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Biosorption of chromium to fungi

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Eighteen fungal strains were isolated from water and soil samples and tested for their ability to enrich chromium. The microorganism with the highest enrichment capacity, a zygomycete (*Mucor hiemalis* MP/92/3/4), was chosen for detailed investigations. Some basic tests such as the pH-dependence, the kinetics of the enrichment and the metal selectivity were carried out with the two most frequent oxidation states of chromium, the trivalent cation (Cr^{3+}) and the hexavalent anion (CrO_4^{2-}). With Cr^{3+} the enrichment showed a saturation kinetic reaching 70% of the maximum capacity after about 30 min, whereas with CrO_4^{2-} a linear time course with a much lower metal enrichment was observed. The highest level of enrichment for Cr^{3+} was observed at pH 5.5 (21.4 mg/g dry wt), and for CrO_4^{2-} at pH 1 (4.3 mg/g dry wt). Investigations concerning the metal enrichment selectivity resulted in the following series of decreasing ion uptake: $Cr^{3+} > Cu^{2+} > Pb^{2+} > Ag^{+} > Al^{3+} > Co^{2+} > Zn^{2+} > Ni^{2+} > Fe^{2+} > Mo^{5+} > Cd^{2+} > Cs^{+} > Cr_2O_7^{2-} > CrO_4^{2-} > VO^{3-}$, calculated on a molar basis. Trivalent chromium caused a staining of the outer cell wall region in transmission electron microscopy. The localization of chromium in the stained outer layers of the cell wall could be verified by electron energy loss spectroscopy. The enrichment of Cr^{3+} by *M. hiemalis* seemed to be mainly a passive biosorption to the cell wall, whereas for the uptake of CrO_4^{2-} intracellular accumulation as well as biosorption is possible.

Keywords: chromium, biosorption, *Mucor hiemalis*, TEM, EELS

Introduction

Chromium, with its great economic importance in industries such as metal plating, leather tanning, paints, pigments and wood preservation, is a major metal pollutant of our environment. Chromium can occur in oxidation states from −2 to +6, but is commonly found in oxidation states of +3 and +6. The oxidation state has important consequences for toxicity, bioavailability and enrichment by microbial biomass (Bartlett 1991). Derivates of Cr^{3+} are water insoluble at neutral pH, in contrast to Cr^{6+} compounds, which are highly soluble (Cary 1982). In animals and humans Cr^{3+} is an essential trace element, for example as 'glucose tolerance factor', and has no or rather low toxicity (Anderson 1981). Cr^{6+} is highly toxic and has been shown to be mutagenic and carcinogenic (Yassi & Nieboer 1988). In *Neurospora crassa* Cr^{6+} uptake is energy-dependent and it is inhibited by sulfate (Roberts & Marzluf 1971). A cellular uptake for Cr^{6+} via the sulfate proton symport is considered. Detoxification of Cr^{6+} by microbial reduction to Cr^{3+} seems to be a widespread resistance mechanism (Silver & Cervantes 1992).

The aim of this study was the isolation of a microorganism which could enrich substantial amounts of chromium and thus be of potential use for clearing waste water.

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Materials and methods

Isolation and culture conditions

Isolation of the fungi from water and soil samples and further cultivation were carried out on Czapek Dox medium (free of chloride), containing 300 mg l^{-1} trivalent chromium as Cr_3NO_3 at 30 °C.

Metal accumulation test

Strains were grown for 5 days at 30 °C on a gyratory shaker. The mycelium was separated, washed with distilled water and incubated for 30 min with 1 mM $Cr(NO_3)_3$ at pH 5.5 and 30 °C. The enrichment procedures for K_2CrO_4 were carried out at the same conditions, except that incubation was at pH 2. The amount of accumulated

metal by the hyphae was determined by measuring the metal content of the supernatant before and after the incubation by atomic absorption spectroscopy (Perkin Elmer 2380). The remaining fungal pellet was then washed and digested with boiling, concentrated HNO_3. Both analyses showed the same results. For further experiments only the fungus with the highest enrichment, *Mucor hiemalis* MP/92/3/4, was chosen.

Tests for metal specificity were carried out with 1 mM solution of the following metal salts (all at pH 5.5, apart from Al^{3+} which was at pH 3): $Cr(NO_3)_3$, K_2CrO_4, $Na_2Cr_2O_7$, $AgNO_3$, CsCl, $Cd(NO_3)_2$, $Cu(NO_3)_2$, $CoSO_4$, $FeSO_4$, $Ni(NO_3)_2$, $Pb(NO_3)_2$, $ZnCl_2$, $Al_2(SO_4)_3$, $MoCl_5$, $NaVO_3$.

Cell disintegration

Cr^{3+} and Cr^{6+} treated and untreated mycelia of *M. hiemalis* were disintegrated with a pestle and a mortar under liquid nitrogen. After redissolution in physiological NaCl, the supernatant was separated from the pelleted fraction by centrifugation (1000 *g* for 5 min). The pellet was digested with boiling concentrated HNO_3. Both the supernatant and the digested pellet were analyzed for chromium and potassium by atomic absorption spectroscopy.

Preparation for transmission electron microscopy (TEM) and electron energy loss spectroscopy (EELS)

Samples of *M. hiemalis* grown for 5 days with (treated) and without (untreated) trivalent chromium were used for TEM observation. For fixation the samples were treated in 2% glutaraldehyde (in 0.1 M phosphate buffer, pH 7.2) and dehydrated in ethanol series. No OsO_4 was used for fixation to avoid interference and background noise when using EELS. After embedding in Spurr's resin (Spurr 1969) the samples were cut with a diamond knife and placed on a copper grid (200 mesh). All observations were done on a Zeiss electron microscope (TEM 902) equipped with a prism-mirror-prism imaging spectrometer for EELS.

EELS

To achieve high resolution with EELS analyses, very thin sections (30–40 nm) were used with no additional staining. The optimum atomic absorption edge for chromium at 580 eV was taken from Ahn *et al.* (1983). The specimen area which can be analysed by EELS is restricted by a constant 100 μm shutter. Thus the area depends on the magnification (diameter of the area: 1.2 μm (× 12000); 0.5 μm (× 30000); 0.18 μm (× 85000). The best results are obtained when the analyzed particle fills the opening of the shutter entirely, as background is then reduced to a minimum (Kottke 1991). All measured EELS-spectra were recalculated to the same amplification and zeroed at 530 nm.

Results and discussion

The primary goal of this work was the isolation of a fungus, which is able to accumulate substantial amounts of trivalent chromium and to find out, if this enrichment is either a biosorption or an intracellular uptake. The two fungi with the highest enrichment of Cr^{3+} were zygomycetes, *M. hiemalis* (MP/92/3/4) and *Rhizopus nigricans* (MP/92/3/11) (Figure 1).

A reason for this improved enrichment of these two zygomycetes compared with the other fungal strains could lie in a different composition of the cell wall. It is known that the homopolymer of *N*-acetylglucosamine, chitin, is principally present in many fungi. Zygomycetes contain chitosan, a heteropolymer of *N*-acetylglucosamine and glucosamine. The deacetylated amino groups of glucosamine may act as possible binding sites for metals, although they are positively charged. In addition, a new siderophore type, named rhizoferrin, has been found in Mucorales (Winkelmann 1992) and may be important for the improved Cr^{3+}-enrichment. Rhizoferrin is a polycarboxylate siderophore that is able to bind trivalent iron, chromium and aluminium. A release of the metal from the siderophore results in reduction to its divalent form. While this is possible for Fe^{3+}, trivalent Cr and Al should not be reduced under physiological conditions. These metals remain bound to the siderophore and may in a further stage be accumulated by the cells; for some microorganisms this can be a resistance mechanism to toxic metals.

Experiments with M. hiemalis *(MP/92/3/4)*

We first studied pH-dependency (Figure 2), and found that Cr^{3+} was enriched best at pH value 5.5. Up to this pH chromium exists in its cationic form, while above pH 6 Cr^{3+} starts precipitating as its hydroxides and above pH 8 anionic hydroxides are found. The best accumulation for Cr^{3+} at pH 5.5 can be explained by the fact that at lower pH more protons would compete for the binding sites. A similar pH for the optimum Cr^{3+}-uptake by algae was

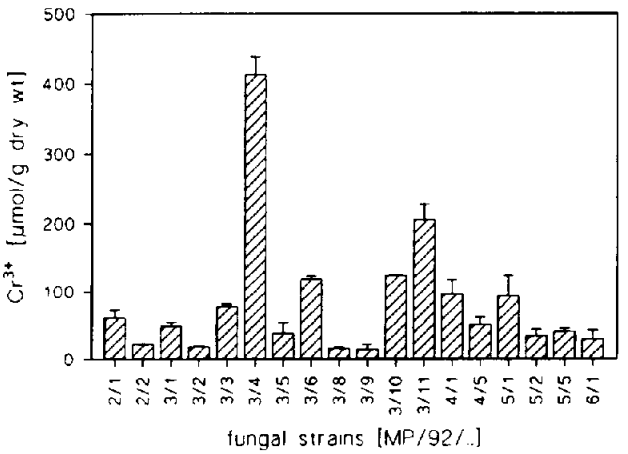


Figure 1. Comparison of Cr^{3+}-enrichment by 18 fungal strains.

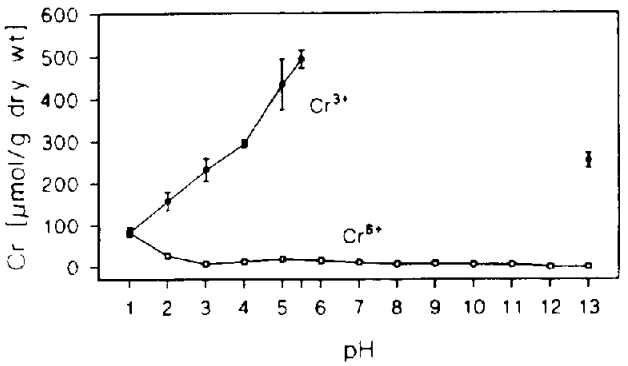


Figure 2. pH effect on the enrichment of Cr^{3+} and Cr^{6+} by *M. hiemalis*.

found by Kuyucak & Volesky (1988) and Pappas *et al.* (1990).

In contrast to trivalent chromium, the hexavalent form showed highest accumulation at pH 1 and no precipitation if the pH was varied. Three possibilities exist for the increased enrichment at this acidic pH: firstly, the binding sites of the cell wall may be saturated with protons and therefore the interaction between protonated binding sites and the hexavalent chromium anions are improved; secondly, a higher uptake can be the result of the increased reduction from Cr^{6+} to Cr^{3+} at acidic conditions (Imai & Gloyna 1990), with the resulting chromium cation perhaps better bound than the chromium anion, thirdly, a possible transport of the anionic CrO_4^{2-} via the sulfate proton symport may be enhanced at acidic pH values.

The cationic Cr^{3+} enrichment at pH 5.5 (21.4 mg/g dry wt) was at least five times higher than for the anionic Cr^{6+} at pH 1 (4.3 mg/g dry wt).

Differences between the oxidation states were also found when the initial rate of the saturation kinetic was examined (Figure 3). For Cr^{3+}, over 70% of the capacity (taken after 1000 min) was bound within 30 min at pH 5.5. In contrast, Cr^{6+} showed a slow initial increase, with 20% bound in the first 30 min. An accumulation maximum could not be reached for both chromium ions after 1000 min (= 16.6 h).

For the estimation of the biomass affinity (1/b) and the maximum Cr^{3+}-uptake (q_{max}) of *M. hiemalis*, the data for an isotherm were fitted to the Langmuir sorption model (Holan *et al.* 1992; Figure 4). The uptake isotherm showed a calculated q_{max} of 424.7 (± 2.87) µmol/g dry wt and a 1/b of 62.7 (± 2.27) µM. The Langmuir equation fitted the experimental data well ($r^2 = 0.9998$), indicating a one site binding of chromium.

Tests for metal selectivity with *M. hiemalis* (pH 5.5) showed a wide metal range enrichment with no metal specifity (Figure 5). As expected, a much lesser binding capacity was observed for anions than cations. The best,

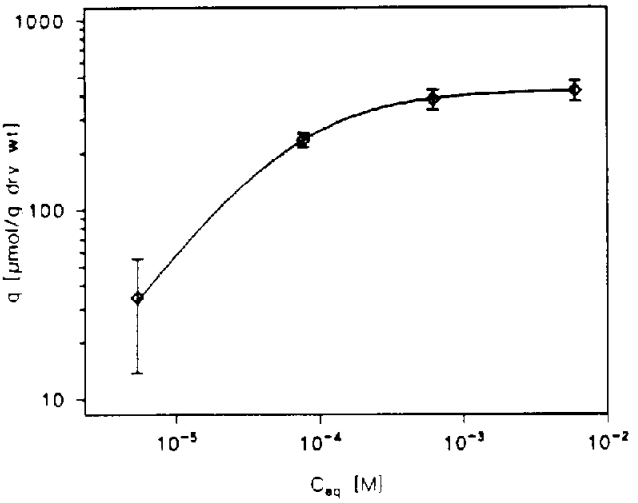


Figure 4. Enrichment isotherm of *M. hiemalis* for Cr^{3+}.

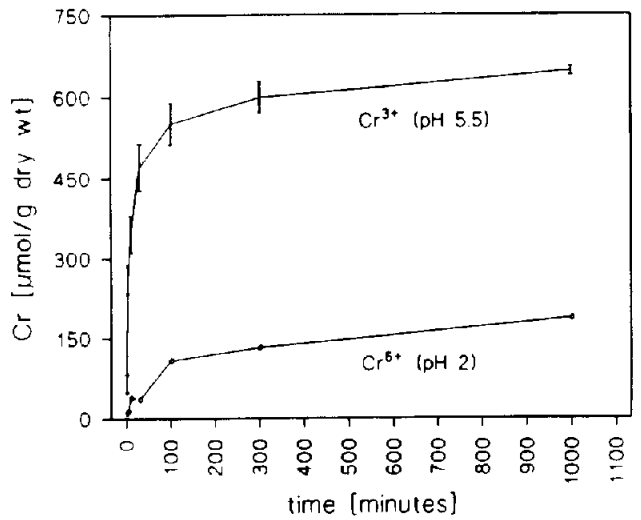


Figure 3. Enrichment kinetics of Cr^{3+} and Cr^{6+} by *M. hiemalis*.

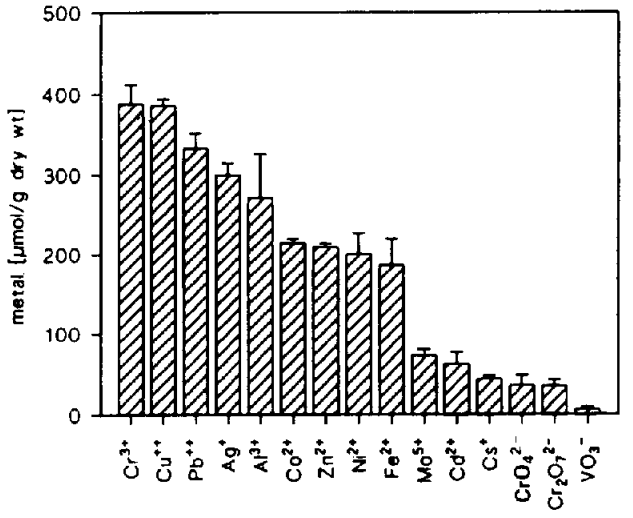


Figure 5. Specificity of metal enrichment of *M. hiemalis*.

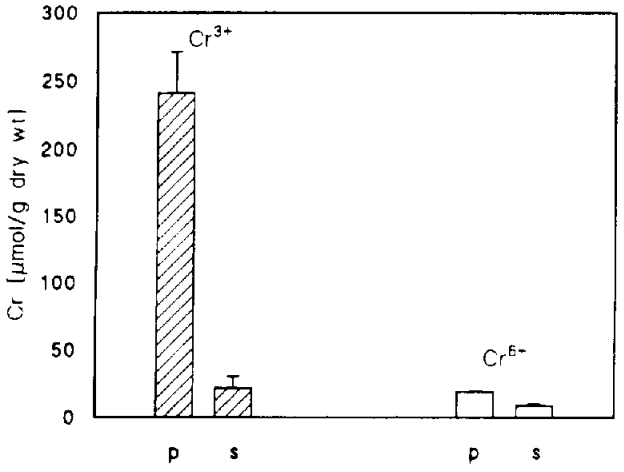
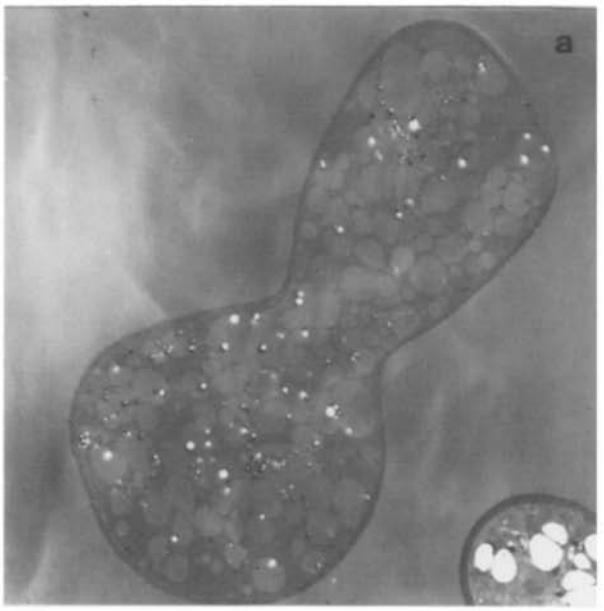
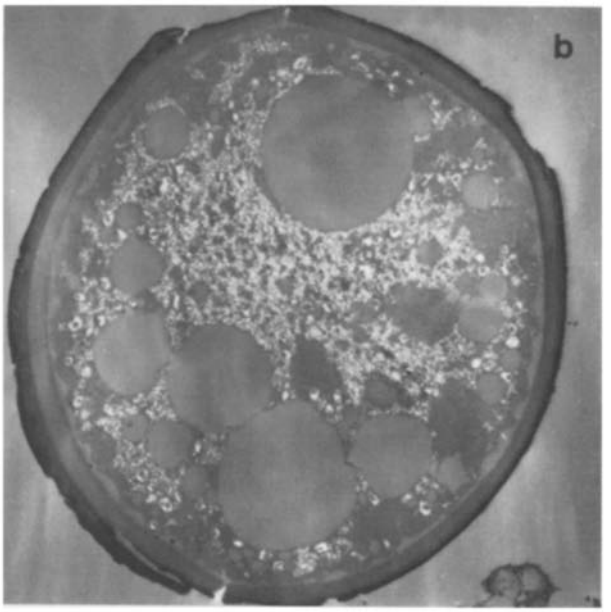
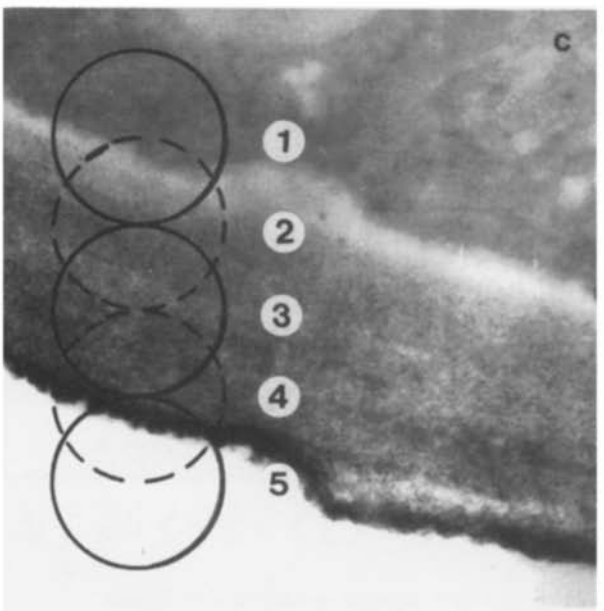


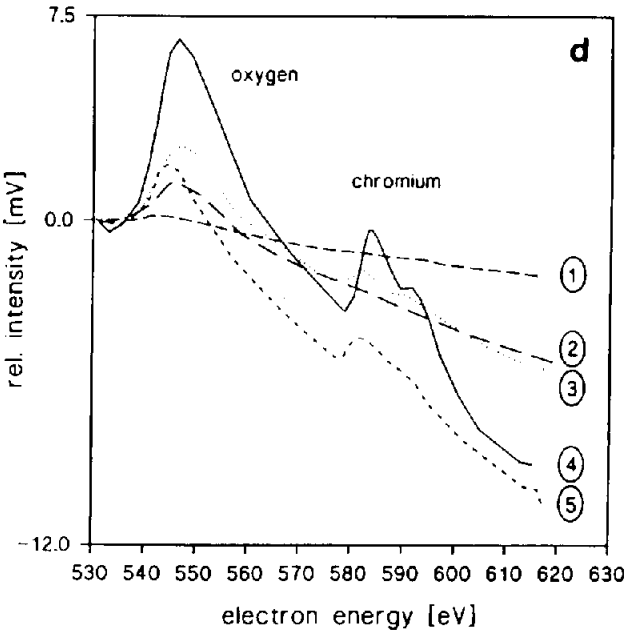
Figure 6. Cr^{3+} (▨) and Cr^{6+} (□) analyses of a pellet (p) and a supernatant (s) fraction of *M. hiemalis* after chromium exposure.

Figure 7. Unstained hyphal cross-sections of *M. hiemalis*: **a** untreated, **b** Cr^{3+}-treated. × 3200. **c** Detail of the cell wall shown in **b**, with five EELS-measurement positions starting from the cytoplasm (position 1) to the outer region of the cell wall (position 5). × 76900. **d** EELS spectra from positions 1–5 in **c**.









almost identical, binding capacities were obtained for Cr^{3+} and Cu^{2+}, followed by Pb^{2-}, Ag^{+}, Al^{3+}, Co^{2+}, Zn^{2+}, Ni^{2+}, Fe^{2+}, Mo^{5+}, Cd^{2+}, Cs^{+}, $Cr_2O_7^{2-}$, CrO_4^{2-} and VO^{3} . These results are very similar to those found by Brady *et al.* (1991). Their enrichment with *Saccharomyces cerevisiae* showed Cu^{2+} followed by Cr^{3+}, Pb^{2+}, Cd^{2+}, Co^{2+}, Ag^{+}, Ni^{2+}, Fe^{2+}, $Cr_2O_7^{2-}$. Except for the deviation of Cd^{2+} our ranking showed good correspondence with this.

After separation of the cell wall from the cytoplasm by centrifugation, the majority of Cr^{3+} was found in the pelleted fraction (Figure 6). The ratios of Cr^{3+} and Cr^{6+} contents of pellet (mostly cell wall) to supernatant were 13:1 and 2:1, respectively.

TEM and EELS

TEM of untreated and Cr^{3+}-treated unstained samples showed clearly a staining of the outer region of the cell wall for the treated fungi (Figure 7a & b). The distribution of the bound chromium seemed to be mainly in a layer of the outer region of the cell wall. We could not verify the presence of bigger aggregates or precipitates, or intracellular accumulation and deposits: if present they may have been washed out by the chemical fixation procedure.

Chromium was successfully detected in the cell wall using EELS. The chromium doublepeak appears, as expected from the *EELS Atlas* (Ahn *et al.* 1983) at 580 eV. At a magnification of 30000 the EELS-measured area has a diameter of 0.5 μm. This defined area allowed us to take five measure positions through the cell wall (detail in Figure 7c). A clear decrease can be seen in the peak maximum from the outer cell region (position 5) to the cytoplasm (position 1; Figure 7d).

Finally, the enrichment of Cr^{3+} by *M. hiemalis* appears to be a passive biosorption to the cell wall rather than an active uptake through the cell membrane. Evidence for this comes from the rapid initial adsorption, the detection of chromium in the pelleted cell wall fraction and the results from the TEM and EELS observations. In contrast to Cr^{3+} an intracellular uptake of Cr^{6+} via the sulfate proton symport is known, which may lead to a main intracellular accumulation (Ono 1988). This is supported by the slow but continuous uptake during the enrichment kinetics (Figure 3) and the proportionally high chromium level in the cytoplasmatic fraction after cell disintegration (Figure 6). Intracellular accumulation by the uptake of Cr^{6+} may be important, but the low uptake of Cr^{6+} by *M. hiemalis* made successful TEM and EELS studies impossible.

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