STUDY ON ISOLATION, IDENTIFICATION AND LEAD BIOSORPTION CAPABILITY OF A LEAD-TOLERANT *PENICILLIUM* SP. Pb-G FROM CONTAMINATED SOIL

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Abstract. To acquire a potential strain that may be used for the bioremediation of lead (Pb^{2+}) contaminations, an indigenous lead-resistant fungus *Penicillium* sp. Pb-G (GenBank No.MK372218) was isolated from lead contaminated soil, and the Pb^{2+} biosorption characteristics were determined in this study. The results showed that *Penicillium* sp. Pb-G was highly tolerant to Pb^{2+}, and it could survive on PDA medium with Pb^{2+} concentration up to 4000 mg/L. Under these circumstances, the spores of the strain Pb-G becomes shrunk and malformed as observed by scanning electron microscopy (SEM). X-ray diffractometer (XRD) analysis further revealed that the *Penicillium* sp. Pb-G's mycelia had a good biosorption capability for Pb^{2+}. The best biosorption effect of strain *Penicillium* sp. Pb-G was recorded at the Pb^{2+} concentration of 1500 mg/L, with the biosorption rate and biosorption amount of 53.05% and 178.02 mg/g, respectively. Collectively, these results demonstrated that the strain of Pb-G had strong Pb^{2+} resistance and biosorption abilities, which provides an attractive application prospect in bioremediation of heavy metal contamination.
Keywords:*maximum resistance level, identification, Penicillium, scanning electron microscopy, X-ray diffractometer*

Introduction

Heavy metal pollution in soil and water has become an increasingly prominent environmental problem, among the heavy metals lead gained special attention as a pollutant due to its high persistence and toxicity. It mainly derives from minerals, metal smelting, leaded gasoline, municipal sewage, industrial waste and paint spraying (Gisbert et al., 2003; Ganesh et al., 2015). It could be taken up by various crops and then threaten human health (Szczygłowska et al., 2011; An et al., 2018). Conventional physical and chemical approaches applied for the remediation of heavy metal have several drawbacks, such as high operational cost, low removal rate and secondary pollution. Microbes are widely present in the soil, due to their large surface area, they have strong adsorption capability for heavy metals and without any undesirable effects, thus, using microorganisms as an alternative biomaterial in dealing with heavy metal contaminated wastewater and soil have been widely concerned by scientists and become the research hot spots in the field of green environmental protection (Velmurugan et al., 2010; Wu et al., 2010;Deng et al., 2011;Kayalvizhi et al., 2019).

Generally, contaminated soils are sources of heavy metal tolerant microorganisms. Recent studies indicated that there existed a certain amount of anti-heavy metal

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microbial groups in soils that have been subjected to one or more heavy metal stresses for a long time, including bacteria, fungi, actinomycetes and algae, and these microbial groups usually have strong biosorption capacity for heavy metals (Wu et al., 2010; Jacob et al., 2013; Iram et al., 2015; Kayalvizhi et al., 2019). Fungi have been recognized as one promising class of low-cost biosorbents for the removal of heavy metal ions from aqueous waste streams. There are several mechanisms in fungi to tolerate and detoxify metals, including extra and intracellular precipitation, transformation of metals, and biosorption to cell wall. The cell walls of fungi are composed of polysaccharides, proteins, and lipids that contain reactive functional ingredients with potential metal binding capacities (Viraraghavan et al., 2011; Mohammadian et al., 2017).

Terry et al. (2018) reported that a total of 425 fungal strains were obtained from tropical forest soil, which showed heavy metal tolerance, the most common and diverse genera isolated were identified as *Penicillium*. Since the fungus *Penicillium* has strong vitality, rapid reproduction and extensive sources in environments, and it had been found as being most tolerant to many kinds of heavy metals, such as Zn, Hg, Cr and Pb (Ye et al., 2018; Kayalvizhi et al., 2019; Chang et al., 2020; Long et al., 2020), its anti-lead characteristics have been studied by some researchers particularly (Zucconi et al., 2003; Say et al., 2003; Velmurugan et al., 2010; Mohammadian et al., 2017;Ye et al., 2018). Zucconi et al. (2003) reported that a strain of *Penicillium* (*Penicillium lilacinus*) can grow in a medium containing Pb^{2+} up to 1434 mg/L. As reported by Sun et al. (2007), *Penicillium* sp. Psf-2 can grow in a solution with Pb^{2+} concentration of 4 mmol/L. Velmurugan et al. (2010) isolated a high lead-resistant strain (*Penicillium* sp. MRF-1) from South Korean mining soil, where the lead content is 357 mg/kg, the strain showed high removal efficiency of Pb^{2+}, so it would be an excellent biosorbent for the removal of lead from aqueous solution. The *Penicillium oxalicum* SL2 had tolerance to 1000 mg/L Cr^{6+} and 2500 mg/L Pb^{2+} in potato-dextrose agar, and had excellent removal efficiency of $Cr^{6+}$and Pb^{2+}via reduction with acidic metabolites and form transformation in the mycelium, the strain showed a promising new candidate for bioremediation of heavy metal pollution (Ye et al., 2018;Long et al., 2020).

Most previous reports on heavy metal resistant fungi have been focusing on the adsorption capacity of dead fungi biomass, and less on the biosorption capacity of living fungi biomass (Say et al., 2003; Fan et al., 2008; Velmurugan et al., 2010). Moreover, although these studies showed that *Penicillium* was tolerant to Pb^{2+} and had Pb^{2+} biosorption capability, their performance about tolerance and biosorption were slightly weak. Therefore, it is far-reaching significant to use living fungi to cope with the seriously lead contaminated soil for long-term bioremediation effect.

In this study, a fungus strain with high Pb-resistance was isolated and acclimated from Pb-contaminated soil, and its biological characteristics were studied. The biosorption effects of Pb^{2+} by living strains were also determined. This study would potentially provide a theoretical basis for the bioremediation of lead pollution in the environment.

Materials and methods

Collection of soil samples and Pb content analysis of soil samples

The soil samples were collected from a demonstration zone of the National Loess Fertility and Fertilizer Benefit Monitoring Base in Wuquan Town, Yangling County,

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Shaanxi Province (34°17′51″N, 108°00′48″E), China. In the test site, 350 mg/kg Pb (in the form of $Pb(NO_3)_2$solution) was artificially added in the soil in May 2010. The surface soil (0-20 cm) was collected with the Z-shape 5-point sampling method in December 2013. After mixed, approximately 0.5 kg of soil from each site was collected. A standard soil corer device was used to collect samples without disturbing plant roots. Five collected soil cores from each plot were pooled as a replicate. A total of 3 sample replicates were transferred to laboratory. The soil samples were sieved through 2-mm mesh to remove plant residues and stones. A subsample of each soil core was sealed in a sterilized self-sealing bag in an icebox and delivered to laboratory, then stored in the refrigerator at 4℃ for isolating fungal strains. The remaining sample was air-dried at room temperature until a stable weight was reached, and air-dried soil samples were ground and passed through a 0.25-mm sieve for Pb content analysis.

Total concentration of Pb in the soils were determined by standard soil testing procedures (Bao, 2000), 5 g soil samples were digested by HNO_3:HCl:$HClO_4$(1:2:2) to extract the total Pb. Total Pb concentration was measured with ICP-MS (Thermo, model Xseries Ⅱ, USA).

Isolation of Pb-tolerant fungal strain

A total of 10 g of the collected soil sample was weighed and dissolved in 100 mL distilled water held in a 500-mL flask. The mixture was shaken at 25 ℃, 120 r/min for 2 h and kept still for 20 min, 1 mL of the suspension was removed and diluted to 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} concentrations with a gradient-dilution method (Li et al., 2010b). With a sterilized pipettor, 100 μL of each dilution was transferred onto potato-dextrose agar (PDA) plates supplemented with 200, 300, 400 and 500 mg/L of Pb^{2+}, respectively. The plates were incubated at 28 ℃ for 5-7 d, three replications were performed for each treatment. According to fungus growing characteristics, the blooming colonies with different morphology were picked and re-inoculated onto new PDA plates until the pure strain was obtained. The obtained strains were further examined on PDA plates that contain 600, 800 and 1000 mg/L of Pb^{2+}, respectively. After isolation, the obtained pure strains were preserved with PDA slant and glycerol media. All the operations were conducted under sterilized conditions.

The above PDA culture medium formula was: peeled potato 200.0 g, glucose 15.0 g, peptone 3.0 g, $MgSO_4$ 2.0 g, KH_2PO_4 3.0 g, agar powder 15.0 g, and distilled water 1000 mL. $Pb(NO_3)_2$ solution was prepared and stored at 4 ℃ for adding to the PDA medium.

Maximum Pb resistance level of strain Pb-G

The maximum concentration of the heavy metal that a strain can tolerated is the maximum resistance level (MRL). To determine the MRL of the isolated strain, the strain was cultivated with PDA containing different concentrations of sterilized $Pb(NO_3)_2$ solution. Five Pb^{2+}concentrations (treatments) were selected, that is, 0 mg/L (CK), 1000 mg/L, 2000 mg/L, 3000 mg/L and 4000 mg/L, three replicates per treatment. After 5 days of cultivation at 28 ℃, the colonies were observed and photographed.

Molecular identification for strain Pb-G

The conservative ITS rRNA gene in fungi was used to identify the screened Pb-G strain. The ITS rRNA gene form Pb-G was amplified using the primer ITS1 (5’-

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TCCGTAGGTGAACCTGCGG-3') and ITS4 5'-TCCTCCGCTTATTGATATGC-3') (Velmurugan et al., 2010). The PCR amplification system was 50 μL, containing 5 μL 10×LA Taq Buffer II (Mg^{2+} Plus (TaKaRa, Japan), 8 μL dNTP, 5 U LA Taq enzyme (TaKaRa), 1 μL of each primer (0.5 mmol/L), and 2 μL 10.3 ng/μL DNA template). The PCR program was as follows: pre-denaturation at 94℃ for 5 min, 31 cycles of denaturation at 94℃ for 45 s, annealing at 58℃ for 45 s, extension at 72℃ for 90 s, and finally preservation at 4℃ for less than 12 h. The PCR products were detected by agarose gel electrophoresis, and the qualified ones were sent to the Beijing Liuhe Huada Gene Technology Co., Ltd. for sequencing the ITS gene.

To analyze the homology of the strain, the sequences of the samples were aligned against NCBI databases (http://www.ncbi.nih.gov/index.html) by BLAST, and a phylogenetic tree was constructed using the software MEGA 5.05, using Neighbor-joining analysis for the ITS rRNA.

Effects of temperature and pH on the growth of Pb-G

Temperature and pH are the decisive factors affecting the growth of the strain, and the metabolic rate and growth rate of the strain will be increased at the appropriate temperature and pH. Therefore, the two factors were selected to determine the optimal growth conditions of the strain.

The *Penicillium* sp. Pb-G strain was rejuvenated on PDA medium without Pb^{2+}, and then agar dishes attached with plenty of mycelia were taken out using a sterilized hole puncher (Φ=1.2 cm). The agar dishes were put onto PDA medium and inoculated for 7 d at 20 ℃, 25 ℃, 30 ℃ and 35 ℃, respectively. The colony diameter was then measured by the Cross-crossing method at a fixed time every day. The colonial average growth rate was calculated according to *equation (1)*:

$$G_t = \frac{D_t - D_0}{t} \quad \text{(Eq.1)}$$

where, G_t (v, cm/d) is the average growth rate; D_t (φ, cm) is the average colony diameter; D_o (φ, cm) is the disk diameter, and t (d) is the culture time.

The pH of solid PDA medium was adjusted to 6.0, 7.0, 8.0 and 9.0 respectively using sterilized 1 mol/L HCl and NaOH solutions. The rejuvenated *Penicillium* sp. Pb-G were then inoculated onto these media by agar dish inoculation method using a sterilized hole puncher (Φ=1.2 cm) and cultivated at 25 °C for 7 d. Every treatment repeats three times. The colonial diameter was measured by the Cross-crossing method at a fixed time every day. The colonial average growth rate was also calculated according to equation (*Eq.1*).

Scanning electron microscopy (SEM) analysis

The scanning electron microscopy (SEM) was used to analyze the morphological characteristics of *Penicillium* sp. Pb-G cultured in different Pb^{2+} concentrations. The sterilized $Pb(NO_3)_2$ solution was added into PDA medium to get the final concentrations of 0 mg/kg (CK), 2000 mg/kg, 3000 mg/kg and 4000 mg/kg. The *Penicillium* sp. Pb-G was then inoculated in these PDA liquid media at 28℃. After 5 d, fungal biomass was harvested by centrifugation at 8000 r/min (15℃) for 10 min. To remove any loosely associated and unsequestered Pb^{2+}, the collected mycelia were soaked in 2% oxalic acid for 10 min and then soaked in distilled water for another 10 min for two times. Then the samples were dried by vacuum freeze drier (Thermo Fisher, USA), coated with gold and

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examined under SEM (FLEXSEM1000, Hitachi, Japan), photographed with optimum magnification (Glukhova et al., 2018).

X-ray diffraction (XRD) analysis

To analyze the Pb^{2+} biosorption capability of *Penicillium* sp. Pb-G, it was cultivated in PDA liquid medium with $Pb(NO_3)_2$concentrations of 0 mg/L (CK) and 4000 mg/L respectively. Every concentration was set for three repetitions. The strain was cultured on an oscillator at 25°C, 120 r/min for 5 d, centrifuged at 6000 r/min for 15 min to precipitate the fungal biomass, the supernatant was discarded and the precipitates were rinsed with distilled water, repeat these steps twice. The precipitates were taken out and dried at 65°C. After grinding the sample was analyzed by an XRD (Nippon science Minniflex 600, Japan), and parameters were set as the light pipe current, 15 mA; the voltage, 40 kV; the width of the slit, 0.02 deg; the scanning angular velocity, 0.25 º/min; and the scanning angle ranging from 3º to 90º. The characteristics of *Penicillium* sp. Pb-G were analyzed and plotted by MDI Jade 6.5 software (Materials Data Inc. Liverpool, CA) and Origin 8.5 (Origin Lab, USA) respectively based on the average value of three repetitions.

Biosorption properties of the strain Pb-G to Pb^{2+}

The mycelium from the vigorous *Penicillium* sp. Pb-G colony was picked, inoculated into 100 mL PDA liquid medium, and cultivated on a shaker at 120 r/min and 25℃. After 5 d of inoculation, the fungus was filtered with four layers of gauze, rinsed three times with deionized water, dried together with filter paper, and then weighted.

To examine the biosorption capability of Pb-G, the collected mycelium was inoculated in $Pb(NO_3)_2$ solution (pH=7) with initial Pb^{2+} concentrations of 0 mg/L(CK), 500 mg/L, 1000 mg/L, 1500 mg/L, 2000 mg/L and 2500 mg/L respectively, three repeats per Pb^{2+} concentration. For inoculation, 1 g fungi were put into 50 mL $Pb(NO_3)_2$ solution contained in a flask, fully dispersed by a glass rod, and shaken on shaker at 25℃, 150 r/min. After 24 h, all the mycelia were filtered with the filter paper, and then the hyphae were dried to a constant weight in a drying oven at 80 ℃ for 12 h with the filter paper together. The weight of the filter paper was then subtracted to obtain the dry weight of the biomass. Finally, the collected mycelia weight was calculated (Pan et al., 2010). The biosorption rate (*Q*) and the biosorption amount (*q*, mg/g) of fungus *Penicillium* sp. Pb-G were calculated based on mycelium dry weight according to the equation (*Eq.2*) and (*Eq.3*), respectively.

$$Q = \frac{(C_0 - C_t)}{C_0} \times 100\% \quad \text{(Eq.2)}$$

$$q = \frac{(C_0 - C_t)}{m} \times V \quad \text{(Eq.3)}$$

where, C_0 (mg/L) is the initial Pb^{2+} concentration before biosorption; C_t (mg/L) is the final Pb^{2+} concentration after biosorption; V (L) is the volume of the reaction solution; and m (g) is the mass of dried biomass of fungi in the reaction solution.

The supernatant was digested by nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) for measuring Pb^{2+}amount.

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Data analysis

The growth rate and biosorption capability were performed with three repetitions and the data were reported as mean ± standard deviation (SD) in the figures and table. The significant differences among the treatments were evaluated using One-way analysis of variance (ANOVA) followed by LSD test at a level of $p < 0.05$ using SPSS 22.0 software for Windows (SPSS Inc., Chicago, IL, USA).

Results

The maximum resistance level of strain Pb-G

The average concentration of total Pb in collected soil samples was 331.13 mg/kg that exerted a selection pressure on microbial communities, including filamentous fungi. After isolation and culturing, a strain of fungus with high resistance capacity to Pb^{2+} was obtained, named Pb-G. The center of the colony was cyan, and the periphery of the colony showed white. The *Figure1* demonstrated that the morphology of the *Penicillium* sp. Pb-G remained same with the blue-green color and no prominent changes were detected under the different concentration of Pb^{2+} from 1000 to 2000 mg/L. Whereas, when the Pb^{2+} concentration was raised to 3000 mg/L, the growth of strain Pb-G slowed down, showing low hyphae amount, deeper color and white colony edge, which indicated that the growth of the fungi was inhibited to a certain extent and when Pb^{2+} reached up to 4000 mg/L in the medium, Pb-G strain still grew, but the number of hyphae was reduced, the colony was obviously small in diameter, and no obvious changes were found for hyphae even after 25 d of cultivation. So the MRL of the Pb-G strain was 4000 mg/L.

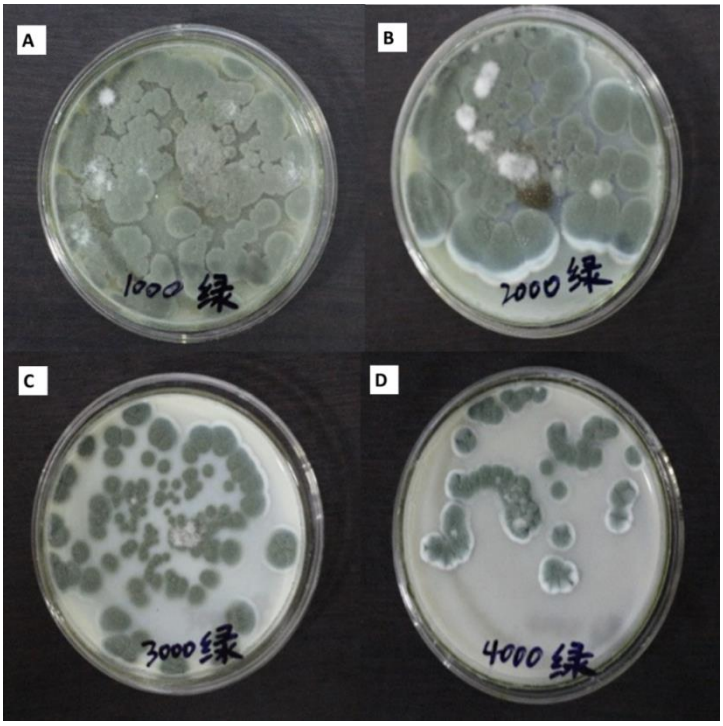

Figure 1. *The growth status of Penicillium sp. Pb-G in medium with different concentrations of Pb^{2+}. A, 1000 mg/L; B, 2000 mg/L; C, 3000 mg/L; D, 4000 mg/L*

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Molecular identification of the Pb-tolerant microorganism (Pb-G)

The ITS rRNA gene was amplified from Pb-G stain and analyzed by software MEGA 5.05. The ITS rRNA gene in Pb-G strain was about 600 bp in length, the ITS rRNA sequence of Pb-G was deposited to GenBank and accession number was obtained, the GenBank accession was No. MK372218 (http://www.ncbi.nih.gov/index.html). A phylogenetic tree was constructed with the obtained homologous sequences from GenBank according to the sequence similarity over 97% using the ITS nuclear ribosomal RNA (Torres et al., 2018). If the similarity of ITS rRNA gene sequences was more than 99% between a studied strain and its nearest neighbor, the strain can be identified as the same species with its nearest neighbor (Mohammadian et al., 2017). In the present study the results of phylogenetic tree showed the strain Pb-G was closely related to *Penicillium* in evolution, and its nearest neighbor was the *Penicillium* JN397373.1 in the phylogenetic tree (*Figure2*). Therefore, based on the fungal morphology and sequence alignment of ITS rRNA gene, the strain Pb-G was identified as *Penicillium* genus.

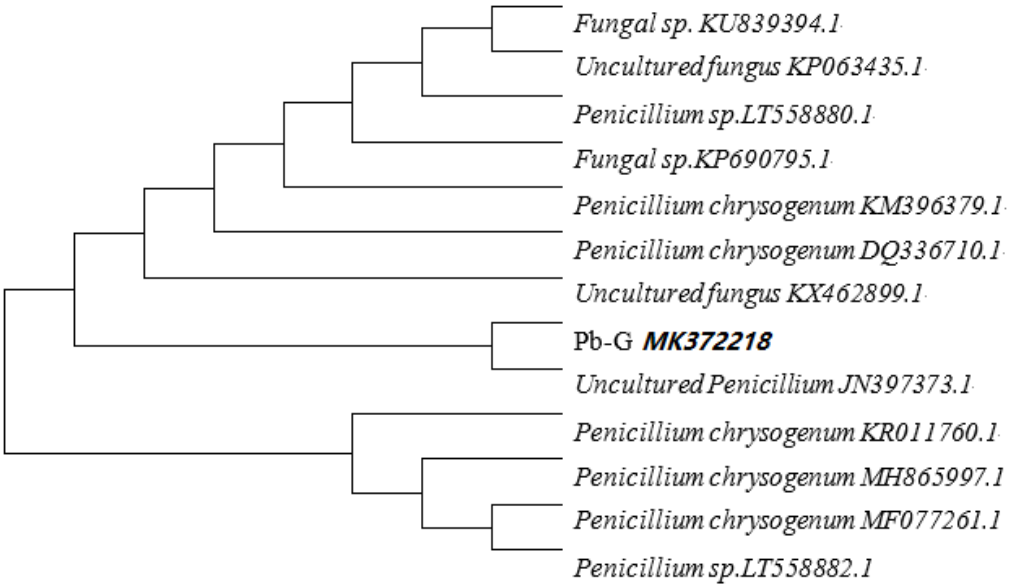

Figure 2. *The phylogenetictree of strainstolerantto* Pb^{2+}

Effects of different temperatures and pH on the growth of Pb-G

The growth rate of *Penicillium* sp. Pb-G changed under the influence of different temperatures (*Figure3*). On the first day, the *Penicillium* sp. Pb-G strain at 25 °C grew slowly, the strain at other temperatures did not change at all. On the 2-4th day, *Penicillium* sp. Pb-G had the highest growth rate at 25 °C, while they grew fast at 20 °C on the 4-7th day. For the *Penicillium* sp. Pb-G at 30 °C, they grew fast on the 2-4th day, but the growth rate decreased on the 4-7th day. The *Penicillium* sp. Pb-G was almost no obvious growth at 35°C. These results indicated that the strain Pb-G grow well within the temperature range of 20-30 °C, but inhibited at 35°C, therefore, 25 °C was the most suitable temperature for its growth.

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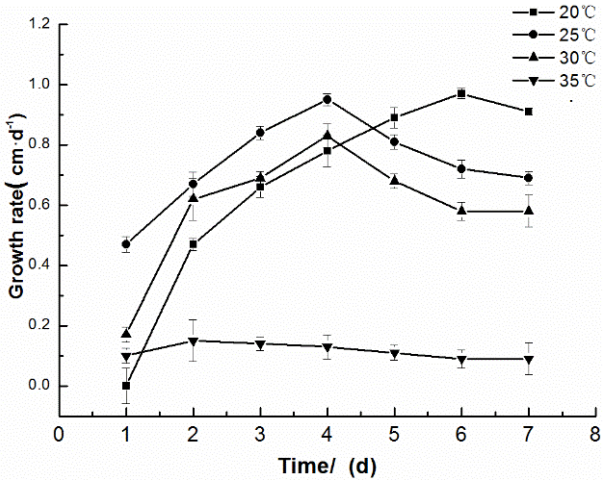

Figure 3. *Effects of different temperatures on the growth rate of Penicillium sp. Pb-G.*

Data are means ± SD (n=3)

The growth rate of strain *Penicillium* sp. Pb-G also changed under the influence of different pH values (*Figure4*). The growth rate of *Penicillium* sp. Pb-G was highest at pH 7 among all pH conditions, followed by the growth rate at pH 6, while it was decreased when the pH reached up to 8 and 9. The results indicated that the pH 7 was the optimal pH value for the strain growth, and the growth rate reached the highest on the 4th day.

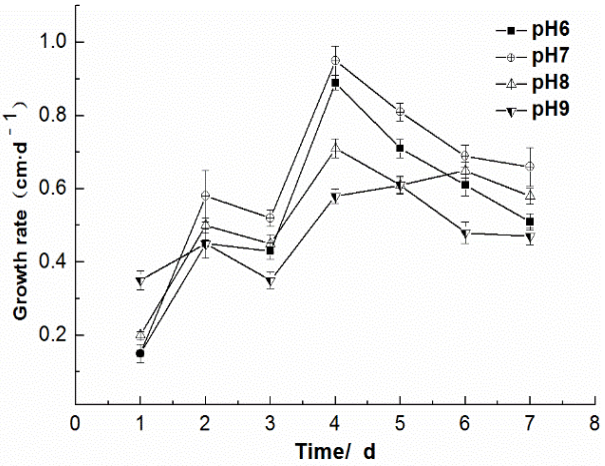

Figure 4. *Effects of different pHs on the growth rate of Penicillium sp.Pb-G.*

Data are means ± SD (n=3)

Morphological characteristics of strain Pb-G's spores with different Pb^{2+} concentrations

The spore morphologies of the *Penicillium* sp. Pb-G in medium with different Pb^{2+} concentrations were observed by scanning electron microscopy. As shown in *Figure 5*, the spores of *Penicillium* sp. Pb-G in the CK group (0 mg/kg of Pb^{2+}) were more than that treated under 4000 mg/kg of Pb^{2+}, and the individuals were full and evenly

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distributed. However, the amount of spores was reduced under different Pb^{2+} treatments, and it showed morphologies of collapse, shrinkage and deformity. Furthermore, some spores dissolved and joined into pieces in "strips" shape (as indicated by the arrows). With the increase of Pb^{2+} concentration, the phenomenon of spore shrinkage and connection into pieces also increased. After magnified, the *Penicillium* sp. Pb-G spores under the 4000 mg/kg of Pb^{2+} treatment showed obvious distortion and deformation compared with the CK group, and some of them had irregularly shaped blocky material attached, as shown by the arrows (*Figure 5*-E and F).

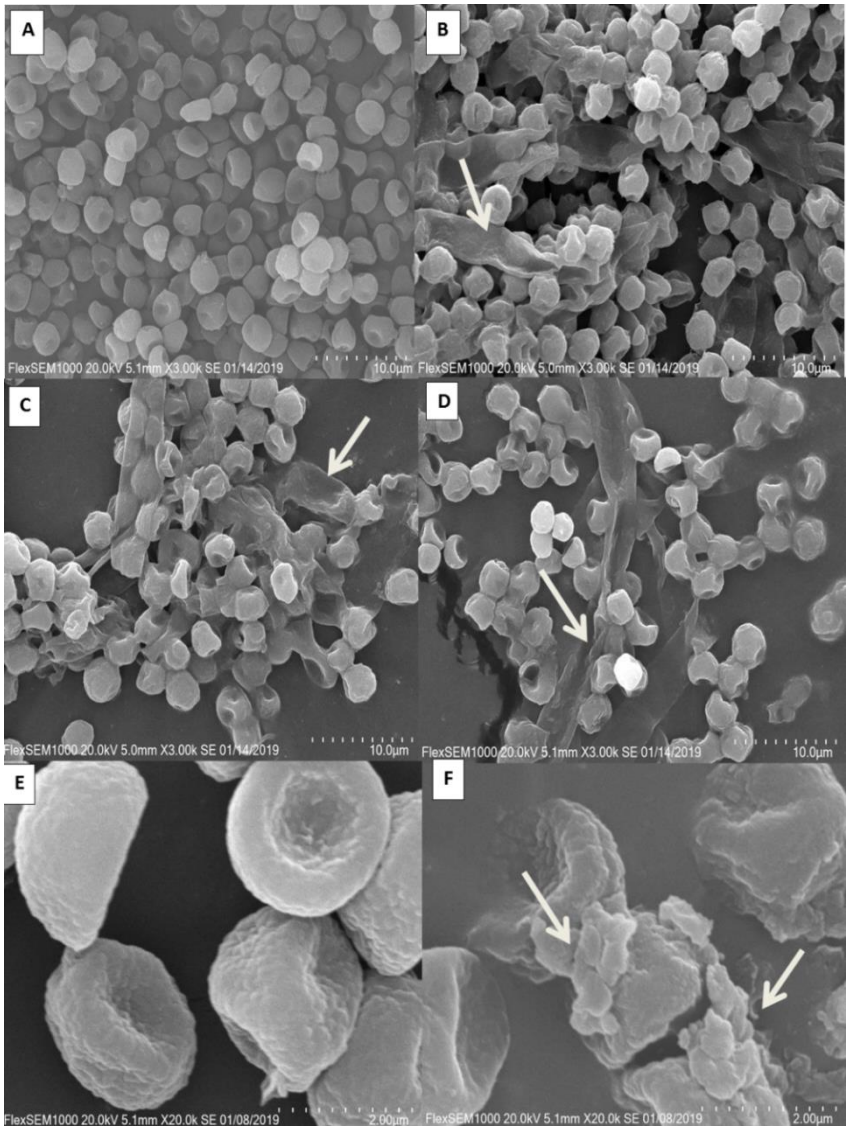

Figure 5.*Scanning electron microscopy (SEM) micrographs of strain Pb-G treated by different concentrations of* Pb^{2+}*. (A and E, 0 mg/kg (CK); B, 2000 mg/kg; C, 3000 mg/kg; D and F, 4000 mg/kg; A, B, C and D were amplified by 3000 times, and E , F were amplified by 20000 times)*

XRD analysis

The *Penicillium* sp. Pb-G grown under 0 mg/L (CK) and 4000 mg/L $Pb(NO_3)_2$ treatments were further analyzed by X-ray diffractometry. The XRD patterns of *Penicillium* sp. Pb-G cells treated with 0 and 4000 mg/L $Pb(NO_3)_2$ were different

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(*Figure 6*). Analysis by using Jade 6.0 software and phase retrieval, it was found that the characteristic peaks of Pb-G cells under the treatment of 4000 mg/L Pb^{2+} (marked by black triangles) contained Pb elements, in which the characteristic peaks were the highest when the diffraction angle was 30.16°-30.30°. But there was no obvious characteristic peak under CK treatment and no Pb elements were found by phase retrieval. In this study, Pb-G cells treated with 4000 mg/L $Pb(NO_3)_2$ contained Pb with different compound forms, of which the characteristic peak was highest and there was no Pb element in the fungi of CK group when the diffraction angle was 30.16°-30.3°. It can be inferred that the Pb elements in Pb-G mainly come from the exogenous $Pb(NO_3)_2$ solution, indicating that the strain *Penicillium* sp. Pb-G had biosorption capability to exogenous Pb^{2+}.

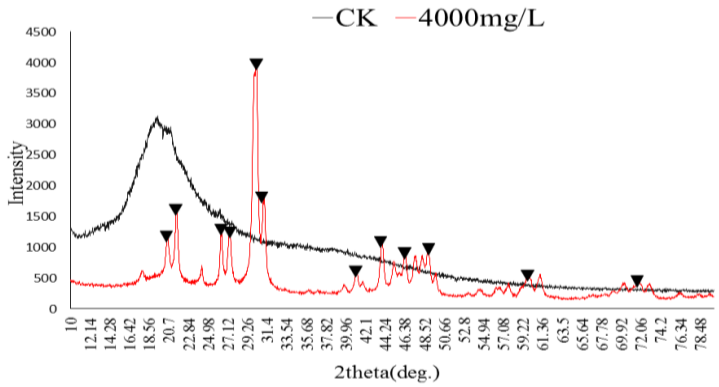

Figure 6. *X-ray diffractogram of strain Penicillium sp. Pb-G treated by different concentrations of* Pb^{2+}

Analysis of the biosorption of Pb^{2+} *by the strain Pb-G*

In this study, as the results shown in *Table 1*, it can be seen that the fungus *Penicillium* sp. Pb-G had good biosorption efficiency at different initial concentration of Pb^{2+}, and the trend of biosorption characteristics of Pb^{2+} by *Penicillium* sp. Pb-G was also be shown in *Figure 7*, both the biosorption capacity and biosorption rate were increasing at first and then decreasing with the increasing of Pb^{2+} concentration. When the Pb^{2+}concentration was 1500 mg/L, the biosorption influence of strain Pb-G was optimal, on which the biosorption rate and biosorption amount was 53.05% and 178.02 mg/g, respectively. When the Pb^{2+} concentration was more than 1500 mg/L, both the biosorption amount and the biosorption rate of *Penicillium* sp. Pb-G showed a rapid decline. Based on these findings, this strain has excellent biosorption capacity for Pb^{2+}.

Table 1. *The biosorption efficiency of* Pb^{2+}*by Penicillium sp.Pb-G*

The Initial concentration of Pb^{2+} (mg/L)	The residual concentration of Pb^{2+} (mg/L)	Biosorptioncapacity (mg/g)	Biosorptionrate (%)
500	426.95 ±0.66	28.25 ± 1.6 e	14.61 ± 1.9 e
1000	729.54 ±0.48	125.45 ± 3.2 b	27.05 ± 1.5 b
1500	704.25 ±0.16	178.02 ±3.9 a	53.05 ± 1.0 a
2000	1509.10 ±1.62	88.51 ± 2.8 c	24.55 ± 0.8 c
2500	2092.70 ±0.68	51.26 ± 0.9 d	16.29 ± 0.9 d

Note: Values are means ± SD, n=3. Different letters indicate significant differences among the treatment means (p *<0.05*)

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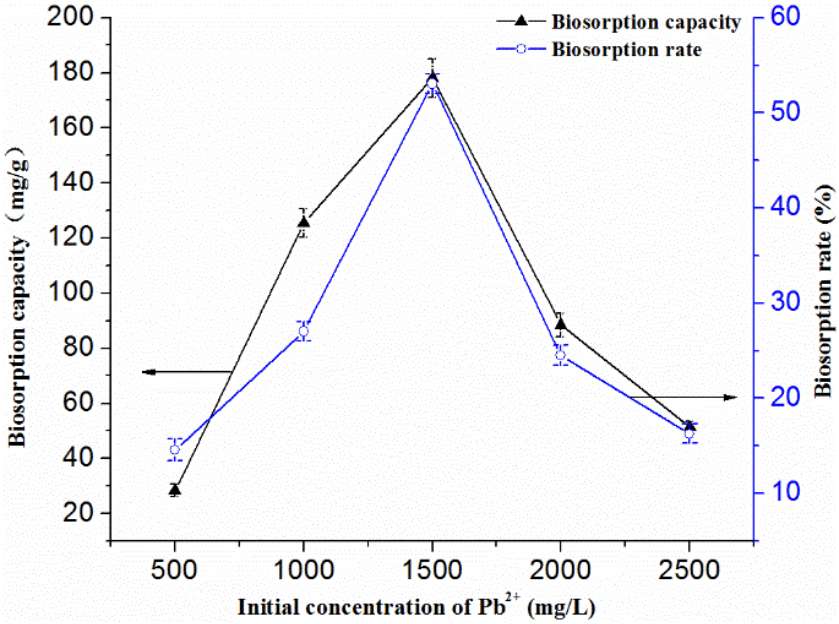

Figure 7. *Biosorption characteristics of Penicillium sp. Pb-G treated by different concentration of* Pb^{2+}

Discussion

Pb is highly toxic to living organisms. In a long-term Pb-contaminated environment, a number of microorganisms have evolved various mechanisms to counteract Pb stress and thereby improved their tolerance to Pb. Jacob et al. (2013) isolated three strains which were highly-resistant to Pb and Se from polluted seawater, namely *Aspergillus*, *Fusarium* and *Penicillium*, which can still grow on the medium contained high concentrations of Pb and Se. Iram et al. (2015) studied two strains of *Aspergillus flavus* and *Aspergillus niger* isolated from soil, which were highly resistant to Pb^{2+} and Cu^{2+}, and the biosorption capacity of *A.niger* to Pb^{2+} was 3.25-172.25 mg/g. Velmurugan et al. (2010) reported that *Penicillium* sp. MRF-1 can grow within the range of Pb^{2+} concentration from 0.31 to 1.24 g/L. Heavy metal resistant strains generally exist in areas contaminated by heavy metals, but the resistance levels of the same genus were inconsistent, and their resistance levels always depend on the level of contamination at the site of separation and the extent of heavy metal contamination in the separation test level (Jacob et al., 2013). The resistance of strain Pb-G to Pb in this study was similar to that of the previous studies. However, due to the high level of contamination at the isolation site, the strain Pb-G in this study can grow at a higher Pb^{2+} concentration and had better biosorption capacity after domestication.

It has been reported that bacteria, fungi and algae often act as biosorbent to remove a variety of heavy metal elements from the environment (Wang et al., 2006; Cain et al., 2008; Wu et al., 2010; Li et al., 2010a; Jacob et al., 2013; Iram et al., 2015), in particular, fungus in the genus of *Penicillium* had a strong biosorption capacity. Due to a large number of extracellular hyphae outside the cell wall of *Penicillium*, it had the function of adsorbing heavy metals, and some heavy metals can precipitate on the surface of the growing hyphae (Sintuprapa et al., 2000; Sun et al., 2007). The biosorption process is closely related to the concentration of heavy metal ions and the

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biomass produced by the fungi, the biosorbent has more adsorption sites. When the adsorption site of the biosorbent reaches the saturation of the heavy metal, the biosorption amount gradually decreased (Fan et al., 2008; Pan et al., 2010; Yang et al., 2012). Therefore, as the concentration of heavy metal ions increases, the biosorption rate of the cells increases, and as the saturation of the adsorption sites increases, the biosorption capacity decreases gradually (Yang et al., 2012; Fan et al., 2013). In this study, the biosorption rate of Pb^{2+} by *Penicillium* sp. Pb-G reached the highest value, and the biosorption amount and biosorption rate began to decrease with the increasing of Pb^{2+}concentration, which indicated that the adsorption site of the added fungi may be close to saturation level in the biosorption test.

In this study, it was observed by SEM that the spores of *Penicillium* sp. Pb-G under high concentration of Pb^{2+} were deformed, collapsed and dissolved, and formed a "strip" shape in parallel. During fungal growth, the heavy metal resistant fungus can produce organic chelators and acids (Liang et al., 2016), the solubilizing effects of these compounds may be a probable reason for the spores of *Penicillium* sp. Pb-G deformed and dissolved. XRD analysis showed that *Penicillium* sp. Pb-G cells with $Pb(NO_3)_2$ treatment contained Pb, but none in CK, which indicated that Pb in *Penicillium* sp.Pb-G cells was derived from exogenously added $Pb(NO_3)_2$ solution, indicating *Penicillium* sp.Pb-G had biosorption function to Pb^{2+}. Raheem et al. (2013) reported that the *Enterobacter* sp. could produce nitrate reductase and denitrify $Pb(NO_3)_2$ to PbO when it was treated by $Pb(NO_3)_2$, and PbO peak appeared at a diffraction angle of about 30° analyzed by XRD. Liang et al. (2016) reported that *Aspergillus niger* and *Paecilomyces* grew in $Pb(NO_3)_2$-containing medium can produce phosphatase, phyticacid and glycerol-2-phosphate can be hydrolyzed by phosphatase, releasing inorganic phosphate and oxalic acid, where by $Pb(NO_3)_2$ in the solution can be precipitated into Pb-oxalate and Pb-chlorite, and the Pb-chloride can be further converted into Pb-oxalate, which is insoluble or weakly soluble. The *Penicillium* sp. Pb-G in this study showed similar characteristics to the reported strains, and the specific biosorption mechanism needs to be further studied.

The mechanism of adsorption of metal ions by fungi mainly included cell surface adsorption or complexation, intracellular enrichment and efflux, among them, enrichment was mainly achieved by transport of cell membranes inside the cells, while the stage of intracellular and extracellular excretion was when heavy metal ions reach a certain concentration, and the fungi prevented more heavy metal ions from entering the cells through efflux (Congeevaram et al., 2007; Sun et al., 2007; Deng et al., 2011). In the range of different concentrations of heavy metals, the living microbial fungus initiated two different mechanisms, one was biosorption by non-living or non-growing biomass, which was a metabolism-independent and passive uptake process, another was bioaccumulation by living and growing cells, which was mainly an intracellular accumulation (Deng et al., 2011). When the concentration of heavy metal reached a certain range that inhibiting microbial growth, the absorbing capacity of the microorganisms showed a downward trend. In this study, the biosorption rate of the strain *Penicillium* sp. Pb-G was measured in a specific concentration of Pb^{2+} solution. Within a certain concentration range, the biosorption of Pb^{2+} by the cell adsorption and enrichment achieved. When the concentration of Pb^{2+} inhibited the activity of fungi, the biosorption rate and the biosorption capacity tended to decrease.

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Conclusions

After domestication and isolation, a strain of fungus (*Penicillium* sp. Pb-G) with high resistance to Pb was obtained, it can grow in a medium with Pb^{2+} content of 4000 mg/L. According to the morphological feature and molecular analysis, *Penicillium* sp. Pb-G had a close homology with *Penicillium*, thus the strain Pb-G was classified into the genus of *Penicillium*. The suitable temperature range for the *Penicillium* sp. Pb-G growth was 20-30°C, the most suitable pH was 7, and it had a biosorption effect on Pb^{2+}. When the Pb^{2+}concentration at 1500 mg/L, the best biosorption rate of *Penicillium* sp. Pb-G was 53.05% and the biosorption amount was up to 178.02 mg/g. This study indicated that *Penicillium* sp. Pb-G strain had high tolerance and outstanding biosorption capacity to Pb^{2+}, it could be as a potential candidate to apply in future for metal remediation from wastewater and heavy metal-contaminated soils.

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