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Remediation of Pb-Resistant Bacteria to Pb Polluted Soil

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ABSTRACT

To show the remediation of Pb-resistant bacteria to Pb polluted soil, several indices including microbial counts, soil enzyme activity, microbial community diversity and soil Pb concentration were investigated. Two Pb-resistant bacteria were filtrated and identified by previous study as Bacillus pumilus and Pseudomonas aeruginosa (*GeneBank Accession No. FJ*402988 *and GU*017676) *and inoculated to soil planted with cabbages. Soil with different Pb application rates were incubated for a period of* 0, 12, 24, 36, 48 *days in greenhouse. Results indicated the count of bacteria in* 1000 *mg/kg Pb treated soil greatly affected by inoculating Pb-resistant bacteria, which was raised about* 237% *and* 347% *compared with control. Soil urease and invertase were intensified* 37.9% *and* 65.6% *after inoculation compared with control. Phosphatase activity was inhibited by inoculation of Bacillus pumilus. Catalase activity was intensified about* 64.2% *in* 24 *days incubation but decrease in the following days. Microbial community diversity analyzed by polymerase chain reaction-denaturing gradient gel electrophoresis* (*PCR-DGGE*) *also proved that the samples inoculated with Pb-resistant bacteria exhibited more bands and intensity in DGGE patterns compared with uninoculated ones. For Pb-resistant bacteria inoculated samples, the reduction of Pb concentration in rhizospheric soil was* 15 *mg/kg at least and* 42 *mg/kg at most, and Pseudomonas aeruginosa showed a better tolerance to high Pb concentration and stronger remediation ability. It was concluded that remediation of Pb polluted soil can be promoted by the two Pb-resistant bacteria.*

Keywords**:** *Pb Pollution, Pb-Resistant Bacteria, Microbial Community Diversity, PCR-DGGE, Enzyme Activity*

1. Introduction

Extensive mining and smelting have resulted in soil contamination which poses risk to human and ecological health. Over 20 000 000 acres of farmland in China have been contaminated by Sn, Cr, Pb and Zn and other heavy metals, accounting for almost one fifth of the total arable farmland [1]. Soil quality in some farmland near a mining site is getting worse and the content of heavy metal has already exceeded the third level of Environmental quality standard for soil in China (GB15618-1995) [2]. Crops harvested in these areas had high concentration heavy metals and their accumulation in living tissues throughout the food chain brought a further health problem.

Different from other organic pollutants, heavy metals are harder to be chemically or biologically degraded. Three methods are usually employed to remediate heavy metal contamination in the soil: excavation-physical removal of the contaminated material, stabilization-amendment of the metals in the soil on site, and phytoremediation-growing plants to uptake the metals from the soil [3]. However, the application of first two methods is sometimes restricted due to technological or economical constrains. Bioremediation is a very efficient method for cleaning up superficially contaminated soils [4]. It makes use of plants and their rhizospheric microbes to degrade or immobilize pollutants in soils [5]. Soil microbes play significant roles in the process of bioremediation [6]. They can absorb, transform, or degrade heavy metals, and they also can reduce the mobility and bioavailability of contaminants reviewed by Wu Gang [5]. Microbes in rhizospheric soil can promote plants to accumulate extra heavy metals [7]. Possible remediation by microbes is based on the concentration of heavy metal and property of microbe. *Bacillus sp.* has been identified as a possible candidate for metal sequestration and has been used in commercial biosorption preparation. Besides the biosorp-

tion of metals using *Pseudomonas sp.*, *Zoogloea ramigera* and *Streptomyces sp*., Ying Ma [8] and Mani Rajkumar [9] reported that the inoculation of plant-growth promoting bacteria can protect the plants against the inhibitory effects of nickel and improving the uptake of heavy metal meanwhile. *Geobacillus thermodenitrificans*, a thermophilic bacteria isolated from Damodar river, was proved by S. K. Chatterjee [10], a potential biosorption to heavy metals by dead biomass. The same conclusion was obtained by Claudio C.V.Cruz and Xiao-na Li *et al*. [11,12].

The efficiency of bioremediation was reflected by the increase of soil enzyme activity, the number of rhizospheric microorganism and diversity of microbial community. Soil enzyme activity indicating the potential ability of soil to support biochemical processes is a sensitive indicator of soil quality [13-15]. Several studies showed the effect of heavy metal pollution on soil enzyme activity, microbes community and heavy metal concentration and the changes of these indics by bioremediation. Khan Sardar *et al.* [16] showed that remediation slightly increased the enzymatic activities in all the samples polluted with heavy metals, and the community structure changed significantly in the Cd resistant bacteria inoculated samples. Urease and invertase were proved exhibiting more sensitivity to Pb pollution than other enzymes [17,18]. Zhaohui Guo [19] proved that the toxicity of Cu in heavily contaminated soils impacted on the quantities of specific microbial populations and had no significant change in the microbial diversity of highly contamination soils. Chiquan He [20] confirmed that Zn-tolerant bacteria isolated from heavy metal-contaminated sludge can increase mobility of Zn in soil and enhance accumulation of Zn by *O. violaceus*.

In this study, pot experiments were conducted to investigate the remediation of Pb-resistant bacteria to Pb polluted soil. Two Pb-resistant bacteria *Bacillus pumilus* and *Pseudomonas aeruginosa* (GeneBank accession No.FJ402988, and GU017676) were filtrated and identified by previous study [21] based on the 16S rDNA gene sequence analysis from soil of Pb mining district (Heilongjiang province, China) and inoculated into soil planted with cabbages and different levels of Pb. Objectives of this work were to 1) evaluate the capability of Pb-resistant bacteria on remediation of soil with different Pb concentration 2) characterize the variation of enzyme activity, microbe counts, microbial community and Pb concentration diversity under remediation.

2. Materials and Methods

2.1. Soil Preparation

Soils used in this study were obtained from experiment station of Northeast Agricultural University. Physicochemical properties of the soil were determined with routine methods recommended by SSSA [22] and listed in **Table 1**.

All pots (20 cm diameter) contained 2 kg soil with different concentration of Pb (0 200 400 600 800 1000 Pb mg kg^{-1} dry soil ($Pb(NO_3)_2$, 99% purity), which were denoted as treatments CK, Pb200, Pb400, Pb600, Pb800 and Pb1000, respectively. Treated soils were stored for a period of 2 weeks to establish equilibrium between the added Pb and soils.

2.2. Plant Culture

Seeds of cabbage were from college of horticultural science of Northeast Agricultural University. Ten seeds were sowed in each pot and thinned to five seedlings after emergence.

2.3. Bacteria Inoculation

Bacillus pumilus and *Pseudomonas aeruginosa* were cultivated in nutrient agar medium at 30℃ for 48 h. Cells were collected in the exponential phase by centrifugation at 8 000 rpm for 10 min. Bacteria were diluted with sterile water at a density of 10^8CFU·ml^{-1} and inoculated to the soil surface (50 ml·pot^{-1}) three times a week after seedling emergence. Non-inoculation pots were made as control. All pot experiments were conducted under greenhouse conditions with constant sterile water (content 60%) and temperature (20℃ - 30℃).

2.4. Sample Collection

Soils were sampled at 0, 12, 24, 36, 48 days after inoculation. Soil samples for microbial community analysis were stored at 4℃. Samples for enzyme activity and Pb concentration analysis were sieved, air-dried and ground into powder by agate mortar.

Table 1. Main physicochemical properties of initial soils.

Properties	Initial soil
pH [H_2O]	6.85 ± 0.15
Silt [%]	31.7 ± 0.36
Clay [%]	39.63 ± 0.53
Sand [%]	29.8 ± 0.22
Bulik density [g·cm^{-3}]	1.15 ± 0.27
SOM [g·kg^{-1}]	4.48 ± 0.64
Cultivable bacteria population [g^{-1} fresh soil]	(5.9 ± 0.42) × 10^8
Cultivable fungi population [g^{-1} fresh soil]	(6.0 ± 0.39) × 10^5
Cultivable actinomycetes population [g^{-1} fresh soil]	(2.1 ± 0.26) × 10^6

2.5. Total Microbial Amounts and Soil Enzyme Activity

2.5.1. Total Microbial Amounts

The amounts of cultivable microbe including bacteria, fungi and actinomyces were determined by plate counting method. One gram of each sample was weighed and added to 9 ml of filter-sterilized saline. Soil suspensions were diluted and plated on bacteria, fungi and actinomyces selective medium. The number of microbial colony was counted after 3 to 7 days incubation at 28℃.

2.5.2. Soil Enzyme Activity

Soil enzyme activity is the direct expression of the soil community to metabolic requirements and available nutrient, and it related to enzyme sources and substrate specificity [23].

Phosphatase activity was determined according to Songyin Guan [24]. Phenol was used as substrate and the intensity of red color of the filtrate was determined using a UV-Visspectrophotometer (Beijing Purkinje General Instrument Co., Ltd. TU-1810) at wavelength of 510 nm, and the results were expressed as mg P_2O_5 produced 100 g^{-1} dry soil in 2h. Urease activity was measured according to the indophenols blue colorimetric method described as Songyin Guan [24], and expressed as μg NH_4-H g^{-1} soil (dry weight basis) h^{-1} at 37℃. Catalase activity was measured by titration method [25] and expressed as ml (0.02 mol/L $KMnO_4$) $g^{-1}{\cdot}h^{-1}$. Anthrone colorimetry was used to determine soil invertase activity according to Songyin Guan's method [24], and expressed as mg glucose produced by 10 g dry soil in 72 h. All the enzyme assays were performed with the dry soil samples in triplicates. The substrate was added to blanks after the reaction stopped before filtration of the soil suspensions.

2.6. PCR-DGGE

For PCR amplification of 16S rDNA, total soil DNA was extracted with modified method described by Zhou *et al.* (1996) [26]. DNA extraction was further purified using DNA purification kit (TIANGEN DNA gel extraction kit) to remove the humic substance according to the instruction manual. The quality of soil DNA was assessed by 1.0% agarose gel electrophoresis stained with ethidium bromide. All DNA samples were stored at –80℃ until use.

The relationships between microbial community diversity and heavy metals were assessed by PCR-DGGE analysis. Bacterial primers F357GC: 5"-CGCCCGCCG CGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCCT ACGGGAGGCAGCAG-3" and R518: 5-"ATT ACCGC GGCTGCT GG-3" were used in this study [27]. This set of primers amplified a 236 base-pair DNA segment. The PCR reaction mixture consisted of 10 ng DNA template, 2 μL 10 mM primers, 5 μL 10 × buffer, 0.5 μL TaqDNA polymersase (Takara) in a total volume of 50 μL. PCR amplification (94℃ for 4min, 30 cycles of denaturation at 94℃ for 45 s; annealing at 65℃ for 45 s; extension at 72℃ for 30 s; and a final extension at 72℃ for 10 min) of the V3-region of 16S rDNA was performed in a Eppendorf Mastercycler (Eppendorf biotech company, Germany). 5 μL PCR products were analyzed by electrophoresis on 1.0% agarose gels stained with ethidium bromide. For DGGE analysis, 1-mm-thick polyacrylamide gels (8% acrylamide-bisacrylamide; Bio-Rad) were prepared and electrophoresed. The DGGE conditions were 30% - 60% urea gradient, 200 V for 15 min, followed by 150 V for 6 h. The gels were stained with SybrR Green I (1:5 000 in 0.5 TAE; FMC BioProducts, Rockland, ME, USA).

2.7. Determination of Pb Concentration

For the total metal analysis, 0.5 g of air dry soil was first treated with 5 ml HCl in a tefolon beaker with low-temperature heating. 5 ml HNO_3, 4 ml of HF and 2 ml of $HClO_4$ were added and heated with medium temperature when about 2 to 3 ml HCl was left. After about 1 h, the thick white smoke and the black organ material were removed, wash the Teflon beaker (include the lid) with distilled water to make up the volume to 50 ml. Data were obtained when the wash liquid was measured by atomic absorption spectrophotometer (Shimadzu atomic absorption spectrophotometer AA-6300C).

2.8. Statistical Analysis

Values of Pb concentration, enzyme activity and cultivable microbe amounts were expressed as means and compared statistically by Tukey's t-test at the 5% level with SPSS 13.0 (SPSS FOR Windows, Version 13.0, USA). Quantity One image analysis software 4.6.2 (Bio-Rad) was used to analyze band migration distance and intensity within each lane of PCR-DGGE fingerprinting. PCA analysis was conducted by SPSS 13.0 to determine the distribution of microorganisms and differences between different treatments. To analyze the effects of the different treatment on the bacterial communities, the Shannon index was used and calculated from DGGE band data as follows:

$$H = -\sum_{i=1}^{s} p_i \ln p_i ,$$

where S is the richness or total number of bands, p_i is the proportion of the total intensity accounted for by the ith band and ln is the natural logarithm.

3. Results and Discussion

3.1. Numbers of Cultivable Microbe

Soil microbes are the most important component of soil

ecosystem, they play a key role in material cycles and energy flow in soil. The microbial community construction and quantity often vary with the soil environment changes, such as soil contamination, flood and drought.

In this study, the numbers of soil bacteria, fungi and actinomyces changed significantly with different concentration of Pb treatment. From **Figure 1**, the bacteria counts were decreased 19.67% (from 5.64×10^8 to 4.53×10^8) when Pb concentration increased from 0 mg/kg to 600 mg/kg. When Pb concentration was increased from 600 mg/kg to 1000mg/kg, bacterial counts decreased 54.95% (from 4.53×10^8 to 1.43×10^8) and showed a significant difference from control ($p < 0.01$) by ANOVA analysis. During the incubation, total numbers of bacteria, fungi and actinomyces decreased from 5.64×10^8, 6.83×10^6 and 2.43×10^6 to 1.43×10^8, 5.17×10^6 and 1.75×10^6, respectively. The numbers of fungi and actinomyces changed less than that of bacterial but still significant ($p < 0.05$). The terminal counts of total microbes were significantly affected by high Pb concentration, which meant that high Pb concentration had a detrimental effect on microbial activity and function. After the inoculation of Pb-resistant bacteria, the counts of bacteria and fungi increased about 290% and 40% than that of control when Pb concentration increased to 200 mg/kg and the decreasing trend was weakened with the increase of Pb concentration. Actinomyces increased 28.4% and 40.7% when Pb concentration increased to 400 mg/kg and decreased to initial level when Pb concentration increased to 1000 mg/kg (showed in **Figure 1**).

Numbers of soil microbes were also altered with different incubation time at 1000mg/kg Pb concentration (Showed in **Figure 2**). The number of bacteria decreased in 24 days and increased faintly later, and it was 17% to 290% higher in Pb-resistant bacteria incubated soil than in control all the time. It is probably because the inhibition of growth by heavy metal toxicity at first, and recovery when bacteria adapt to the polluted environment. Fungi counts decreased sharply (12% and 45.3%) in first 24 days during the incubation, but it was decreased by 22.7% and 42.1% compared with control later. The actinomyces count was not affected significantly ($p > 0.05$) by inoculation of Pb-resistant bacteria, while a higher level of number could be found during the whole incubation.

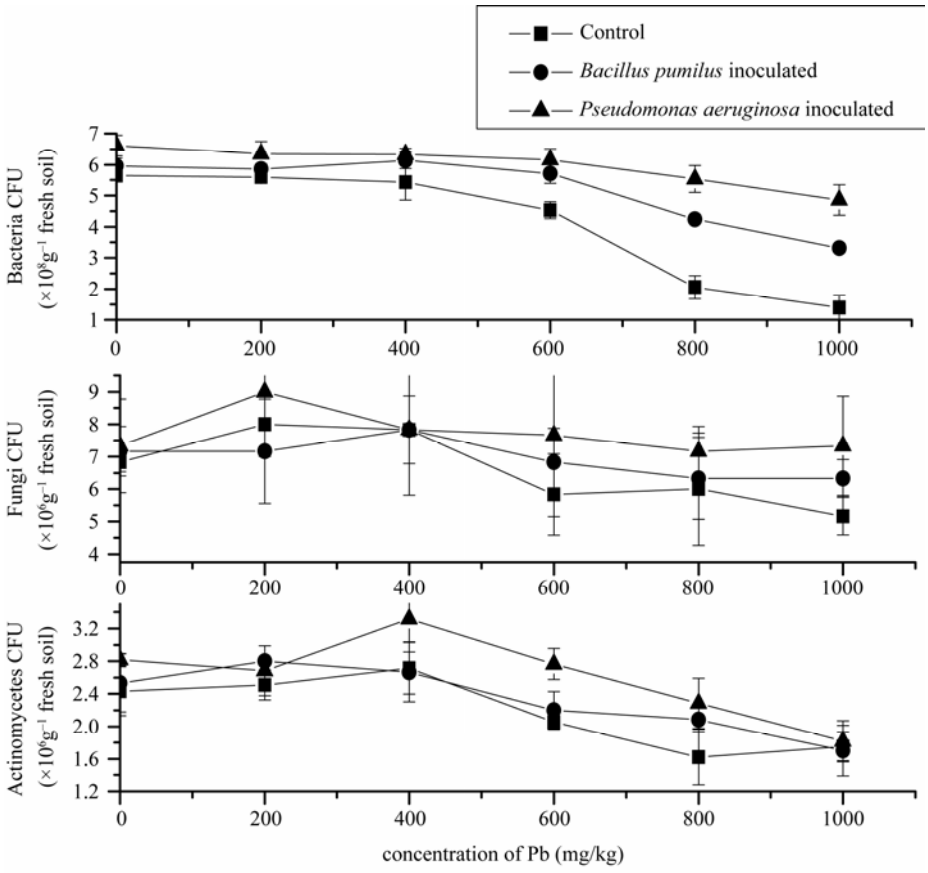

Figure 1. Numbers of bacteria, fungi and actinomycetes under different Pb concentrations.

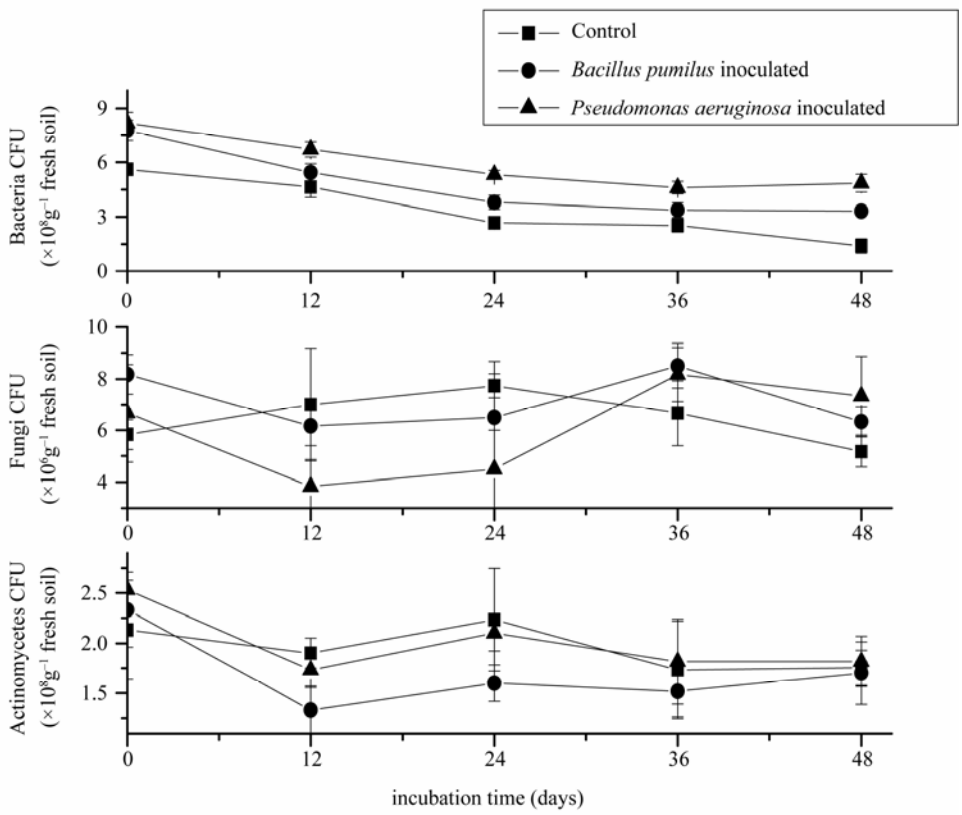

Figure 2. Numbers of bacteria, fungi and actinomycetes at different incubation time under 1000 mg Pb /kg fresh soil.

In this study, soil microbial numbers showed a decreasing trend resulted from increasing level of Pb, but it was weaken by inoculating Pb-resistant bacteria, which indicated two resistant bacteria can remediate the heavy metal contamination and recover the activity of microbe. Remediation by microbes may be effective on the recovery of bacterial activity at low concentration of Pb (showed in **Figure 1** and **Figure 2**). The inconsistent relationship between total microbes counts and different management practices may be the consequence of large-scale heterogeneity within samples taken across the experiment in the same test-site, due to an irregular distribution of microbial population sizes in the soil. However, this approximate trend still proved the effect by differently processed samples. Moreover, from the two graphs, the increasing range of bacteria was 1.89×10^8 and 3.43×10^8 respectively, much more than the initial inoculated number of bacteria (1×10^8). Compared with control, Pb-resistant bacteria can decrease microbe quantity in contaminated soil by releasing the toxic effect on microorganism through some unclear mechanisms.

3.2. Enzyme Activities

Soil environment has a significant impact on soil enzyme activities. In this study, urease activity increased rapidly over time, which illustrated that Pb pollution didn't have a detrimental effect on the activity of urease and inoculation of Pb-resistant bacteria can intensify its activity. Soil phosphatase activity decreased in first 24 days and increased to initial level after 36 days. The opposite trend could be found in catalase activity, which increased in the first 24 days and decreased in later 24 days. The effect of heavy metals on soil enzyme activities may be due to the sudden exposure to polluted environment in the first few days, thus resulted in a shortly decrease of enzyme activities. Later on, when microbes adapted to the polluted environment, the enzyme activity tended to recover. Soil invertase activity also exhibited the same pattern, the activity greatly decreased from 51.63 mg/5 g·24 h to 31.17 mg/5 g·24 h in first 24 days, and increased to 48.88 mg/5 g·24 h later.

As a whole, soil enzyme activities showed a significant difference ($p < 0.05$) between the inoculated treatment and control based on ANVOA analysis, which demonstrated that Pb-resistant bacterial inoculation in soil may raise soil urease and invertase activity. The very significance of urease activity was showed between inoculated and uninoculated samples ($p < 0.01$). The

maximum increase of soil urease activity was up to 204%, which is about 70% higher than that of control. Invertase activity also showed an increasing difference between inoculated and uninoculated soils, in **Table 2**, there was no difference in first 12 days, while a difference ($p < 0.05$) and a significant difference ($p < 0.01$) showed in 36 days and 48 days incubation respectively. Similar with urease activity, the average increasing rate of invertase activity was up to 119% by inoculating the Pb-resistant bacteria and decreasing rate was 95% in control samples. As to catalase activity, 9.6% and 7.4% increasing were detected in two different treatments compared with initial soil, while 3.3% and 5.1% decreasing were detected compared with control after 48 days incubation (**Table 2**). Phosphatase activity of all treatments were inhibited by Pb pollution in first 24 days, and recovered to initial level after 48 days incubation, so it was deduced phosphatase was not affected by inoculation during the incubation.

Soil enzymes play an essential role in catalyzing reactions necessary for organic matter decomposition, nutrient cycling, energy transfer, environmental quality and crop productivity [28]. Soil enzyme activities are greatly affected by organic matter content in the soil and often used as indices of soil fertility and soil pollution [29]. According to the studies conducted by Tyler (1974) [30] and Kízílkaya *et al.* (2004) [31], soil enzyme activities diminished with increasing concentrations of available heavy metals.

Urease activity rose immediately after Pb treatment, which has proved by many previous researches. Youn-Joo An *et al.* [32] proved the increase of urease acitivty in soil was up to 168% when antimony treatment was at 800 mg/kg. However, Caravaca *et al.* (2005) [33] reported that plant type mediated the urease activity and soil microbial community structure. In this study, the similar result with Youn-Joo An [32] was obtained and the urease activity of inoculated samples reached a higher level than uninoculated control reflected in **Table 2**, which proved a promoting function of Pb-resistant bacteria.

Invertase activity is another important index of Pb contamination in soil. Although some previous research has documented that there was no significant effect on the samples processed with different Pb concentration over time [34], but a significant effect was proved in this study. It may be affected by different types of soil, pH, incubation environment and so on [35-37].

Soil phosphatases are important in soil P cycling, involving in mineralization of organic P and releasing phosphate for plants [32,33]. In this study, it was not greatly affected by different treatment. Catalase is an intracellular enzyme involved in microbial oxidoreductase metabolism [38]. A slightly rise of catalase activity was measured in inoculated samples after 48 days incubation.

Table 2. Change of soil enzyme activities in different incubation time.

Enzyme activity	Sample processing [1 g/kg ($Pb(NO_3)_2$)]	Sample time [days]				
		0	12	24	36	48
Urease activity (mg NH_3-N produced/(g·24h) dry soil)	Control	9.77 ± 0.13	10.72 ± 0.19A	11.33 ± 0.49A	12.74 ± 0.45A	13.17 ± 0.62A
	Bacillus pumilus inoculated	9.77 ± 0.13	11.67 ± 0.25B	12.87 ± 0.23B	17.81 ± 0.46B	18.16 ± 0.34B
	Pseudomonsa inculated	9.77 ± 0.13	12.97 ± 0.18B	13.06 ± 0.31B	19.06 ± 0.22B	21.72 ± 1.45B
Phosphatase activity (mg P_2O_5 produced/(g·2h) dry soil)	Control	1.31 ± 0.07	1.04 ± 0.07a	1.10 ± 0.03a	1.15 ± 0.04a	1.23 ± 0.03a
	Bacillus pumilus inoculated	1.31 ± 0.07	1.12 ± 0.04a	1.16 ± 0.03a	1.27 ± 0.04b	1.29 ± 0.02ab
	Pseudomonsa inculated	1.31 ± 0.07	1.13 ± 0.03a	1.16 ± 0.04a	1.26 ± 0.04b	1.33 ± 0.04b
Invertase activity (mg reducing sugar produced/(5g·24h) dry soil)	Control	51.63 ± 0.21	40.24 ± 1.57a	31.17 ± 1.29Aa	45.17 ± 3.36a	48.88 ± 2.11A
	Bacillus pumilus inoculated	51.63 ± 0.21	42.78 ± 2.18a	33.67 ± 2.11ABb	51.44 ± 1.82b	61.44 ± 2.66B
	Pseudomonsa inculated	51.63 ± 0.21	42.33 ± 1.89a	37.33 ± 0.77Bb	52.45 ± 2.24b	61.17 ± 2.01B
Catalase activity (ml 0.1 N $KMnO_1$ consumed by 1 g dry soil in 20 min)	Control	1.87 ± 0.41	2.64 ± 0.07a	3.62 ± 0.12Aa	2.63 ± 0.08a	2.12 ± 0.06a
	Bacillus pumilus inoculated	1.87 ± 0.41	2.48 ± 0.46a	3.07 ± 0.10Bb	2.31 ± 0.18b	2.05 ± 0.06a
	Pseudomonsa inculated	1.87 ± 0.41	2.67 ± 0.20a	3.00 ± 0.16Bb	2.6 ± 0.10a	2.01 ± 0.12a

Data in the table are Mean ± SE, n = 3; lowercase letters represent the significant difference at $p < 0.05$, capital letters represent the significant difference at $p < 0.01$.

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3.3. Pb Concentration

The decrease of Pb concentration in rhizospheric soil is an important indicator of remediation by the Pb-resistant bacteria.

From **Figure 3**, the remediation to Pb contaminated soils promoted by Pb-resistant bacteria varied with different Pb concentration treatment. On one hand, the Pb concentration in rhizospheric soil inoculated with

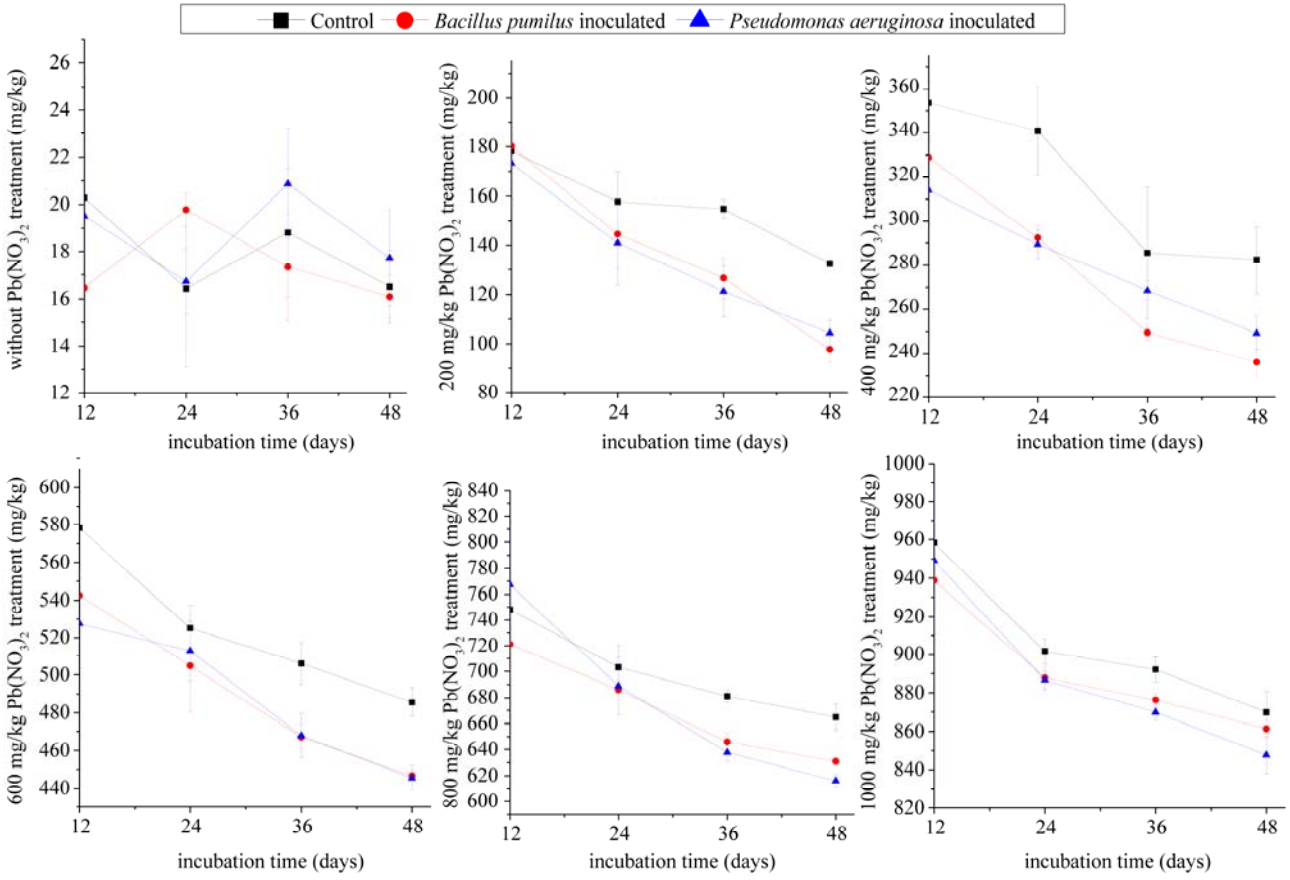

Figure 3. Pb concentration at different incubation time.

Pb-resistant bacteria was significantly lower than those in uninoculation ones, and with the increasing of heavy metal concentration, the curves of different incubation time with the same Pb treatment got closer over time, which meant the remediation of Pb-resistant bacteria was inhibited by high heavy metal concentration. The terminal Pb concentration differences were 34.8, 46.2, 39, 34.1, 8.6 mg/kg and 28.1, 33.13, 40.1, 48.9, 22.1 mg/kg between control and the two Pb-resistant bacteria (*Bacillus pumilus* and *Pseudomonas aeruginosa* respectively) inoculated samples after 48 days incubation when Pb treatments were 200 mg/kg, 400 mg/kg, 600 mg/kg, 800 mg/kg and 1000 mg/kg. From the above data, on one hand, remediation of inoculated samples was inhibited at a Pb concentration of 1000 mg/kg, which only 8.6 mg/kg and 22.13 mg/kg Pb was decreased. On the other hand, *Bacillus pumilus* exhibited more power of remediation when Pb concentration was less than 800 mg/kg, while *Pseudomonas aeruginosa* performed well when Pb concentration was less than 1000 mg/kg. The best Pb concentration for soil remediation by *Bacillus pumilus* was 400 mg/kg at which about 46.2 mg/kg Pb was decreased, while 48.9 mg/kg was decreased at Pb concentration of 800 mg/kg by *Pseudomonas aeruginosa*.

Both the Pb-resistant bacterial strains showed a remediation to Pb-contamination soil. According to Weibin Lu *et al.* (2006) [39], it may be resulted from a reversible process of adsorption and desorption. But only part heavy metal can be removed from soil by this process as earlier studies. The most feasible remediation is to combine microremediation with phytoremediation, as reviewed by Gang Wu [5], which can get over the disadvantages of microremediation. In this study, it was inferred that these two strains may have the ability to enhance remediation through promoting adsorption of heavy metal by plants and heavy metal resistant bacteria, same with Jiang's (2008) result [7]. We also found that Pb concentration of the uninoculated samples showed a decreasing trend, it may be resulted from the loss of pretreatment and sampling.

3.4. Analysis of DGGE Patterns

From the result of plate counting, we deduced that bacteria was affected mostly by Pb pollution, so PCR-DGGE analysis of bacterial community structure was conducted based on bacterial 16S rDNA gene sequence in the following study. Individual banding patterns from different treatment were showed in **Figure 4** and **Figure 5**.

An obvious regular pattern could be found in **Figure 4**, The DGGE profiles displayed a Pb concentration ladder from low to high (CK (control, 0 mg/kg), L(low concentration, 400 mg/kg) and H (high concentration, 1 000 mg/kg)) and four sampling times of each concentration. The numbers of bands decreased with Pb concentration increasing, and the intensity of bands faded obviously with the increase of Pb concentration over time. Only 5 bands in lane 12 were detected while more than 10 bands were detected in lane 4 and 8 by other treatments when Pb concentration is 1000 mg/kg, which proved that high concentration could impact on microbial community diversity and Pb was the main factor influencing bacteria diversity by changing species composition and richness. The PCR-DGGE patterns provided the evidence that DGGE patterns varied with different levels of Pb contamination and high dose of Pb caused a greater change in soil bacterial diversity. Similar as this study, Khan Sardar (2007) [18] proved that high concentration of heavy metal can decline soil community structure and quantity.

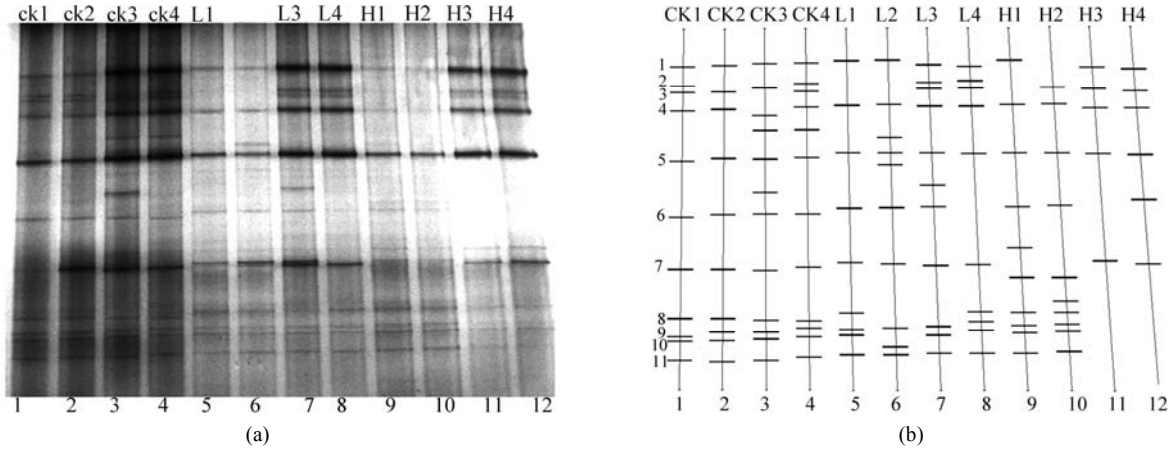

Figure 4. DGGE profiles of V3 region of the 16S rDNA gene amplified by PCR from soil DNA in different incubation time (ck: 0 mg/kg, L: 400 mg/kg, H: 1000 mg/kg; lane 1, 2, 3, 4 means incubation time as 12, 24, 36, 48 days respectively); A: DGGE patterns; B: Comparison of DGGE patterns using Quantity One 4.6.2 software.

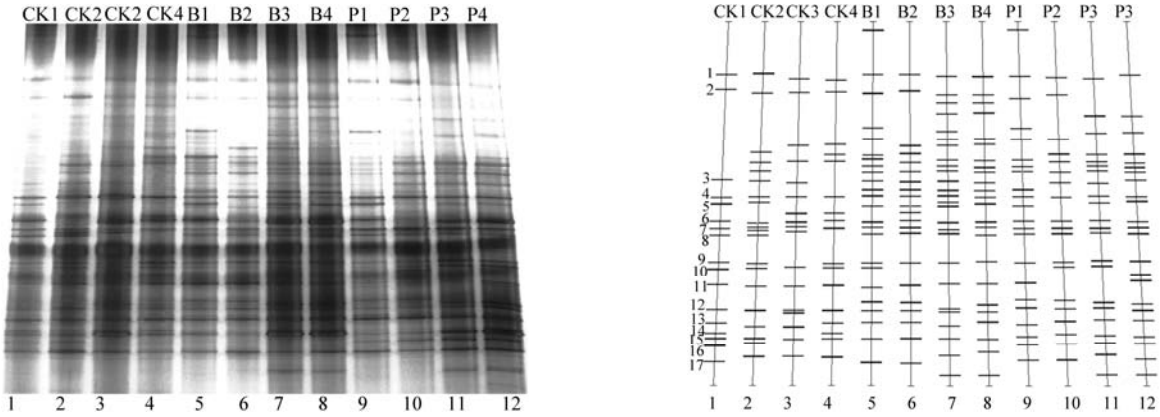

Figure 5. DGGE profiles of V3 region of the 16S rDNA gene amplified by PCR from DNA extracts with different treatments. CK: Control; B: *Bacillus pumilus* inoculated; P: *Pseudomonas aeruginosa* inoculated; 1, 2, 3, 4: incubation time as 12, 24, 36, 48 days respectively; A: DGGE patterns; B: Comparison of the DGGE patterns using Quantity One 4.6.2 software.

DGGE profiles were analyzed by Quantity One software (**Figure 5**) and Shannon-Wiener index was calculated to measure the size and importance of bacterial community (**Table 3**). Shannon-Wiener index showed the diversity of Pb-resistant bacteria treated samples were more abundant than control, which proved that inoculation of Pb-resistant bacteria could raise the diversity of soil bacteria. Similar conclusion can be obtained from DGGE profiles (**Figure 5**), although the PCR-DGGE patterns showed numerous bands were common to all treatments, there were also changes in band presence and the band number of Pb-resistant bacteria treated samples were about 5 to 10 bands more than control. So inoculation of Pb-resistant bacteria can complicate soil bacteria community structure, especially after 24 days incubation.

For a further conclusion of the relation among different treatments, cluster analysis was conducted by Quantity One software with UPGMA method (**Figure 6**). The dendrogram showed that at each sampling time molecular patterns of Pb-resistant bacteria inoculated soils could be well discriminated from patterns of control soils. It was obvious that two clusters were divided, one cluster represented control including sample 2, 3 and 4, and the other represented Pb-resistant bacteria treated samples including two subsets B and C (**Figure 6**). In one word, all treatments were well divided by cluster method, and the Pb-resistant inoculated samples showed the least similarity to control (60%), which confirmed that the addition of Pb-resistant bacteria had a negative impact on the microbial community structure of heavy metals contaminated soil. Otherwise, the first lane was shown much similar with subsets B, it was concluded that the bacteria community structure in Pb-resistant bacteria inoculated soils had a better recovery to initial soil.

Table 3. Shannon's diversity index (*H*) of different treatments based on PCR-DGGE analysis.

Treatment	Shannon's diversity Index (*H*)
CK	1.3744 ± 0.1409a
Bacillus pumilus	1.6654 ± 0.1177b
Pseudomonas aeruginosa	1.6381 ± 0.1371b

Data in the table are Mean ± SE, n = 3; different letters represent the significant difference at $p < 0.05$.

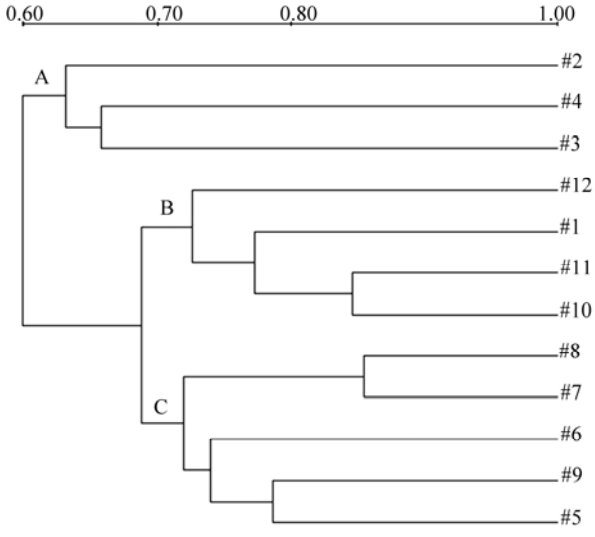

Figure 6. Cluster analysis (UPGMA, Dice coefficient of similarity) generated by PCR-DGGE profile.

Principal component analysis for the DGGE patterns showed that the first, second and third principal components explained 54.48%, 12.88% and 9.47% of the variance respectively, and the three principal components explained 76.83% of the total variance. Since the third component only explained less than 10% variance and it was hard to investigate by the 3D load diagram, the first two components were extracted and formed a new load diagram (**Figure 7**). It can be seen clearly that the significant difference between the control and treatment samples. The control group (CK2, CK3, CK4) was distributed on the positive part of the first principal component (PCA2), while other treatments were distributed on the negative part of PCA2, and the treatments with *Bacillus pumilus* and *Pseudomonas aeruginosa* were clustered together. Effect of the Pb-resistant bacteria inoculated samples on bacterial community structure showed in DGGE profiles (**Figure 5**, B1-B4, P1-P4) appeared similar patterns to each other, which proved that these two bacteria had a positive impact on microbe community structure in Pb contaminated soil. From the cluster analysis and principle component analysis, the same conclusion was drawn.

4. Conclusions

In this study, we illustrated remediation of two Pb-resistant bacteria, *Bacillus pumilus* and *Pseudomonas aeruginosa,* through four aspects such as culturable microbes, soil enzyme activity, heavy metal concentration and microbial community diversity in Pb polluted soil. Results indicated the quantity of culturable bacteria, fungi, actinomyces from the soil samples that were decreased from 1% - 93%, 12% - 25% and 16% - 33% respectively with the increasing of metal concentration. The count of three microbes after inoculation were 5% - 137%, 6% - 23% and 12% - 28% increasing with *Bacillus pumilus* inoculated, 14% - 246%, 13% - 44% and 7% - 35% increasing with *Pseudomonas aeruginosa* inoculated. Soil enzymes like urease, invertase and catalase were intensified by 37.9%, 65.6% and 9.6% after inoculating Pb-resistant bacteria compared with control. And phosphatase activity showed no difference with initial soil and higher than uninoculated soils. For Pb-resistant bacteria inoculated samples, the reduction of Pb concentration in rhizospheric soil was 15 mg/kg at least and 42 mg/kg at most, and *Pseudomonas aeruginosa* showed a better tolerance to high Pb concentration and stronger remediation ability. DGGE patterns showed that inoculation of Pb-resistant bacteria can intensify soil bacteria community diversity. In conclusion, both Pb-resistant bacteria showed a remediation to Pb polluted soil.

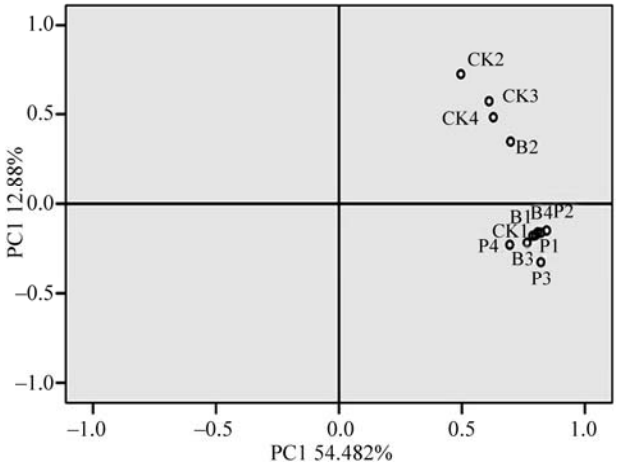

Figure 7. Principal Component Analysis of DGGE patterns data with different treatments.

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