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Bioleaching mechanism of heavy metals in the mixture of contaminated soil and slag by using indigenous *Penicillium chrysogenum* strain F1

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HIGHLIGHTS

- We use *Penicillium chrysogenum* growth experiment data to fit Gompertz model.
- We compared the removal efficiencies of bioleaching with chemical bioleaching.
- The morphology and resistant mechanism of *P. chrysogenum* were preliminary examined.
- Glucose oxidase activity produced by *P. chrysogenum* during bioleaching was studied.

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ABSTRACT

The ability and bioleaching mechanism of heavy metals by *Penicillium chrysogenum* in soils contaminated with smelting slag were examined in this study. Batch experiments were performed to investigate the growth kinetics of *P. chrysogenum*, organic acids production and to compare the removal efficiencies of heavy metals between bioleaching with *P. chrysogenum* and chemical organic acids. The results showed that the bioleaching had higher removals than chemical leaching, and the removal percentages of Cd, Cu, Pb, Zn, Mn and Cr reached up to 74%, 59%, 24%, 55%, 57% and 25%, respectively. Removal efficiencies of heavy metals (15.41 mg/50 mL) by bioleaching were higher than chemical leaching with 0.5% of citric acid (15.15 mg/50 mL), oxalic acid (8.46 mg/50 mL), malic acid (11.35 mg/50 mL) and succinic acid (10.85 mg/50 mL). The results of transmission electron microscope (TEM) showed that no damage was obviously observed on the surface of the living cell except for thinner cell wall, discontinuous plasma membrane, compartmentalized lumen and concentrated cytoplasm during bioleaching process. The activity of extracellular glucose oxidase (GOD) produced by *P. chrysogenum* is influenced severely by the multi-heavy metal ions. The result implied that *P. chrysogenum* can be used to remove heavy metals from polluted soil and smeltery slag.

1. Introduction

Most of the smelting slag produced by mining and metallurgical activities has been deposited without any management at mines and smelters in China. Their improper management in the past resulted in the pollution of heavy metals to the surrounding environment such as soil and groundwater. For soil contamination, the typical properties include soil texture destruction, short of nutrient, ecological landscape destruction, and decrease in biological diversity [1]. In the heaping site, there are a large amount mixture of soil and slag, which differs from arable soils and pure slag. It is difficult to treat the above mixture. In order to resolve the above problems, it is important to develop a suitable and economical technology for removal of heavy metals from the smelting slag and the contaminated soil.

Physico-chemical processes to remove heavy metals in soils were documented in plenty of literatures. In these processes, chelator was commonly used. Both solubility and bioavailability of heavy metals are improved when synthetic chelator such as ethylenediamine-tetracetic acid (EDTA) was added into the soil. In particular, Na salt of EDTA was employed to chelate some metal ions strongly bonds to the soil phase and the metal ions chelating with Na salt of EDTA are less bio-available. However, excessively usage of chemical chelates has been proven to pollute the ground water and negatively affect soil quality because many necessary ions are also chelated unselectively [2]. Therefore, bioremediations are more concerned by scientists due to their more economical and

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Table 1
Physico-chemical characteristics of the mixture of slag and contaminated soil.

Item	pH (U)	Total Pb (mg/kg)	Total Zn (mg/kg)	Total Cd (mg/kg)	Total Cu (mg/kg)	Total Mn (mg/kg)	Total Cr (mg/kg)
Value	6.7	1889.6	5682	48.4	1848.6	3045	103.99

eco-friendly advantages than physico-chemical processes [3,4]. Especially, microbial metal-extraction processes i.e. bioleaching has been rapidly developed in recent decades [5–8]. Bioleaching is based on the ability of microorganisms to transform solid compounds into soluble and extractable elements. The advantages of bioleaching technology include mild reaction condition, low energy consumption, simple process, low environmental impact and being suitable for low grade mine tailings, residues and contaminated soils.

Several species of microorganism have been reported for bioleaching of heavy metals in soils, including *Aspergillus niger*, *Penicillium simplicissimum*, *Penicillium purpurogenum*, *Rhodotorula rubra*, *Acidithiobacillus thiooxidans* and *Acidithiobacillus ferrooxidans* [9–11]. The mechanism of metal solubilization during bioleaching is related to a chemical process, although attachment of microbes to the mineral can enhance dissolution [12]. Generally, microorganisms are involved in the mobility of metals usually through their oxidation, reduction, accumulation and their metabolites during the bioleaching. The bioleaching mechanism of *A. niger*, *P. simplicissimum*, *P. purpurogenum* was related to the production of low molecular weight metabolites, mainly organic acids, such as gluconic acid, pyruvic acid, citric acid, oxalic acid, malic acid and succinic acid [13,14], but it is different from that of *R. rubra*, *A. thiooxidans* and *A. ferrooxidans*. The bioleaching of *R. rubra* was related to its metabolic activity and to structural macromolecules of the capsule and the cell wall [9]. The bioleaching of *A. thiooxidans* and *A. ferrooxidans* contributed to "contact" and "non-contact" mechanisms. The contact mechanism took into account that most cells attach to the surface of bioleaching substrates. The non-contact mechanism was related to the redox reaction such as the reduction of iron (II) ion and the oxidation of sulfur (S) [15,16,25].

In our previous research, one strain *Penicillium chrysogenum* was isolated from the contaminated soils under smelter heap. It has strong ability for leaching of heavy metals in the contaminated soils [17]. In order to obtain the highest bioleaching efficiency of heavy metals, it is important to understand bioleaching mechanism of *P. chrysogenum*. Therefore, the objectives of this study were to (i) evaluate the growth of *P. chrysogenum* under heavy metals stress; (ii) identify organic acids produced by *P. chrysogenum*; (iii) investigate the activity of extracellular glucose oxidase (GOD) under heavy metal stress; (iv) assess the leaching ability of heavy metals by the strain.

2. Methods

2.1. Description of P. chrysogenum and the mixture of slag and soil

A fungi strain (*P. chrysogenum*) has been isolated from the polluted soil under the slag heap at a Smelting Industry in Zhuzhou, Hunan Province, central-south China, which has been identified as *P. chrysogenum* by sequencing 18S rDNA and ITS [17].

The total content of heavy metals in soils was determined by subjecting to acid digestion mixture (HCl, HNO_3, $HClO_4$ and HF) on an electric heating plate. The digestion solution was diluted with 1% (v/v) nitric acid for heavy metals analysis and then determined by inductively coupled plasma-optical emission spectrometer (ICP-OES, Perkin-Elmer Optima 3000 V). The characteristic of the soil is shown in Table 1.

2.2. Growth of P. chrysogenum under heavy metal stress

Measuring the colony diameter of fungi to evaluate the heavy metal tolerance of fungi was used to represent the growth of *Penicillium funiculosum*, *P. simplicissimum*, *Aspergillus foetidus* and *A. niger* by several scientists [18,19]. The effects of pH and heavy metals concentration on *P. chrysogenum* growth were evaluated through comparing the diameter of fungal colony in LB plates. The pH of LB was adjusted to 5, 7, 9 and 11 using 0.1 mol/L HCl and NaOH before innoculation of *P. chrysogenum*. LB agar media consisted of tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L. Assuming that the heavy metal in the soil were all leached out, different ratios of the slag and soil mixture (5–12.5%) to culture medium (w/v) were calculated according to the data in Table 1, and the heavy metal concentrations was shown in Table 2. Based on the total concentrations of Pb, Zn, Cd, Cu and Mn in soils, the desired metal concentrations ($Pb(NO_3)_2$, $Zn(NO_3)_2{\cdot}6H_2O$, $Cd(NO_3)_2{\cdot}4H_2O$, $Cu(NO_3)_2{\cdot}3H_2O$ and 50% $Mn(NO_3)_2$ solution) were added to the LB media prior to sterilization of the solution at 121 °C for 15 min. After sterilization, the medium was allowed to cool down to around 60 °C and poured into a 12 cm diameter petri dish for culture growth. The plates were inoculated by dropping 0.5 μL spores suspension liquid onto the plates and then incubated at 30 °C to establish their growth. A culture in the absence of the metal elements was also conducted as a control.

The growth of *P. chrysogenum* was monitored by measuring the diameter of the colony from the end of the longest to smallest hyphae. The tolerance index, an indication of *P. chrysogenum* response to metal stress, was calculated from the growth of the strain exposed to the metals divided by the growth in the control plate.

2.3. Bioleaching experiment

The spores for inoculation were collected from the Czapek agar media consisting of 1.0 g/L K_2HPO_4, 3.0 g/L $NaNO_3$, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.5 g/L KCl, 0.01 g/L $FeSO_4{\cdot}7H_2O$, 30.0 g/L sucrose and 15.0 g/L agar. The pH value of liquid medium was adjusted to 6.7 with 0.1 mol/L HCl. The number of spores was counted using a Neubauer counting chamber and adjusted to approximately 10^7 spores/mL using sterilized physiologic saline. Then 1 mL of spore suspension was added into 49 mL liquid medium in a 250 mL flask for inoculation.

2.3.1. Bioleaching under different ratios of soil and slag mixture

1 mL of spore suspension was inoculated in 49 mL of modified Czapek liquid medium for 7 days. Thereafter, different amount of slag and soil was added into the flasks containing media to obtain the soil and slag ratios of 5%, 7.5% and 10% (w/v), respectively. The mixture was continuously incubated in the shaker (120 rpm, 30 °C) to initiate bioleaching. In the course of bioleaching, the released concentrations of Zn, Cu, Pb, Cd, Mn and Cr in leachate were measured. The sterile controls were performed in the absence of fungi. All the experiments were carried out in triplicates.

2.3.2. Bioleaching and chemical leaching

Bioleaching experiments were carried out in 250 mL autoclaved conical flasks. 1 mL of spore suspension was inoculated in 49 mL of modified Chashi liquid medium for 7 days cultivation. Thereafter,