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Dynamics of augmented soil system containing biphenyl with *Dyella ginsengisoli* LA-4

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ABSTRACT

One high efficient biphenyl-degrading strain *Dyella ginsengisoli* LA-4 was inoculated into biphenyl-contaminated soil for bioaugmentation in this study. The results showed that bioaugmentation could accelerate the startup period of the biphenyl bioremediation process compared with the non-augmented one. PCR-DGGE fingerprints demonstrated that both of the diversity and pattern of microbial community were affected by the addition of strain LA-4 and biphenyl. Biphenyl-utilizing populations gradually increased and become the dominant species. The introduced strain LA-4 could be persistent and co-exist well with the indigenous populations. However, both of the strain LA-4 and indigenous microorganisms in the bioaugmented system would be partially inhibited by Zn^{2+} and Ni^{2+}. This study suggests that it is feasible and potentially useful to remediate biphenyl-contaminated soil using bioaugmentation with *D. ginsengisoli* LA-4.

1. Introduction

Biphenyl, one of the polycyclic aromatic hydrocarbons (PAHs) that originate in petroleum refining, coal mining and wood processing factories, is a widely distributed environmental pollutant. According to its toxicological properties, biphenyl in the diet has been reported to cause kidney disorders, result in bladder cancer [1], and even reduce life span [2]. And it also could cause slight eye irritation, hepatotoxicity and toxic effects on the central and peripheral nervous systems [3]. Soil was considered to be the biggest sink of biphenyl [4]. Though biphenyl can be degraded aerobically by a variety of soil bacteria capable of degrading low chlorinated PCBs [5], the bioremediation of biphenyl-contaminated soils is very often adversely affected by the low bioavailability of biphenyl and/or the scarcity of autochthonous pollutant-mineralizing microorganisms [6].

Bioaugmentation by inoculated specific compounds degraders, which help conventional biodegradation processes run faster, is expected as the most straightforward strategy to remedy such systems [7]. Bioaugmentation has been used as a tool for bioremediation of xenobiotic- contaminated soil [6,8,9]. However, microbial resources suitable for bioaugmentation are limited. Recently, a new bacterial strain identified as *Dyella ginsengisoli* LA-4 possessed high efficiency for biphenyl removal and the previous study showed that it was candidate for bioaugmentation of biphenyl-contaminated soil [10].

The diversity of the microbial community in soil is an important issue in modern soil microbiology. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA gene is considered as a powerful tool in characterizing the microbial community, monitoring the dominant population and identifying species from the analysis of the sequences [11]. Although the PCR-based method is susceptible to PCR bias and may not represent a complete or accurate picture of the bacterial populations, the abundance of DGGE bands still can reflect the higher proportion of microorganisms present in samples [12].

The aim of this study was to determine the effects of bioaugmentation with *D. ginsengisoli* LA-4 on biphenyl-contaminated soil remediation and to monitor shifts in microbial community in the presence or absence of strain LA-4 by PCR-DGGE. Additionally, the effects of Zn^{2+} and Ni^{2+} (1.0 mmol kg^{-1}) on the augmented system were also investigated.

2. Material and methods

2.1. Chemicals

Biphenyl (>99% purity) was purchased from Sigma–Aldrich (Shanghai, China). All other chemicals were of the highest purity, commercially available and used without further purification.

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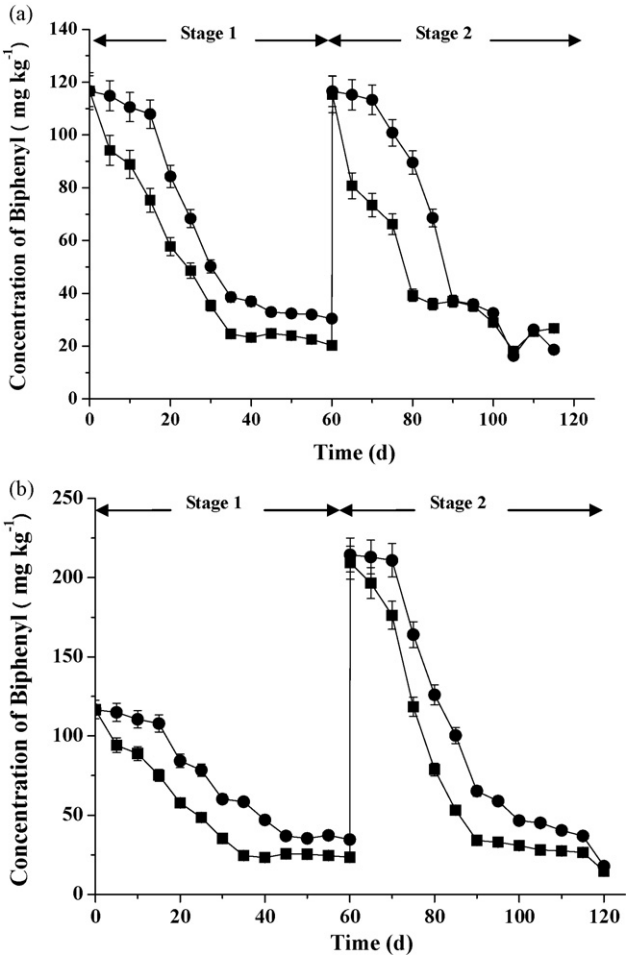

Fig. 1. Removal of biphenyl in bioaugmented system (■) and non-augmented system (●). In the second stage, biphenyl was added to about 116.64 mg kg^{-1} (a) and 209.42 mg kg^{-1} (b), respectively, initially.

2.2. Soil samples

The soil sample used in this study was from an agricultural loam in Shenyang City (Liaoning Province, China) without a history of biphenyl contamination. The soil was taken from the top layer (0–10 cm) and sieved in a 2 mm mesh before being stored moist at 4 °C until use. The soil had a total nitrogen content of 0.04%, a total potassium content of 0.18% and a total phosphorus content of 0.04%. Other parameters were: pH 6.22, organic matter content 1.65%, cation exchange capacity (CEC) 12.26 per 100 g, and total background values of Cu, Zn, Pb, Cd were 32.9 mg kg^{-1}, 28.1 mg kg^{-1}, 11.1 mg kg^{-1}, 0.17 mg kg^{-1}, respectively. Besides, its moisture was 22%.

2.3. Experimental design

All experiments were performed in 1 L glass bottles containing 519 g of native soil, corresponding to 400 g soil dry weight, to which added 100 mg kg^{-1} of biphenyl. The biphenyl-degrading strain *D. ginsengisoli* LA-4 was cultivated aerobically in 100 mL LB medium at 30 °C and the shaking speed of 150 rpm until it reached the late exponential growth phase. Cells were harvested by centrifugation (8000 rpm, 10 min at 4 °C), washed with phosphate buffer solution (pH 7.0) at least twice and re-suspended in the same buffer. Bioaugmented systems were inoculated with stain LA-4 to a final concentration of approximately 10^7 colony forming units (CFU) g^{-1} (dry weight).

To assess the bioaugmentation effects of strain LA-4 in soil contaminated by biphenyl, two simulated batch reactors were operated, all in triplicate, with following treatments: (1) native soil (non bioaugmentation) (2) native soil with LA-4 added (bioaugmentation), respectively. Biphenyl of certain initial concentration would be supplied for the next round of bioremediation process when the residual biphenyl concentration became stable.

For investigating the effects of metal ions on bioaugmented system, four systems were designed with following treatments: (1) with no additional metal ions; (2) with 1 mmol kg^{-1} of Zn^{2+}; (3) with 1 mmol kg^{-1} of Ni^{2+}; (4) with 0.5 mmol kg^{-1} of Zn^{2+} and Ni^{2+}, respectively. The metal ions were added in the forms of their chlorides.

The same volume of sterile water was added to soil to adjust the final moisture content (50%) in each reactor. After vigorous stirring, the systems were operated at room temperature for bioremediation. And certain water would be added regularly to maintain the moisture content of 50%.

2.4. Analytical methods

Biphenyl extraction from soil sample (5 g each) was done twice by ultrasonic extraction (80 kHz) for 30 min. The same volume of acetone and soil samples was added into the 100 mL ground conical flask and mixed sufficiently under the action of ultrasonic. Then the extract was filtered for gas chromatography (GC) analysis.

The biphenyl concentration was analyzed using an Agilent 6890 Series Gas Chromatography (Agilent, USA), equipped with a flame ionization detector (FID) and capillary column (30 m × 0.32 mm × 0.25 μm HP-5) (Agilent, USA). The following temperature program was performed: initial column temperature 80 °C for 5 min, 5 °C min^{-1} to 100 °C, 15 °C min^{-1} to 280 °C holding for 5 min. The injector and detector temperature were all 280 °C. The carrier gas was N_2 at a constant flow rate of 1.0 mL min^{-1}. The injection volume was 1 μL using split (50:1).

2.5. DNA extraction and PCR-DGGE analysis

The total genomic DNA was extracted from the samples by the method described previously [13] and purified by TaKaRa Biotechnology Co., Ltd., Dalian, China. The bacterial universal primers GC338F and 518R [14] were used and the PCR amplification of 16S rDNA was conducted in a total volume of 50 μL containing 0.5 pmol of each primer per μL, 4 μL (each) dNTP, 4 μL dNTP mixture (2.5 mmol L^{-1} each), 5 μL 10 × PCR buffer II (Mg^{2+} plus), 0.5 μL rTaq DNA polymerase. DGGE was performed using a BioRad Dcode system (BioRad Co., Ltd., The USA). Acryl amide gel (8%, w/v) was used and run in 1 × TAE buffer. The denaturing gradient ranged from 30% to 60% (100% is 7 mmol L^{-1} urea and 40% (v/v) formamide). Electrophoresis was run at 200 V and 60 °C for 5 h. Then the gel was stained with GeneFinder (BIO-V Co., Ltd., Xiamen, China) at 10,000-fold dilution, and the gel's UV transillumination image was captured using a gel imaging instrument (BioRad Co., Ltd., The USA). DGGE profiles were analyzed with the software "Quantity One".

3. Results and discussion

3.1. Biphenyl removal in the non-augmented system and augmented system

The time courses of biphenyl concentration in two different systems were shown in Fig. 1. In the non-augmented system, the better biphenyl removal ratio was obtained and became stable after 45 d, however, removal ratio of the bioaugmented system was stable after 35 d. In addition, during the startup period, biphenyl removal

rates of the two systems were obviously different. In the bioaugmented system, about 35.5% of biphenyl was removed within 15 d (2.367 mg L^{-1} d^{-1}), by contrast, only about 0.74% of biphenyl was removed at the same time interval (0.062 mg L^{-1} d^{-1}) in the non-augmented system. Besides, it was demonstrated that the maximal biphenyl removal ratio of bioaugmented system was about 78.85%, which was higher than that of non-augmented system (71.80%). These results indicated that the introduction of strain LA-4 actually promoted the remediation of biphenyl-contaminated soil. It was proved that bioaugmentation with specific microorganism was an effective tool in remediation of contaminated soil. When the residual biphenyl concentration became stable, biphenyl of about 116.64 mg kg^{-1} was once more supplied to the system for another round of remediation (stage 2). The results showed that after the acclimatization in stage 1, both of the bioaugmented and non-augmented systems displayed higher performance for biphenyl removal. And the remediation performance of bioaugmented system was also better than that of the non-augmented one. However, at the 30th day of stage 2 (90 d from the beginning), the biphenyl removal ratio of the two systems became equal. This result was different from that of stage 1, which might due to that after acclimatization for about 1 month, the microbial community became to adapt the biphenyl-contaminated environment and some biphenyl-degrading bacteria became the dominant members in the consortium gradually. High efficiency of complex treatment system was always associated with longer acclimatization time [15].

The impact of higher initial biphenyl concentration (about 209.42 mg kg^{-1}) on remediation in stage 2 was also investigated. It was demonstrated in Fig. 1(b) that biphenyl removal rate was faster than that of stage 1, it was shown than about 30 d for the bioaugmented system and 40 d for the non-augmented system. However, the remediation rate of bioaugmented system was always higher than that of non-augmented system until they were operated for 120 d (Fig. 1(a)). It was suggested that when organic shock loading increased, the bioaugmented system showed more stability than original soil system. This result indicated that bioaugmentation with specific microorganisms could also enhance the shock resistance of natural microbial community to environmental pollutants.

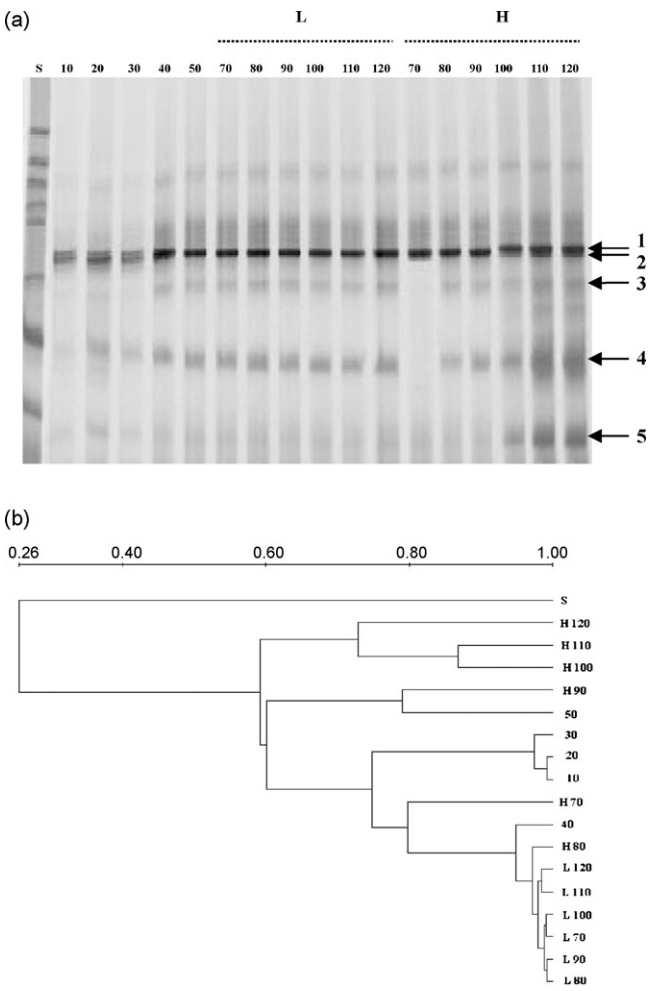

Fig. 2. DGGE fingerprints of non-augmented system (a) and corresponding cluster analysis based on "UPGMA" method (b). Lane designations: S, native soil; L, system was added with concentration of biphenyl 116.64 mg kg^{-1}; H, system was added with concentration of biphenyl 209.42 mg kg^{-1}. The numbers "10, 20, . . ., 120" indicate the sampling time (days).

3.2. DGGE analysis of the population dynamics during the processes

The population dynamic changes of the non-augmented system were presented in Fig. 2(a). It was showed that comparing with the original fingerprint, DGGE patterns dramatically shifted and the genetic diversity was declined. Results of cluster analysis showed that the similarity coefficient between native soil and each sample was lower than 0.4. It was demonstrated that with the addition of biphenyl, microbial community structure of native soil was changed and biphenyl-utilizing populations gradually increased and became the dominant species.

During 70–120 d operation, the similarity between each sample was more than 0.95. It was indicated that during the acclimation in the first stage, microbial population had adapted to the biphenyl concentration of about 100 mg kg^{-1}, the predominant population structure was developed and the structure seemed not to change evidently. With biphenyl concentration added two times higher than the initial concentration, several bands disappeared and some others appeared later. An approximate similarity coefficient of about 0.54 was observed between day 70 and the end of first stage (day 50). It was indicated that the high concentration would have impact on community structure.

Figs. 2 and 3 showed the DGGE fingerprints of the microbial communities at different time and stage for the non-augmented and bioaugmented systems, respectively. Lane "S" was the fingerprint of original soil and the only band in lane "LA" was that of strain LA-4. It was shown that LA-4 was always existent in the DGGE fingerprint patterns and the number of dominant bands obviously decreased compared with that of original soil (Fig. 3(a)), suggesting that biphenyl significantly affected the microbial structure of original soil consortium. Some bands entirely disappeared in the fingerprints of the augmented system, while several bands appeared in the later samples and became brighter. At the end of each stage, relatively stable microbial community was formed in which strain LA-4 was one of the dominant members throughout the whole process. Comparatively, the DGGE fingerprint of Fig. 2(a), which represented the microbial communities in the non-augmented system at different stage, contained relatively few dominant bands. Hence, the comparison of DGGE fingerprint from the two systems suggested that bioaugmentation could impact the microbial community structure and then promoted the bioremediation process. The reason was that inoculation of strain LA-4 led to decrease or increase of certain populations in the community, which is different from the earlier study [16]. Therefore, it was concluded that the strain LA-4 could steadily survive in the system and compatible well with soil indigenous microorganism for utilization of biphenyl as carbon sources.

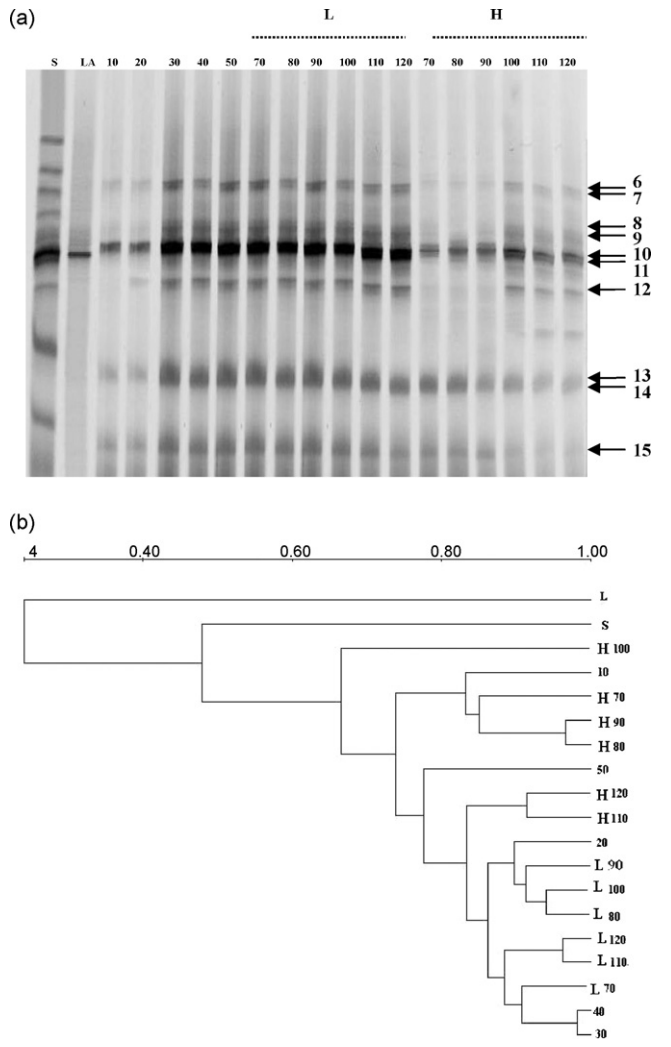

Fig. 3. DGGE fingerprints of bioaugmented system (a) and corresponding cluster analysis based on "UPGMA" method (b). Lane designations: S, native soil; LA: Strain LA-4; L, system was added with concentration of biphenyl 116.64 mg kg^{-1}; H, system was added with concentration of biphenyl 209.42 mg kg^{-1}. The numbers "10, 20, . . ., 120" indicate the sampling time (days).

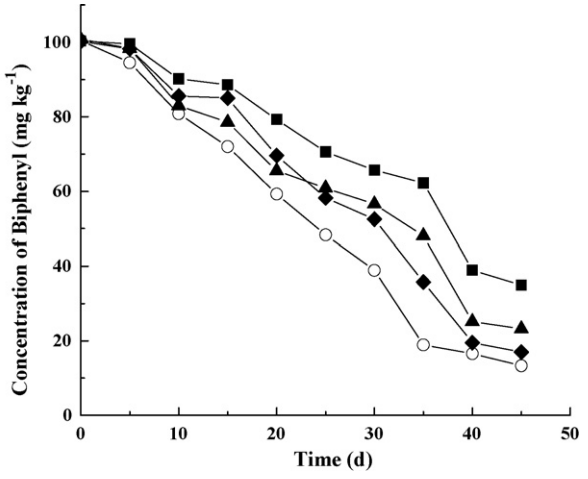

Fig. 4. Time course of biphenyl removal by bioaugmented system which were added with different metal ions: 1 mmol kg^{-1} of Zn^{2+} (♦), 1 mmol kg^{-1} of Ni^{2+} (▲), 0.5 mmol kg^{-1} Zn^{2+} and Ni^{2+}, respectively (■), and control (○).

When biphenyl concentration was supplied to the same level as stage 1, DGGE fingerprints appeared extremely similar with a similarity coefficient of more than 0.8 between each two samples. However, when biphenyl concentration was added to about 200 mg kg^{-1} at stage 2, several bands disappeared in both of the two systems at the beginning and appeared again after a period time of acclimatization. The reason might be that the microbial communities would be relatively stable under constant conditions, whereas, it would be easily affected by the outer conditions. When exposed to high-loading environment, some previous dominant microorganisms would be inhibited and became non-dominant members at the beginning. However, after a period time of acclimatization, the microbial community would again adapt the environment and new consortium would be formed. Additionally, the augmented strain LA-4 existed in all the lanes of DGGE fingerprint, and the samples between each other were relatively similar according to the cluster analysis (Fig. 3(b)). It further proved that the biphenyl-degrading strain LA-4 could promote the remediation of biphenyl-contaminated soil through accelerating the acclimatization of microbial community, and it could co-exist well with indigenous microorganisms.

3.3. Effects of metal ions on augmented system

In the previous study, it was proved that Zn^{2+} and Ni^{2+} took different influence on strain LA-4, therefore, both Zn^{2+} and Ni^{2+} (total concentration of 1.0 mmol kg^{-1}) were selected as type metal ions to test effects on biphenyl removal in the bioaugmented system in this study. As shown in Fig. 4, during the 45-d operation, the systems containing different metal ions, including the mixture of two ions, were all affected in different extent compared with the control system.

Among the tested metal ions, Zn^{2+} showed less inhibition effect than that of Ni^{2+}. And the mixture of Zn^{2+} and Ni^{2+} displayed more negative effects on the removal of biphenyl than others. Besides, though all the systems were affected, there were still more than 60% removal ratio within 45 d, which suggested that the microbial communities were just partially inhibited.

It was reported that Zn^{2+} was one of the necessary trace elements and it would promote the activity of various microorganisms. However, it would also inhibit microbes when concentration exceeded the specific limitation [17]. Additionally, Ni^{2+}, which was always discharged into the environment such as soil and river by the industries of electroplating, metallurgy, etc., could affect the activity of microorganisms at a relatively low concentration [18]. Both of them could inhibit the microorganisms through the inhibition of important functional macromolecules such as enzyme and other useful proteins [19]. However, there were still some microbes which could tolerate specific metal ions [20] and some even could transform toxic metal ions to nontoxic state [21]. Thus, when these metal ions were added to the soil in the form of single compound or mixture, some of the indigenous microorganisms would be inhibited and there might be still some specific metal-ion-tolerant ones survived and became the dominant members to maintain the activity of the microbial community.

3.4. DGGE analysis of the microbial communities affected by different metal ions

Effects of metal ions on microbial community structures were shown in Fig. 5(a). The DGGE profiles displayed dynamic shifts in the microbial community and the genetic diversity was declined compared with the original soil system and most of the lanes (samples) contained fewer bands than those in Fig. 2(a) and Fig. 3(a). Results of cluster analysis showed that the similarity coefficient between each DGGE pattern was very low, which suggested that

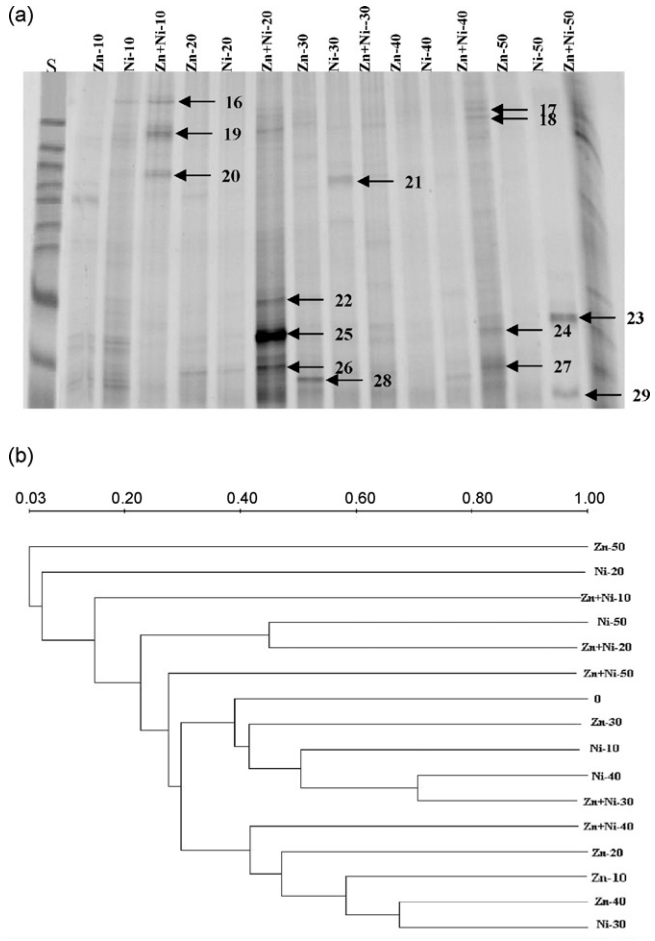

Fig. 5. DGGE fingerprints of bioaugmented system with different metal ions (a) and corresponding cluster analysis based on "UPGMA" method (b). Lane designations: Zn, addition of Zn^{2+} (1 mmol kg^{-1}); Ni, addition of Ni^{2+} (1 mmol kg^{-1}); Zn + Ni, addition of Zn^{2+} and Ni^{2+} (0.5 mmol kg^{-1}, respectively). The numbers "10, 20, . . ., 50" indicate the sampling time (days).

different metal ions performed different effects on microbial community structures. In addition, band of strain LA-4 disappeared from all the samples, suggesting that it was inhibited by these metal ions completely. However, a few bands could also be detected in each sample, which might be the ones could tolerate or even transform certain metal ions.

It was indicated that DGGE bands dramatically changed because the microorganisms capable of tolerating metal ions became developing [22]. For instance, band 28 obviously appeared in the lane "Zn-30" showed that it might be Zn^{2+}-tolerant microorganism and band 23 in lane "Ni-50" showed that it might be a Ni^{2+}-tolerant member. Also, band 25 in lane "Zn + Ni-20" suggested that it was probably microorganism which could tolerate both of the two metal ions. However, it became dark with time prolonged, suggesting that it was inhibited by other microbes in the consortium. And there were very few bands always appeared in the same sample, which suggested that the concentration and form of metal ions might change during the process and caused complex effects on microbial community structure. Therefore, it could be concluded that the bioaugmented system would be inhibited by high concentration of by Zn^{2+}, Ni^{2+} and their mixture could inhibit some dominant members in the microbial community including stain LA-4. Some specific metal-ions-tolerant microorganisms should be introduced to deal with the influence of metal ions pollution in future investigation.

4. Conclusions

In this study, bioaugmentation with an efficient biphenyl-degrading strain LA-4 was used to remediate a biphenyl-contaminated native soil. The results showed that the bioaugemented system performed better in biphenyl removal than the non-augmented system. And strain LA-4 could steadily survive and compatible with the indigenous populations. However, both of the strain LA-4 and indigenous microorganisms were affected by Zn^{2+}, Ni^{2+} and their mixture at different level. Results of PCR-DGGE displayed that there were dynamic shifts in the bioaugmented and non-augmented systems along with time. Both Zn^{2+} and Ni^{2+} could completely inhibit activity of strain LA-4.

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