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A possible mechanism of Zn^{2+} uptake by living cells of *Penicillium* sp.

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Abstract

Metabolically-active mycelia of *Penicillium* sp. PT1 took up Zn^{2+} in a biphasic mode, involving an initial energy-dependent binding of Zn^{2+} to the cell surface, followed by a slower intracellular accumulation. The independent binding probably involved a simple ion exchange, as indicated by the pH decrease during the initial adsorption from 4.55 to 3.28. Intracellular accumulation probably involved polyphosphate precipitation as suggested by transmission electron microscopy

Introduction

Generally, living microorganisms accumulate heavy metal ions via two distinct processes. Firstly, there is an initial rapid step which is metabolism- and temperature-independent and is thought to involve heavy metal cation binding to cell surfaces. The second step is metabolism-dependent, which is believed to transport heavy metal ions into the cells. Certain metal ions, such as Zn^{2+} and Cu^{2+}, are essential for normal physiological functioning of living organisms, whereas others such as Cd^{2+} and Hg^{2+} are non-essential and toxic. Even essential metal ions can cause toxicity if the intracellular concentration of the ion rises above physiologically required levels. The survival of cells depend on their ability to limit the intracellular concentration of essential as well as toxic metal ions (Mehra & Winge 1991). The toxicity of heavy metals is mainly due to their interference with microbial metabolism or their altering of the physiochemical environment of cells (Mago & Srivastava 1994).

The detoxification of metal ions is achieved either by regulating uptake and/or efflux, or by intracellular sequestration and compartmentalization. The most commonly found mechanism of plasmid-controlled bacterial metal ion resistance are (Gadd 1994), synthesis of binding proteins (Remacel & Vercheval 1991), complexation, crystallization, transformation of metal species by enzymatically convert it from a more toxic to a less toxic form (Silver & Phung 1996), biosorption to functional groups of cell wall (Tobin *et al.* 1984, Fouerst & Roux 1992, Chen & Ting 1995), pigments and polysaccharide (Gadd 1990) and precipitation, e.g., cell-bound cadmium phosphate, probably $CdHPO_4$ (Macaskie *et al.* 1987). Tantiwachwuttikul *et al.* (1993) have shown that the presence of polyphosphate granules in the growing hyphal tips was precipitated in the form of nickel phosphates in *P. simplicissimum* strain P23. Boswell *et al.* (1995) have also reported that the precipitation of heavy metal phosphates on the surface of actively metabolisming *Acinetobacter* spp.

In previous work, we observed that 66% (w/w) of Zn^{2+} was solubilised by a bioleaching process and 17% (w/w) of Zn^{2+} was adsorbed onto fungus mycelium simultaneously (Tungkaviveshkul *et al.* 1995). In this paper we have investigated Zn^{2+} uptake by *Penicillium* sp. PT1. Under similar conditions during a bioleaching process such as pH of solution 4.55, initial concentration of 1000 mg $Zn^{2+} l^{-1}$ was also used in a study of Zn^{2+} uptake by *Penicillium* sp. PT1. Mechanism for Zn^{2+} adsorbed by *Penicillium.* PT1 is determined by transmission electron microscopy.

Materials and methods

Microorganism

Penicillium sp. PT1 was isolated from zinc silicate residue containing 1.2% (w/w) Zn^{2+}. This organism was routinely maintained on potato dextrose agar (PDA) and incubated at 30 °C for 7 days. One ml of fungal spore suspension (1×10^9 spores) was inoculated into a conical flask containing 100 ml of sucrose medium. The sucrose medium contained the following constituents (g l^{-1}): $NH_4H_2PO_4$ 1.6; KH_2PO_4 0.25; $MgSO_4 \cdot 7H_2O$ 1.2; $FeSO_4 \cdot 7H_2O$ 49.8×10^{-6}; $ZnSO_4 \cdot 7H_2O$ 44×10^{-6}; and $CuCl_2$ 21.2×10^{-6}. After one week, the fungal biomass was maximal and the Zn^{2+} adsorption experiments were conducted. The biomass of dead cells was filtered, dried at 80 °C for at least 24 h and powdered in an agate pestle and mortar to obtain an approximate diameter size of <75 μm.

Biosorption of Zn^{2+} by Penicillium *sp. PT1*

The Zn^{2+} adsorption experiments with dead cells and living cells of *Penicillium* sp. PT1 were investigated by adding 1000 mg Zn^{2+} l^{-1} (added as $ZnSO_4 \cdot 7H_2O$) in deionized water at pH 4.55. Samples were taken immediately to determine initial Zn^{2+} concentration and again after various periods of time for final Zn^{2+} concentration by flame atomic absorption spectrophotometery. The biomass was filtered, dried at 80 °C for at least 24 h or until the weight was constant.

Electron microscopy

Transmission electron micrographs of fungi were obtained by using a JEOL JEM 1210 transmission electron microscope. The samples were prepared by equilibrating the fungal mycelium with a solution containing 1000 mg Zn^{2+} l^{-1} and collecting it in Eppendorf tubes by centrifuging at $380 \times g$ for 10 min. After washing, the mycelium was fixed with 2.5% (v/v) glutaraldehyde in a mixtures of 0.1 M sodium cacodylate and 13% (w/v) sucrose at 4 °C for 24 h, then washed with 0.1 M sodium cacodylate 3 times (15 min each). The fixed mycelium was embedded in 1.5% (w/v) agar, cut in 1 mm^2 and fixed with 1% OsO_4 (w/v) in 13% (w/v) sucrose at room temperature for 1 h and washed with distilled water. The mycelium was dehydrated by 35% (v/v), 70% (v/v) and 95% (v/v) ethanol (15 min each). The dehydrated mycelium was washed in absolute ethanol 2 times (15 min each) and then infiltrated in a series of mixtures of 100% (v/v) ethanol and Spurr resin (3:1, 1:1 and 1:3, v/w respectively) for 2 h each. Embedding was then continued with a series of pure Spurr resin 3 times (2 h each). The resin was then polymerised in an oven at 70 °C for 8 h. Samples of the embedded mycelium were cut with a ultramicrotome and mounted on copper grids. The thin sections (90 nm) on copper grids were stained with uranyl acetate and lead citrate, then ready for TEM.

Determination of Zn^{2+} and PO_4^{3-} in the biomass

The samples were prepared as the following: 0.5 g of dry biomass before and after exposure to 1000 mg Zn^{2+} l^{-1} were pressed into a 20 mm diameter steel disc at a force of 25 tonnes for 5 min and examined by a Kevex 771-EDX superdry X-ray fluorescence spectroscopy.

Extraction of polyphosphate

The procedure for extraction of polyphosphate was modified from Rao *et al.* (1985). Approximately 3.0 g of wet weight of *Penicillium* sp. PT1 was lysed by liquid nitrogen and suspended immediately in ice-cold 10% (w/v) trichloroacetic acid (TCA) with 1 mM NaF. The precipitated cells were sonicated for 15 min, four times in an ice-bath. The sonicated extraction was centrifuged at $15\,000 \times g$ for 10 min, and the supernatant fluid and the pellet were separated. The TCA-soluble fraction of polyphosphate was adjusted to pH 4.50, and then precipitated with saturated $BaCl_2$. The precipitate was dissolved in 1 M HCl containing 1 mM NaF. A portion of this polyphosphate was heated at 100 °C for 15 min to hydrolyse the polyphosphate to phosphate for assay. The PO_4^{3-} concentration was determined by the ascorbic acid method (Rao *et al.* 1985).

Results and discussion

Zn^{2+} uptake by Penicillium *sp. PT1*

During a bioleaching process of zinc silicate residue by *Penicillium* sp. PT1, Zn^{2+} was adsorbed onto fungus mycelium and leaching in the medium simultaneously (Figure 1). Zn^{2+} uptake by *Penicillium* sp. PT1 was investigated under the same conditions used in the leaching process (pH of solution 4.55, initial concentration of 1000 mg Zn^{2+} l^{-1}). As can be seen (Figure 2), dead cells adsorbed up to 10 mg Zn^{2+} g^{-1} biomass. Under similar conditions, living

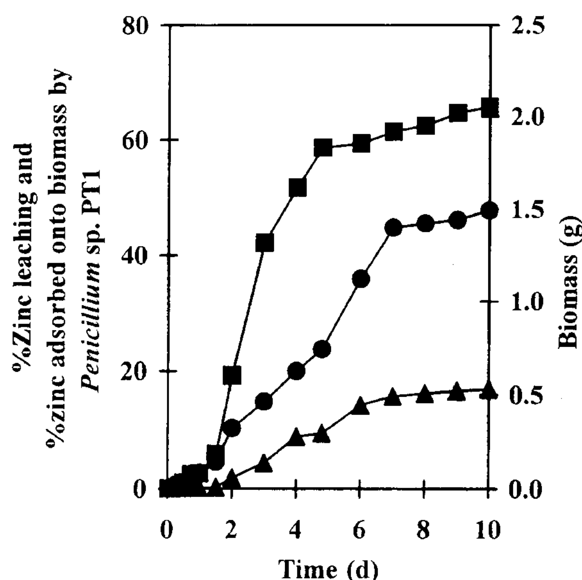

Fig. 1. Percentage of Zn^{2+} leaching and Zn^{2+} adsorbed onto biomass in a bioleaching process by *Penicillium* sp. PT1: (■), Concentration of Zn^{2+} in medium, (▲), concentration of Zn^{2+} adsorbed onto biomass, (●), biomass.

cells of *Penicillium* sp. PT1 adsorbed 16 mg Zn^{2+} g^{-1} biomass. The higher Zn^{2+} adsorption in living cells of *Penicillium* sp. PT1 was expected to contribute to intracellular uptake of Zn^{2+} occurring in metabolically active cells in combination with extracellular

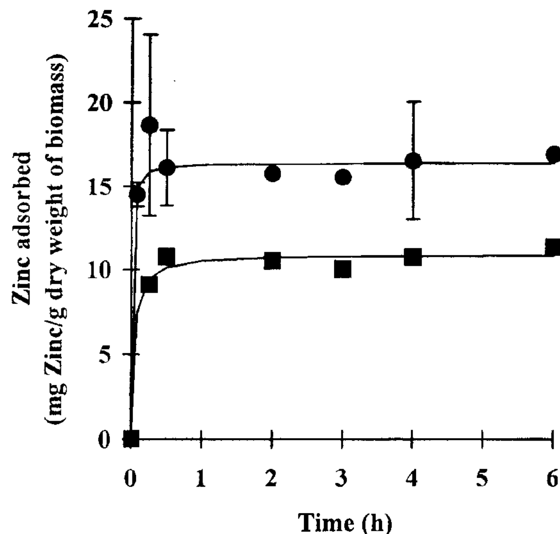

Fig. 2. Biosorption of Zn^{2+} by dead cells and living cells of *Penicillium* sp. PT1 at initial concentration of 1000 mg Zn^{2+} l^{-1}, initial pH of 4.55, biomass 0.5 g dry weight: (●), Living cells, (■), dead cells.

adsorption. This result is similar to work of Tantiwachwuttikul *et al.* (1993) that it took less than one day to achieve the accumulation inside the cell membrane and metal ion uptake by living cells was higher than that of dead cells.

Biosorption of Zn^{2+} by dead cells of *Penicillium* sp. PT1 involved independent binding (probably a simple ion exchange), as the pH of the solution decreased during the initial adsorption from 4.55 to 3.64. Whereas, Zn^{2+} uptake by living cells of *Penicillium* sp. PT1 may also attribute to independent binding because the pH of the solution also decreased from 4.55 to 3.28 and intracellular accumulation. Adsorption of Zn^{2+} by a simple ion exchange of dead cells and living cells of *Penicillium* sp. PT1 resulted from functional groups of cell wall containing negative charges, e.g., phosphate groups, hydroxyl groups and carboxyl groups that reacted with Zn^{2+} in the solution causing a decrease in pH.

Transmission electron microscopy, X-ray fluorescence spectroscopy and 10% trichloroacetic acid (TCA)

Transmission electron microscopy was expected to indicate the presence of Zn^{2+}, particularly if present as an insoluble complex. An examination of cells before and after exposure to Zn^{2+}, revealed spherical electrondense granules within the cell (Figure 3). It was assumed that the granules observed were similar to the polyphosphate granules because these granules were extremely opaque. Examination of *Penicillium* sp. PT1 before exposure to Zn^{2+} by X-ray fluorescence spectroscopy, shows only a phosphorus peak, while *Penicillium* sp. PT1 after exposure to Zn^{2+} contained both zinc and phosphorus peaks. The results were confirmed by extraction of polyphosphate from cells before and after exposure to Zn^{2+} by 10% TCA containing 0.5% and 0.7% dry weight (w/w) polyphosphate, respectively. Evidence of the combination of PO_4^{3-} and Zn^{2+} can be precipitated as zinc-phosphate detected by X-ray diffraction (Tungkaviveshkul *et al.* 1995). Therefore, the results indicated that Zn^{2+} was bound to polyphosphate granules and precipitated within the cell.

These results suggested a plausible mechanism of Zn^{2+} uptake by living cells of *Penicillium* sp. PT1 involving extracellular adsorption and intracellular uptake in the form of zinc phosphate precipitates/complexes within the cell. The function of polyphosphate in *Penicillium* sp. PT1 may be (i) an energy storage polymer, (ii) a regulator of metabolic

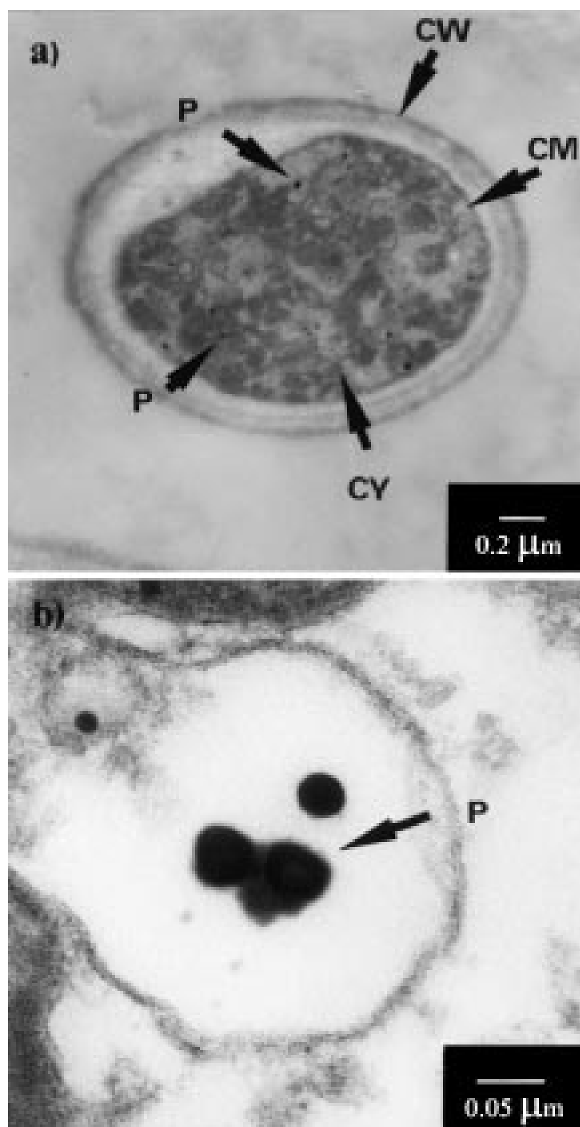

Fig. 3. Electron photomicrographs of a thin section of *Penicillium* sp. PT1: (a) after exposure to 1000 mg Zn^{2+} l^{-1}, (b) black spot from (a); P = polyphosphate; CW = cell wall; CM = cell membrane; CY = cytoplasm.

processes, mainly through control of the energy charge, and (iii) a specfic role for polyphosphate as a phosphate storage polymer, namely as a defense reserve against heavy metal (Aiking *et al.* 1984). Thus, polyphosphate accumulation may be a detoxificating mechanism, precipitating inside the cell.

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