Genetic diversity of bacteria in bioremediation system of iron mine tailing by *Robinia pseudoacacia - Ryegrass - Trifolium repens*

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Abstract. To exploration the change of microorganism in iron tailing sand restoration, a bioremediation system of iron tailing sand by combination of *Robinia pseudoacacia - Ryegrass - Trifolium repens* pus soil or bio-matrix flowerpot was designed, the change of bacterial quantity and genetic diversity at different time was determined. The tailings and soil samples C1 (tailings), C2 (tailings adjacent to biologic pot) and C3 (*Robinia pseudoacacia* rhizosphere soil), were collected on the 180th d, 270th d and the 360th d. The bacterial quantity was determined, the bacterial strains were isolated and purified, and genetic diversity was studied by BOXA1R-PCR and 16S rDNA sequencing. The results showed that along the restoration time increasing, the number of bacteria in C2 and C3 increased from 4.81×10⁵ cfuꞏg⁻¹ and 11.82×10⁵ cfuꞏg⁻¹ to 5.99×10⁵ cfuꞏg⁻¹ and 15.67×10⁵ cfuꞏg⁻¹ at 270th d, then to 8.96×10⁵ cfuꞏg⁻¹ and 21.96×10⁵ cfuꞏg⁻¹ at 360th d, respectively, while there was little change in C1. From these samples, a total of 32, 46 and 52 bacterial strains were isolated, and the genetic diversity was determined by BOXA1R-PCR, the results showed that there were 7, 9 and 10 BOXA1R groups formed, respectively; phylogenetic analysis of representatives suggested that genus *Bacillus* were the predominant, and bacteria with biological nitrogen fixation ability, distributed in *Rhizobium, Ensifer, Azotobacter*, etc. were founded during the iron tailing sand bioremediation. The results suggests that bio-matrix flowerpot combining *Robinia pseudoacacia - Ryegrass - Trifolium repens* cultivar can increase the bacterial quantity and genetic diversity in iron tailing sand significantly, and probably has a good application potential for tailings restoration.

1. Introduction

Tailings are particulate solid powder left after the ore has been crushed and flotations concentrate. Tailings are usually discharged into low-lying areas or piled up by mountain dams to form tailings ponds [1]. In 2016, the total tailings pile stock has reached 14.6 billion tons in China, of which, about 7.5 billion tons are iron tailings, and the annual growth amount is 500 million tons [2, 3]. At the same time, about 900,000 m² was covered per year due to tailings stacking [4, 5]. Tailings sand has small particle size and poor structure. With the increase of dam height, tailings pond is prone to dam break and other major natural disasters [6-7]. Therefore, it is of great significance to carry out tailings restoration.
The existing tailings pond treatment methods mainly include tailings re-dressing, used as construction materials, glass raw materials, making fertilizer, filling mine goafs and using physical methods for in-situ stabilization. There are many problems in these methods, such as high investment cost, complex manufacturing process, immature technology, and results in secondary pollution and so on \cite{8-10}. Therefore, the environment friendly processing technology is urgently needed. Several studies suggested that phytoremediation can significantly improve the physical and chemical properties of tailings soil, and has the advantages of low cost, good effect, less field disturbance and easy integration with other restoration technologies \cite{11-12}. Li et al. reported that *Imperata cylindrica* was associated with the sulfur-oxidizing bacterial abundance, and vegetation growing on the tailings effectively prevented the oxidization of sulfide minerals \cite{13}. Xie et al. demonstrated that the *M. amophae/A. tumefaciens* – *R. pseudoacaciac*, *M. lupulina* – *S. meliloti* symbiosis provides a potential tool for soil fertility restoration in the metal tailing area by growing plants \cite{14}.  

In this study, we designed a combining phytoremediation system of *Ryegrass, Trifolium repens* and *Robinia pseudoacacia* plus soil or bio-matrix flowerpot for restoration of iron tailings sand because of its low organic matter, nitrogen, and other nutrients. We presumed that this combination system can provide nutrients for plant growth, and then affect the soil microbial quantity and community. Hence, the bacterial quantity was determined, and bacteria strains were isolated and their genetic diversity at different time was explored.

2. Materials and methods

2.1. Materials description

Iron tailing sand was provided by Chengdu Leejun Industrial Co., Ltd. The organic matter is 3.45 g·kg\(^{-1}\), pH is 7.90, total nitrogen, total phosphorus and total potassium is 0.12 g·kg\(^{-1}\), 8.92 g·kg\(^{-1}\) and 7.96 g·kg\(^{-1}\); Alkaline nitrogen, available phosphorus and available potassium is 16.64 mg·kg\(^{-1}\), 1.78 mg·kg\(^{-1}\) and 9.13 mg·kg\(^{-1}\), respectively. The contents of available iron, available zinc and exchangeable magnesium were 0.19 mg·kg\(^{-1}\), 0.014 mg·kg\(^{-1}\) and 47.20 mg·kg\(^{-1}\), respectively.

Plants cultivars: *Trifolium repens*, *Lolium perenne* seeds and *Robinia pseudoacacia* seedlings were bought from market. The seeds were disinfected with 10% \(\mathrm{H}_2\mathrm{O}_2\) for 10 minutes, washed with distilled water 5 times, placed in a dish on the sterilized wet filter paper, and incubated 2 days at 25 °C for germination. The *Robinia pseudoacacia* seedlings with a height of 30 cm were chosen for planting.

Bio-matrix flowerpot was made by corn cob with size of 30 \(\times\) 25 \(\times\) 25 cm, and 2 cm thickness.

2.2. Experimental design and sample collection

Pot experiment was carried out in a plastic bucket with a size of 100 \(\times\) 50 \(\times\) 50 cm, and about 540 kg iron tailing sand was put inside, then the bio-matrix flowerpot containing 6.5 kg of soil was put in the middle, and a *Robinia pseudoacacia* seedling was planted into soil in each bio-matrix flowerpot; finally, a 2 cm thick soil was covered on the surface of tailing sand, and sowed 0.5 g perennial *Lolium perenne* and 0.3 g *Trifolium repens* seeds (Figure 1).

The humidity of tailing and soil was maintained at 20% - 22%. Samples were collected at 180\(^{th}\) d, 270\(^{th}\) d and the 360\(^{th}\) d, respectively. When sampling, the grass cortex was uncovered, then the soil and tailings samples were taken and named as C1 (tailings sample), C2 (the tailings closely adhering to the bio-matrix flowerpot), and C3 (*Robinia pseudoacacia* rhizospheric soil).
2.3. Research method

2.3.1. Determination of bacterial quantity and isolation and purification. Bacteria amount was detected by using dilution plate method. 10 g of the sample was weighed and placed into a conical flask containing 90 mL sterile water, then mixed thoroughly, the suspension in 10\(^{-1}\) to 10\(^{-7}\) was prepared. The dilution series of 10\(^{-5}\), 10\(^{-6}\) and 10\(^{-7}\) was used for check the bacterial number with 3 repeats. The petri dishes with beef extract-peptone medium were incubate at 25 °C for 2 days, and the bacterial amount was counted the bacterial strains were picked and purified by stroke method according to the colony aspects, and purity of bacteria were determined by gram staining and microscopy. The purified bacterial strains were obtained and stored at 4 °C[15].

2.3.2. Analysis of bacterial genetic diversity. Bacterial DNA was extracted by GUTC method [16], and BOXA1R-PCR was done by using BOX primer (5’-CTA CGG CAA GGC GCT GAC G-3’). The reaction system was: template DNA 1 μL, BOXA1R primer 0.2 μL, 2 × PCR TaqMix 5 μL, and addition of ddH\(_2\)O to 10 μL. PCR products were detected in 2% agarose gel by 80 V for 1 h, and stained with EB [17].

Representative bacteria were obtained based on BOXA1R PCR analysis, and 16S rDNA fragments were amplified by using primers 27f (5’-AGA GTT TGA TCCTGG CTC A-3’) and 1492r (5’-GTT TAC CTT GTT ACG ACT T-3’) [18]. The products were checked, and then sequenced by Beijing Qingke zixi biotechnology co., LTD.

2.4. Statistical analysis
The data were analyzed by Microsoft Excel 2010, and the significant difference was analyzed by one-way ANOVA and Duncan. BOXA1R PCR patterns were analyzed by NTSYSpc2.1 software with average linkage method (UPGMA). The 16S rRNA sequence of the representatives was submitted to NCBI and accession number. The phylogenetic tree was constructed by MEGA7.0.

3. Results

3.1. Changes of bacteria number
The amount analysis (Figure 2) indicated that the bacterial quantity in C3 was the highest, and was 11.82 \times 10^5 \text{cfu} \cdot \text{g}^{-1}, 15.67 \times 10^5 \text{cfu} \cdot \text{g}^{-1} and 21.96 \times 10^5 \text{cfu} \cdot \text{g}^{-1} at 180\(^{th}\) d, 270\(^{th}\) d and the 360\(^{th}\) d, respectively; While was the minimal in C1, and number was 2.02 \times 10^5 \text{cfu} \cdot \text{g}^{-1}, 2.08 \times 10^5 \text{cfu} \cdot \text{g}^{-1} and 2.10 \times 10^5 \text{cfu} \cdot \text{g}^{-1}, respectively. Interestingly, the amount of bacteria in C2 increased with the extension.
of restoration time; and bacterial quantity at 360<sup>th</sup> d increased by 86.28% and 49.58%, compared with that of 180<sup>th</sup> d and 270<sup>th</sup> d, respectively.

![Figure 2. Bacteria amount at different time.](image)

**Table 1. The aspects of tested bacteria strains.**

| Strain number | Gram staining | Shape | Spore | Colony aspects | Isolate time |
|---------------|---------------|-------|-------|----------------|--------------|
| WTB12, WTB13, WTB14, WTB22, WTB23, WTB25, WTB36, WTB37, WTB38, WTB41, WTB5, WTB16, WTB18, WTB27, WTB28, WTB39, WTB40 | G<sup>+</sup> rod + circular, white or milky | 180 d |
| WTB19 | G<sup>+</sup> rod - circular, white or milky | |
| WTB2, WTB6, WTB10, WTB15, WTB21, WTB24, WTB30, WTB31, WTB32, WTB33, WTB35 | G<sup>+</sup> rod + circular, white or milky | 270 d |
| WTB65, WTB82, WTB85, WTB105, WTB107, WTB108, WTB109, WTB110, WTB111, WTB113, WTB114 | G<sup>+</sup> rod + circular, white or milky | |
| WTB79, WTB87, WTB94, WTB100, WTB101, WTB103, WTB106 | G<sup>+</sup> rod + circular, orange | |
| WTB52, WTB59, WTB60, WTB63, WTB83, WTB104 | G<sup>+</sup> rod + circular, white or milky | |
| WTB61, WTB70, WTB73, WTB80, WTB95 | G<sup>+</sup> rod + circular, white or milky | 360d |
| WTB116, WTB119, WTB122, WTB127, WTB129, WTB137, WTB143, WTB149, WTB153, WTB156, WTB158, WTB160, WTB171, WTB172, WTB177, WTB178, WTB180, WTB184, WTB186, WTB187 | G<sup>+</sup> rod + circular, white or milky | |
| WTB163, WTB181 | G<sup>+</sup> rod + circular, white or milky | |
| WTB147, WTB166, WTB170, WTB174 | G<sup>+</sup> rod - circular, white or milky | |
| WTB117, WTB118, WTB125, WTB126, WTB133, WTB135, WTB139, WTB140, WTB141, WTB142, WTB144, WTB145, WTB146, WTB148, WTB173, WTB154, WTB155, WTB157, WTB167, WTB168, WTB169, WTB176 | G<sup>+</sup> rod - circular, white or milky | |
| WTB130, WTB138, WTB151, WTB162 | G<sup>+</sup> rod - circular, white or milky | |
3.2. Purification and morphology of the tested bacteria

Using streak plate method, a total of 32, 46 and 52 bacterial strains were isolated and purified from the samples collected at 180\textsuperscript{th}, 270\textsuperscript{th} and 360\textsuperscript{th} d according to colony characteristics, respectively. The colony morphology, gram staining and microscopic observation were performed (Table 1).

From the sample at 180\textsuperscript{th} d, a total of 32 bacteria were isolated. Of them, 18, 11 isolates were from C3, C2, and the occupied rates were 53\%, 34\%, respectively; only 4 strains were from C1 (Figure 3a).

From the sample at 270\textsuperscript{th} d, there were 46 bacteria strains isolated, including 35 gram-positive and 11 gram-negative; and 15 strains were from C2, and still only 4 strains were from C1 (Figure 3b). A total of 52 strains were isolated from the sample at 360\textsuperscript{th} d, including 26 gram-positive and gram-negative strains both (Figure 3c). There were 30 and 16 bacteria in C3 and C2. Though the ratio of bacteria between each sample was not significantly changed, the number in C2 and C3 increased, and the amount in C3 was still the highest.

Figure 3 shows the ratio of bacteria occupied.

![Figure 3. The ratio of bacteria isolated from each sample at different time. 3a: sample of 180\textsuperscript{th} d, 3b: sample of 270\textsuperscript{th} d, 3c: sample of 360\textsuperscript{th} d.](image)

3.3. Genetic diversity of the tested bacteria

3.3.1. BOXA1R - PCR analysis. Clustering analysis of BOXA1R-PCR showed that there was significant genetic diversity among the tested strains. 32 strains from the 180\textsuperscript{th} d sample were clustered together at 54\% similarity, and were further divided into 7 genetic groups at 72.6\% (Figure 4a). Group II and group VI were the largest and both consisted of 9 strains; followed by group I containing 7 strains. The 46 bacteria from the 270\textsuperscript{th} day’s samples clustered together at 64\% similarity, and were divided into 9 groups at level of 76.6\% similarity. Group I was the largest one, consists of 14 bacteria, followed by group IX containing 8 bacteria (Figure 4b). In the 360\textsuperscript{th} day’s samples, all the 52 bacteria clustered together at 64\% similarity, and were divided into 10 genetic groups at 75.86\% boundary. Group IV was the biggest one containing 22 strains, followed by group I and group VI, both consist of 7 strains. Group III, V, IX and X were formed by single strain (Figure 4c).

Figure 4 shows the dendrogram.
3.3.2. Represents strain phylogenetic analysis. Based on the BOXA1R-PCR cluster analysis, 15, 20 and 22 representatives were selected from the samples at 180th d, 270th d and 360th d, and 16S rDNA fragments were amplified and sequenced and submitted to NCBI, the accession numbers were MK240431-MK240449, MK241850-MK241872 and MK734308-MK734336, respectively. Reference strains were blasted from GenBank, and the phylogenetic tree was constructed by MEGA7.0. The results showed that the tested bacteria distributed in 17 phylogenetic branches, and were very abundant in population (Figure 5). The predominant one was genus Bacillus containing 17 strains, and further formed by 8 sub-branches. The next one was genus Enterobacter containing 18 bacteria and occupied 12.28%. There were 6 bacteria belong to genus Rhizobium and Pseudomonas, accounted for as 10.53%.

Simultaneously, many bacteria with biological nitrogen fixation were appeared. Including *Ensifer* sp. WTB2, WTB30, WTB142 and WTB144; *Rhizobium* sp. WTB3, WTB29, WTB59, WTB70, WTB157, WTB167 and WTB176; *Phyllobacterium* sp. WTB32 and *Azotobacter* sp. WTB24, WTB155. In addition, Enterobacteria were isolated only in C1 and C3, but not found in C2. Bacteria such as *Microbacterium* sp., *Rheinheimera* sp., and *Acinetobacter* sp. were only found in C2 and C3, not in C1. Bacteria such as *Aeromonas* sp., *Olivibacter* sp., and *Chryseobacterium* sp. were only found in C2, not in C1 and C3 samples. The results showed that phytoremediation had a great effect on the microbial community diversity in tailings and soil environment.

Figure 5 shows the phylogenetic tree of the tested strains.
Figure 5. Phylogenetic tree of the tested strains based on 16S rRNA gene sequences.
4. Discussion

Soil microbial diversity and life activities are strongly affected by soil physical and chemical properties including soil nutrition, pH, water porosity, particle size and vegetation secretion. There is a positive correlation between vegetation and microbial community diversity in natural soil [19]. Vegetation affects microbial communities through roots and also by influencing soil physical and chemical properties. Study by Cao et al. showed that the structure of fungal community was mainly affected by soil factors, while the number and diversity of non-rhizosphere and rhizosphere microorganisms were greatly affected by total nitrogen, carbon and pH [20]. In turn, microorganisms play a certain role in the survival and growth of plants, which can significantly increase the biomass, growth rate and resistance [21].

In this paper, the number of cultivatable bacteria in the samples treated with phytoremediation was significantly higher than that of the control sample. In C1, the bacteria number at three periods was 2.02×10⁵ cfuꞏg⁻¹, 2.08×10⁵ cfuꞏg⁻¹ and 2.10×10⁵ cfuꞏg⁻¹, and there was no significant difference. While in C2 and C3, bacteria number, 4.81×10⁵ cfuꞏg⁻¹, 11.82×10⁵ cfuꞏg⁻¹ at 180th d, and 5.99×10⁵ cfuꞏg⁻¹, 15.67×10⁵ cfuꞏg⁻¹ at 270th d, 8.96×10⁵ cfuꞏg⁻¹, 21.96×10⁵ cfuꞏg⁻¹ at 360th d, was significantly increased. Apparently, after planting Robinia pseudoacacia, ryegrass and clover, the root exudates improved the micro-ecological environment of tailings, which promoted bacterial growth [22]. Yan [23] studied the mechanism of soil microorganisms participating in the natural settlement of vegetation, the results showed that there were significant differences in the number of microorganisms in the soil of different natural settlement vegetation and bacteria were the main microorganisms in the soil.

The structure and function of microbial community can be used as a quantitative index to evaluate the process of recovery and development in tailings remediation [24]. In this study, the bacterial richness and diversity of samples in the three periods were significantly different, suggested that bacteria diversity increased with time, and the number of strains isolated from C2 and C3 increased (Figure 3). The same as BOXA1R-PCR analysis, there were 7 and 9 genetic groups in the 180th d and 270th d samples respectively, while 10 genetic groups were obtained in the 360th d samples, which showed that the genetic diversity of bacteria increased with the extension of remediation time. Similar results of bacteria communities was got by Illumina sequencing technique (data not showed).

The phylogeny analysis showed that the bacteria at different time were distributed in 17 phylogenetic branches, which revealed the bacterial community diversity. The dominant bacterial flora was Pseudomonas sp., Bacillus sp., and Rhizobium sp. at 180th d; though the dominant was still Bacillus sp. at 270th d, the diversity of Rhizobium sp. increased. Meanwhile, Rhizobium sp., Phyllobacterium sp., Ensifer sp. and Azotobacter sp. included 9 bacterial species and 14 strains with nitrogen fixation ability increased, among which, 6 strains were isolated from C2 and 6 strains were isolated from 360th d samples. The results showed that the iron tailing environment was improved with the recovery time, suggesting that the environment was becoming more hospitable to bacterial growth. Strains in Aeromonas sp., Olivibacter sp. and Chryseobacterium sp., include WTB5, WTB148 and WTB514, were isolated in C2 only, but not in C3, which suggested that these strains may be more adaptable for the restoration environment.

Due to the nutrient deficiency of tailings, leguminous plants were planted as pioneers to improve the nitrogen nutrition of tailings. The results showed that strain WTB2, WTB24, WTB29, WTB30 and WTB32, belong to Rhizobium, Phyllobacterium, Azotobacter and Ensifer in classification, presented in the 180th d sample, they could form symbiosis with legumes and increase environmental nitrogen content. In the 270th day’s sample, the number of Rhizobium sp. bacteria (WTB59 and WTB70) increased, but the bacteria of Phyllobacterium sp., Ensifer sp. and Azotobacter sp. could not be isolated. This indicated that the bacterial population changed with the environmental condition. At 360th d, the number of Rhizobium and Azotobacter was further increased, including WTB157, WTB176, WTB167, WTB142, WTB144 and WTB155. The results suggested that the legume-rhizobia symbiotic nitrogen fixation system is an important resource for vegetation restoration of iron tailings, and can play a key role in increasing soil nitrogen supply, organic matter content and soil enzyme activity [25-27]. Xie et al. have been sequenced the genomes of a large number of rhizobia from
different species, the *Mesorhizobium amorphae* CCNWGS0123 (from *Robinia pseudoacacia*) provides the unique possibility for species comparison of metal resistance determinants among species and showed a good biological nitrogen fixation ability. Rhizobia, as a subset of PGPB, thus play a key role in the phytoremediation by legume plants [28].

In addition, strains from the genus *Bacillus* have been shown to enhance the growth of agricultural crops, wild plants, trees, microalgae, and model plants, through different mechanisms of plant growth-promotion [29]. The research of Govarthanan et al. suggested that the soil enzyme activity was increased significantly in bioaugmented mine soil, and the observations indicate the potential role of indigenous *Bacillus* sp. KK1 for the bioremediation of mine tailings [30]. *Bacillus* has the characteristics of strong resistance, fast reproduction speed and so on. It is easy to colonize in plant rhizosphere and occupy a dominant position in extreme environment. In this study, the similarity between WTB22 in C3 and *Bacillus altitudinis* 41KF2bT was 99.86%, and WTB23 in C2 sample and *Bacillus tequilensis* KCTC13622T was 99.52%. *Bacillus altitudinis* has been proved to produce xylanase with straw as substrate [31], and *B. tequilensis* can produce hemicellulase that decomposes hemicellulose and xylose [32]. In this study, strains WTB53 and WTB89, close to *Bacillus altitudinis* and *B. tequilensis*, were isolated from C2 at 270th d, maybe because the biological flowerpot made by straw was added in the experiment.

5. Conclusions
Using combination of *Robinia pseudoacacia*, *Ryegrass*, *Trifolium repens* plus soil or bio-matrix flowerpot to repair iron tailings, the number of bacteria and population diversity increased obviously, and with the restoration time extending, the number of functional bacteria such as *Bacillus* and *Rhizobium* increased. This suggested that the combined restoration system can provide excellent environment and nutrient conditions for bacterial colonization, so as to improve the ecological characteristics of iron mine tailings, and has a good application prospect.

Acknowledgement
This work was supported by Students’ innovation training program of Sichuan Province (201610626083).

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