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Solubilization and Transformation of Insoluble Zinc Compounds by Fungi Isolated from a Zinc Mine

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Abstract

Fungi were isolated from zinc-containing rocks and mining soil. They were screened for the ability to solubilize and transform three insoluble zinc compounds: ZnO, $Zn_3(PO)_4$, and $ZnCO_3$. Fungi were plated on potato dextrose agar (PDA) medium which was supplemented with 0.5% (w/v) of insoluble zinc compounds. Of the strains tested, four fungal isolates showed the highest efficiency for solubilizing all the insoluble zinc compounds, producing clearing zone diameters > 40 mm. These were identified as a *Phomopsis* spp., *Aspergillus* sp.1, *Aspergillus* sp.2, and *Aspergillus niger*. Zinc oxide was the most easily solubilized compound and it was found that 87%, 52%, and 61% of the tested fungi (23 isolates) were able to solubilize zinc oxide, zinc phosphate, and zinc carbonate, respectively. Precipitation of zinc-containing crystals was observed in zinc oxide-containing agar medium underneath colonies of *Aspergillus* sp.1, and these were identified as zinc oxalate. It is suggested that these kinds of fungi have the potential application in bioremediation practices for heavy metal contaminated soils.

Keywords: solubilization; transformation; zinc compounds; fungi; bioremediation

1. Introduction

Contamination of soil by heavy metal through industrial and human activities, especially from metalliferous mining and smelter sludges, may result in extremely high levels of toxic metals including zinc (Alloway, 1995; Khan *et al*., 2008; Zhang *et al*., 2010). Zinc is an essential metal for all organisms. However, in humans, manifestation of toxic symptoms such as nausea, vomiting, and epigastric pain can occur with very high zinc intake. Moreover, zinc toxicity may cause adverse reactions in different organs and biological functions, including reproduction and inactivation of enzymes (Fosmire, 1990; Malik, 2004). Conventional methods for removing heavy metals from aqueous streams include chemical precipitation and membrane technologies, and these techniques are of very high efficiency. However, they are extremely expensive so cannot be widely used in large scale applications. These are the reasons why biological methods are now considered more seriously (Wang and Chen, 2006; Volesky, 1994).

The introduction of heavy metal compounds into the environment can result in changes in the microbial communities present, including selection of metal tolerant and resistant microorganisms (Gadd, 1993). Fungi have important roles in biogeochemical processes and are involved in solubilization of insoluble metal compounds (Gadd, 2004; 2007). In fact, fungal solubilization also has the potential to release essential metals and phosphate into the soil (Sayer *et al*., 1995). In addition, fungi are able to immobilize a number of metal compounds by a variety of mechanisms (Sayer and Gadd, 1997; Gadd, 2000). Immobilization, including oxalate crystallization, immobilizes heavy metals and may limit bioavailability (Gadd, 2000). Metal oxalate complex and crystal formation is a process of environmental significance in connection with fungal survival, biodeterioration, pathogenesis, soil weathering, and metal detoxification (Dutton and Evans, 1996; Gadd, 1999; 2000; Wei *et al*., 2013). The objective of this research was to study the ability of fungi isolated from a zinc-contaminated environment to solubilize and transform insoluble zinc compounds in order to understand their possible roles in such an environment in affecting zinc bioavailability.

2. Materials and Methods

2.1. Fungal isolation

Natural zinc-containing mineral rocks were collected from a zinc mining site in Tak province, northern Thailand. All samples were stored in sterile polythene bags. The isolation method was based on the method of Adelake *et al*. (2010) and carried out under sterile conditions. The isolated fungi were maintained on potato dextrose agar (PDA) in the dark at 25ºC. Fungi were identified according to their macro- and microscopic structures. The taxa were assigned to genera following Von Arx (1981) and Barnett and Hunter (1998).

2.2. Preparation of zinc-containing media and culture conditions

Commercial preparations of ZnO, $Zn_3(PO)_4$, and $ZnCO_3$ were used, these were included in the media to 0.5% (w/v) final concentration. Fungal inoculations were carried out with 7 mm diameter discs of mycelium excised from actively-growing cultures which were then placed on the surface of zinc compound-amended plates. These were incubated at 25ºC for 7 days in the dark (Sayer *et al*., 1995; Fomina *et al*., 2005).

Table 1. Clear halo zone diameters produced by fungi grown on insoluble zinc compounds

Isolate origin	Isolate	Insoluble zinc compounds 0.5% (w/v)		
		ZnO	$Zn_3(PO)_4$	$ZnCO_3$
Hemimorphite	HM1	+++	+++	+++
	HM2	+	-	-
	HM3	+++	+++	+++
	HM4	+++	+++	+++
	HM5	+++	++	++
	HM6	+++	-	++
Zinc silicate	ZS1	-	-	-
	ZS2	+++	++	++
	ZS3	+++	++	+++
	ZS4	-	-	-
Smithsonite	SS1	+++	+++	+++
	SS2	+++	++	++
	SS3	+++	-	++
	SS4	++	-	-
Mining soil	MS1	+	-	-
	MS2	+++	++	++
	MS3	+++	-	++
	MS4	++	-	-
	MS5	+	-	-
	MS6	+++	++	+++
	MS7	+++	++	++
	MS8	-	-	-
	MS9	+++	++	++

(-) = no clear zone, (+) 7-20 mm, (++) 20-40 mm, (+++) > 40 mm

2.3. Investigation of solubilizing ability

The magnitude of solubilizing ability was assessed by measuring the diameter of solubilization halo zones in the agar medium (Sayer *et al*., 1995; Fomina *et al*., 2005). At the end of the incubation period (7 days), the diameters of any clear solubilization zones were measured in three replicate plates.

2.4. Evaluation of culture medium acidification

For pH measurements, fungal strains were cultured in 250 ml Erlenmeyer flasks containing 100 ml potato dextrose broth (PDB, pH 7). Fungal cultures were inoculated and grown in a shaker (150 rpm) at 25ºC. An appropriate amount of zinc compound was added to the liquid media to give the desired final concentration. The pH value was measured after seven days, and measurements were taken in triplicate using a Mettler-Toledo pH electrode (Model S20) (Fomina *et al*., 2005; Yazdani *et al*., 2010).

2.5. Analysis of mycogenic crystals

Mycogenic crystals formed in the culture medium were extracted from the agar according to the procedure described by Sayer and Gadd (1997). The crystals were examined using a scanning electron microscope (SEM, JEOL: JSM-5410LV). The samples were covered with carbon and gold layers, and finally observed in the secondary electron mode at an acceleration voltage of 15 kV. Crystals were identified by X-ray powder diffraction (XRPD, Bruker AXS: D8-Discover) and elemental composition determined using the SEM equipped with energy dispersive X-ray micro-analysis (SEM-EDS, JEOL: JSM-6400 LV- ISIS Series 300) (Sayer and Gadd, 1997; Joseph *et al*., 2012).

3. Results and Discussion

Twenty-three fungal isolates were tested for the solubilization and transformation of insoluble zinc compounds (Table 1). Zinc oxide was the easiest compound to be solubilized by 87% of the fungal isolates followed by zinc phosphate (52%) and zinc carbonate (61%). *Phomopsis* spp. (HM1), *Aspergillus* sp.1 (HM3), *Aspergillus niger* (HM4), and *Aspergillus* sp.2 (SS1)

Table 2. Solubilization halo diameters (mm) of selected strains and final pH values

Zinc compounds	Isolate	Fungal strains	Clear zone diameter (mm)	Final pH
ZnO	HM1	*Phomopsis* spp.	64.1±3.3	5.0±0.0
	HM3	*Aspergillus* sp.1	63.3±2.5	5.0±0.0
	HM4	*Aspergillus niger*	70.5±1.8	4.5±0.0
	SS1	*Aspergillus* sp.2	60.5±2.5	5.7±0.0
$Zn_3(PO)_4$	HM1	*Phomopsis* spp.	56.8±1.2	3.7±0.0
	HM3	*Aspergillus* sp.1	60.0±1.5	3.6±0.0
	HM4	*Aspergillus niger*	55.0±3.5	3.7±0.0
	SS1	*Aspergillus* sp.2	44.8±2.7	4.2±0.0
$ZnCO_3$	HM1	*Phomopsis* spp.	50.5±2.1	5.0±0.0
	HM3	*Aspergillus* sp.1	55.0±1.8	4.2±0.0
	HM4	*Aspergillus niger*	59.3±1.0	4.1±0.0
	SS1	*Aspergillus* sp.2	41.8±1.2	5.7±0.0

Each value is the mean of three replicates ± standard error of the mean.

showed the highest efficiency for solubilizing all the different insoluble zinc compounds (halo diameters > 40 mm) and these strains were selected for further studies. Clearing zone diameters and final medium pH values for selected fungi are shown in Table 2. *Aspergillus niger* produced the largest solubilization zone diameters for both zinc oxide (70.5±1.8 mm) and zinc carbonate (59.3±1.0 mm); while *Aspergillus* sp.1 showed the greatest halozone for zinc phosphate (60.0±1.5 mm). The pH of fungal growth media decreased after 7 days growth of the selected strains which indicated that they can acidify the zinc compound-containing medium during cultivation. Fungi from the *Aspergillus* and *Penicillium* genera are common in contaminated soil (Zdanova *et al*., 2000; Levinskaite *et al*., 2009) and they frequently produce organic acids which are directly involved in metal solubilization (Gadd, 2010). Fungal organic acid secretion during growth decreases the pH of the system and can increase metal solubility by metal-complex formation (Gadd and Griffiths, 1978; Bosecker, 1997; Gadd, 1999).

After incubation, mycogenic crystals were found in the agar medium after growth of *Aspergillus* sp.1 on zinc oxide (Fig. 1). The crystals were purified and analysed by XRPD and SEM-EDS. Comparison of XRPD patterns of crystals extracted from the agar medium with standard zinc oxalate are shown in Fig. 2 and revealed that zinc oxalate was produced by *Aspergillus* sp.1 grown on PDA amended with zinc oxide. Further elemental analysis with SEM-EDS confirmed the presence of zinc, oxygen, and carbon (Fig 3). Oxalate production was responsible for metal immobilization in this case. These results are in agreement with results obtained for the ericoid mycorrhizal fungi *Oidiodendron maius* Cd8, which could also transform zinc oxide into zinc oxalate (Martino *et al*., 2003). The production of metal oxalates by fungi also occurs for other heavy metals such as Co, Mn, Ni, Pb, Sr and Cu (Gadd, 1999; Jacobs *et al*., 2002; Wei *et al*., 2012; Joseph *et al*., 2012). The formation of oxalates containing potentially toxic metals

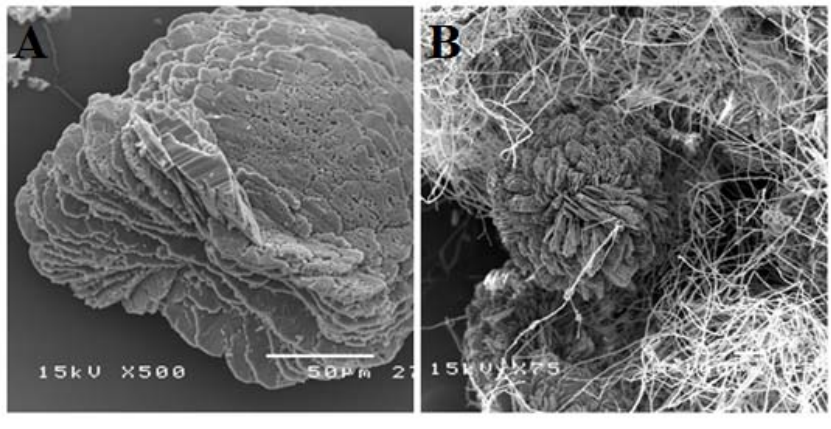

Figure 1. Scanning electron micrographs of crystals produced by *Aspergillus* sp.1. (A) Crystals purified from agar media. (B) Crystals associated with the fungal mycelium. Scale bars: (A) 50 µm (B) 100 µm.

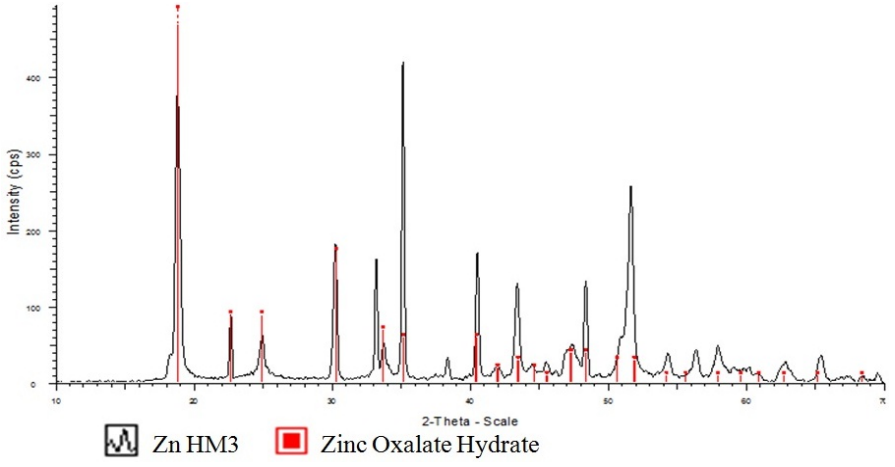

Figure 2. XRPD pattern of zinc oxalate crystals precipitated by *Aspergillus* sp.1.

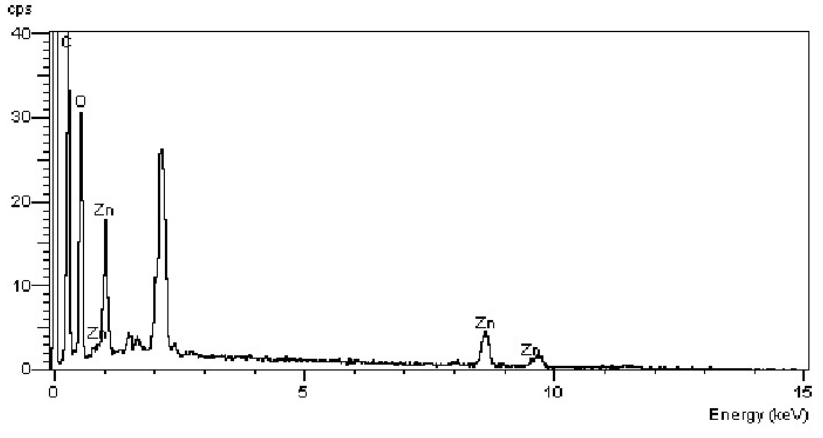

Figure 3. Spectrum obtained by SEM-EDS of purified crystals.

may provide a mechanism whereby oxalate-producing fungi can tolerate metal-rich environments (Sayer and Gadd, 1997). It is possible that solubilization and immobilization are key fungal processes with potential for metal recovery and reclamation from contaminated soil, solid wastes, and low grade ores (Gadd, 1999; 2000). In conclusion, we have shown that fungi with high level of zinc transformation ability can be isolated from polluted sites, and these are capable of zinc solubilization as well as immobilization of zinc by means of zinc oxalate production. Whether this is a process of significance *in situ* remains to be ascertained.

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