Pilot-scale field studies on activated microbial remediation of petroleum contaminated soil

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Keywords: Microbial remediation, Petroleum contaminated soil, In situ bioremediation, Biodegradation, Bioaugmentation, Microbial activator

Posted Date: July 24th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3181844/v1

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Abstract

A simple and cost-effective microbial remediation process has been developed for the treatment of petroleum contaminated soil by adding microbial activators to active the native microorganisms for enhancing the degradation of petroleum hydrocarbon. The microbial activator is formulated to provide nitrogen sources, phosphorus sources, trace elements, growth factors, biosurfactants, and soil pH regulators. The field trials, involving two 500 m$^3$ oily soil samples with initial oil content of 5.01% and 2.15%, respectively, show that the petroleum hydrocarbon content can be reduced to 0.41% and 0.02%, respectively, in 50 days, reaching the national standard of cultivated land category II. The treatment period is significantly shorter than the commonly used composting and bioaugmentation methods. The remediation effect of microbial activator on oily soil was investigated through the germination experiment of rye seeds. The results showed that the activator itself could not only activate the functional microorganisms in the soil, but also reduce the biological toxicity of oily soil. After 40 days of treatment, the germination rate of rye seeds increased from 20–90%, indicating that the microbial activator could be effectively used for rapid in-situ remediation of oil contaminated soil.

1. Introduction

Microbial remediation of pollutants generally refers to the use of natural or artificially cultivated functional microorganisms to transform pollutants either completely to CO$_2$ and H$_2$O (for organic pollutants) or into less harmful substances (Tekere et al., 2019; Mishra et al., 2001; Silva-Castro et al., 2013). Such bioremediation approach has been widely accepted as a technique for decontaminating a polluted environment including air, water and soil owing to its environmentally friendly and sustainable nature (Sharma et al., 2022; Tiwari et al., 2020; Chen et al., 2020). When it is applied in the microbial remediation of petroleum contaminated soil (also referred to as oily soil), the polluting petroleum hydrocarbons are used as the carbon source for microbial metabolism to degrade the pollutants into less toxic forms. Additionally, the use of pollutants as the carbon source provides a promising route to on-site, or in situ remediation of petroleum contaminated soil (Mafiana et al., 2021; Singh et al., 2022). As a result, the degradation or removal of the pollutants can improve the soil ecosystem by effectively reducing the toxicity, an important evaluation index of soil health status (Saterbak et al., 2000). For soil bioremediation, there are three main types of microorganisms employed, according to their sources, namely, indigenous, exogenous and genetically engineered microorganisms (Hamidi et al., 2021; Moliterni et al., 2012; Yaashikaa et al., 2022). Indigenous microorganisms are a group of innate microbial consortium that are native to the soil environment they inhabit. After the soil is polluted, some specific indigenous microorganisms in the soil can respond and produce an enzyme system in order to degrade and/or transform the pollutants under the induction of such pollutants (Mishra et al., 2001; Sharma et al., 2022). Although indigenous microorganisms widely exist in the soil, their growth rate and metabolic activity are mostly low. The number of indigenous microorganisms may reduce significantly mainly due to the stress of the pollutants (Liu et al., 2010). Therefore, it is sometimes necessary to introduce from the external world highly effective bacteria, i.e. exogenous microorganisms, to the closed biological system in
order to enhance the soil bioremediation process. However, the inoculation of exogenous microorganisms can be affected by the competition of indigenous microorganisms, that necessitates the inoculation of a large number of microorganisms in order to form a dominant flora (Hamid et al., 2020; Karamalidis et al., 2010). The whole process also involves multiple steps including strain screening, domestication, scale-up fermentation, transportation and on-site construction, leading to increased treatment costs. In recent years, the advancement in genetic engineering has enabled the provision of highly efficient genetic engineered bacteria which has attracted attention from researchers worldwide. However, due to challenges in terms of adaptability, biosafety and environmental concerns, its practical application is still limited (Liu et al., 2019; Singh et al., 2011).

Currently, there are a range of microbial remediation technologies investigated for decontaminating oily soil, mainly including tillage, composting and bioaugmentation (Ventorino et al., 2019; Aguelmous et al., 2019; Koolivand et al., 2020; Obi et al., 2020; Ke et al., 2021). For example, the most commonly used tillage and composting methods mainly involve the addition of inorganic or organic fertilizers and leavening agents to the soil, combined with tillage aeration and watering to achieve the degradation of petroleum hydrocarbons by indigenous microorganisms in the soil. However, this treatment process has been found to be very slow. For instance, in a two-step composting process to repair oil-bearing soil with petroleum hydrocarbon content of 1%-5%, it took 16 weeks to achieve the petroleum hydrocarbon removal rate of 67.64%-89.56% (Poorsoleiman et al., 2020). Microbial augmentation or bioaugmentation involves the addition of exogenous microorganisms in order to speed up the rate of degradation of contaminants. As discussed above, this process with the introduction of exogenous microorganisms suffers from drawbacks including process effectiveness mainly due to the competition between indigenous and exogenous microorganisms, and process complexity (Obi et al., 2020; Ke et al., 2021; Qu et al., 2022). As a result, the large-scale industrial application of such technologies in the field for effective soil remediation is still limited largely due to their slow treatment and operational impracticalities (Mridul et al., 2021).

In the petroleum contaminated soil there mostly are abundant bacteria which are capable of degrading petroleum hydrocarbons. Due to the lack of balanced nutrients necessarily required for the growth and reproduction of microorganisms, however, these bacteria mostly are insufficiently active thus inefficient for degrading oil (Nwaichi et al., 2015; Cai et al., 2018; Wang et al., 2017). Therefore, there has been research effort to activate the indigenous bacteria in the oil contaminated soil to induce the degradation of the contaminating petroleum hydrocarbons in situ (Xu et al., 2019; Yamazaki et al., 2011; Zhang et al., 2013; Zhang et al., 2013). Specifically, by introducing reagents as microbial activators, the indigenous microorganisms activation strategy can not only eliminate the competition between native and foreign bacteria, but also effectively reduce the treatment cost by improving the whole bioremediation process. However, there are few studies and reports on this technology.

In our previous work, we pioneered the method of formulating and introducing microbial activators to the indigenous microorganisms environment in order to provide appropriate nutrient ratio for microbial growth, quickly activate indigenous microorganisms in oily soil, and enhance the bacteria’s capability of
degrading petroleum hydrocarbons and other indigenous bacteria's activities to co-metabolize and synergize petroleum hydrocarbon degradation.

The aim of this study was to evaluate the bioremediation process assisted by microbial activators through the enhancement of petroleum hydrocarbon degradation on a large scale. The effects of the addition of microbial activators was compared with the introduction of exogenous microorganisms, in terms of bacterial growth, oil degradation and toxicity reduction. Furthermore, pilot-scale field studies were carried out using two 500 m$^3$ oily soil samples with initial oil content of 5.01% and 2.15%, respectively. The contaminated soil left over from the past was treated on-site in Well Pad Liu 64 – 04 of Dingbian No. 9 Oil Extraction Plant.

2. Materials and methods

2.1. Materials

All chemicals and reagents were obtained commercially and used as received without further treatment. The biochemical reagent kits were purchased from Beijing Leadman Biochemical Ltd, China. Glucose, peptone, yeast extract, urea (CH$_4$N$_2$O), ammonium sulfate, potassium dihydrogen phosphate (KH$_2$PO$_4$), dipotassium hydrogen phosphate (K$_2$HPO$_4$), ammonium nitrate (NH$_4$NO$_3$), magnesium sulfate (MgSO$_4$), calcium chloride (CaCl$_2$), sodium citrate and sodium chloride were purchased from Tianjin Tian Da Chemical Factory, China. Bran was purchased from Hunan Huinong Technology Co., Ltd. Other reagents used in this study were analytical grade, and the water was deionized.

Instrument and apparatus used in this study included GC-MS (Agilent 6890N-5975, Agilent), automatic infrared oil detector (FLY6800, Beijing Fly Seth Technology Co., Ltd), Constant temperature shaking table (TY-70B, Suzhou Jiangdong Precision Instrument Co., Ltd), Biochemical incubator (SPX- 250BIII, Tianjin Taisite), Automatic fermenter (RZY-SJB-50L, Naijing Runzhe Biology Engineering Facility Co., Ltd.) and digital pH meter (PHSJ-3F, Rex Electric Chemical).

Samples of oil contaminated soil (or oily soil) were collected with plastic containers (1 L capacity) from Well Pad Liu 64 – 04 of Dingbian No. 9 Oil Production Plant (located in Shaanxi Province, China), and analyzed before experiments. The oily soil samples were milled to pass through a 2-mm sieve and kept at 4 °C for within 48 h before use in experiments.

The microbial activators (Bran 60%, modified bentonite 10%, Urea 10%, calcium peroxide 5%, calcium hydrogen phosphate 4%, sodium glutamate laurate 3%, rhamnolipid 3%, magnesium sulfate 2%, ferrous sulfate 2%, manganese chloride 0.5%, zinc chloride 0.5%) used in the experiment were formulated nutrients, mainly containing nitrogen sources, phosphorus sources, trace elements, growth factors, biosurfactants, soil pH regulators and volatile petroleum hydrocarbon collectors. The formulation was optimized especially the nutrient ratio and composition. In addition, the introduction of petroleum hydrocarbon trapping agents in the formula provided a means to immobilize and trap the volatile
petroleum hydrocarbons in the soil, and also reduce the potential secondary pollution during the process of cleaning, mixing and tillage of contaminated soil.

The exogenous microorganisms introduced was a complex microbial community composed of Proteus mirabilis, strain Luteimonas huabeiensis sp., Acinetobacter and Candida tropicalis in equal proportions, that was showed to have the ability of quickly emulsify and degrade petroleum hydrocarbons (Ke et al., 2021, 2018; Sun et al., 2022).

2.2. Experimental methods

2.2.1. Analysis of indigenous microbial community

After the total DNA of the oily soil sample was extracted, the primer was designed according to the conservative region. A sequencing connector was added at the end of the primer, and PCR amplification was performed. The products were subsequently purified, quantified and homogenized to form a sequencing library. The library quality inspection was carried out with the constructed library first, and then the qualified library was sequenced with Illumina Novaseq 6000. The original image data files obtained by high-throughput sequencing (such as Illumina Novaseq and other sequencing platforms) were converted into original sequenced reads through base calling analysis (Wu et al., 2013).

2.2.2. Determination of bacterial density in oily soil

5 g oily soil sample was added to 95 g sterile water. Following 5 min fully stirring and then 5 min standing, the upper suspension was taken to determine the bacterial density by the method of a serial dilution \(10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}\) and \(10^{-8}\) on agar plates, where the bacterial density was determined by a flat colony counting method (Baron et al., 2006). Each dilution was plated in triplicate on a nutrient agar plate and incubated at 37 °C for 24 h. The number of CFU at each dilution rate was counted after incubation, and the average bacterial density (CFU/g) was determined.

2.2.3. Determination of petroleum hydrocarbon content in oily soil

The oily soil sample was firstly dried at 50 °C in oven. The oil in 2 g Sample was extracted with 20 mL petroleum ether under ultrasonic stirring for 4 times. The extraction solution was then dehydrated with anhydrous sodium sulfate, and filtered to a constant volume of 100 mL. Finally, the solution’s absorbance was measured at the maximum absorption wavelength with petroleum ether as the reference, and converted to oil concentration according to the standard calibration curve. By comparing the initial and residual oil concentrations, the oil degradation rate (%) was obtained (Eq. 1)

\[
\text{Degradation rate} \% = \frac{(C_0 - C_d)}{C_0} \times 100\% (1)
\]

Where \(C_0\) is initial oil concentration, mg/L; \(C_d\) is residual oil concentration after degradation, mg/L.
2.2.4. Germination test of bioremediated oily soil (Zhang et al., 2013)

2g Oily soil sample was mixed with 20 mL sterile water in a Centrifuge tube, and shaken for 1 h. Then it was centrifuged at 6000 r/min for 15 min., and the supernatant was put into the culture dish covered with filter paper. Finally, 20 intact rye seeds were evenly placed on the filter paper, covered with breathable film and placed in the biochemical incubator for cultivation at 20 °C. During the culture, sterile water was refilled every 10 h and the number of germinated seeds were counted over 48 h. The test was run in triplicate and the results were averaged of three independent experiments. The average germination rate $\mu$ (%) was determined by Eq. 2.

$$\mu = \frac{N}{N_0} \times 100\% \quad (2)$$

where N is the number of germinated seeds and $N_0$ is the total number of seeds placed.

2.2.5. Comparison of different formulas for microbial activated bioremediation of petroleum contaminated soil

The oily soil was crushed and sieved (5mm) first. Four samples (namely, Exp 1, Exp 2, Exp 3 and Control) were prepared for comparison, containing 500 kg oily soil each mixed with the additional components (wt.% of oily soil), as summarized in Table 1. The selected formulas were based on the laboratory work for optimization. The purpose of adding sawdust was to increase the porosity of soil as a bulking agent, and provide an additional carbon source.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Oily soil (kg)</th>
<th>Sawdust (%)</th>
<th>Microbial activator (%)</th>
<th>CMC (%)</th>
<th>Other addition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp 1</td>
<td>500</td>
<td>2%</td>
<td>2%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Exp 2</td>
<td>500</td>
<td>2%</td>
<td>2%</td>
<td>2%</td>
<td>-</td>
</tr>
<tr>
<td>Exp 3</td>
<td>500</td>
<td>2%</td>
<td>-</td>
<td>2%</td>
<td>0.5% urea; 0.2% KH$_2$PO$_4$</td>
</tr>
<tr>
<td>Control</td>
<td>500</td>
<td>2%</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

After the sample was mixed thoroughly, it was piled up (1.0 m × 0.8 m × 0.4 m) on a piece of plastic sheet put underneath. Then water was added with an amount of 20% of the soil mass. For keeping heat and moisture, straw curtains were covered on the top of the sample. The samples were kept outside door under natural conditions for 60 days. During the process, these mounds were turned over every 5 days, and water was added to maintain the content at 20%-25%. Throughout the process, changes in bacterial concentration and petroleum hydrocarbon content in the soil were monitored.
2.2.6. Field trials

For field trials, the petroleum contaminated soil, generated during the previous oil production process in Well Pad Liu 64 – 04 of Dingbian No. 9 Oil Extraction Plant, was treated on site with the bioremediation process in the field. Firstly, the oily soil was excavated, crushed and screened. Then, 2% microbial activator and 2% sawdust were added to the oily soil. Upon fully mixing, the mixture was placed on a plastic sheet (5 m long × 25 m wide) laid on the ground with a sample thickness of 50–80 cm, having a total volume of 500 m$^3$. Care was taken when piling the sample to avoid additional compaction. Then, water sprayed from the top of the soil pile and controlled the water content to be 20% of the total soil mass. Finally, the surface of the soil pile was covered with straw curtains for retaining heat and moisture. During the bioremediation process, the temperature in the soil pile was monitored to be 30–40 °C and the pH, at 6.5–7.5, while the oxygen distribution within the pile was reasonably uniform (varying less than 7%). Sampling and analysis were carried out every 5 days over the 2-month experiment to monitor the process for changes in bacteria concentration, oil content and pH.

3. Results and discussion

3.1. Characterization of petroleum contaminated soil

The chemical and microbiological properties of the oily soil used were characterized before the bioremediation experiments, and the results are presented in Table 2. It was found that the oily soil contained a very high concentration of total petroleum hydrocarbon (TPH) together with low contents of water heavy metal ions. The TPH content was 51.23 g/kg, including 45.18% saturated hydrocarbons, 31.25% aromatic hydrocarbons, and 23.57% of resins and asphaltenes. The water content was 8.5%. Compared to the “Soil environmental quality - Risk control standard for soil contamination of development land” (GB36600-2018), some important indicators were found to be over or close to the standard limits, in particular for TPH, water and benzo[a]pyrene, whilst the contents of heavy metal ions were lower than the reference values. Nevertheless, the results suggested that the petroleum contaminated soil must be effectively treated before disposal or further utilization.
Table 2
Chemical and microbiological characteristics of the oily soil

<table>
<thead>
<tr>
<th>Parameter (unit)</th>
<th>Content measured</th>
<th>Reference value a</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPH (g/kg)</td>
<td>51.23</td>
<td>≤ 4.5</td>
</tr>
<tr>
<td>Water content (wt.%)</td>
<td>8.5</td>
<td>60</td>
</tr>
<tr>
<td>pH</td>
<td>7.10</td>
<td>2.0-12.5</td>
</tr>
<tr>
<td>Salinity (mg/L) b</td>
<td>1047</td>
<td>/</td>
</tr>
<tr>
<td>N (g/kg)</td>
<td>59.2</td>
<td>/</td>
</tr>
<tr>
<td>P (g/kg)</td>
<td>7.8</td>
<td>/</td>
</tr>
<tr>
<td>Copper (mg/kg)</td>
<td>218.25</td>
<td>18000</td>
</tr>
<tr>
<td>Cadmium (mg/kg)</td>
<td>12.58</td>
<td>65</td>
</tr>
<tr>
<td>Nickel (mg/kg)</td>
<td>84.75</td>
<td>900</td>
</tr>
<tr>
<td>Chromium (mg/kg)</td>
<td>4.46</td>
<td>5.7</td>
</tr>
<tr>
<td>Arsenic (mg/kg)</td>
<td>15.45</td>
<td>60</td>
</tr>
<tr>
<td>Mercury (mg/kg)</td>
<td>0.02</td>
<td>38</td>
</tr>
<tr>
<td>Lead</td>
<td>116.38</td>
<td>800</td>
</tr>
<tr>
<td>Benzo[a]pyrene (mg/kg)</td>
<td>1.45</td>
<td>1.5</td>
</tr>
<tr>
<td>Bacteria (CFU/g)</td>
<td>2.5 ×10^5</td>
<td>/</td>
</tr>
</tbody>
</table>

a Reference values - “Soil environmental quality - Risk control standard for soil contamination of development land” (GB36600-2018). b The values of salinity were measured by leaching 10 g soil with 100 mL solution while stirred and soaked for 24 h.

As seen in Table 2, the content of TPH in the soil was significantly high at 51.23 g/kg. That was mainly the result of long-term deposition and accumulation of petroleum contamination in the area. In addition, the covering of the surface of soil particles by oil turned the soil surface to be strong hydrophobic. Furthermore, this hydrophobicity made it difficult for water to be absorbed by the soil particles, resulting in a very low water content in the soil of 8.5%.

The contents of total nitrogen (N, 59.2 g/kg) and total phosphate (P, 7.8 g/kg) in the oily soil sample were relatively very low compared to carbon content. It is generally understood that the lack of N and P in nutrients can limit the TPH biodegradation, and a C/N ratio ranging between 25 and 30 was found to be suitable for enhancing the biodegradation (Wu et al., 2017). However, the C/N/P ratio in the soil sample was found to be greater than 100/5/1. By taking into account the function of P, the addition of both N
and P nutrients was also needed for stimulating growth of exogenous and/or indigenous microorganisms (Devlin, 2007; Ma et al., 2016).

A low number of microorganisms in the soil was also observed, i.e. $2.5 \times 10^5$ CFU/g. That can be attributed to the findings of the high level TPH content and great C/N/P ratio. Thus, it became clear that in order to facilitate bioremediation through activation of microorganisms in soil, it was necessary to supplement the N and P sources needed by microorganisms, and modify the soil wettability for supplementing water.

3.2. Effect of activators on native microbial community

To examine the activation effect of the activator on the native microbial community, the microbial community structure in the oil contaminated soil was analyzed by high-throughput sequencing. The changes in the microbial community structure before and after activation are shown in Fig. 1.

It can be seen from Fig. 1 that prior to the introduction of microbial activator, the dominant bacteria groups in the oily soil within this oilfield area were mainly *Nocadioids, Sphingomonas, Ochrobactrum, Micrococcaceae, Burkholderia* and *Bacteroidia*, accounting for 82% of the total number of bacteria in the microbial community. There were 14 bacterial genera accounting for more than 0.1%, among which there were 5 types of microorganisms with petroleum hydrocarbon biodegradation function (names in red). *Nocadioids* and *Ochrobactrum* were common petroleum hydrocarbon degrading bacteria, accounting for 31% and 12% respectively. In addition, there were other petroleum hydrocarbon degrading bacteria, including *Acinetobacter, Pseudomonas* and *Arthrobacter*, though the abundance of these bacteria appeared relatively low. The results indicated that the oil contaminated soil has over a long term produced characteristic functional bacteria coupled with the soil environment of the oilfield area during the long-term underground process, showing a certain degree of environmental bioremediation response.

After 7 days of treatment with the microbial activator, the microbial community structure in the soil changed significantly. Specifically, the number of petroleum hydrocarbon degrading bacteria *Acinetobacter, Arthrobacter* and *pseudomonas* bacteria increased remarkably, from 1.5%, 0.25% and 1–32%, 17% and 9%, respectively, whilst their numbers increased though the percentages of *Nocardioides* and *Ochrobactrum* decreased relatively. In addition, some new petroleum hydrocarbon degrading bacteria emerged including *Saccharomyces* and *Rhodococcus*. As a whole, the number of activated petroleum hydrocarbon degrading bacteria increased significantly and became the dominant group, accounting for more than 78%. Moreover, the total number of bacteria in oily soil increased from $10^5$ to $10^9$ CFU/g after 7 days microbial activation, which indicated that the activator could not only rapidly increase the numbers of native microorganisms in soil, but also significantly change the microbial community structure while selectively activate some functional bacteria in soil.

Due to the long-term petroleum hydrocarbon pollution, some microorganisms (i.e. oleophobic bacteria) in the soil were unable to adapt to the polluted soil environment, thus their numbers gradually decreased. On the other hand, other indigenous microorganisms were capable of using carbon, nitrogen and other components of the polluting oil as nutrients for growth and metabolism, while producing metabolites
such as biosurfactants and biological enzymes which conducive to the degradation of petroleum hydrocarbons (Aguelmous et al., 2019). However, due to the lack of balanced nutrients including low contents of N and P in the oil-polluted soil, the total number of bacteria was still small. When the activator was added to the oily soil, the N source, P source, growth factor and microelements in the activator promoted the rapid growth of functional microorganisms especially that took petroleum hydrocarbons as the only carbon source. At the same time, the number of other indigenous microorganisms being unable to degrade petroleum hydrocarbons had insignificant changes, thus resulting in significant increases in the percentages of petroleum hydrocarbon degrading bacteria in the microbial community upon activation. This showed the potential of the native indigenous bacteria for degrading petroleum hydrocarbons in the oil contaminated soil when selectively activated by microbial activators.

3.3. Comparison of different formulas for microbial activated bioremediation of petroleum contaminated soil

In order to evaluate the effects of microbial activators and exogenous microorganisms on the remediation of oily soil, three formulas were investigated together with a control experiment for comparison, as detailed in Table 1. The changes in the number of microorganisms and the residual oil content were measured under identical operational conditions. The results are depicted in Figs. 2&3.

It can be seen from Fig. 2 that as expected the total bacterial density in all three samples (Exp 1 - Exp 3) increased in two stages, i.e., a rapid rising (at different rates) followed by leveling off. In Exp 1, where 2% activator and 2% bulking agent were added to the oily soil, total bacterial density increased rapidly with time, and reached $10^{11}$ CFU/g on the 7th day. That was clear indicative that the native microorganisms in the soil were effectively activated by the activator which provided a set of necessary nutrient supplements including N and P sources lacked in the oily soil. In Exp 2 with an additional of 2% CMC to Exp 1, the density of microorganisms appeared to be high at beginning, that was $10^8$ CFU/g on the first day that was largely due to the addition of exogenous microorganisms. The bacterial density also increased with time to $10^{11}$ CFU/g on the 7th day. Following that the density remained relatively stable at the level similar to Exp 1, further supporting the existence of competition between indigenous and exogenous microorganisms which hindered further increase in total bacterial density upon addition of exogenous microorganisms (Hamid et al., 2020; Karamalidis et al., 2010).

In Exp 3 without microbial activators, where 2% of CMC together with N and P supplements were added to the oily soil, the bacterial density rising trend in the first stage was found to be similar to that in Exp 2, increasing from $10^8$ CFU/g on the first day to $10^{11}$ CFU/g on the 7th day. That was likely attributed to the addition of N and P sources which needed for microorganisms growth thus showing a similar effect to that of microbial activator. In contrast, from Day 8 the bacterial density started to drop gradually and on Day 50 reached the lowest level of $10^9$ CFU/g which was followed by a bouncing back. The bacterial density drop was likely due to the lack of other necessary nutrient supply in a sustainable way. The final bouncing back in bacterial density following its decline might be related to the induction time needed for the exogenous microorganisms to adapt to the environment, however, the exact causes remained to be
further investigated. Overall, the results showed clear effect of microbial activator on microorganism growth providing a simple and easy-to-operate way for enhancing bioremediation activities.

As a result, the different formulas had different effect on the petroleum hydrocarbon degradation process in oily soil, as shown in Fig. 3. Overall the TPH content gradually decreased with time in all three formulas added tough to different extent, while kept almost unchanged in Control. The general trend of decline also showed two stages, i.e., a rapid approximately linear drop within the first week followed by a lower declining rate. In Exp 1, TPH decreased from 5.01–3.75% on Day 7, and to 0.3% after 55 days treatment. The final degradation reached over 94%, well meeting the agricultural land standard (< 0.45%) (GB 36600 – 2018). This corresponded to the rapid increase in bacterial density with the microbial activator added (Fig. 2). The oil degradation effect shown in Exp 2 was found to be approximately identical to that in Exp 1. As discussed above, although the initial bacterial density was significantly high in Exp 2 than that in Exp 1 owing to the introduction of exogenous microorganisms (Fig. 2), it had insignificant contribution to the overall oil degradation mainly due to the competition between indigenous and exogenous microorganisms (Fig. 3) (Hamid et al., 2020; Karamalidis et al., 2010).

In Exp 3 in the absence of microbial activator, it showed a similar oil degradation trend with time by adding exogenous degradation bacteria and supplementing N and P sources, but the degradation performance was less effective than that in Exp 1 and Exp 2. The TPH content in the oily soil decreased from 5.01–4.55% on Day 7, and after 55 days of microbial remediation, it decreased to about 0.8% with a degradation rate of 84.37%. In the control experiment, the degradation rate of TPH was only 8.2%, indicating that the degradation of TPH in soil was very slow under natural conditions.

In summary, the results showed that all three formulas introduced to the oily soil were able to degrade the contaminating petroleum hydrocarbons, while the addition of microbial activator performed more effectively than that with introduction of exogenous. This was of significance in large-scale industrial application for rapid remediation of petroleum contaminated soil by simply adding microbial activators to the oily soil to activate the native indigenous bacteria without the need for introducing exogenous microorganisms. That held great potential to not only greatly shorten the preparation and operation time for microbial remediation, but also reduce the cost in materials, equipment, and skilled labor.

### 3.4. Effect of bioremediation on reducing toxicity of petroleum contaminated soil

It is well understood that the petroleum contaminants have adverse effects on soil ecosystem throws special concern of researchers globally (Khan et al., 2016; Obida et al., 2018; Rodrigo et al., 2014). Therefore, the degradation of such petroleum hydrocarbons can be a direct way to reduce their toxicity leading to soil remediation ultimately, where seed germination ability is a direct indicator of the health status of soil ecosystems (Javaid et al., 2022). Seed germination is also considered as acute toxicity assay of various pollutants (Varjani et al., 2020). To characterize the effect of the bioremediation process on toxicity reduction in soil, the germination rate of grass seeds was measured over different time periods
with oily soil being treated with the three formulas (Table 1) along with Control for comparison. The results are shown in Fig. 4.

It can be seen from Fig. 4 that in the control experiment, the germination rate of grass seeds was low (<20%) while remained stable over time. That clearly indicated the significant toxicity of the oily soil mainly due to the high content of TPH in the soil. For all three formulas added (Exp 1, Exp 2 and Exp 3) all germination rates increased with time though to different extents.

After the addition of 2% microbial activator (Exp 1), the seed germination rate started to rise approximately linearly, reaching about 90% by Day 40. This suggested that the toxicity was significantly reduced. Even within a short period (i.e., the first 1–2 days)) a 50% germination rate was observed likely due to some reagents such as bentonite in the added microbial activator acting as petroleum hydrocarbon immobilizers, which can immobilize and capture volatile petroleum hydrocarbons in the soil (Shao et al., 2015). With the increase of microbial remediation time, germination rate raised accordingly that was attributed to the decrease in the content of TPH in soil (Fig. 3), reflecting reduction in biological toxicity of the oily soil. Exp 2 exhibited a similar behavior compared to Exp 1 in terms of both upward trend and germination level, that corresponded to the comparable TPH contents (Fig. 3) as a main contributor to biotoxicity. Similarly, relatively lower germination rates in Exp 3 compared to Exp 1 and Exp 2 were associated with the higher TPH contents (Fig. 3) indicting higher degrees of biotoxicity.

Overall, the reduction in toxicity in affection of Exp 1, Exp 2 and Exp 3 was largely attributed to the addition of microbial activators or N and P sources that provided necessary nutrients required for seed germination. The observation that the germination rates in Exp 1 and Exp 2 were significantly higher than that of Exp 3 was consistent with the trend of TPH degradation (Fig. 3). It indicated that the degradation of TPH by microorganisms directly or indirectly detoxified pollutants, which was reelected on the germination performance in the treated soil. In the control experiment, the germination rate remained at a low level of about 20% throughout the experiment period indicating an unchanged biological toxicity, associated with the weak degradation of TPH by microorganisms during the natural remediation process without activation (Fig. 3). The experiment results showed that the remediation processes assisted by the addition of microbial activators and microorganisms was able to not only achieve the degradation of TPH in oily soil, but also effectively reduce the biological toxicity of soil towards soil ecological remediation.

3.5. Field trials

Microbial remediation field studies were carried out on-site the oil production area with two oily soil samples (50 m³ each) having initial TPH contents of 5.01% and 2.15%, respectively, with 2% microbial activator and 2% sawdust added. Figure 5 shows the treatment site and steps including dosing and mixing oily soil, underlying, and shaping and covering the sample. During the 60-day treatment, soil samples were analyzed every five days to investigate the changes of TPH content and microbial density. The measurement results are shown in Fig. 6.
It can be seen from Fig. 6 that the TPH content in both samples showed a linear drop over the first 2 weeks, followed by a slower decline. In the sample with a high initial TPH content of 5.01%, the TPH content dropped to 3.2% on Day 15, showing a 36.1% degradation rate, and further decreased to 0.41% on Day 50 giving a degradation rate of 91.8%. That was closely associated with the increase in bacterial density. Particularly, within the first stage of two weeks bacterial density raised in approximately a linear way, to about $10^{11}$ CFU/g on Day 15, and then remained at the high level throughout the process. The decline of TPH content and bacterial density profiles showed similar trends as observed in the formulation tests (Figs. 2 & 3).

In the sample with a low initial TPH content of 2.15%, the TPH content declining trend followed that in the high TPH content sample, dropped to 0.52% on Day 15, showing a degradation rate of 75.8% doubling that in the high TPH content sample. That was well related to the rapid increase in bacterial density, reaching to $10^{11}$ CFU/g on Day 15. Further, after 50 days treatment, the TPH content in the oily soil decreased to a very low level of 0.02% with a remarkable degradation rate of 99%. Related to the oil degradation process was the bacterial density profile that increased over the first two weeks to the highest level of $10^{11}$ CFU/g. Following that, however, the bacterial density started to drop gradually, which was likely due to the unavailability of hydrocarbons as metabolic carbon source that was degraded along the process.

It was noticed that, after 25 days the TPH content in the oily soil with initial content of 2.15% decreased to 0.41%, which satisfactorily reached the national standard in China for cultivated land category II (GB 36600 – 2018). That clearly demonstrated the effectiveness of the remediation enhanced by the addition of microbial activator, where the activator was able to quickly activate the functional degrading bacteria in the soil, and achieve rapid degradation of TPH in the petroleum contaminated soil. The remediation speed was related to the content of TPH; the lower of the initial TPH content, the shorter of the remediation time needed. For oily soil with a TPH content lower than 6%, this process required about two months, that was significantly shorter than the current commonly used composting and bioaugmentation methods (Hamid et al., 2020; Koolivand et al., 2017; Asgari et al., 2017).

4. Conclusions

By introducing microbial activators, the microorganisms taking petroleum hydrocarbons as carbon sources were selectively activated to grow as the dominant bacteria group in oil contaminated soil. The microbial activator was formulated to provide nitrogen sources, phosphorus sources, trace elements, growth factors, biosurfactants, and soil pH regulators. In the petroleum contaminated soil, the microbial activator was found to not only rapidly increase the numbers of native microorganisms in soil, but also significantly change the microbial community structure while selectively activate the oil degrading bacteria. As a result, the addition of microbial activators enabled the TPH content to decrease from 5.01–0.28% after 55 days treatment, well meeting the agricultural land standard. At the same time, it effectively reduced the biological toxicity of the petroleum contaminated soil as evidenced by the increase in seed germination rate, reaching 90% after 40 days treatment with 2% microbial activator added. It was also
found that the introduction of exogenous microorganisms together with microbial activator had insignificant further enhancement for oil degradation and toxicity reduction compared to the addition of microbial activator alone, mainly attributed to the competition between the indigenous and exogenous microorganisms. By applying the microbial activator enhanced bioremediation process, the pilot-scale studies successfully demonstrated the feasibility for potential industrial application. With 2% microbial activator added to 500 m³ oily soil, the TPH content decreased from 5.01% initially to 0.41% after 50 days treatment giving a degradation rate of 91.8%, whilst dropping from initial 2.51% TPH to a very low level of 0.02% with a remarkable degradation rate of 99%. The development provided a simpler and more cost-effective approach for bioremediation of petroleum contaminated soil compared to the currently used technologies.

Declarations

Authors Contributions

Cong-Yu Ke: Conceptualization, Methodology, Writing - original draft. Wu-Juan Sun: Formal analysis, Investigation, Writing - review & editing. Qian Li: Participated in the design and coordination of the study, performed the experiments and interpreted the data. Rui Sun: Formal analysis, Investigation, Writing - review & editing. Bo-Yun Luo: contributed to experiment design and data analysis. Si-Chang Wang: Formal analysis, Investigation, Writing-review & editing. Qun-Zheng Zhang: Formal analysis, Investigation, Writing - review & editing. Xun-Li Zhang: Conceptualization, Methodology, Writing - original draft.

Fundings This work was supported by the Key Research and Development Program of Shaanxi (Program No. 2022ZDLSF07-04), Xi’an Science and technology project (2022JH-RYFW-0114) and Shaanxi Engineering Research Center of Green Low-carbon Energy Materials and Processes.

Data availability The data are available upon request from the corresponding author.

Conflict of interest The authors declare that they have no conflict of interest.

References


Figures
Figure 1

Microbial community structures in oil contaminated soil before and after 7 days activation

Figure 2

Change of total bacterial density in oily soil under different treatment conditions
Figure 3

Remediation effect of different conditions on historical oily soil

Figure 4
Variation of seed germination rate in bioremediated oily soil

Figure 5

Remediation process of oily soil on-site

Figure 6

Graph showing TPH content and bacterial density over time.
Changes of TPH content and bacterial density during remediation of oily soil