Mobilization and Immobilization of Zinc Oxide Nanoparticles by *Phomopsis* sp. Isolated HM1

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Abstract

Phomopsis sp. isolated HM 1 was isolated from zinc-containing rocks (Hemimorphite). It was screened for the ability to solubilize and immobilize zinc oxide nanoparticles (ZnONPs). Fungal strain was plated on potato dextrose agar (PDA) medium, which was supplemented with various concentrations of zinc oxide nanoparticles. *Phomopsis* sp. isolated HM 1 showed the highest efficiency for solubilizing zinc oxide nanoparticles, producing clearing zone diameters more than 40 mm in 0.1, 0.3 and 0.5% (w/v) of ZnONPs amended plates. Mycogenic crystals were observed in the agar medium underneath the fungal colonies of tested strain. The crystals were identified by scanning electron microscope (SEM) and X-ray powder diffraction (XRPD) and were identified as zinc oxalate hydrate ($C_2O_4Zn{\cdot}2H_2O$). Therefore, it could be suggested that this fungal strain might has the potential application in agriculture and bioremediation practice of heavy metals contaminated soils.

Keywords: Mobilization, Immobilization, Zinc oxide nanoparticles, Fungi

1. Introduction

Zinc oxide nanoparticles are widely used in many fields such as industrial coating, cosmetic, semiconductor, pharmaceutical industry and agriculture [1, 2]. Apart from these industries, high concentration of zinc oxide nanoparticles can be contaminated in the environment and can be harmful effect in soil microorganisms, plants and human health [1], [3].

Fungi are a key role in biogeochemical cycle. Many soil fungi can survive and grow in high concentration of toxic metal, and are involved in mobilization of insoluble metal compounds [4, 5]. Indeed, mobilization is a process of fungal physiology for releasing phosphate and other insoluble micronutrients. Fungal solubilization potential may also release metal cations into the soil [6]. Organic acid such as citric acid and oxalic acid produced by fungi could have direct effect on solubilization activity. They provide both proton (H^+) and metal complexing anion ($C_2O_4^-$), and mediate release of available phosphate and metal ion from insoluble compounds [7]. Moreover, fungi are able to immobilize metals ion into metal oxalate complex. Immobilization by insoluble metal oxalate complex formation is a process of marked environmental significance on both regarding fungal survival and metal detoxification [8, 9]. The production of metal oxalate complex might provide a mechanism whereby oxalate produced fungi could tolerate environment containing high concentration of heavy metals [5],[10, 11]. Mobilization and immobilization

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of insoluble metal compounds by fungi could be applied to remediate the contaminated site because of its potential low cost application in bioremediation and recovery of metal [12]. Phomopsis sp. isolated HM 1 had been reported with the ability to solubilize insoluble heavy metals compound such as ZnO and $PbCO_3$ [13]. However, it has not been reported about the ability to solubilize and immobilize zinc oxide nanoparticles; therefore, the objectives of this research was to investigate the ability of Phomopsis sp. (HM 1) to solubilize and characterize zinc biomineral produced by tested fungi.

2. Experimental details

2.1. Fungal strain, culture condition and solubilization ability assessment

Phomopsis sp. isolated HM 1 was isolated from zinc-containing rocks (Hemimorphite) at Padaeng zinc mine, Tak province, northern Thailand [13]. Commercial zinc oxide nanoparticles (Sigma-Aldrich) were supplemented in PDA medium to 0.1-0.5% (w/v) final concentration. Fungal inoculation was carried out with 7 mm diameter discs of fungal mycelium excised from actively-growing cultures which were then placed on the surface of zinc oxide nanoparticles amended plates. Phomopsis sp. isolated HM 1 was incubated at 25^{o}C for 7 days in the dark. The magnitude of solubilizing ability was assessed by the diameter of solubilization halo zones in the agar medium. At the end of incubation period (7 days), the diameters of any clear solubilization zones were measured in three replicate plates [5, 6].

2.2. Evaluation of culture medium acidification

Tested fungal strain was cultivated in 250 ml Erlenmeyer flasks containing 100 ml potato dextrose broth (PDB). The initial pH of culture medium was adjusted prior to inoculation to 7.0. Fungal cultures were inoculated and grown in rotary shaker with speed of 150 rpm at 25^{o}C. An appropriate amount of heavy metal compounds was added to the liquid media with various concentrations of zinc oxide nanoparticles with concentration of 0.1, 0.3, 0.5 and 0.7 % (w/v). The pH value was measured after seven days, pH measurement was done in the triplicate using a pH electrode (Mettler-Toledo, Model S20) [5],[14].

2.3. Analysis of mycogenic oxalate crystals

The crystals were examined using a scanning electron microscope (SEM, JSM-6400 LV). The samples were mounted on double-sided carbon adhesive tape on 1.0 cm diameter carbon stubs and these were dried in vacuum desiccators at room temperature for at last 24 h and the samples were coated with gold by using a sputter coating machine (Balzer model SCD 040). The prepared samples were observed in the secondary electron mode at an acceleration voltage of 15 kV. Zinc biomineral were identified by X-ray powder diffraction (XRPD). Lyophilized samples were mounted onto crystal silicon substrates and examined using X-ray powder diffraction (XRPD, Bruker AXS: D8-Discover) equipped with a VANTEC-1 detector. Samples were scanned from 2-theta = 10° - 80°. Diffraction patterns were identified by reference to patterns in the international centre for diffraction data (ICDD) [15].

3. Results and discussion

After incubation period, Phomopsis sp. isolated HM 1 showed high efficiency for solubilizing zinc oxide nanoparticles (halo diameters > 40 mm.) at concentration of 0.1, 0.3

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and 0.5 % (w/v) (Table 1). Final pH was dropped from the initial pH (7.0) in every concentration of zinc oxide nanoparticle amended media (Table 1). The pH of fungal media was decreased during fungal growth, which showed that they became acidity and solubilized zinc oxide nanoparticles in fungal medium. The acidification had strong effect on metal mobilization. Generally, fungi can produce citric and oxalic acid, which are directly involved in metal solubilization [16]. Fungal organic acid secretion during growth decreases the pH of the system and can increase metal solubility by metal-complex formation [9], [17, 18].

Table 1. Solubilization halo zone and final pH of *Phomopsis* sp. (HM 1)

ZnO NPs % (w/v)	Halo zone diameter (mm)	Final pH
0.1	61.25±1.25	2.26±0.02
0.3	54.00±2.56	2.45±0.01
0.5	41.50±2.28	3.34±0.02
0.7	25.50±1.17	5.23±0.05

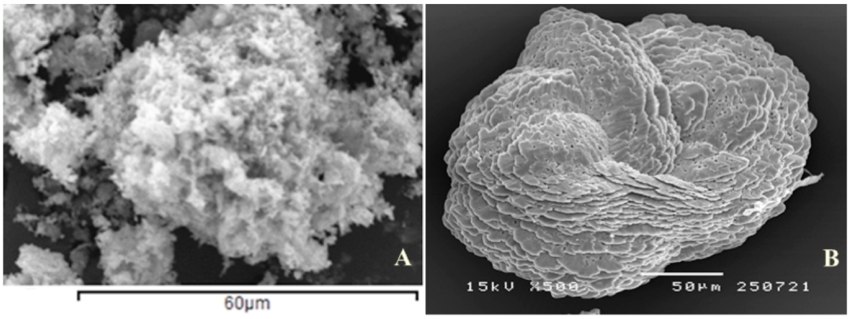

Fig. 1 Scanning electron micrographs of zinc oxide nanoparticles and mycogenic crystals produced by *Phomopsis* sp. (HM1). (A) Zinc oxide nanoparticles, scale bar = 60 µm. (B) Zinc biomineral crystals, scale bar = 50 µm.

The formation of mycogenic crystals was observed in the agar medium underneath the growing colonies. The crystals formation in the agar medium underneath the growing colonies and on the fungal mycelia may be related to the production of organic acids such as citric acid and oxalic acid, which were previously found to be the major role in immobilizing soluble metal ions by formation of insoluble metal oxalate complexes [9],[19]. Scanning electron micrographs of the mycogenic crystal and zinc oxide nanoparticles were shown in Fig. 1. The result showed that the crystals produced by tested fungi showed the different form of crystals when compared with the original crystal of zinc oxide nanoparticles. X-ray powder diffraction (XRPD) analysis of biomineral crystals produced by tested fungi showed the presence of a crystallized compound with an excellent match to standard pattern of zinc oxalate hydrate ($C_2O_4Zn \cdot 2H_2O$) (Fig. 2). Sayer and Gadd (1997) reported that Aspergillus niger, a fungus capable of oxalic acid production, was therefore capable of transforming inorganic insoluble metal compound such as zinc oxide (ZnO), zinc phosphate ($Zn(PO_4)_2$) and cobalt phosphate ($Co_3(PO_4)_2$) into insoluble oxalate complexes [10]. Sutjaritvorakul et al. (2016) also reported the ability of Aspergillus nomius to transform zinc oxide into zinc oxalate dehydrate [20]. Gharieb et al. (2004) found that in

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copper oxychloride amended medium, Aspergillus niger would excrete oxalic acid and transformed inorganic copper compound into copper oxalate [21]. Fomina et al. (2005) suggested that the amount of oxalic acid produced by entomopathogenic fungi, Beauveria caledonica was the main metal transforming agent, which transformed zinc, lead, copper and cadmium minerals, transforming them into the metal oxalate complexes [22]. The formation of oxalates containing potentially toxic metals may provide a mechanism whereby oxalate-producing fungi can tolerate metal-rich environments [10].

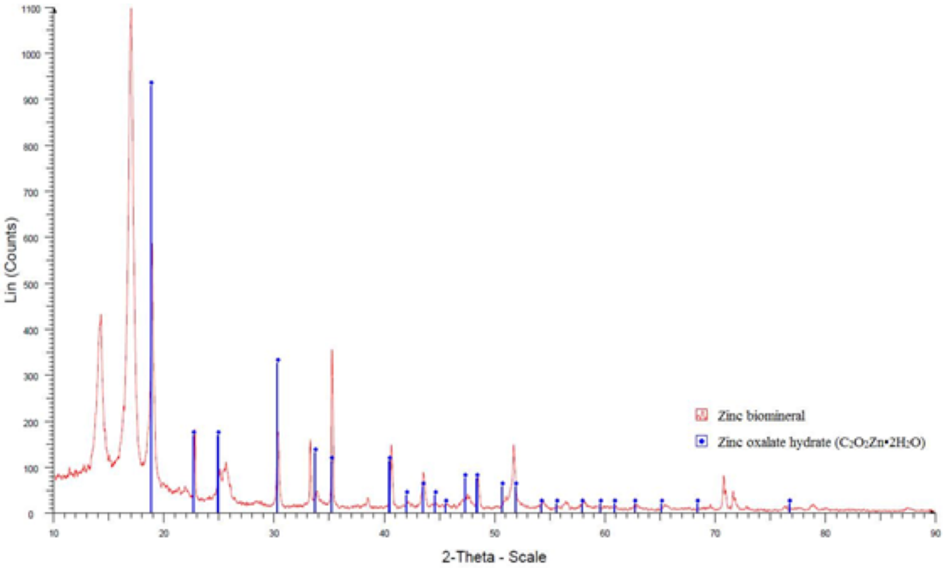

Fig. 2 XRPD pattern of zinc oxalate crystals produced by *Phomopsis* sp. (HM 1)

4. Conclusions

Phomopsis sp. isolated HM 1 showed high efficiency to solubilize and produce the metal crystals in the medium amended with zinc oxide nanoparticles. Organic acids produced by tested fungi have directly involved in metal mobilization. This research has shown that fungi with high level of heavy metal transformation ability can be isolated from mineral rocks, and these are capable of heavy metal mobilization as well as immobilization of zinc oxide nanoparticles by means of metal oxalate production. This study provided the evidence that fungi could detoxify heavy zinc oxide nanoparticles by mobilization and immobilization. Whether this is a process of significance in situ remains to be ascertained.

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