for studying Cr(VI) microbial bioremediation would not be a minor point, particularly if Cr(III) oxidation methods are applied. It was clearly demonstrated that Cr(VI) determinations may be the target of significant biases and erroneous interpretations if performed with no preliminary control tests, as those herein described. For instance, some culture media like YEPD or MOPT particularly interfered with Cr(VI) quantification, not only hampering Cr(III) oxidation in a great extent but also leading to values of Cr(VI) quite lower than expected (<0.5 mM) or even nil (Figs. 2 and 3). Consequently, to minimize these biases, subsequent experiments were carried out starting with YNB culture medium composition.

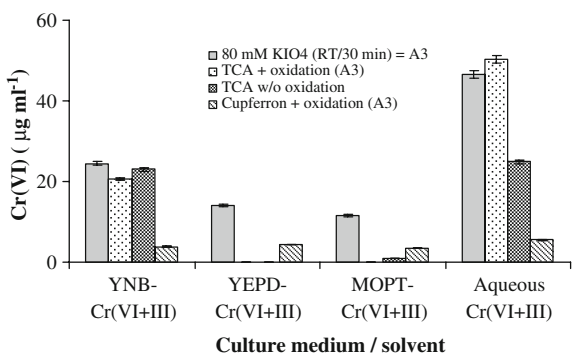

Fig. 3 Incidence of TCA deproteinization and cupferron treatment on Cr(III) oxidation protocol A3. Media or bd water were amended with 1 mM Cr(VI+III) mixture prior to oxidation. For further details, see Section 2. Post oxidation Cr(VI) values were obtained by DPC quantification

3.2 Influence of Culture Medium Components on Cr(III) Oxidation and Cr(VI) Quantification

In order to systematically assess the influence of some culture medium components on Cr(VI) determination, independent variations in the C and N sources were assayed. The starting basal medium was the YNB without amino acids and ammonium sulfate, in order to have a precise control over the supplemented N concentration. Chromium(VI) concentrations after oxidation (protocol A3) both with basal YNB (without C and N source) and with 2.25 g L^{-1} $NaNO_3$, either for 1 mM Cr(VI) or for the mixture (Cr(VI+III)=1 mM), were a little lower than the theoretical Cr(VI) value in water (aqueous Cr(VI) without oxidation, $P<0.01$; Fig. 4a). However, the values were similar to those of aqueous chromium solutions subjected to oxidation (aqueous Cr(VI) and aqueous Cr(VI+III) with oxidation, $P>0.05$; Fig. 4a). As discussed above, the impossibility to reach the theoretical Cr(VI) concentration after oxidation, even for aqueous solutions, might be related to the interference caused by the oxidants applied (Bobrowski et al. 2004). On the other hand, when aqueous chromium solutions were added with sucrose (150 g L^{-1}, as in MOPT), Cr(III) oxidation demonstrated to be significantly affected ($P<0.001$; Fig. 4a).

In a second instance, the three variables (basal YNB, $NaNO_3$, and sucrose) were combined in pairs (Fig. 4b). Once again, $NaNO_3$ did not show a marked influence on Cr(VI) determination, either for Cr(VI) or for Cr(VI+III) mixtures subjected to oxidation ($P>0.05$). However, all combinations with sucrose led to more or less diminished Cr(VI) values ($P<0.01$–0.001; Fig. 4b). Lower values when only Cr(VI) was present in the

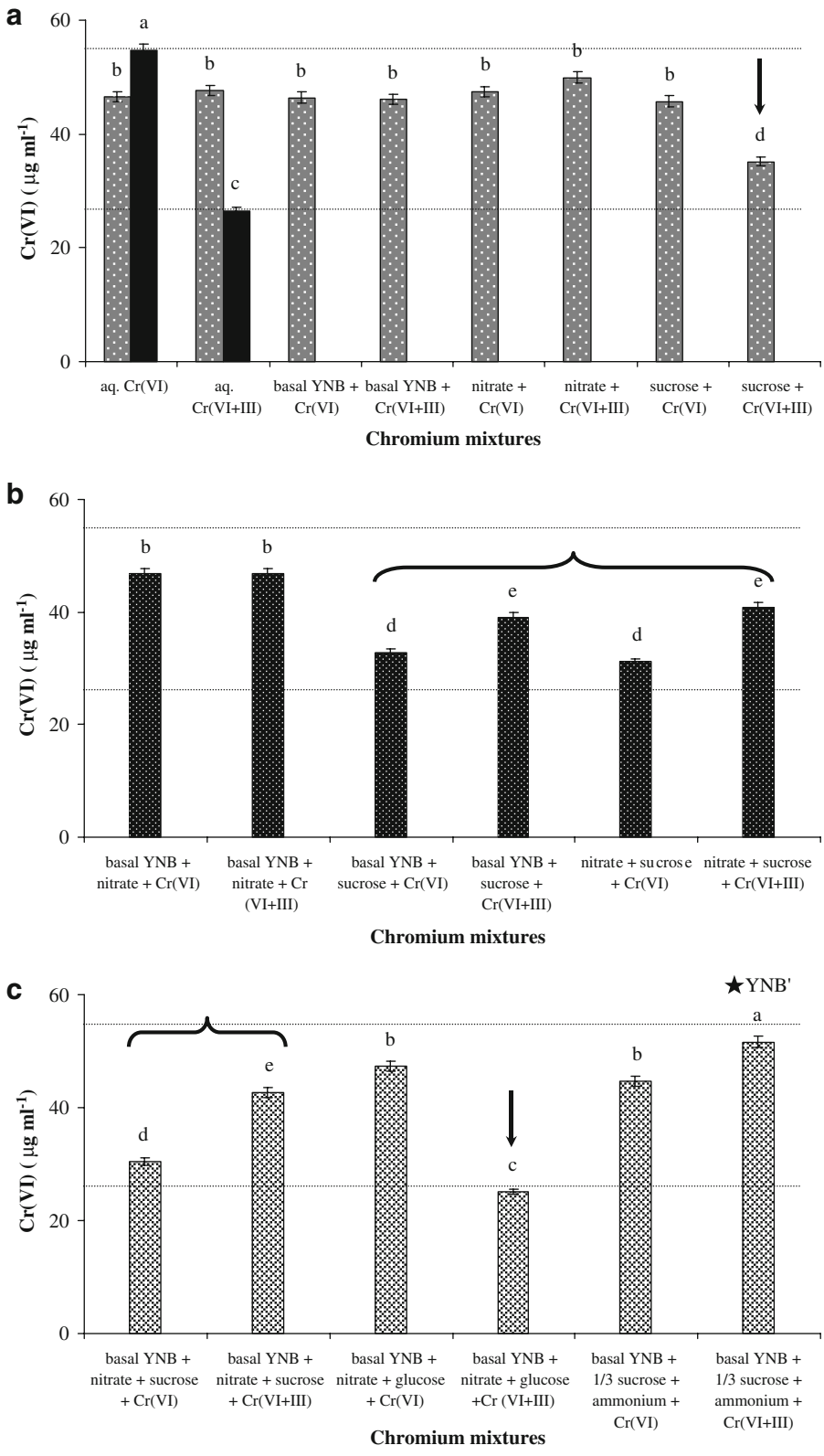

Fig. 4 Interference of culture medium components on the Cr(III) oxidation and Cr(VI) quantification. **a** Basal YNB (without amino acids and ammonium sulfate), C and N source were independently assessed. **b** Two-component combinations. **c** Three-component combinations. Media or bd water were amended with 1 mM Cr(VI+III) mixture prior to oxidation (protocol A3). For further details, see Section 2. Post oxidation Cr(VI) values were obtained by DPC quantification. *Black bars* represent the measured Cr(VI) values in aqueous solutions without oxidation. *Bars with the same letter* indicate no significant differences. Significantly lower values are denoted by a *downward arrow* or under a *horizontal bracket*. Selected medium (YNB′) is marked with a *black star*

mixtures might be related to the higher interference of unconsumed Cr oxidant.

Finally, variables were combined all at once, i.e., basal YNB + $NaNO_3$ + sucrose (Fig. 4c). Also, this trial included changes of sucrose by glucose as C source, $NaNO_3$ by $(NH_4)_2SO_4$ as N source, and the simultaneous reduction in C and N source concentrations (from 150 to 50 g L^{-1} and from 0.37 to 0.13 g N L^{-1}, respectively; Fig. 4c).

Comparing the oxidation efficiency of the Cr mixture (aqueous Cr(VI+III)), the medium with basal YNB plus $NaNO_3$ and sucrose led to better Cr(VI) levels than the one with glucose at equivalent concentration (150 g L^{-1}, $P<0.01$; Fig. 4c). This result revealed the convenience of using sucrose instead of glucose for avoiding eventual misleading interpretations. Surprisingly, the usual tendency of Cr (VI) values to increase after oxidation for Cr(VI+III) mixtures in comparison to Cr(VI)-amended media (Fig. 4b, c) was inverted for the case of glucose. Values were higher when only Cr(VI) species was present (Fig. 4c). It was suspected that glucose may act as a strong reducing agent thus avoiding the interference of the oxidant on the subsequent Cr(VI) quantification step.

When sucrose was threefold reduced at 50 g L^{-1} and N source was changed to $(NH_4)_2SO_4$, a further improvement in the oxidation efficiency could be achieved thus getting closer to the theoretical Cr(VI) value (Fig. 4c). In order to analyze Cr(VI) removal kinetics, especially when applying Cr(III) oxidation methods, media with sucrose seemed to be more appropriate than glucose-containing ones. The extremely marked decrease of Cr(VI) titers after oxidation of culture medium in the presence of glucose (basal YNB + nitrate + glucose + Cr(VI+III); Fig. 4c) might indicate a false significant Cr(VI) removal. Accordingly, a medium containing basal YNB (without amino acids and ammonium sulfate) supplemented with 50 g L^{-1} sucrose and 0.6 g L^{-1} $(NH_4)_2SO_4$ (YNB′) was considered the one leading to lower interferences for Cr bioremediation assays.

Considering the complexity of Cr(III) oxidation and as another attempt to perform Cr(III) quantification in culture medium, a method based on the reaction with EDTA was also evaluated. Results showed a linear relationship between Cr(III) and optical density up to 250 µL mL^{-1} (~5 mM). However, absorbance readings were so extremely low in the concentration range of usual Cr bioremediation assays (~1 mM) that the method was considered not as sensitive as required for determining Cr(VI) biospeciation (Fig. 5). Most of the microbial isolates usually reported exhibit Cr(VI)-reducing activities within the range of 0.1–0.5 mM (Ksheminska et al. 2003; Juvera-Espinosa et al. 2006).

All the preceding results concerning the influence of culture medium composition on the subsequent Cr(VI) quantification by DPC, as well as those on the Cr (III) oxidation protocols, would be valid independently of the microorganism used in Cr(VI) removal studies.

3.3 Evaluation of Selected Conditions for Cr(III) Oxidation During Cr(VI) Removal Kinetics

As a mode to check the selected culture medium (YNB′) for Cr(VI) removal assays, yeast isolates were grown in this medium plus 1 mM Cr(VI) and compared to the metal-deprived biotic controls. Cultures were analyzed for biomass production, pH, sucrose and ammonium consumption, and Cr(VI) removal (Figs. 6 and 7). When biotic cultures were compared to Cr(VI)-amended cultures, growth was significantly restricted in the presence of Cr(VI), especially for the fungal isolates PMF-1 and *Lecythophora* sp. NGV-1. In the case of *A. pullulans* VR-8, despite of a significant lag phase observed at the beginning of cultivation, biomass production approached almost normal levels thereafter (Fig. 6). That was in agreement with the literature which refers that heavy metal-tolerant yeasts in metal-amended media could reach 80% of growth under metal-deprived conditions (Vadkertiová and Sláviková 2006).

Evolution of pH as well as sucrose and ammonium consumption accompanied growth, showing a higher

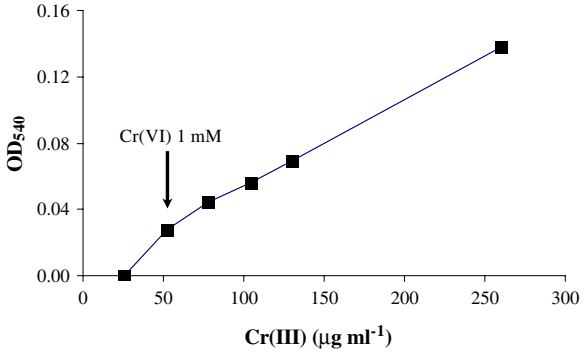

Fig. 5 Cr(III) quantification with EDTA. For details, see Section 2. Standard errors are below 5%

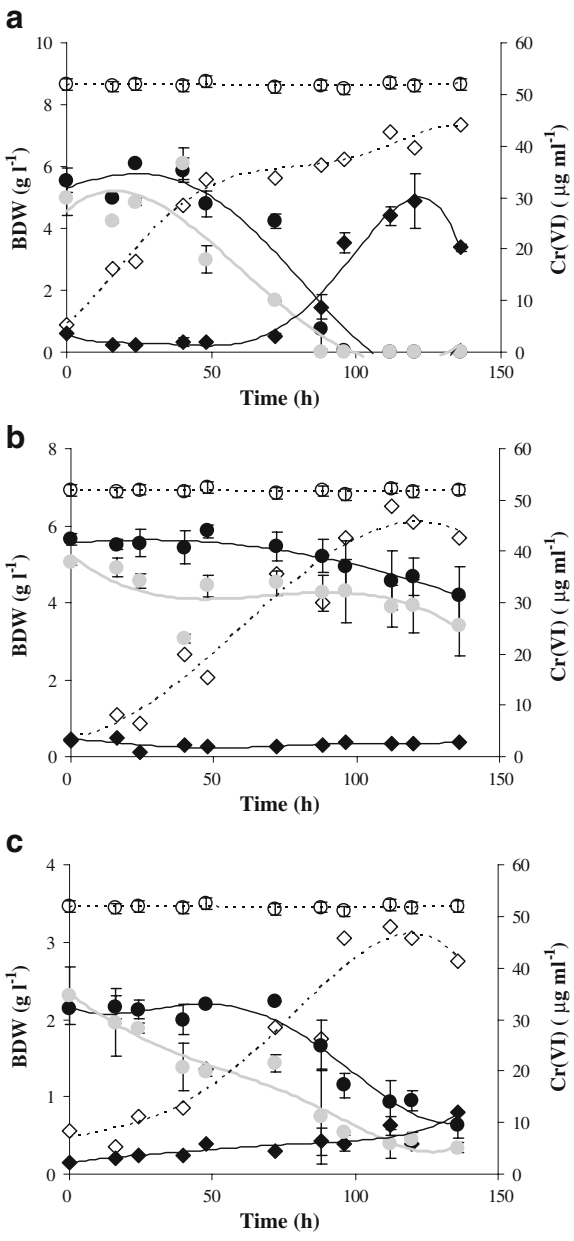

Fig. 6 Kinetics of growth and Cr(VI) removal for YNB′ cultures of fungal isolates: **a** *A. pullulans* VR-8, **b** PMF-1, and **c** *Lecythophora* sp. NGV-1. *Open* and *filled diamonds* BDW without and with Cr(VI), respectively, *open circles* Cr(VI) in abiotic control, *filled black circles* Cr(VI) in metal-amended cultures, *filled gray circles* Cr(VI) in metal-amended cultures after oxidation (protocol A3). No visible error bars indicate that they do not exceed symbol size

decrease when significant growth was observed (see Fig. 7 for ammonium, pH not shown). With respect to Cr(VI) removal, the best performance was exhibited by *A. pullulans* VR-8, reaching zero values at around 96 h of cultivation. In second place was the strain *Lecythophora* sp. NGV-1 and last was the fungus PMF-1 (Fig. 6).

Finally, analysis of Cr(III) according to the oxidation protocol A3 was not as good as expected. Despite the

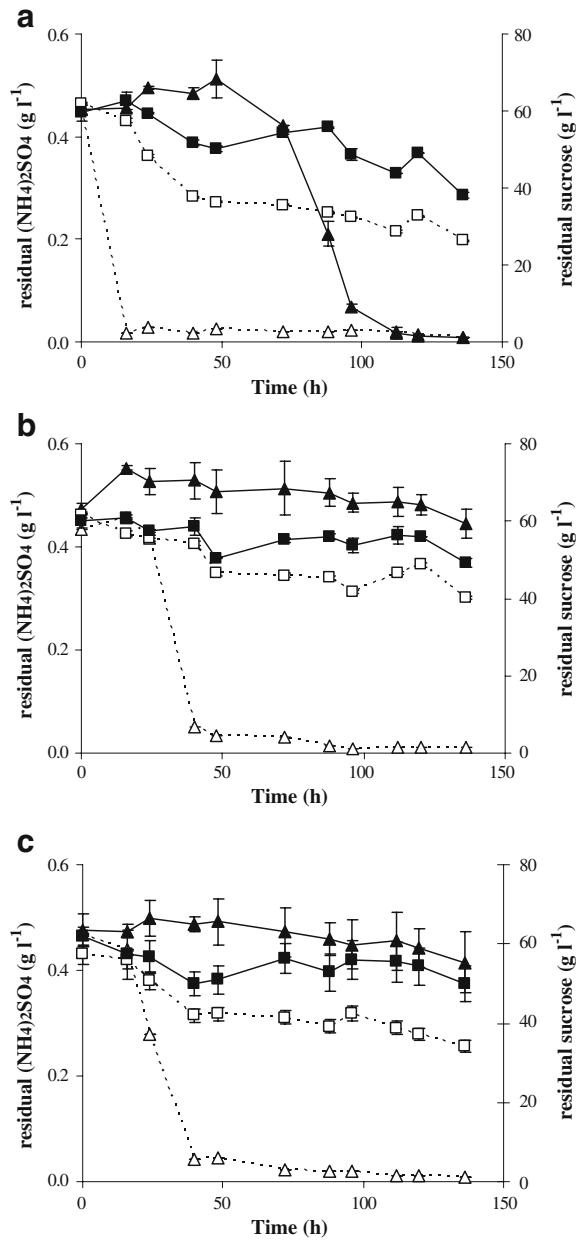

Fig. 7 Kinetics of $(NH_4)_2SO_4$ and sucrose consumption for YNB′ cultures of fungal isolates: **a** *A. pullulans* VR-8, **b** PMF-1, and **c** *Lecythophora* sp. NGV-1. *Open* and *filled triangles* residual $(NH_4)_2SO_4$ without and with Cr(VI), respectively, *open* and *filled squares* residual sucrose without and with Cr(VI), respectively. No visible error bars indicate that they do not exceed symbol size

fact that this oxidation protocol worked efficiently for uninoculated culture medium YNB′ (Fig. 4c), the procedure was not so competent when applied to culture broths (i.e., post inoculation; Fig. 6).

As it can be deduced from the Cr(VI) values after Cr(III) oxidation (Fig. 6a–c), they should have been higher than the ones without oxidation; however, the oxidation step prior to DPC was unsuccessful. Consequently, Cr(III) could not be indirectly estimated, i.e., substracting Cr(VI) values without oxidation to the Cr(VI) values after oxidation. Probably, this fact revealed a further interference from the inoculum or its interaction with the culture medium components at the time of Cr(III) oxidation.

Moreover, Cr(VI) initial values at time 0 should have approached the abiotic control value; however, they were markedly lower (Fig. 6a–c). A similar interpretation as the abovementioned for Cr(III) oxidation interference was suspected. On the other hand, the decrease at time 0 was different among fungal isolates, being lower for PMF-1 than for *A. pullulans* VR-8 and *Lecythophora* sp. NGV-1 (Fig. 6a–c). Therefore, some interference related to the inoculum source might be speculated.

4 Conclusions

Results have shown that when optimizing culture conditions for the study of chromium bioremediation, the influence of some culture factors on the nonmicrobial Cr(VI) disappearance should not be ignored. Nevertheless, scarce literature has put emphasis on this topic. It could be concluded that culture medium formulation becomes essential at the time of evaluating chromium bioremediation mechanisms, independently of the microorganism used.

Furthermore, results seem to show that extreme care should be also taken at the time of analytical measurements, such as Cr(VI) colorimetric quantification and, particularly, Cr(III) estimations based on oxidation methods prior to DPC. In spite of the fact that some literature refers to Cr(III) oxidation protocols before DPC quantification, our results seem to indicate that this procedure should be cautiously considered and that AAS cannot still be surpassed in order to get more reliable results and proper mechanistic interpretations about Cr(VI) removal.

Knowledge on Cr(VI) bioremediation by fungal isolates is still in its beginnings and these results may warn about certain critical aspects that should be taken into account at the time of performing analytical determinations, thus avoiding misleading results and interpretations. This may contribute to the development of appropriate methodologies for chromium analytical measurements in order to identify promising microbial candidates for alleviating chromium environmental pollution.

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References

APHA, AWWA, & WPCF. (1975). Determination of metals (part 307): chromium. In M. C. Rand, A. E. Greenberg & M. J. Taras (Eds.), *Standard methods for the examination of water and wastewater* (pp. 192–195). Washington, DC: American Public Health Association.

Baldi, F., Vaughan, A. M., & Olson, G. J. (1990). Chromium (VI)-resistant yeast isolated from a sewage treatment plant receiving tannery wastes. *Applied and Environmental Microbiology, 56*, 913–918.

Bobrowski, A., Mocak, J., Dominik, J., Pereira, H., Baś, B., & Knap, W. (2004). Metrological characteristics and comparison of analytical methods for determination of chromium traces in water samples. *Acta Chimica Slovenica, 51*, 77–93.

Fariña, J. I., Siñeriz, F., Molina, O. E., & Perotti, N. I. (1998). High scleroglucan production by *Sclerotium rolfsii*: influence of medium composition. *Biotechnology Letters, 20*, 825–831. doi:10.1023/A:1005351123156.

Guillén-Jiménez, F. M., Morales-Barrera, L., Morales-Jiménez, J., Hernández-Rodríguez, C. H., & Cristiani-Urbina, E. (2008). Modulation of tolerance to Cr(VI) and Cr(VI) reduction by sulphate ion in a *Candida* yeast strain isolated from tannery wastewater. *Journal of Industrial Microbiology & Biotechnology, 35*, 1277–1287. doi:10.1007/s10295-008-0425-7.

Jamnik, P., & Raspor, P. (2003). Stress response of yeast *Candida intermedia* to Cr(VI). *Journal of Biochemical and Molecular Toxicology, 17*, 316–323. doi:10.1002/jbt.10093.

Juvera-Espinosa, J., Morales-Barrera, L., & Cristiani-Urbina, E. (2006). Isolation and characterization of a yeast strain capable of removing Cr(VI). *Enzyme and Microbial Technology, 40*, 114–121. doi:10.1016/j.enzmictec.2005.10.045.

Kaszycki, P., Fedorovych, D., Ksheminska, H., Babyak, L., Wójcik, D., & Koloczek, H. (2004). Chromium accumulation by living yeast at various environmental conditions. *Microbiological Research, 159*, 11–17. doi:10.1016/j.micres.2003.12.002.

Koroleff, F. (1976). Determination of ammonia. In K. Grasshof (Ed.), *Methods of seawater analysis* (pp. 127–133). Weinheim: Verlag Chemie.

Ksheminska, H., Jaglarz, A., Fedorovych, D., Babyak, L., Yanovych, D., Kaszycki, P., et al. (2003). Bioremediation of chromium by the yeast *Pichia guilliermondii*: toxicity and accumulation of Cr(III) and Cr(VI) and the influence of riboflavin on Cr tolerance. *Microbiological Research, 158*, 59–67. doi:10.1078/0944-5013-00178.

Ksheminska, H., Fedorovych, D., Babyak, L., Yanovych, D., Kaszycki, P., & Koloczek, H. (2005). Chromium(III) and (VI) tolerance and bioaccumulation in yeast: a survey of cellular chromium content in selected strains of representative genera. *Process Biochemistry, 40*, 1565–1572. doi:10.1016/j.procbio.2004.05.012.

McLean, J., & Beveridge, T. J. (2001). Chromate reduction by a pseudomonad isolated from a site contaminated with chromated copper arsenate. *Applied and Environmental Microbiology, 67*, 1076–1084. doi:10.1128/AEM.67.3.1076-1084.2001.

Miller, G. L. (1959). Use of dinitrosalycilic acid reagent for determination of reducing sugars. *Analytical Chemistry, 31*, 426–428. doi:10.1021/ac60147a030.

Morales-Barrera, L., & Cristiani-Urbina, E. (2006). Removal of hexavalent chromium by *Trichoderma viride* in an airlift bioreactor. *Enzyme and Microbial Technology, 40*, 107–113. doi:10.1016/j.enzmictec.2005.10.044.

Morales-Barrera, L., & Cristiani-Urbina, E. (2008). Hexavalent chromium removal by a *Trichoderma inhamatum* fungal strain isolated from tannery effluent. *Water, Air, and Soil Pollution, 187*, 327–336. doi:10.1007/s11270-007-9520-z.

Morales-Barrera, L., Guillén-Jiménez, F. M., Ortiz-Moreno, A., Villegas-Garrido, T. L., Sandoval-Cabrera, A., Hernández-Rodríguez, C. H., et al. (2008). Isolation, identification and characterization of a *Hypocrea tawa* strain with high Cr(VI) reduction potential. *Biochemical Engineering Journal, 40*, 284–292. doi:10.1016/j.bej.2007.12.014.

Muter, O., Patmalnieks, A., & Rapoport, A. (2001). Interrelations of the yeast *Candida utilis* and Cr(VI): metal reduction and its distribution in the cell and medium. *Process Biochemistry, 36*, 963–970. doi:10.1016/S0032-9592(01)00136-4.

Panreac Química SA (2008). Cromo (VI). Kit para la determinación de Cromo (VI) en aguas superficiales y residuales. Retrieved from http://www.panreac.es/new/esp/productos/docs/931020.pdf.

Ramírez-Ramírez, R., Calvo-Méndez, C., Avila-Rodríguez, M., Lappe, P., Ulloa, M., Vázquez-Juárez, R., et al. (2004). Cr(VI) reduction in a chromate-resistant strain of *Candida maltosa* isolated from the leather industry. *Antonie Van Leeuwenhoek, 85*, 63–68. doi:10.1023/B:ANTO.0000020151.22858.7f.

Roldán, V. P. (2005). Estudio de la interacción entre el cromo y la lactosa. Retrieved from http://www.e-campo.com/?event=news.display&id=1DF45439-F492-4F4C-867076AB6D9C7FB8&,Lechería-Tambo-Sanidad2005-02-14.

Suh, J. H., & Kim, D. S. (2000). Comparison of different sorbents (inorganic and biological) for the removal of Pb^{2+} from aqueous solutions. *Journal of Chemical Technology and Biotechnology (Oxford, Oxfordshire), 75*, 279–284. doi:10.1002/(SICI)1097-4660(200004)75:4<279::AID-JCTB217>3.0.CO;2-A.

Tseng, J. K., & Bielefeldt, A. R. (2002). Low-temperature chromium(VI) biotransformation in soil with varying electron acceptors. *Journal of Environmental Quality, 31*, 1831–1841.

Urone, P. F. (1955). Stability of colorimetric reagent for chromium, *S*-diphenylcarbazide, in various solvents. *Analytical Chemistry, 27*, 1354–1355. doi:10.1021/ac60104a048.

Vadkertiová, R., & Sláviková, E. (2006). Metal tolerance of yeasts isolated from water, soil and plant environments. *Journal of Basic Microbiology, 46*, 145–152. doi:10.1002/jobm.200510609.

Villegas, L. B., Fernández, P. M., Amoroso, M. J., & Figueroa, L. I. C. (2008). Chromate removal by yeasts isolated from sediments of a tanning factory and a mine site in Argentina. *Biometals, 21*, 591–600. doi:10.1007/s10534-008-9145-8.

Vitale, R. J., Mussoline, G. R., Petura, J. C., & James, B. R. (1997). Cr(VI) soil analytical method: a reliable analytical method for extracting and quantifying Cr(VI) in soils. *Journal of Soil Contamination, 6*, 581–593.

Zetic, V. G., Stehlik-Tomas, V., Grba, S., Lutilsky, L., & Kozlek, D. (2001). Chromium uptake by *Saccharomyces cerevisiae* and isolation of glucose tolerance factor from yeast biomass. *Journal of Biosciences, 26*, 217–223. doi:10.1007/BF02703645.