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Fungal biomineralization of lead phosphates on the surface of lead metal

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ABSTRACT

The present work describes lead phosphate biomineralization by a wood-decaying fungal isolate, *Penicillium chrysogenum* A15, after incubation in solid medium with metallic lead (Pb shot pellets). This fungal isolate showed high tolerance to this element, being able to grow on solid medium containing 8 mM Pb(II). Environmental Scanning Electron Microscope (ESEM) observations of abiotic controls showed the presence of lead oxide crystals on the lead shot surface after 8 weeks incubation. However, in presence of *P. chrysogenum*, lead phosphate mineral phases were detected as aggregates on the surface of lead shots after 2-weeks and 8-weeks incubation. The morphology of the biotically deposited secondary minerals showed significant differences in comparison to those produced under abiotic conditions, appearing globular, prismatic and acicular crystals. High Resolution Transmission Electron Microscope (HRTEM) analysis indicated deposition of lead phosphates on the cell surface which could be considered as Pb resistance mechanism used by this isolate to cope with toxicity of lead. This study extends the spectrum of fungal isolates with potential on the biomineralization of lead phosphates useful for the bioremediation of lead contaminated sites.

1. Introduction

Lead is a well-known and hazardous heavy metal that may cause serious ecological and human health problems as was reported by European Commission (2002). This element is widely used in several industrial activities such as lead smelting, leaded petrol production, and reactional shooting and hunting products (Dermatas et al., 2006; Sullivan et al., 2012). Many occidental countries like USA, Canada, England, etc. reported high annual deposition rates of metallic Pb in the environment through anthropogenic activities associated with angling, hunting and reactional shooting (Sullivan et al., 2012). In soil, the speciation of metallic lead used in shooting activity, is controlled by several processes such as sorption, precipitation and complexation with various organic and inorganic soil colloids (Zhang et al., 1997). However, in highly heavy metal contaminated soils, the mobilization of metallic lead may occur through processes such as dissolution, hydration, carbonation or oxidation mediated by abiotic (exposure to air, water, and varying pH conditions) or biotic factors (Cao et al., 2003; Dermatas et al., 2004; Vilomet et al., 2003).

Regarding the biotic processes, it is well-known that soil microbial populations play an important role in the biogeochemical cycle of metals through different processes including biosorption (Merroun and Selenska-Pobell, 2008; Morcillo et al., 2014), intracellular accumulation (Brookshaw et al., 2012), biomineralization (Merroun et al., 2011; Mondani et al., 2011) and chelation by organic acid production, leading to the immobilization or mobilization of heavy metal (Gadd et al., 2012). Among the soil microorganisms, indigenous fungi are able to solubilize metal compounds releasing phosphates and other ligands, which in turn lead to the liberation of metal cations (e.g. Pb). Conversely, fungi interact with lead through immobilization processes such as biomineralization of lead oxalates, phosphates and carbonates (Gadd et al., 2012).

Lead phosphate biomineralization by fungi and bacteria is considered an attractive and efficient bioremediation strategy, due to the long-term stability of this mineral phase. Pyromorphite ($Pb_5[PO_4]_3X$, where X = F, Cl, Br, OH) is known as the most stable lead mineral occurring in terrestrial environments (Casas and Sordo, 2006; Debela et al., 2010; Miretzky and Fernandez-Cirelli, 2008), with a very low solubility product (K_{sp}) of $10^{-84.4}$, (Miretzky and Fernandez-Cirelli, 2008; Nriagu, 1974). Therefore, pyromorphite formation has previously been proposed as a remediation treatment for immobilization of contaminant lead (Cao et al., 2003; Hettiarachchi et al., 2001; Ma et al., 1993; Miretzky and Fernandez-Cirelli, 2008). In this respect, Rhee et al. (2014) described pyromorphite within a fungal biofilm community growing on Pb sheeting in natural environment, thus providing novel evidences on the fungal involvement in lead biogeochemistry.

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The present study describes the ability of a fungal strain isolated from decayed woods to transform metallic lead into lead phosphates. The resulting lead mineral phase was characterized by applying a multidisciplinary approach combining Environmental Scanning Electron Microscopy (ESEM) and High Resolution Transmission Electron Microscopy (HRTEM).

2. Material and methods

2.1. Fungal strains and culture conditions

Fungal strains used in the present work, *Penicillium chrysogenum* A15, *Aspergillus niger* A43 and *Trichoderma viride* B30, were previously isolated from decayed wood collected from historic edifications (Castro-Rodriguez, 2013). These fungal strains were maintained on potato dextrose agar (PDA: 4 g/L potato extract, 20 g/L dextrose, 15 g/L agar) and grown in malt extract agar (MEA: 5 g/L peptones, 15 g/L malt extract, 15 g/L agar) during 14 days at 28 °C.

2.2. Metal stock solution preparation

A stock solution of $Pb(NO_3)_2$ at 0.1 M was prepared by dissolving the appropriate quantity of metal salt in 0.1 M $NaClO_4$. The stock solution was sterilized by filtration through 0.22 μm nitrocellulose filters and stored at 4 °C until use. Working solutions were prepared by dilutions of the stock solution.

2.3. Determination of lead minimal inhibitory concentration (MIC)

Tolerance of the tested fungi to Pb(II) was determined as the Minimal Inhibitory Concentration (MIC). The MIC is defined as the lowest metal concentration where no visible fungal growth occurs. Discs of fungal mycelium (5 mm diameter) were placed on MEA, solid medium containing increasing concentrations of Pb(II) nitrate (1, 2, 4 and 8 mM). After inoculation, all plates were incubated in the dark at 25 °C for 7 days.

2.4. Interaction of metal lead with Penicillium chrysogenum

For Pb-fungal interaction study, individual oven-sterilized (105 °C for 3 days) lead shots (4.5 mm in diameter) were placed aseptically at regular intervals on the surface of MEA plates leaving free the center of the plate to place the fungal disc, as described by Rhee et al. (2012). Afterwards, the plates were inoculated with 5 mm diameter discs of two weeks-old fungal mycelium. The plates were incubated for 2, 4 and 8 weeks in the dark at 25 °C. Abiotic control consisted of lead shots placed on the surface of MEA culture medium and incubated at the same conditions as described above. Experimental and control plates were prepared in triplicate. After incubation, the lead shots were aseptically recollected and prepared for the different microscopic analyses.

2.4.1. Environmental scanning electron microscopy and energy-dispersive X-ray analyses

Lead shots and their surrounding fungal hypha collected after 2, 4 and 8 weeks incubation were fixed in 3% glutaraldehyde prepared in cacodylate buffer at 4 °C. Then, they were washed three times for 15 min with cold-cacodylate buffer. Afterwards, the samples were post-fixed in 1% OsO_4 for one hour in the dark, at room temperature. Next to washing was performed with distilled water (3 × 5 min), samples were dehydrated through gradients of ethanol (50, 70, 90 and 100%) for 15 min each wash, followed by three additional times in 100% ethanol for 15 min each. The dehydrated samples were then critical point dried (Anderson, 1951) using a *Leica EM CPD300* Critical Point Dryer. Samples were immediately mounted on aluminum stubs using carbon adhesive tapes and coated with carbon by EMITECH K975X coater. The coated samples were observed using a *Quanta 400 FEI* ESEM operating at an accelerating voltage of 15 kV and 20 kV for the observation of biocorrosion products formed on the surface and around the lead shots.

The elemental composition of the secondary minerals formed on the surface and the periphery of lead shots was determined by energy-dispersive X-ray (EDX) analysis system. Spectra were acquired using EDX analysis system embedded within an environmental scanning electron microscope (Quanta 400 FEI ESEM) operating at an accelerating voltage of 20 kV. EDX provides elemental information via analysis of the X-ray emission caused by a high electron energy beam.

2.4.2. High resolution transmission electron microscopy and energy-dispersive X-ray analyses

Lead-treated fungal biomass was fixed in 2% glutaraldehyde (in 0.1 M cacodylate buffer, pH 7.4) for 2 h at 4 °C and then washed with the same cacodylate buffer three times for 10 min. Samples were dehydrated with ethanol gradients from 30% to 100%, followed by propylene oxide treatment for 10 min, then embedded in a mixture of propylene oxide-resin (EPON) (1:1) and kept overnight in pure resin, to be afterwards polymerized in heater at 60 °C for 14 h. The samples were thin-sectioned using a Reichert Ultracut S ultramicrotome, and the sections were supported on copper grids and coated with carbon. Samples were examined using a *Philips CM 200* high-resolution transmission electron microscope at an acceleration voltage of 200 kV and *MegaViewIII* camera under standard operating conditions with the liquid nitrogen anticontaminator in place. EDX analysis was also performed at 200 kV using a spot size of 7 nm and a live counting time of 20 s.

3. Results

3.1. Tolerance of fungal strains to Pb(II): minimal inhibitory concentration (MIC)

The MIC of Pb(II) for the growth of the three tested fungal isolates is presented in Table 1. The results indicated that the strains *A. niger* A43 and *P. chrysogenum* A15 were able to grow at 8 mM lead-amended MEA medium. The MIC of Pb for the growth of *T. viride* B30 was 8 mM.

The contact of the mycelium with incremented Pb concentrations resulted in phenotypic changes including sporulation inhibition (more relevant in *P. chrysogenum*), colored-mycelium changes and variations in the morphology and the size of the fungal colonies (*T. viridae*) (Figs. 1 and 2).

3.2. Interaction of Penicillium chrysogenum A15 with metallic lead

The interaction of fungal hyphae with the lead shot was macroscopically monitored for a total of two months (Fig. 3). After 9 days incubation, the mycelium covering lead shots presented a greenish color in comparison to the free-lead shot mycelium, which

Table 1
Minimal inhibitory concentration (mM) of lead for the growth of the three tested fungal species.

Fungal strain	MIC (mM Pb)
Aspergillus niger A43	>8
Penicillium chrysogenum A15	>8
Trichoderma viride B30	8