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Characterization on the rhizoremediation of petroleum contaminated soil as affected by different influencing factors

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Abstract

In this paper, pilot experiments were conducted to analyze the effect of different environmental factors on the rhizoremediation of petroleum contaminated soil. Different plant species (cotton, ryegrass, tall fescue, and alfalfa), addition of fertilizer, different concentration of TPH in soil, bioaugmentation with effective microbial agent (EMA) and PGPR, and remediation time were tested as influencing factors during bioremediation process of Total Petroleum Hydrocarbon (TPH). The result shows that the remediation process can be enhanced by different plants species with the following order: tall fescue > ryegrass > alfalfa > cotton. The degradation rate of TPH increased with increased fertilizer addition and moderate level of 20 g/m^2 urea is best for both plant growth and TPH remediation. High TPH content is toxic to plant growth and inhibits the degradation of petroleum hydrocarbon with 5% TPH content showing the best degradation result in soil planted with ryegrass. Bioaugmentation with different bacteria and plant growth promoting rhizobacteria (PGPR) showed the following results for TPH degradation: cotton+EMA+PGPR > cotton+EMA > cotton+PGPR > cotton > control. Rapid degradation of TPH was found at the initial period of remediation caused by the activity of microorganisms, continuous increase was found from 30–90 d period and slow increase was found from 90 to 150 d. The result suggests that rhizoremediation can be enhanced with the proper control of different influencing factors that affect both plant growth and microbial activity in the rhizosphere environment.

1 Introduction

With the development of economy and petroleum exploration, contamination of soil with petroleum compounds is of concern worldwide (Banks et al., 2003; Rojo, 2009). Bioremediation of contaminated soil is supposed to be low cost, less interference to soil structure and higher public acceptability as compared to other approaches such as soil thermal desorption and soil leaching treatment. There are two different approaches for bioremediation of petroleum contaminated soil: microbial remediation and

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phytoremediation. Phytoremediation is a strategy that uses plants to degrade, stabilize, and/or remove soil contaminants. Phytoremediation of TPH has the potential to be a sustainable waste management technology if it can be proven to be effective in the field (Gurska et al., 2009). Recently, combination of microbial remediation and phytoremediation has become a general practice in the field treatment of petroleum contaminated soil. This technique can be defined as rhizoremediation, which is a specific type of phytoremediation that involves both plants and their associated rhizosphere microbes, and can occur naturally or can be actuated by deliberately introducing specific microbes (Gerhardta et al., 2009).

The addition of crude oil results in an immediate change in bacterial community structure, increasing abundance of hydrocarbon-degrading microorganisms and a rapid rate of oil degradation, which suggests the presence of a pre-adapted oil-degrading microbial community and sufficient supply of nutrients (Coulon et al., 2006; Hamamura et al., 2006). The degradation rate of microbial remediation and phytoremediation differed greatly depending on different conditions. Microbial degradation can be accomplished by different species of microorganisms both native to the soil and added as effective degrading strains. The microbial degradation is generally higher than 40% within 1 y of disposal and may be as high as over 70% in some cases (Sathishkumar et al., 2008). Influencing factors for microbial remediation included soil moisture content, temperature, soil pH, oxygen supply, nutrient, oxidation-reduction potential, soil texture and structure (Riser-Roberts, 1998). However, the degradation rate in phytoremediation is generally low, which maybe only 9.1%~15.5% or 20% higher than that in the control soil (Brandt et al., 2006; Euliss et al., 2008). In this account, bioaugmentation is needed to introduce effective microorganisms to improve the efficiency of rhizoremediation. By the synergistic reaction of the plant and microorganisms, rhizoremediation showed higher degradation rate of petroleum pollutants as compared to microbial remediation and phytoremediation (Gurska et al., 2009; Xin et al., 2008; Escalante-Espinosa et al., 2005). Several plant species were proved to be more effective in degrading TPH including: ryegrass, sorghum, maize, alfalfa, Bermuda grass, rice, legume, sorghum and beggar ticks (Nedunuri et al., 2000; Kaimi et al., 2007; Merkl et al., 2005; Shirdam et al., 2008). In different influencing factors of rhizoremediation, the TPH content is an important factor influencing bioremediation process as high TPH content is toxic to both microorganisms and plants. Some plants are sensitive to oil pollution, and plant growth may be greatly reduced in a high TPH soil (Peng et al., 2009). In a rhizoremediation process by using ryegrass and plant growth promoting rhizobacteria (PGPR), the degradation rate in soil TPH content of 13% is 61.5% during 3 y of remediation (Gurska et al., 2009). At a low TPH content of 5% TPH, the process removed 90% of all fractions of TPHs from the soil, and phytoremediation alone was able to remove only about 50% of TPHs in the same time period (Huang et al., 2005). The result suggests that high TPH content inhibits plant growth and microbial activity in the rhizosphere environment and results in low TPH degradation. Other factors affecting the rhizoremediation process include inoculation, addition of nutrients, soil organic content, soil depth and salt content and etc (Mishra et al., 2001; Margesin et al., 2003; Lin and Mendelssohn, 1998; Hutchinson et al., 2001; Keller et al., 2008).

Despite our understanding of the mechanisms of remediation and the success of studies in the laboratory and greenhouse, efforts to translate bioremediation research to the field have proven challenging (Gerhardta et al., 2009). This is partially because that the plant growth in the field experiment is generally different from the laboratory conditions, and the field remediation can be affected by many different factors. Until now, although different rhizoremediation techniques for petroleum contaminated soil have been applied in both laboratory and field experiment, systematic research on the influencing factors for rhizoremediation has been seldom conducted. For a better understanding of the mechanisms of remediation and enhancement of the remediation efficiency, a series of rhizoremediation experiments were conducted and compared in order to further understand how different factors affect the rhizoremediation process and how remediation process can be controlled for a better disposal of TPH contaminated soil.

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2 Materials and methods

2.1 Petroleum contaminated soil

Petroleum contaminated soil was brought from Shengli Oil Field of China. The soil was air dried and ground to 20 meshes before using for experiment. The chemical properties of the soil can be seen in Table 1. The pH value was relatively high and a higher TPH and Zn content were found in the soil. The TPH content is about 10%, and different concentration of petroleum contaminated soil was prepared by mixing the contaminated and un-contaminated soil at different ratios.

2.2 Experiment for different influencing factors of rhizoremediation

Five experimental series were designed to check different influencing factors on the effect of rhizoremediation: (1) Comparision of different species on the remediation of TPH. Plants were selected based on the literature reports that have been generally used for the remediation of petroleum pollutants. Four plant species were used and compared: cotton (*Gossypium hirsutum Linn*), ryegrass (*Lolium perenne* L.), tall fescue (*Festuca arundinacea*), and alfalfa (*Medicago sativa*). The soil TPH content is 5%, the experiment was carried out in flower pot with 750 g soil and lasted for 150 d. The management of plant growth was the same as their general requirements. (2) Effect of chemical fertilizer addition on the remediation process. Urea was added to the TPH contaminated soil at the rate of 0, 5, 10, 20 and 50 g/m^2 using the same soil as described in the above experiment. (3) Effect of TPH content on the growth of plant and remediation effect. TPH content of 2%, 5% and 10% were prepared and tall fescue was planted for a period of 150 d and the degradation rates were calculated based on the change of TPH content before and after remediation. (4) Effect of bioaugmentation on the remediation process. Pot experiment was carried out using 750 g petroleum contaminated soil at a TPH concentration of 5%. Four different treatments was as the followings: (a) control, (b) planting cotton, (c) planting cotton and addition of effective microbial agent (EMA, 2%), EMA consists of 2 kinds of bacteria: *Acinetobacter radioresistens*, *Rhodococcus erythropolis*, (d) cotton+PGPR, (e) cotton+EMA+PGPR, (5) Degradation of pollutants at different time of rhizoremediation process. Three treatments were conducted as the followings: control, addition of 2% EMA (*Acinetobacter radioresistens* and *Rhodococcus erythropolis*), 2% EMA with planting of tall fescue. Samples were taken after 0, 15, 30, 45, 60, 90 and 150 d to check the dynamic change of TPH content during the remediation process.

2.3 Analysis of TPH

TPH content analysis: 5 g sub samples of air-dried underwent ultrasound extraction with 15 mL chloroform for 15 min. Then centrifugation is carried out at 4000 rpm. The supernant is then filtrated and put into a flask. The extraction procedure repeated 3 times and the extracts were concentrated to dryness with a rotary evaporator at 40 °C. After drying to constant weight at 60 °C, the flask was reweighed to determine the oil contents.

2.4 Dehydrogenase activity analysis

5 g air-dried soil sample was put in the flask, then 5 mL 0.1% TTC (2,3,5-triphenyltetrazolium chloride) solution and 2 mL pH 7.6 of 0.2 mol/L Tris-HCl buffer solution was added and shook. A blank was carried out without addition of TTC. Then the prepared samples were incubated under 37 °C for 24 h. After incubation, 1M H_2SO_4 solution was used to stop the reaction and 5 mL toluene was added and incubated for 30 min with shaking. After centrifugation, the absorbance of organic solution under 492 nm was detected and the dehydrogenase activity was expressed as the amount of TPF (triphenyl formazan) produced by reduction of TTC.

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2.5 Denaturing gradient gel electrophoresis (DGGE) analysis of partial 16S rRNA genes

DNA extraction was carried out by ZR Soil Microbe DNA Kit™ (Orange, CA). The procedure of the DNA extraction from soil was according to the manufacturer's instructions. The 16S rDNA genes were amplified with PCR using the following primers, 357f-GC clamp (forward, 5′-CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCA CGGGGGGCCTACGGGAGGCAGCAG-3′, Escherichia coli position: 341–357), 517r (reverse, 5′-ATTACCGCGGCTGCTGG-3′, Escherichia coli position: 517–534). The reaction solution for PCR contains 0.5 µl of each primer, 2.5 µl 10× Ex Taq buffer, 2.5 µl of 2.5 mM dNTP mixture, 2.0 µl bovine serum albumin (BSA), 1.5 µl of 25M Mg^{2+}, 0.25 µl of 5 U/µl Ex Taq DNA polymerase (TaKaRa, Otsu, Japan), 1.0 µl DNA template and 14.75 µl ultrapure sterile water. PCR was carried out by a TaKaRa PCR Thermal Cycler Dice Model TP600 (TakaRa, Otsu, Japan) with the following conditions for amplification: initial denaturation at 94 °C for 3 min, 25 cycles of 1 min of denaturation at 94 °C, 1 min of annealing at 55 °C, and 2 min of extension at 72 °C, and then final extension at 72 °C for 7 min.

DGGE analysis was used to obtain fingerprintings of the PCR products of partial 16S rDNA genes. The denaturing gradient of polyacrylamide ranged from 30% to 60%. After application of PCR products in the gel, electrophoresis was performed in an electrophoresis cell D-code TM system (Bio-Rad laboratories, Hercules, CA, USA) at 60 °C and with 200 V for 4 h. The gel then was stained with ethidium bromide (EB) for 30 min and photographed under UV light. The result of the DGGE bands was analyzed by Quantity One (Bio-Rad laboratories, Hercules, CA, USA).

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3 Results

3.1 Effect of different plant species on the remediation process of TPH

Different plant species have been used in the phytoremediation process of petroleum contaminated soil. Grass of tall fescue and ryegrass and alfalfa are generally used and have been proved to be effective in enhancing bioremediation of TPH. Cotton is a kind of economic crop and is adapted to the saline and alkaline land. The Effect of different plant species on the remediation of TPH is shown in Fig. 1. The degradation of TPH is 33.1% to 48.6% with the following order: tall fescue > ryegrass > alfalfa > cotton. The value of degradation in tall fescue and ryegrass is almost the same and slightly higher than that of alfalfa. The different degradation rates of TPH in different plants were probably caused by the different physiological functions of root in different plants and suggest that proper selection of plant species is an important strategy in bioremediation process of TPH.

3.2 Effect of chemical fertilizer addition on the remediation process

As both microbial activity and plant growth can be affected by addition of fertilizer, fertilizer addition is an important factor in affecting the efficiency of bioremediation process. Fig. 2a shows the degradation rate of TPH under different addition rate of urea. A positive relationship between degradation rate of TPH and addition rate of urea indicating that fertilizer is effective in enhancing rhizoremediation process of TPH. On the other hand, Fig. 2b shows the change of biomass weight with addition of different amount of urea. Under urea application of 20 g/m^2, both wet weight and dry weight of tall fescue increased with the increase of urea addition. However, low biomass weight was found under higher urea application rate of 30 g/m^2. The highest biomass value of 6.28 g was achieved under urea addition of 20 g/m^2, which is about 6 times higher than that under urea addition of 30 g/m^2.

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3.3 Effect of TPH concentration on the growth of plant and remediation effect

Petroleum is toxic to plant thus inhibits plant growth at high concentration of TPH. In Fig. 3, the degradation of TPH is 60.3%, 48.4% and 14.9% at different concentration level of 2, 5 and 10%, respectively during phytoremediation process by tall fescue for 150 d. This indicates that low concentration is favorable for the TPH degradation as evaluated by degradation rate. However, the degradation amount of THP is the highest in a moderate concentration as shown in Fig. 3. Proper concentration of TPH should be considered during phytoremediation process to achieve the best remediation result.

3.4 Effect of bioaugmentation on the rhizoremediation process

Rhizoremediation process is a combined effect of microbial degradation and plant growth. Figure 4 compared the effect of plant growth, addition of EMA and addition of PGPR on the bioremediation process. The degradation of TPH is as the following order: cotton+EMA+PGPR > cotton+EMA > cotton+PGPR > cotton > control. The highest degradation rate 29.8% was found in the treatment of cotton with addition of both EMA and PGPR indicating that bioaugmentation with EMA and PGPR is effective in promoting rhizoremediation process of TPH. As comparing cotton planting with control, a 5% higher degradation in cotton planting treatment suggested the effectiveness of phytoremediation. On the other hand, cotton+EMA and cotton+PGPR showed further higher degradation rate than cotton planting only. The bioaugmentation with petroleum degrading bacteria is supposed to be able to enhance the rhizoremediation process. Dehydrogenase activity was higher in the 4 treatments than that of control and showed the highest value by addition of EMA. It is showed that PGPR did not improve the dehydrogenase activity during the remediation process.

From the DGGE analysis result in Fig. 5a, a more complex microbial community was found in lane 2–6 compared with lane 1, indicating that rhizoremediation of TPH with cotton and different microorganisms allows the proliferation of the complex microbial community. Some new bands developed during remediation process. Cluster analysis

(Fig. 5b) indicated that the microbial community of control was different from that of cotton planting, while the two treatments with addition of PGPR were grouped in one category in cluster analysis result.

3.5 Degradation of TPH pollutants at different time of rhizoremediation process

At different time of rhizoremediation process with tall fescue, the process can be divided into 3 periods: a rapid increasing of degradation rate was found at the initial period of remediation of 0–30 d, continuous increase from 30–90 d period, and slightly increase from 90 to 150 d. At the initial period of 15 d, the degradation rate is at the following order: EMA > EMA+plant > control. While the degradation rate gradually increased in the treatment of EMA+plant and became higher than that of EMA in TPH degradation after 30 d. An enhanced remediation effect by planting tall fescue coupled with addition of EMA (7% higher than control and EMA) was shown after 90 d in Fig. 6.

4 Discussion

Regulation on the different influencing factors during rhizoremediation process is important for a better degradation of TPH. In this research, plant species, fertilizer addition, TPH concentration, inoculation of effective microorganisms and remediation time are considered to be the main influencing factors and studied in detail. Petroleum hydrocarbon contamination reduced the growth of the plants significantly, correspondingly the degradation efficiency of different plant species on the soil TPH may differ greatly (Shirdam et al., 2008; Euliss et al., 2008). In our research, 4 typical plant species tall fescue, ryegrass, cotton and alfalfa were used and compared in rhizoremediation process because these plants may grow in a wide range of soil conditions, especially they can stand high saline-alkali stress and can be applied in the oil field where the soil property is saline-alkali in many parts of China.

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The different TPH degradation rate among various plant species may be dependent on the microbial population in the rhizosphere of these plants. Many different plant species have been reported to be effective in remediation of TPH soil including grass, alfalfa, poplar, ryegrass etc (Phillips et al., 2009; Euliss et al., 2008; Kechavarzi et al., 2007). It was reported that perennial ryegrass and alfalfa increased the number of rhizosphere bacteria in the hydrocarbon-contaminated soil (Kirk et al., 2005). Tall fescue also showed high degradation rate of TPH in petroleum contaminated soil (Ezzatian et al., 2009). Cotton may grow under saline conditions of about 0.5% salinity (Sacchi et al., 2000) and thus can be possibly applied in the saline-alkaline environment of oil field in China. However, the degradation rate of TPH by cotton planting is lower than that by other three plants species. Microbial degradation rates in the TPH contaminated soil were more affected by soil properties and the chemical characteristics of the contaminant than the presence of roots (Song et al., 2004). This means that research on soil TPH remediation should be concentrated more on the effect of different soil properties rather than the selection of different plant species. Addition of NPK fertilizer and compost has greatly enhanced the hydrocarbon degradation (Palmroth et al., 2006). A positive relation between TPH degradation rate and fertilizer addition also supports this conclusion and requires that moderate fertilizer must be used for better plant growth and TPH degradation.

The effect of The TPH concentration on the degradation of TPH is probably caused by the toxicity of petroleum hydrocarbons on the plants and related rhizosphere microorganisms. TPH concentration was the major determinant of total bacterial abundance and had positive effects on abundances of hydrocarbon degraders (Nie et al., 2009). As evaluated by different organisms, earthworms were 1.4 to 14 times more sensitive than Microtox and 1.3 to >77 times more sensitive than plants to the oily soils (Dorn et al., 1998). This means that plants can stand high concentration of TPH than other organisms. A preference degradation of saturated hydrocarbon has been found by Peng et al. (2009) during phytoremediation process of TPH using *Mirabilis Jalapa L.* As light oil was generally more toxic than heavy oil fractions (Dorn et al., 1998), the preferential degradation of light oil generally causes a decrease in soil toxicity during TPH degradation process. On the other hand, degradation of old soil contaminated by TPH is generally difficult than that fresh contaminated soil. Research result has indicated greater rates of hydrocarbon loss for the soils containing the fresh petroleum product compared with the aged product (Parker and Burgos, 2001). Our result proved that a moderate TPH concentration (about 5%) is favorable for petroleum hydrocarbon degradation. This means that biological remediation is most effective in a moderate TPH concentration of contaminated soil at the beginning time of spilling.

As TPH reduction was positively correlated to culturable hydrocarbon degraders (Philiip et al., 2006), it is possible that addition of effective microorganisms enhanced the rhizoremediation process of TPH (Gurska et al., 2009; Mishra et al., 2001). Different plant species may introduce different microbial communities and increase the total culturable microbial amount during their growth process. Cotton root can be colonized by a variety of microorganisms including *Fusarium culmorum*, *F. solani*, *F. oxysporum*, *Macrophomina phaseoli* and *Bacillus* sp. (Ghaffar and Parveen, 1969). Some bacteria such as *Rhodococcus* sp. strain can grow at the oil-water interface and produce a mycolic acid-containing capsule to enhance TPH degradation (Van Hamme et al., 2001). A high degradation rate of 85.67% within 120 d was reached in combination of cotton with native microorganisms as compared to plant species of sunflower, bermuda grass and sudan grass (Liu et al., 2009). It is proved by DGGE analysis in this research that cotton growth increases microbial diversity and alters microbial community structure. However, some research reported that the introduction of exotic micro-organisms did not improve the remediation, and that inoculation of oil-contaminated sites with non-indigenous species is likely to fail (Li et al., 2002; Thomassin-Lacroix et al., 2002; Cavalca et al., 2002). It is suggested that the effect of inoculated microorganism depends on the environment conditions such as the existence of indigenous microorganisms, nutrient level and amount of inoculation. As microbial degradation happened at the early stage of remediation, an early period of microbial remediation followed by phytoremediation is supposed to be effective in the field remediation practice.

It usually takes several months for the bioremediation of TPH to reach a reasonable end point. Our result suggests that the degradation rate of TPH is different at different time of the bioremediation process. Among different influencing factors, TPH content is the most important factor for rhizoremediation and the degradation rate can also be affected by the amount of microbial agent, addition of fertilizer and enhanced by plant growth at the late period of time. As microbial degradation is most effective at initial period of remediation, second time addition of microbial agent may be a good strategy during the course of remediation. At the end time of 150 d, the degradation rate becomes stable even in combination of microbial remediation with planting tall fescue. At this point, research should be carried out to further enhance the bioremediation by multi-process phytoremediation system such as addition of fertilizer, management of plant growth and increasing aeration of the rhizosphere environment.

5 Conclusions

Grass plants like tall fescue and ryegrass are better choices for bioremediation of TPH as these plants grow well under different environmental conditions. Addition of fertilizer enhanced the degradation of TPH and positive relation between amount of fertilizer addition and degradation rate of TPH can be found. Higher TPH content inhibited bioremediation process probably caused by the toxicity of TPH to the plant and bacteria. Bioaugmentation with different bacteria and PGPR bacteria was proved to be able to enhance the rhizoremediation process. Bioaugmentation is generally effective at the initial period and plant growth enhances the TPH degradation at a late period of remediation. The result suggests that rhizoremediation can be enhanced with the proper control of different influencing factors that affect both plant growth and microbial activity at the rhizosphere environment.

As long time period is needed to conduct bioremediation process, it is difficult to check different influencing factors at the same time. TPH content is crucial for rhizoremediation as its toxicity on plants. Plant selection is important in that crop management

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will be different greatly for different plant species. Finally, fertilizer and EMA addition should be generally applied in the field remediation practice for a better degradation of TPH.

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Table 1. Chemical properties of the soil used in the experiment.

pH	TPH%	Total N (g/kg)	Total N (g/kg)	Heavy metal (mg/kg)					
				Zn	Cd	Ni	Cu	Pb	Cr
7.9	10	2.75	0.11	666	–	6.5	16.5	–	12

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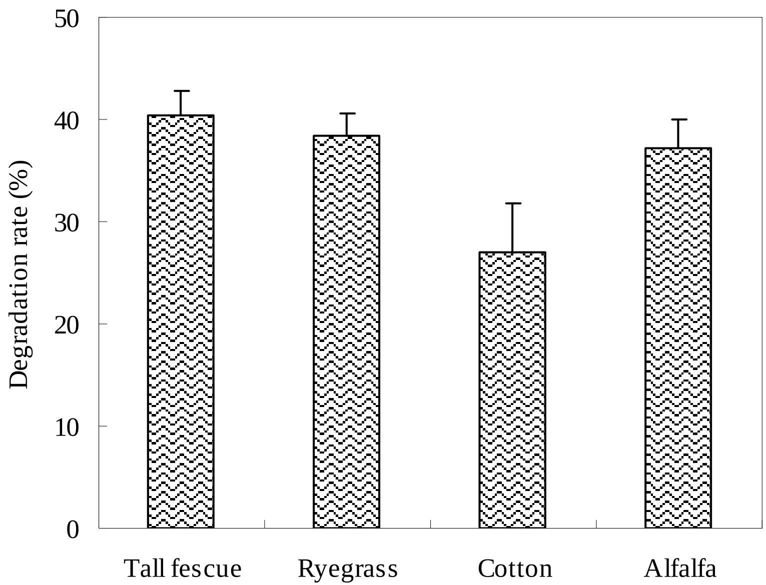

Fig. 1. Comparison of different plant species on the bioremediation of TPH.

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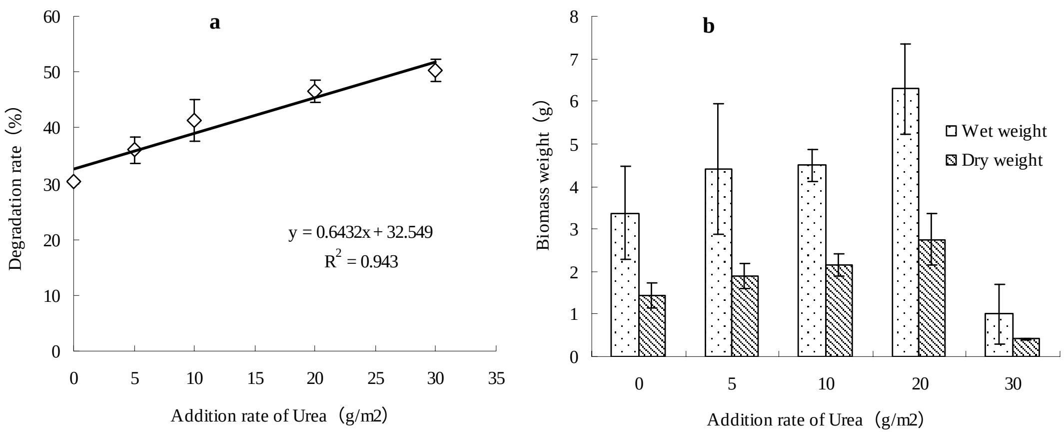

Fig. 2. Effect of urea addition on the bioremediation process of TPH.

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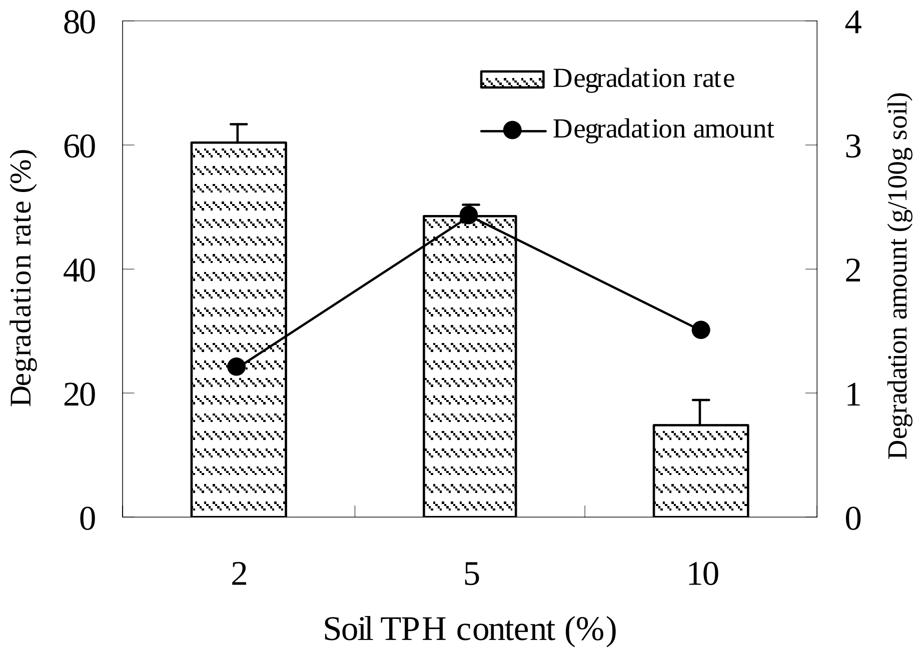


Fig. 3. Effect of soil TPH concentration on the degradation rate.

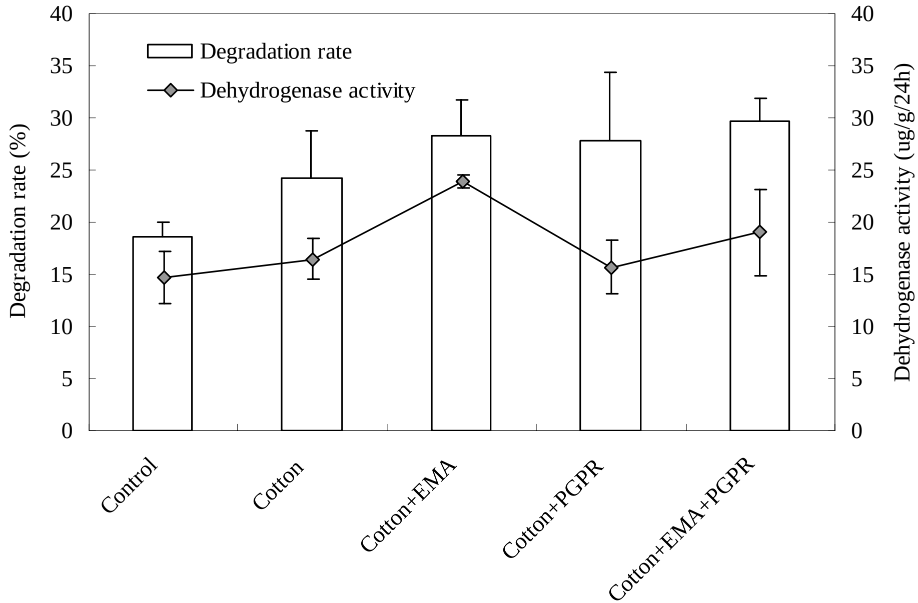
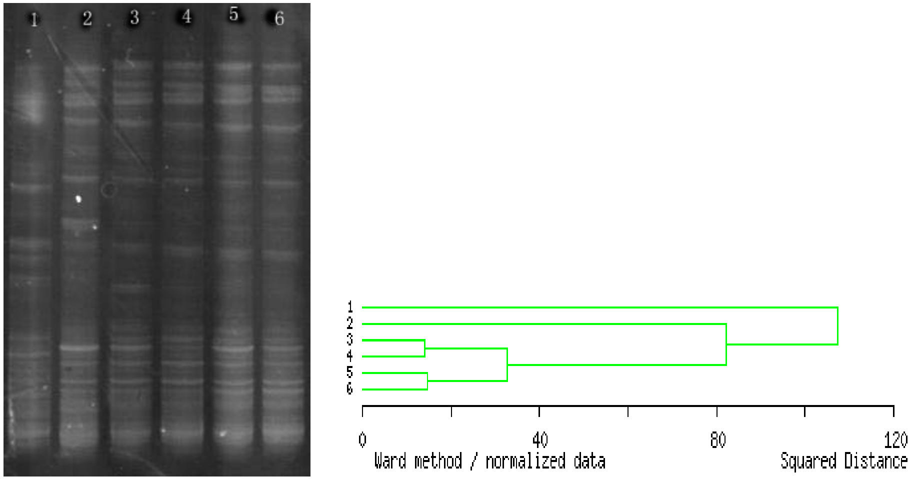


Fig. 4. Addition of EMA and PGPR bacteria on the remediation process.



a b

Fig. 5. DGGE analysis result of TPH bioremediation process **(a)** and cluster analysis **(b)**. 1: soil before remediation; 2: control; 3: cotton; 4: cotton+EMA; 5: cotton+PGPR; 6: cotton+PGPR+EMA.

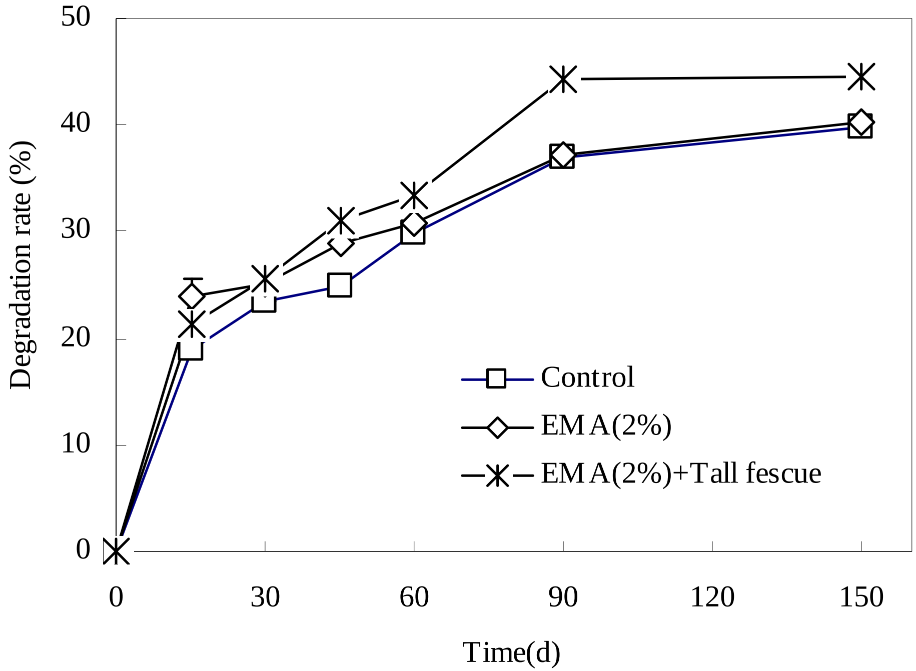


Fig. 6. Degradation of TPH pollutants at different time of rhizoremediation process with *Tall fescue*.

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