The use of *Aspergillus niger* (Strain 4) biomass for lead uptake from aqueous systems

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Abstract

The potential of *Aspergillus niger* Strain 4 pellets to remove lead (Pb^{2+}) from solution was determined. *Aspergillus niger* Strain 4 was cultured in Currie's liquid medium as mycelial pellets for 5 d. Pellets were washed in water, and some were dried before exposure to varying concentrations of lead (Pb^{2+}) ion solutions. Various masses of dried mycelial material were exposed to different concentrations of Pb^{2+} solutions to determine the effect of biomass concentration on lead uptake. A mycelial biomass of 2 mg·mℓ$^{-1}$ was found to be optimal for Pb^{2+} uptake at all the lead concentrations tested. Drying of the mycelial pellets did not affect the uptake of Pb^{2+}. Scanning electron microscopy and energy dispersive X-ray micro-analysis of the fungal biomass, indicated that the lead was more or less evenly distributed within both the dried and undried mycelial pellets, and transmission electron microscopy confirmed that lead was present in the cell surface layers of the hyphal strands, i.e. the mechanism of uptake was determined to be biosorption onto the cell surface layers. *Aspergillus niger* Strain 4 pellets show potential for use in the removal of lead from industrial waste waters.

Introduction

Many heavy metals cause pollution in the environment and are toxic to living organisms. Conventional methods, such as filtration, chemical precipitation, ion exchange and electrolytic treatment, are becoming increasingly expensive to operate, and also have additional limitations (Wood, 1992). Micro-organisms have the potential for use as an alternative method of heavy metal removal from polluted waters and industrial effluents (Gadd and Griffiths, 1978).

Fungi are known to have good metal uptake systems (Gadd, 1986), with metabolism-independent biosorption being the most efficient mechanism (Tobin et al., 1994). Biosorption has been defined by Shumate and Strandberg (1985) as "a non-directed physico-chemical interaction that may occur between metal species and the cellular compounds of biological species". This may involve several chemical processes such as ion exchange, adsorption, co-ordination and covalent bonding, with the cell walls playing an important role, due to the presence of various uptake sites containing electronegative, anionic and N-containing groups (Tobin et al., 1994). Fungi often have greater tolerance than bacteria and algae towards metals and other adverse external conditions, such as low pH (Gadd, 1990). Some fungi produce spherical mycelial pellets with high metal uptake capacities, for example *Aspergillus*, *Penicillium* and *Rhizopus* species (Tobin et al., 1994). Immobilised *Rhizopus arrhizus* was found to effectively remove low concentrations of Cu^{2+} ions from aqueous solutions (Zhou and Kiff, 1991), a *Penicillium* sp. isolated from soil was found to accumulate large amounts of copper on the cell surface (Mitani and Misic, 1991), and waste mycelium from several industrial fermentation plants (*A. niger*, *P. chrysogenum*, and *Claviceps paspali*) has been used to remove zinc ions from aqueous solutions (Luef et al., 1991).

Biomass-related technologies will not necessarily replace existing metal-ion removal treatments but may complement these chemical treatment processes. A knowledge and understanding of the mechanisms controlling metal sorption by micro-organisms will aid in the optimisation of metal recovery processes (Fourest and Roux, 1992).

In this study, *A. niger* Strain 4 was cultured as submerged mycelial pellets, and then exposed to heavy metal solutions of Pb^{2+} to determine its metal uptake capacity over time. *A. niger* has previously been shown to be capable of removing Cu, Cd, Au, Ag, La and U from solution (Kapoor and Viraraghavan, 1995) but its ability to remove Pb from solution, as well as the mechanism of uptake, has not been demonstrated. Dried mycelial pellets were also used to investigate the effect of biomass concentration on metal uptake. Scanning and transmission electron microscopy and energy dispersive X-ray micro-analysis were used to investigate the uptake mechanism of the Pb^{2+} ions, i.e. whether intracellular uptake or simple biosorption to the cell surfaces was involved. A preliminary report on the ability of this fungus to take up lead has been published (Meyer et al., 1994). The quantitative and qualitative results obtained from a combination of these techniques and atomic absorption spectrophotometry should make it possible to determine the metal uptake potential of a specific biomass and to calculate the amount of biomass required to efficiently remove the metal ions present at known concentrations in a given volume of effluent.

Experimental

A previously isolated and identified *A. niger* (Strain 4) (Meyer et al., 1994) was used in all the experiments. Fungal pellets were cultured in Currie's liquid medium (Currie, 1917) for 5 d at 30°C on a rotary shaker at 200 r·min^{-1}. The resultant fungal pellets were washed twice in sterile distilled water, drained and weighed out into 100 mℓ Erlenmeyer flasks. To obtain the exact dry mass (i.e. 50, 100, 200 and 500 mg) some of the pellets were dried in an oven at 60°C under vacuum overnight before weighing. Aliquots (50 mℓ) of a lead nitrate solution (Pb^{2+} concentrations of 50, 100, 200 and 500 mg·ℓ$^{-1}$), adjusted initially to approximately pH 4 with 1 M HCl and with no nutrients present, were added to each of the flasks containing the dried and undried mycelial pellets. These experiments were carried out in triplicate. The pellets were left in contact with the metal solutions for 24 h on a rotary shaker (200

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r·min^{-1}) at 30°C. Liquid samples (1.5 mℓ) for metal analysis were taken immediately following exposure of the mycelium to the metal solutions and 30, 60, 120 and 180 min and 24 h thereafter. Samples were also taken from experimental controls which contained no biomass.

Supernatant samples were analysed for residual (equilibrium) metal concentration using a Varian AA-275 Series atomic absorption spectrophotometer (AAS). Samples were diluted with distilled water to a range suitable for AAS detection (standard range of 2 mg·ℓ$^{-1}$ to 20 mg·ℓ$^{-1}$), and EDTA was added to obtain a final concentration of 0.1 M to reduce interference.

The uptake of lead ions (q) by the biomass was calculated from a metal mass balance (Holan et al., 1993) determined as follows:

$$q = V(C_i - C_f)/1000M$$

where:

q is the metal uptake (mg Pb^{2+} g^{-1} of dry biomass),
V is the volume of solution in the contact batch flask (mℓ),
C_i and C_f are the initial and final Pb^{2+} concentrations in solution respectively (mg·ℓ$^{-1}$),
M is the dry mass of the pellets (g).

Dried and undried mycelial pellet samples were prepared for scanning electron microscopy as follows: fixed overnight in 3% glutaraldehyde; washed in cacodylate buffer; dehydrated in an alcohol series; and critical-point dried in a Hitachi HCP-2 CPD. The samples were then mounted on carbon stubs with colloidal graphite and carbon-coated before viewing. A Hitachi S-570 scanning electron microscope fitted with a Link eXL II EDX system was used. Specimens in the SEM were tilted to an angle of 15° towards the electron gun. The working distance in the microscope was set at 15 mm and the accelerating voltage at 20 kV. Spectra showing which elements were present in the samples were obtained after exposing the samples for 100 s under the above microscopy conditions. A dot-mapping technique was also used to map selected areas on the specimen for the presence of lead. The site(s) of metal concentration were depicted as a white area on the map.

Transmission electron micrographs were produced in a Jeol 100CX TEM and in a Philips CM120/STEM with a BioTWIN objective lens, equipped with an EDAX energy dispersive X-ray detector. Following glutaraldehyde fixation (3% (v/v) solution), washing in 0.05 M cacodylate buffer, fixation in osmium tetroxide, a second washing in 0.05 M cacodylate buffer, and ethanol dehydration, the pellets from both the control and experimental flasks were embedded in Spurr's resin and polymerised at 70°C for 16 h. Sections 100 to 150 nm thick were cut with an LKB Ultrotome III ultramicrotome. The sections were not stained, except for some control sections which were stained with uranyl acetate. EDX investigations were carried out at 80 kV.

Results and discussion

EDX analyses

Lead uptake by the pre-grown fungus varied depending on the initial metal concentration and the amount of biomass present. Energy dispersive X-ray micro-analysis of the dried mycelial pellets exposed to all selected concentrations of Pb^{2+} showed the presence of the metal. The EDX spectrum obtained from a 500 mg fungal biomass sample exposed to 500 mg·ℓ$^{-1}$ Pb^{2+} for 24 h is shown in Fig. 1. The various other elements also detected, viz.

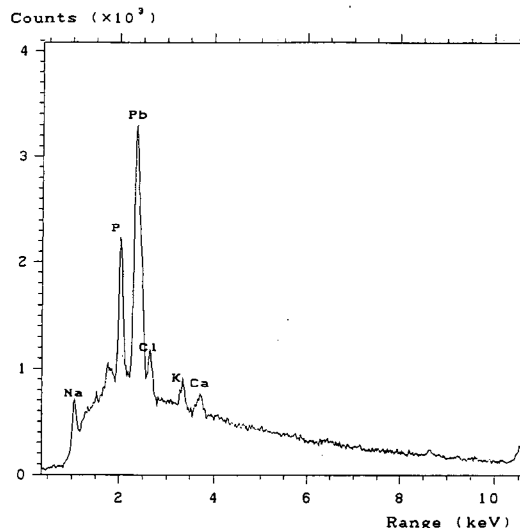

Figure 1
EDX spectrum of dried fungal pellets exposed for 24 h to 500 mg·ℓ$^{-1}$ Pb^{2+}

Na, Cl, K and Ca (Fig. 1), are commonly found in fungal cells.

Undried pellets exposed to 100 and 200 mg·ℓ$^{-1}$ Pb^{2+} were also analysed using the EDX dot mapping technique. Figure 2 is the dot map obtained from an undried sample exposed to 100 mg·ℓ$^{-1}$ Pb^{2+} for 24 h and indicates the presence of lead on and/or in the hyphae of the mycelial pellet. The location of lead is distinguished as the white dots on the map (Fig. 2A), which corresponds to the mycelial strands of the pellet shown in the electron micrograph (Fig. 2B). This technique does not show the precise location of the Pb^{2+} in/on the mycelial strands as electrons are able to penetrate into the hyphae. Hence, the actual mechanism of Pb^{2+}-uptake, i.e. biosorption onto the cell surface or intracellular uptake cannot be determined by this method, but it allows for simple preliminary investigations to determine whether a specific element is present and visualisation of its distribution. For example, in the present study an even pattern of lead accumulation is evident, and metal ions are not restricted to areas such as the growing hyphal tips.

Effect of biomass concentration

Dried mycelial pellets were used to assess the effect of biomass concentration on Pb^{2+} uptake as the exact mass could be obtained. The 100 mg biomass samples exhibited the highest uptake of lead with an average uptake of 36 mg Pb^{2+}·g^{-1} of dried mycelial biomass, followed by the 200 mg biomass samples (Fig. 3). The 500 mg samples exhibited good Pb^{2+} uptake at the lower initial Pb^{2+} concentrations. Little lead was taken up by the 50 mg samples.

A similar effect was observed by Gadd and White (1989) with thorium uptake by a *Penicillium* sp., *A. niger*, and *Saccharomyces cerevisiae*. They concluded that the decrease in uptake at higher biomass concentration was probably due to interference between binding sites and reduced mixing during exposure to the metal-ion solutions (Gadd and White, 1989). The 50 mg sample was too small to demonstrate significant uptake of Pb^{2+} ions, and low biosorption of Pb^{2+} was observed. The biosorption isotherm of the 500 mg samples was steep at the lower initial Pb^{2+} concentrations and then flattened out, indicating that at the higher lead concentrations, blocking of the active metal binding sites in the presence of greater biomass was more pronounced than at the

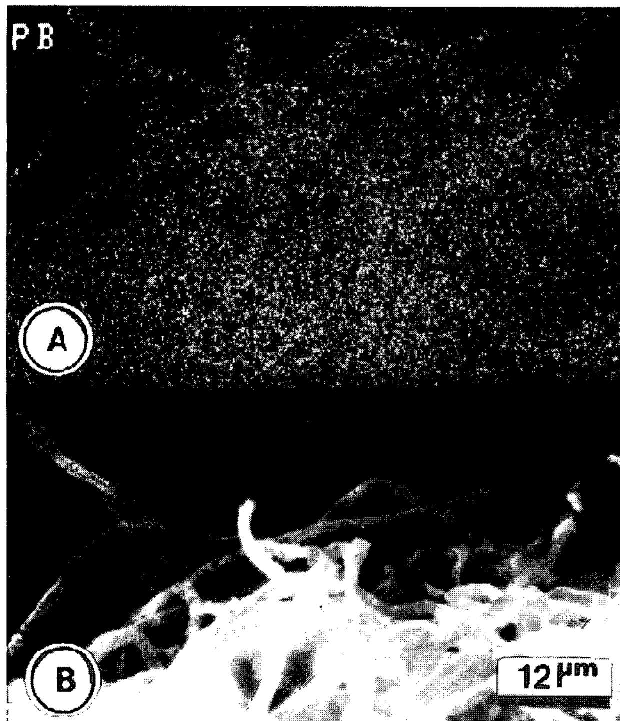

Figure 2A-B
EDX dot map of part of a fungal pellet exposed to 100 mg·ℓ⁻¹ Pb^{2+} for 24 h;
A. dot map indicating the presence of lead
B. scanning electron micrograph corresponding to dot map

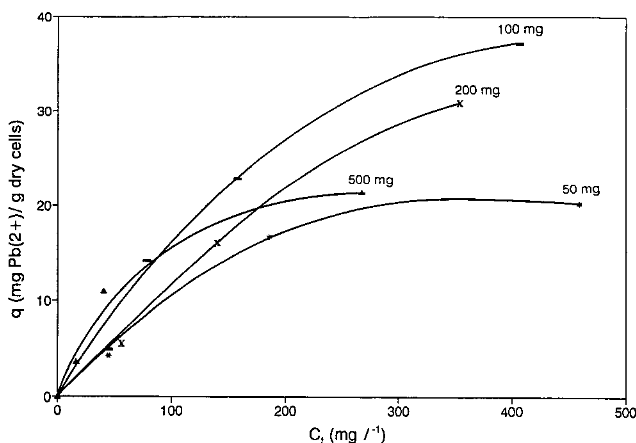

Figure 3
Biosorption isotherms of various biomass concentrations of A. niger *Strain 4 pellets exposed to Pb^{2+} solutions:*
** 50 mg, - 100 mg,* **x** *200 mg, ▲500 mg. q = metal uptake (mg Pb^{2+} g^{-1} dry biomass); Cf = the final concentration of Pb^{2+} in solution*

lower lead concentrations (Fig. 3). Although a greater percentage of lead was removed by the greater masses of mycelium, not all the active metal-binding sites were used, i.e. at higher biomass concentrations (4 to 10 mg·mℓ⁻¹) the uptake efficiency is reduced. The metal/biosorbent ratio is enhanced when the biomass concentration is reduced at a given metal concentration, and the metal uptake per gram of biosorbent increases as long as the biosorbent is not saturated (Fourest and Roux, 1992). This suggests that an optimum biomass concentration needs to be determined for each system to ensure maximum metal uptake efficiency.

Effect of drying on the lead uptake potential of *Aspergillus niger* (Strain 4) mycelium

When using killed cells it is important to remember that the method employed to inactivate the biomass (heat or solvent) may modify the surface properties of the biomass and thus affect its metal-binding properties (Brown, 1991). When comparing the uptake of lead by the dried and undried pellets, very little difference in their metal uptake capabilities was observed (result not shown). Drying at 60°C for 12 h apparently had no effect on the metal adsorption sites on the surface of the fungal mycelium.

The Pb^{2+} uptake capacity of *Penicillium digitatum* was also found to be unaffected by heat (100°C for 5 min) or any other pretreatments, while the uptake of other metals was affected by the various pretreatments (Galun et al., 1987).

Mechanism of Pb^{2+} uptake

Since scanning electron microscopy could not indicate the precise location of the metal within/on the mycelial strands, transmission electron microscopy investigations were carried out. Figure 4A shows a cross-section through a typical hyphal strand (unstained) which was not exposed to lead, i.e. a control. The cell wall is visible and has no electron dense material associated with it. Figure 4B shows a cross-section of a control hypha which was stained with uranyl acetate. Staining served only to slightly darken and define the cell membranes and therefore subsequent samples were not stained.

A longitudinal section of a hypha exposed to lead for 24 h (Fig. 5A) clearly shows the adsorption of the metal onto the cell surface (compare to the control (Fig. 5B) which has no electron dense material associated with it). The lead appears to adsorb to the outer surface of the cell wall and to penetrate the wall fabric to the cell membrane, but was not observed in the cytoplasm.

Lead uptake appeared to be progressive. Initially the metal ions were adsorbed onto the outer surface of the cell wall (Fig. 6A) and then penetrated through the cell wall and collected at the outer layer of the unit cell membrane (Fig. 6B). Some cells showed penetration of the lead throughout the entire cell wall layer (Fig. 6C). It was again observed that no metal ions penetrated the cell membrane, indicating that Pb^{2+} uptake was independent of cell metabolism. This phenomenon was observed even in cells (Fig. 6D) where the membrane had pulled away from the cell wall and the cytoplasm had collapsed inwards (see arrows Fig. 6D). In all cases the intact mycelial pellets used were immersed in the lead solutions for 24 h. However, actual metal contact times of individual hyphae comprising the pellets could not be determined. Dead or non-growing biomass could, therefore, be used to remove lead, and possibly other heavy metals, from polluted waste waters.

A possible explanation for the lower proportion of lead observed on the outer surface of the collapsed (older?) hyphal cell (Fig. 6D) in comparison with that on the cell shown in Fig. 6C (a younger cell?) in which the lead has penetrated into the entire cell surface layer, could be the position of the respective hyphal

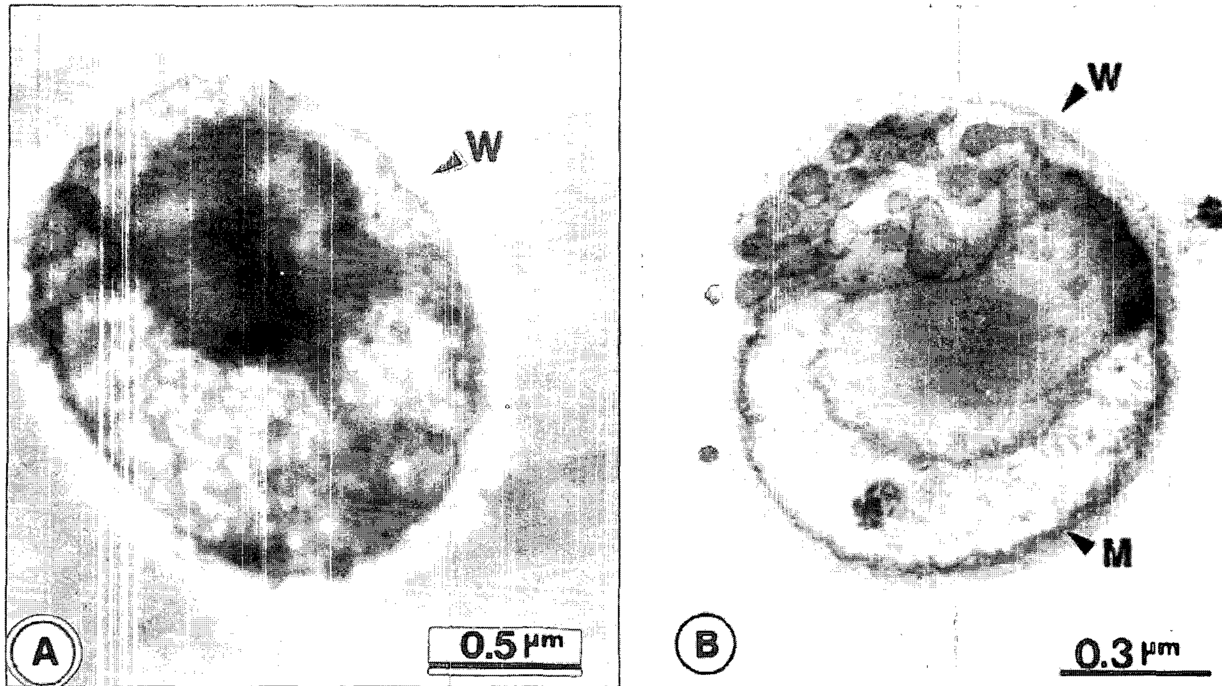

Figure 4A-B
Transmission electron micrographs of sections through hyphal strands of A. niger *Strain 4 which were not exposed to lead*
A. *unstained control strand (W = cell wall)*
B. *uranyl acetate-stained control strand (W = cell wall; M = cell membrane)*

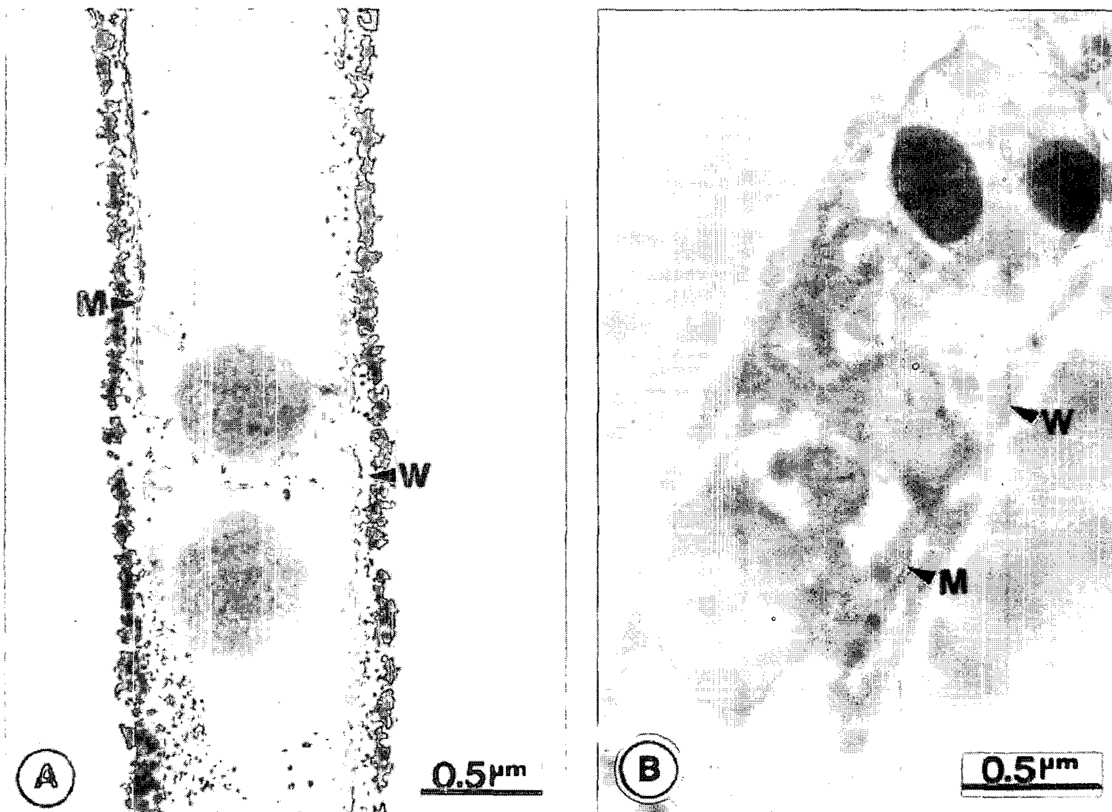

Figure 5A-B
Transmission electron micrographs of longitudinal sections through a hyphal strand of A. niger *Strain 4*
A. *exposed to 100 mg·ℓ$^{-1}$ Pb^{2+} (W = cell wall; M = cell membrane)*
B. *control not exposed to lead (W = cell wall; M = cell membrane*

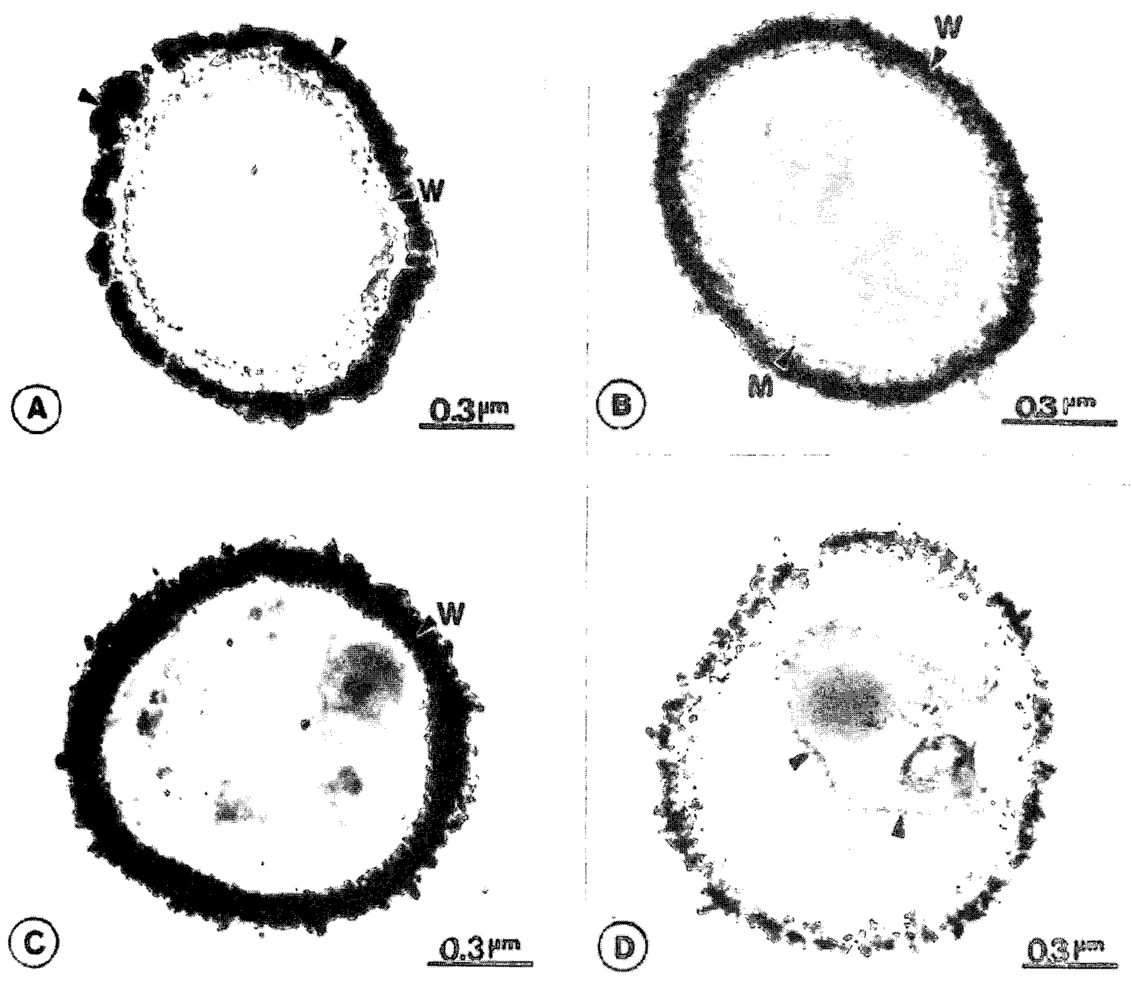

Figure 6A-D
Transmission electron micrographs of cross-sections through hyphal strands of A. niger *Strain 4 exposed to 100 mg·ℓ⁻¹ Pb^{2+}*

A. *first stage of lead adsorption onto outer surface (arrows) of cell wall (W)*
B. *second stage of adsorption showing lead penetration through wall (W) to cell membrane (M)*
C. *third stage of adsorption showing lead saturation of the entire cell wall (W)*
D. *lead adsorption to an "older" cell with collapsed cell contents, arrows indicate lead adsorbed to the outer surface of the shrunken cell membrane*

strands within the pellet. The older hyphae would be found in the centre of a pellet and would thus not be exposed to the same amount of lead as the young hyphae in the outer regions of the pellet. It has been reported that the outer layer of pellets of *Penicillium chrysogenum* contains growing hyphae, while the inner layer consists of hyphae showing signs of autolysis (Prosser and Tough, 1991).

The diameters of individual hyphae varied within all the pellets examined. It has been reported that mycelial pellets lack uniformity, as both "young" apical regions and "older", possibly less active, distal regions are present within the pellets (Prosser and Tough, 1991). Intracellular organelles were not visible in all the hyphae examined. This may be due to differences in age and location of the respective hyphal strands within the pellets examined. X-ray energy dispersive micro-analysis confirmed the presence of lead in the cell surface layers (Fig. 7). Other elements are also indicated in this EDX spectrum (Fig. 7). The nickel is from the grid and phosphorus, calcium and chlorine are elements commonly found in fungal hyphae. No Pb^{2+} was detected in the control samples which were not exposed to lead (EDX spectrum not shown).

Transmission electron microscopy together with energy dispersive X-ray micro-analysis showed that lead was adsorbed onto the cell walls of the hyphal strands of *A. niger* Strain 4 (Figs. 6A to C). No electron dense areas were observed in the interior of these hyphal strands, suggesting that under the present experimental conditions intracellular uptake played little or no role in the bioaccumulation of Pb^{2+} ions in *A. niger* Strain 4.

Transmission electron microscopy of thin sections of *Rhizopus*

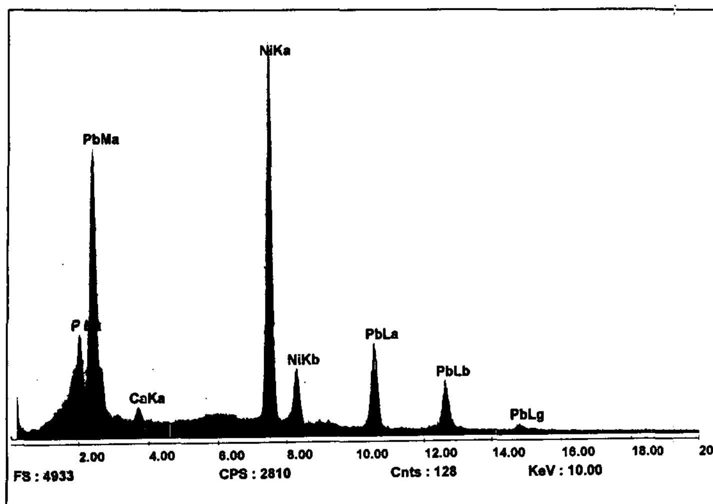

Figure 7
EDX spectrum of the cell wall of a hyphal strand illustrating the presence of lead (Pb)

arrhizus non-living cells exposed to uranium solutions also revealed electron-dense layers throughout the fungal cell wall, which were identified as uranium using X-ray energy dispersive micro-analysis (Tsezos and Volesky, 1982). *Penicillium chrysogenum* biomass was likewise shown to develop a higher electron density in the cell walls due to the concentration of lead (Nui et al., 1993). It is clearly shown in Figs. 5 and 6 that *A. niger* Strain 4 accumulated lead on its cell surface by a similar mechanism to that reported for *Penicillium* and *Rhizopus* biomass (Nui et al., 1993; Tsezos and Volesky, 1982).

Conclusions

Fungal biomass, especially mycelial pellets, can act as adsorbents or ion-exchangers for the removal of heavy metals, including lead, from polluted waters and shows great potential for exploitation, although further research and development are needed. The EDX mapping technique employed here clearly shows the metal-ion biosorption capacity of *Aspergillus niger* Strain 4 biomass and illustrates its potential for use in clean-up systems for metal-containing effluents and water resources. Using a combination of AAS and EDX the metal uptake potential of a specific micro-organism, as well as the amount of biomass necessary to adsorb a known concentration of solubilised metal ions, can be determined. Transmission electron microscopy and EDX micro-analysis can be used to investigate the biosorption distribution of various metal ions in micro-organisms. Knowledge of the uptake mechanism employed by these micro-organisms could be used to optimise waste-water treatment processes.

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