Effects of Lead on Petrol Degradation Efficiency of Bacteria Isolated from Soils in Zhuhai, Guangdong, China

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Abstract Soil pollution, particularly of petroleum hydrocarbons and lead contamination, has become increasingly concerned due to rapid urbanization and industrial development. Bioremediation of petroleum hydrocarbon pollution using microorganisms is a promising solution due to the absence of secondary contamination. In this study, two bacterial isolates (B-7 and B-10), with distinctive colony characteristics, were screened from petroleum contaminated soil collected in Zhuhai, Guangdong Province, China. Using 16S rRNA sequencing technique and biochemical analyses, B-7 and B-10 were identified to be closely related to *Achromobacter denitrificans*, and *Mycolicibacterium phocaicum* N4, respectively. Petrol degrading rates of the two isolates were determined by UV-visible spectrophotometer in this study. With consideration of recovery rate and background evaporation rate, in the absence of lead, in a five-day interval study, the petrol degradation rate of B-7 and B-10 was observed at approximately 2.4g petrol/L for both isolates. Co-contamination with lead at 1, 5, 10 and 20ppm significantly inhibited petrol degrading potential of both isolates, with B-10 demonstrated significantly higher lead tolerance. Future studies are needed to evaluate the effects of other abiotic factors, such as pH, temperature, nutrient contents and concomitant exposure to other pollutants and biotic factors, such as microbial community, on the petroleum hydrocarbons degrading efficiency of these isolates.

Keywords Bioremediation, Lead Contamination, Petroleum Hydrocarbons

1. Introduction

Due to rapid development of the economy, the demand of petrol has been increasing steadily in the past few decades. The main components of petrol include petroleum hydrocarbons, particularly the C5-C12 aliphatic, cycloalkanes, and aromatic hydrocarbons [1-2]. Each year, it was estimated that more than 600,000 tons of petrol are released to the environment, threatening health of humans and our ecosystems [3-4]. Yu et al. [5] and Shen [6] reported that in each year, an additional 100,000 tons of soils in China are polluted by petrol. Once soils are polluted by petroleum hydrocarbons, the efficiency on nutrient absorption by the root system of plants will be affected, inhibiting the growth of vegetation, thereby resulting in a reduction of crop yield, which significantly affects the livelihood of farmers [7-8]. In addition, oil contamination may spread as petroleum hydrocarbons infiltrate into underground water systems or transfer with

flowing surface water due to soil erosions. Once the petroleum hydrocarbons enter the water system, an oil film will be formed on the surface, which may arrest or reduce gas exchange between the water body and the atmosphere, thus decreasing the amount of dissolved oxygen and shifting the pH value of the waterbody. Consequently, petroleum hydrocarbon pollution of topsoil may result in upsetting both soil and water environment and influence the normal activities of the relevant organisms [7,9]. Moreover, some ingredients in petrol, including benzene and aromatic compounds, are also recognized as environmental carcinogens, and have been found to exert both teratogenic and mutagenic effects on different organisms [1,10-11].

Currently, three main approaches, namely physical, chemical, and biological, are employed in the remediation of petroleum polluted soil [12]. Incineration, isolation, and absorption are the common conventional physical approaches used in remediating petroleum contamination. However, physical remediation often involves the use of heavy machines and is very costly, thus it is only suitable for the use in temporary treatments of small areas [13]. As for chemical treatment, dispersion, oxidization, and electrolysis are methods which are commonly employed to treat oil polluted soil. Nevertheless, as with physical approaches, chemical treatment is often costly and contains potential threats of secondary pollution which may exacerbate the pollution scenario [14]. With a research history of less than 30 years, compared with physical and chemical approaches, bioremediation is a relatively new approach in the treatment of petroleum hydrocarbons pollution in China [13,15]. Although this method is relatively new in the field of remediation, it has since gained wide acceptance as bioremediation results in the least disturbance to the ecosystems [13]. Bioremediation currently involves the utilization of plants, animals and/or microorganisms in treating polluted air, water bodies or soil [11,16]. Among these approaches, microbial bioremediation appears to be most promising due to its attributes in low cost, environmental friendliness and comparatively low demand of operational skills [17-18]. Bioremediation uses the metabolic capacity of microorganisms, namely by transporting the degradable proportion of petroleum across the membrane and activating enzymatic reaction to change it into harmless matters such as water and carbon dioxide [7,10].

Before its banning in most countries before 2000 due to its high toxicity, the heavy metal, lead (Pb), was commonly added to petrol for performance improvement via increasing the antiknock ability and octane number [19,20]. Therefore, combustion of petrol was a major source of lead contamination to the environment. Despite the Chinese Government has banned the production of leaded gasoline before the introduction of the China I (Euro I) vehicle tailpipe emission standards in 2000, lead content in surface soil remains considerably higher than

the permissible level in many parts of China (Table 1) [21,22]. The high level of lead detected in surface soil could have been the result to an overestimation of the effectiveness of phasing-out of leaded petrol, and an underestimation of the ever-increasing relative contributions from other potential sources of pollution, including coal combustion, industrial emissions of local Pb-ores, non-additive Pb contents of crude oils and lead contaminated fertilizers [23]. The presence of lead in soil not only threatens human and environmental health, but it may also affect the growth of petrol degrading microorganisms, thereby reducing bioremediation efficiencies of these microbes [24].

Table 1. The distribution of lead in surface soil reported in major Chinese cities. (adopted from Han et al. [22])

City	Lead Concentration (mg/kg)
Anyang	41
Lanzhou	29
Beijing	37
Yulin	21
Jiaozuo	31
Tianjing	121
Xining	33
Guangzhou	57

Due to the wide distribution of oil and lead in the environment, together with their high toxicity, it is of imminence for us to expand our knowledge in bioremediation using native petroleum degrading microorganisms with high lead tolerance. This study aimed to (i) isolate and characterize petrol degrading soil microorganisms collected from the Greater Bay Area, and (ii) evaluate lead tolerance of the isolated bacterial isolates. As no research has considered the joint pollution scenario of lead and petrol in the environment while the scenario is highly probable due to rapid industrialization and urbanization, results of this study would be of high ecological significance and provide insights into the applicability of these bacterial isolates in the actual environment.

2. Material and Methods

Soil Sample Collection

Soil samples were collected in contaminated sites in Zhuhai, Guangdong, China, from July to August, 2020. In brief, approximately 200g of topsoil samples were collected in depth between 2 to 15cm from four selected locations in Zhuhai with known history of petroleum pollution. Soil samples were brought back to the laboratory immediately after collection from selected sites, sieved by 2mm pore size sieves, placed in aseptic bags

and stored at 4℃ for further analysis [25-28].

Enrichment and Identification of Petrol Degrading Bacteria

In brief, 5g soil samples were incubated with 250mL Minimal Salt Medium (MSM) (3.5 g/L $Na_2HPO_4 \cdot H_2O$, 1 g/L KH_2PO_4, 0.5g/L NH_4Cl, 0.1 g/L, $MgCl_2 \cdot 6H_2O$, 0.05 g/L $Ca(NO_3)_2 \cdot 4H_2O$. 1ml/L Trace Element Solution SL-4, pH 7.4) with shaking at 200 rpm for 10 days at room temperature. 2g of sterilized filtered petrol was added to 250 mL MSM as the sole source of carbon and energy. Spread plating technique was applied to further inoculate the isolates onto MSM plates (MSM supplemented with agarose) with petrol (5 g/L) as the sole source of carbon and energy. By examining and collecting the single colonies and further inoculating them on MSM agar for enrichment and on nutrient agar for final isolation and characterization. Selected strains with different colony morphology were identified by biochemical analyses and subsequently sent to Shenzhen National Genebank for 16S rRNA gene sequencing.

Biodegradation of Petrol

$1*10^9$ cells of each bacterial isolate were transferred to 100mL MSM supplemented with 2g sterilized petrol as the sole source of carbon and energy to determine the petrol degrading rate in a five-day study period. In addition, cell growth was determined by OD600 at every 24 hours. After 5 days of incubation, the remaining petrol in MSM was extracted by petroleum ether and the absorbance of extracted petrol was determined under a UV-visible spectrophotometer at optimal wavelength (214nm). The degradation rate of each bacterial isolate was determined by Formula 1:

$$R_i = [M_0 - (M_E - M_i) * r]/ M_O * 100\% \quad \text{(Formula 1)}$$

Where R_i represented the degrading rate of the ith bacteria; M_E represented the actual amount of petrol remained in the evaporation group (minus bacteria) at the end of the experiment; M_i represented the amount of petrol remained in the ith bacteria culture medium at the end of the experiment; r represented the recovery rate; M_0 represented the initial weight of the petrol in MSM before the start of the experiment.

Biodegradation of Petrol with Lead Exposure

The effects of lead exposure on bacterial growth and petrol degrading rates were determined by adding $1*10^9$ cells to the petrol containing MSM supplemented with 1mg/L, 5mg/L, 10mg/L, or 20mg/L Pb^{2+} and using the same method as mentioned above (Formula 1).

Statistical Analysis

One-way analysis of variance was used to test the null hypotheses that exposure to different concentrations of lead did not cause significant changes in petrol degradation. If a significant difference ($p \leq 0.05$) between the control and lead treatment groups was identified, pairwise comparisons between each individual treatment and control group were carried out using the Holm-Sidak method. In cases where data failed to follow Gaussian distribution, log_{10} or arcsin squart root transformation would be performed prior to analysis. Statistics were performed using the statistical software SigmaPlot, Version 12 (Systat, USA). Graphs were plotted using Graphpad Prism, Version 7.00 (Graphpad Software, Inc.).

3. Results and Discussion

Identification of the Screened Bacteria

After screening and isolation, colonies with distinct morphology were subjected to species identification. Two promising bacterial strains, namely B-7 and B-10, were selected due to their fast growth rates in MSM with petrol as the sole carbon and energy source. The colonies of B-7 in MSM agar were translucent with glistening surface, in the form of punctiform with convex elevation and entire margin (Fig. 1a). B-10 colonies were opaque in colour, irregular, with circular elevation and undulate margin (Fig. 1b). B-7 was observed to be a Gram-negative rod-shape bacillus (Fig. 2a), while B-10 was a Gram-positive coccobacillus (Fig. 2b). By 16S rRNA sequencing and biochemical analyses, B-7 was found to show the highest homology to *Achromobacter denitrificans*; while B-10 was found to have 99.71% sequence homology to *Mycolicibacterium phocaicum* N4. Similar strains of *A. denitrificans*, such as *A. denitrificans* strain SP1, *A. denitrificans* ASU-035, *A. xylosoxidans* subspecies *denitrificans* strain EST4002 and *A. denitrificans* strain TB, have recently been identified to be able to degrade di(2-ethylhexyl)phthalate, pyrene, 2, 4-dichlorophenoxyacetic acid and reduce nitric oxide, respectively [29-32]. Although the genera *Mycolicibacterium*, such as *M. mucogenicum, M. frederiksbergense IN53, M. vanbaalenii*, have been reported to be able to degrade methy *t*-butyl ether, polychlorinated biphenyls and petroleum hydrocarbons [33-35], the strain *M. phocaicum* N4, which shows 99.71% sequence homology to the isolated B-10 strain in this study via 16S rRNA sequencing technique, has recently been isolated from human bronchial aspirate from patients showing tuberculosis symptoms [36].
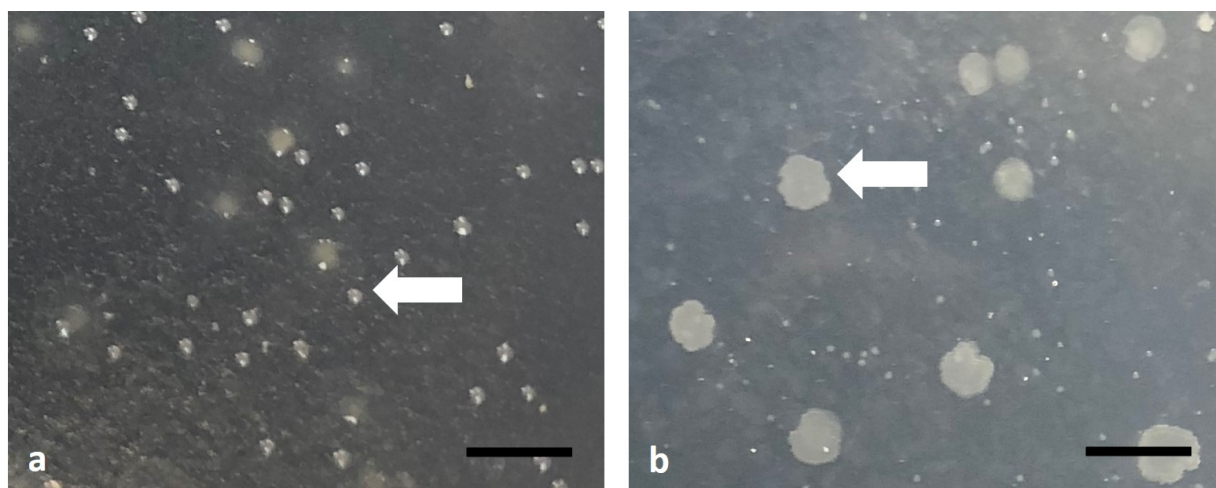
Figure 1. Colony morphology of (a) B-7 and (b) B-10 isolated from MSM enriched with petrol as the sole source of carbon and energy. Scale bar = 1cm.
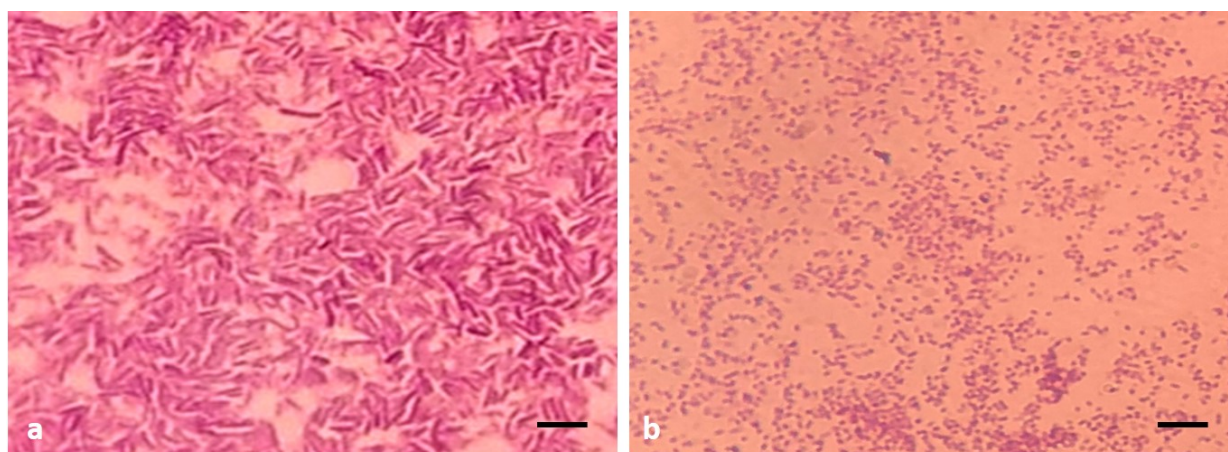

Figure 2. Results of Gram staining. (a) B-7 is a Gram-negative, rod-shaped bacterium whereas (b) B-10 is a Gram-positive, coccobacillus. (magnification x1000; scale bar = 5 mm).

Determination of Petrol Degradation Rates of B-7 and B-10 Bacterial Isolates

After considering the amount of background evaporation and recovery rates, the degradation rates of petrol with lead concentrations ranging from 0 to 20ppm were determined on the two bacterial isolates, B-7 and B-10. As shown in Table 2 and Fig. 3, over a 5-day study period, in the absence of lead, both strains demonstrated similar petrol degradation rate of approximately 12% (i.e., 2.4 g/L/5 days). Bacterial isolate B-10 demonstrated higher tolerance to lead as significant inhibition in its petrol degradation rate was only observed when lead was present at 5 ppm or higher, while significant inhibition in petrol degradation was observed in B-7 with 1ppm lead exposure.

Table 2. Petrol degrading rates of B-7 and B-10 over a 5-day study period under different lead concentrations (g petrol/L/5 days)

Lead concentration	B-7 (g/L)	B-10 (g/L)
0ppm	2.41 ± 0.00	2.43 ± 0.01
1ppm	1.48 ± 0.08**	2.00 ± 0.08
5ppm	0.14 ± 0.12**	1.35 ± 0.62**
10ppm	-0.02 ± 0.28**	0.19 ± 0.00**
20ppm	-0.05 ± 0.09**	-0.18 ± 0.32**

Note: Asterisks (**) indicates significant difference ($p < 0.001$) was detected between the lead treatment group and the control (0 ppm).
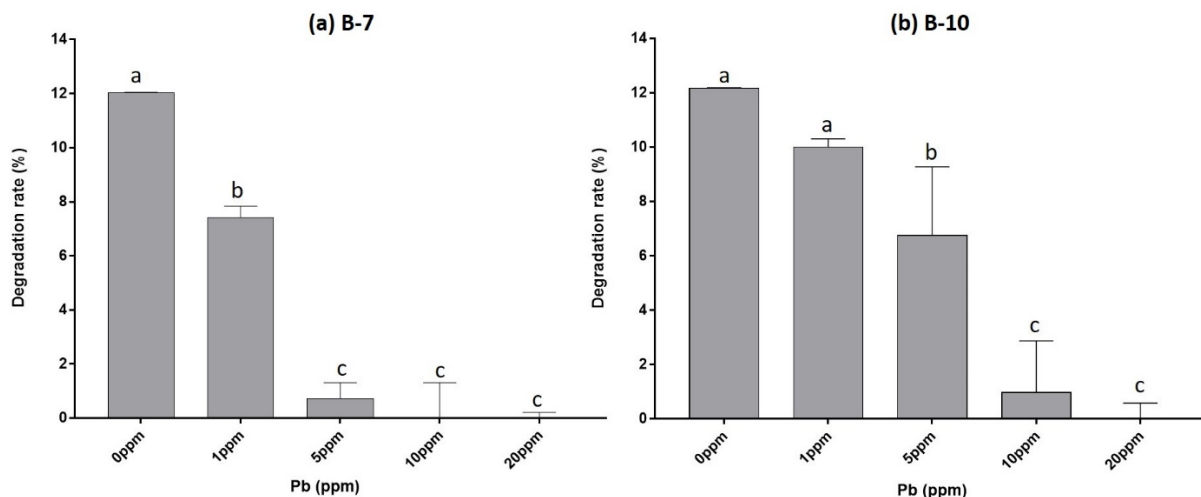
Figure 3. Petroleum degradation rates of bacterial isolate (a) B-7 and (b) B-10 upon exposure to 0, 1, 5, 10 and 20 ppm lead for 5 days. Data are expressed as mean ± S.D. (n=3). Bars (i.e., the control (0 ppm) and different treatment groups (1, 5, 10, 20 ppm) marked with the same English letter are not significantly different from each other ($p > 0.05$).
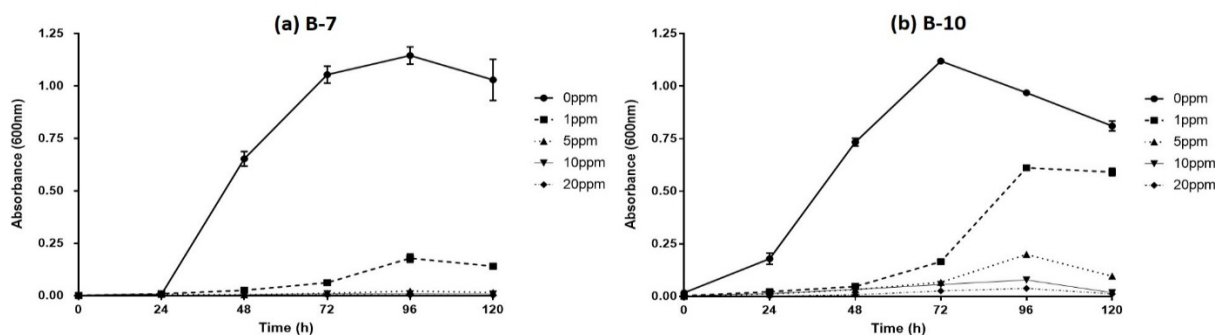

Figure 4. Growth response curves of (a) B-7 and (b) B-10 upon exposure to 0, 1, 5, 10 and 20ppm Pb for 5 days. (n=3)

Growth Response of B-7 and B-10 under Lead Exposure

Higher tolerance of B-10 to lead was also observed in the growth response curve study. Exposure to 1ppm lead resulted in over 80% growth reduction in the B-7 culture (Fig. 4a) while less than 30% reduction in growth was observed in the B-10 culture under identical exposure regime (Fig. 4b). Cell growth was completely abolished at 5ppm lead for B-7 and 10ppm for B-10. Although Shi et al. [37] reported that the average lead concentrations in agricultural soil in China were around 50 mg/kg. Although significant inhibitions in growth and petrol degrading rate were observed at 1 ppm for B-7 and 5 ppm for B-10 this study, however, since the physico-chemical properties of soil are very different from that of the liquid medium, particularly in the accessibility and availability of lead ions, further studies should be carried out in simulated soil environment for more accurate and environmentally realistic results.

4. Conclusions

Two petroleum degrading bacteria, B-7 and B-10 were

screened, enriched and identified in this study. The bacterial isolate, B-7, was a rod-shaped, Gram-negative bacterium showing high similarity to *Achromobacter denitrificans*, while B-10 was a Gram-positive coccobacillus showing 99.7% sequence homology to *Mycolicibacterium phocaicum* N4. Both strains exhibited petrol degradation rate of 2.4 g/L during a 5-day study period. Significant inhibition in growth and petrol degradation rate was observed in B-7 and B-10 at 1ppm and 5ppm lead, respectively, indicating B-10 exhibited higher tolerance to lead exposure. Unfortunately, since *M. phocaicum* N4 may be a potential human pathogen, cautions should be taken in future studies when considering B-10 as potential candidate for petrol remediation. Due to the wide range of organic pollutants that *A. denitrificans* can biodegrade and its absence of pathogenicity, B-7 appears to be a more promising candidate for the remediation of soils polluted by organic pollutants. Further studies should be conducted to improve the lead resistance of B-7, i.e., continuous cultivation of B-7 in the presence of lead ions in MSM having petrol as its sole source of carbon and energy may increase its tolerance to lead and petrol degradation efficiency. In addition, future experiments should be

carried out in simulated soil environment as the complexity of soil may directly affect heavy metals bioavailability. Investigations on the effects of abiotic factors, such as pH, temperature, moisture contents, nutrient levels and co-contamination with other pollutants on the petrol degradation efficiency of B-7 and B-10 should also be conducted in the future. Finally, microbial ecological studies should also be performed to evaluate the effects of microbial consortium on petrol degradation so as to provide environmentally realistic information for future applications of these bacterial isolates in contaminated sites *in situ*.

Acknowledgement

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