https://doi.org/10.17221/433/2022-PSE

Bioremediation approaches for oil contaminated soils in extremely high-mountainous conditions

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Citation: Totubaeva N., Tokpaeva Zh., Izakov J., Moldobaev M. (2023): Bioremediation approaches for oil contaminated soils in extremely high-mountainous conditions. Plant Soil Environ., 69: 188–193.

Abstract: Development of methods for bioremediation of soils contaminated with petroleum products is one of the most urgent tasks of our time. This task is more difficult to perform in high-mountainous landscapes, at an altitude of more than 4 000 m a.s.l. Moreover, these high-mountain ecosystems are the most vulnerable to various kinds of anthropogenic impacts, and therefore the relevance of bioremediation is obvious. The research was conducted in the high-altitude ecosystems of the Kyrgyz Republic at the Kumtor mine. In this study was carried out on the bioremediation of oil contaminated soil using biostimulation, bioaugmentation and biostimulation + bioaugmentation remediation techniques for 90 days in the climatic conditions of high mountain region. The biostimulation treatment showed the highest total petroleum hydrocarbons (TPH) biodegradation percentage 62.78% compared to the bioaugmentation 50.63% and biostimulation + bioaugmentation 49.11%. Thus, the method of biostimulation proved to be the most effective method for bioremediation of soils contaminated with petroleum products. The application of this method could be one of the successful methods of recycling contaminated soils. This study demonstrated the possibility of restoring TPH-polluted soils using biological methods of soil treatment in climatic cold conditions of high mountains.

Keywords: soil contamination; oil products; hazardous waste landfill; soil microorganism; cold region

Pollution of soil ecosystems by petroleum-based contaminants is a global problem that requires urgent measures to resolve it, especially the pollution of soils in the subarctic and high-mountain regions (Filler et al. 2009, McDonald and Knox 2014, Adipah 2019). Petroleum pollution causes oxidative stress, changes in soil chemistry and low nutrient availability. Petroleum-based contaminants reduce the number and metabolic activity of aerobic soil microorganisms and affect plant growth and germination, creating an impermeable membrane that impedes water and oxygen circulation. Especially high molecular weight petroleum hydrocarbons negatively influence soils organisms for a long time (Chen and Zhong 2019).

Sites in cold climatic zones experience temporally variable temperatures, and these variations may have an impact on the local soil microbial activity (Chang et al. 2011). Implementation of bioremediation techniques to accelerate natural biodegradation rates is an economically and ecologically effective method (Kumar et al. 2019). Nowadays bioremediation approaches have been studied by many researchers in numerous laboratory and field experiments, and approved as simple to maintain, applicable over large areas, cost-effective and environmentally friendly technologies to remediate oil contaminants (Adams et al. 2015, Koshlaf and Ball 2017, Wu et al. 2019). There are *in-situ* and *ex-situ* techniques, the first one involves treatment the contaminated soil without excavation whilst the latter does. *In-situ* soil remediation techniques are rare in many countries due to uncertainty about their effectiveness of this technique and possible adverse environmental impacts, especially in cold regions due to lack of knowledge

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(Gomez and Sartaj 2013, Polyak et al. 2018). *Ex-situ* techniques are Landfarming, Windrows and Biopile used more often (Simpanen et al. 2016).

This paper considered Kumtor Gold Company in high mountain area of Kyrgyz Republic. This company has been operating since 1997, and pollutes soils with oil products during refuelling, repair of vehicles as well as leaks during oil storage. The Kumtor Gold Company uses an average of 128 303 750 L of oil per year. This company excavates oil-contaminated soils and moves them to the hazardous waste landfill next to the gold in order to prevent the seepage of oil to deeper soil layers (Kumtor Gold Company 2019). Incidentally, this strategy is costly while also, it causes secondary contamination of soil. Given these deficiencies, bioremediation strategies are thought to provide the best contamination treatment therapies for total petroleum hydrocarbons (TPHs) as established by several studies elsewhere. Biostimulation, bioaugmentation and their combined techniques were chosen for use in natural conditions of the high mountain region. These techniques permit the use of indigenous microorganisms, which are psychrophilic, non-invasive to the treatment site and prolonged exposure to cold conditions (Yap et al. 2021).

Biostimulation involves optimising the conditions that are required for indigenous microorganisms to remove contaminants or the stimulation of the degrading abilities of microorganisms by introducing additional nutrients such as nitrogen, phosphorus and potassium (Goswami et al. 2018). The biostimulation technique is effective if the soil initially contains microorganisms with oil degradation ability (Okoh et al. 2020).

Bioaugmentation involves addition of microorganisms with the ability to degrade petroleum hydrocarbons or the usage of previously isolated and cultivated native microorganisms (Goswami et al. 2018). The effectiveness of bioaugmentation depends on abiotic factors such as the chemical structure of contaminants, their concentration and bioavailability as well as temperature, pH and nutrient level and biotic factors such as interactions between autochthonous and added microorganisms (Mrozik 2016).

The aim of this study was to find more effective bioremediation technique to remediate petroleum contaminated soils exactly in the permafrost area of Kyrgyz Republic.

MATERIAL AND METHODS

Site description. The Kumtor mine is located southeast of Kyrgyzstan at an altitude of 4 000 m a.s.l. in a partially glaciated permafrost zone in the Central Tien Shan Mountains. It lies between 41°52'N and 78°11'E. The field experiments were carried out at Kumtor mine hazardous waste landfill (KMHWL) for 90 days with the onset of the warm season (June–August). The average monthly precipitation of the area is 59.6 mm, an average monthly temperature of 5.23 °C with a maximum of 18.8 °C and a minimum of –11.10 °C (Kumtor Gold Company 2019).

Soil samples collection. Soil samples for physicochemical analysis, determination of initial TPH concentration, detection and isolation of bacteria were gathered from the KMHWL. Soil samples were collected from five random points at the site at a depth of 0–30 cm using shovel. The samples were combined, dried, sieved (2 mm mesh), placed in a plastic bag and stored at 4 °C until used. Soil samples for bioremediation experiments were collected from KMHWL and were placed into three plastic boxes, with dimensions: 0.3 m height × 1.25 m length × 1.25 m width, which were located near the landfill.

Isolation and detection of bacteria from soil samples. To isolate hydrocarbon-degrading bacteria from KMHWL, soil samples were taken on the Voroshilova-Dianova (VD) medium which contained per litre: NH_4NO_3 1.0 g, K_2HPO_4 1.0 g, KH_2PO_4 1.0 g, $MgSO_4$ 0.2 g, $CaCl_2$ 0.02 g, $FeCl_2$ – 2 drops of concentrated solution, with sterile 1% crude oil as the sole carbon source. In addition, 5 mL of crude oil and oil-contaminated soil sample was added in a 250 mL Erlenmeyer flask with a liquid medium. The suspension was shaken in an orbital shaker at 200 rounds per minute and incubated at 30 °C for 15 days. Pure bacteria strains were isolated by traditional spread technique on meat-and-peptone agar (MPA). For selection of oil-degrading microorganisms, each isolate was suspended in 100 mL of VD medium with 1% oil. Bacteria were grown for 7 days on the shaker. The bacteria that were able to grow on oil contained medium were monitored spectrophotometrically at 590 nm (Specord 50, Analytik Jena GmbH) (Totubaeva et al. 2019).

Physicochemical analyses. The total petroleum hydrocarbons in soil samples were analysed using GC with flame ionisation detection (Shimadzu GC-FID, Kyoto, Japan). The physicochemical characteristics of the soil such as soil texture, pH, total nitrogen, mobile forms of phosphorus, exchangeable potassium, and soil organic carbon were determined in the laboratory at Kyrgyz State Soil Institute under the Ministry of Agriculture of the Kyrgyz Republic.

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The soil texture class was defined by soil texture triangle classification method. The pH was estimated according to Jackson (1958) method using digital pH meter. The 20 g soil sample was poured into 40 mL of distilled water, after shaking it was kept undisturbed for half an hour. After that pH value was estimated by using digital glass electrode pH meter (Eutech pH 150, Thermo Scientific). Total nitrogen was determined by Kjeldahl method. With this method, 1 g of soil sample was weighed into a digestion tube. 2 g of a catalyst mixture (6 g $CuSO_4 \cdot 5 H_2O$ + 94 g K_2SO_4) and 5 mL of concentrated H_2SO_4 were added to digestion tube. It was boiled on 390 ± 10 °C until the digestion mixture became clear and then boiling was continued for 10 min. It was then distilled to 20 mL of 2% H_3BO_3 by adding 20 mL of water and 20 mL of 10 mol/L NaOH solution to the distillation unit. After distillation it was titrated with 0.01 mol/L HCl. Mobile forms of phosphorus and exchangeable potassium in soil samples were determined according to standard method by Machigin (GOST 26205-91, 1993). The method is based on the extraction of mobile compounds of phosphorus and potassium from the soil. The method involved using a solution of ammonium carbonate at a concentration of 10 g/L at a ratio of soil to a solution of 1:20 and the subsequent determination of phosphorus was done using a spectrophotometer (710 nm) and potassium on a flame photometer (766–770 nm).

Determination of the soil organic carbon was carried out by the modified Walkley-Black method. With this method, a sample amount containing 20 mL of concentrated H_2SO_4 was added quickly from a fast-delivery burette after being weighed into a 250 mL conical flask and containing around 10 mg of organic carbon. On a hot plate, the flask was heated until it reached 135 °C. The mixture was brought to boiling point rapidly and then simmered gently for 20 min and then allowed to stand on a sheet of asbestos for 30 min. Afterwards, it was treated with water (200 mL) and H_3PO_4 (10 mL) (Walkley 1947).

The total bacterial counts were determined by plate counting method. With this method, 10 g of each soil sample was added to 90 mL of distilled water. The solution was diluted (10^{-1} to 10^{-6}) and aliquots of the resulting solutions placed on appropriate culture media. Meat-peptone agar (MPA) medium, consisting NaCl – 5 g/L, meat extract – 1.50 g/L, algae extract – 1.5 g/L, agar – agar 15 g/L, pH 7.0–7.2 was used for bacteria growth. All experiments were performed in triplicate. After incubation at 30 °C for up to 10 days, the colony forming units (CFU) were counted (Lorch et al. 1995).

Bioremediation treatment experiments. Various bioremediation treatments (biostimulation, bioaugmentation, and biostimulation + bioaugmentation) were implemented according to Table 1. Biostimulation and bioaugmentation were applied in the first and second containers while biostimulation + bioaugmentation were applied in the third container, respectively. Mineral fertilisers in the ratio N:P:K = 16:16:16 containing 16% inorganic N, 16% phosphorous pentoxide and 16% potassium oxide were added as ameliorant's at the rate of 60 g/m^2. These nutrients were added to contaminated soils and properly homogenised mechanically. Consortium of previously isolated indigenous microorganisms; *Pseudomonas fluorescens* P1, *Rhodococcus rhodococcus* R3 and *Flavobacterium* K1 (Brown et al. 2017) was kept in nutrient broth (Totubaeva et al. 2019). Consortium of these microorganisms was inoculated on the surface of bioaugmentation, biostimulation + bioaugmentation treatments and mixed mechanically. In all experiments, the humidity was maintained at 80%, aeration was ensured by loosening the container covers every 14 days.

RESULTS AND DISCUSSION

The results of the physico-chemical characteristics of soil sample before and after treatments are presented in Table 2. Basing on the soil classification

Table 1. Various bioremediation treatment methods

Treatment	Identity	Composition
Control	C	soil
Biostimulation	BS	soil + N. P. K
Bioaugmentation	BA	soil+ MO^X
Biostimulation + bioaugmentation	BS + BA	soil + MO^X + N. P. K

^{X}MO – microorganisms: (*Pseudomonas fluorescens* P1, *Rhodococcus rhodococcus* R3 and *Flavobacterium* K1)

https://doi.org/10.17221/433/2022-PSE

Table 2. Physicochemical characteristics of soil sample

	Soil pH	Total nitrogen (%)	Mobile forms of phosphorus	Exchangeable potassium	Soil organic carbon (%)
			(mg/kg)		
Initial content	8.05	0.130	6.0	88.0	2.86
BS	7.5	–	25.6	376.0	6.55
BA	6.5	–	90.0	300.0	6.81
BS + BA	6.0	–	47.2	352.0	7.17

Physicochemical characteristics of studied soil sample after 90 days of the bioremediation procedure. BS – biostimulation; BA – bioaugmentation

triangle, the investigated soil was light loamy. The pH for all treatment options ranged between 6.0 and 7.5. This pH range fell within the optimum required for the effective bioremediation process (Abdulsalam and Omale 2009).

The examined soil sample was poor in phosphorus. The initial content of mobile phosphorus was 6 mg/kg. After remediation, its content increased in all three treatment, a significant increase ($P < 0.05$) was observed in the bioaugmentation treatment up to 90 mg/kg. The reason for this increase is attributed to the activity of phosphate-solubilising bacteria that must have released organic acids and phosphatase enzymes, which enhance the solubilisation of insoluble phosphorus compounds (Sahu and Jana 2000). The content of exchangeable potassium before experiment was 88 mg/kg and after biological treatment, it increased in all variants by an average of 300 mg/kg. Bioremediation techniques also increased soil organic carbon, which shown in Table 2. It is noted that in bioremediation treatment the increase in soil organic carbon is determined by microbial activity and its mineralisation (Kisić et al. 2022).

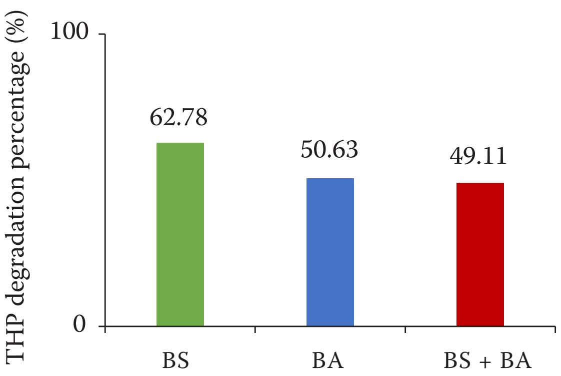

Figure 1. Total petroleum hydrocarbons (TPH) degradation percentage after 90 days of remediation. BS – biostimulation; BA – bioaugmentation

The initial content of TPH in the studied soil was 2 633 mg/kg (Figure 1). After 90 days of field experiments, the initial content of TPH reduced to 980 mg/kg in the biostimulated treatment. The effect of addition pre-selected bacteria consortium promoted degradation of TPH up to 1 300 mg/kg from initial 2 633 mg/kg. The decrease in TPH after 90 days of experiments was up to 1 340 mg/kg from initial 2 633 mg/kg in biostimulation + bioaugmentation treatment (Figure 1).

When using only BS (biostimulation) and BA (bioaugmentation), after 90 days, the degradation percentage of pollutants reached 62.78% and 50.63%, respectively. With biostimulation + bioaugmentation, the decomposition efficiency was 49.11%. It is due to high mountainous climatic conditions i.e. inoculants introduced into the new medium could not adapt and multiply under difficult climatic conditions.

At the initial stage of the experiment, we have studied the change in the total number of bacteria in various experiments of soil remediation for 30 days. The initial number of bacteria was 6.4×10^6 CFU/g (colony forming unit)/g), after 14 and, 30 days in biostimulation the total bacterial number increased up to 19×10^6 CFU/g and, 54×10^6, respectively. CFU/g. In bioaugmentation, a tendency to an increase in the total bacterial number was also traced, but it turned out to be much less than the method of biostimulation, amounting to 26×10^6 CFU/g by the 30th day of exposure. However in combined treatment (BS + BA) the total bacterial number on the 30th day decreased up to 2.9×10^6 CFU/g from initial 6.4×10^6 CFU/g (Figure 2).

Biostimulation (adding mineral fertiliser) accelerated the process of decomposition of hydrocarbons: the content of TPH in the soil decreased by 62.78% in 90 days and the total number of bacteria increased by 8.5 times. Bioaugmentation (addition pre-selected bacteria consortium) reduced the content of TPH in the soil by 50.63% in 90 days and caused an increase in

https://doi.org/10.17221/433/2022-PSE

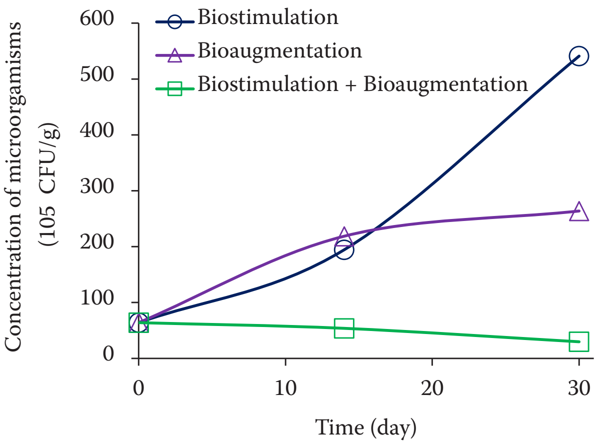

Figure 2. Change the total number of bacteria in contaminated soils during bioremediation treatments. CFU – colony forming unit

the total number of bacteria by 4.1 times. The results of the BS + BA method turned out to be less effective compared biostimulation alone. In the treatment of BS + BA, the degradation percentage of TPH was 49.11% in 90 days of field experiments. While we expected a high percentage of TPH degradation in the treatment of biostimulation + bioaugmentation as was the case elsewhere (Gomez and Sartaj 2013, Cui et al. 2020). However, our results showed that biodegradation was lower in BS + BA compared with biostimulation or bioaugmentation alone. This can be due to competition for nutrient utilisation among indigenous and exogenous bacteria (Abdulsalam and Omale 2009).

The total number of bacteria in biostimulation and bioaugmentation alone showed an increase, in other hand in their combined method there was a decrease. With the combined methods, the acclimatisation of the inoculant might have failed. Thus, much as both biostimulation and bioaugmentation are known to increase the efficiency of soil cleansing processes from oil products, the choice of the appropriate method depends on the environmental conditions as professed by Gutiérrez et al. (2020). Taking into account the costly methods of enrichment of the decomposer and their inoculation, we believe that the use of biostimulation in such difficult climatic conditions is the most appropriate.

In this work, we have demonstrated the possibility of using methods of biological soil treatment in climatic cold conditions of high mountains. In all variants of bioremediation treatments, a decrease in the content of oil products in the soil was observed. The best result in reduction TPH in soil was shown by the biostimulation method.

Acknowledgement. Special thanks to the management and staff of the Kumtor Gold Company for their assistance and support in the execution of experiments. Thanks are also extended to the reviewers and editors who provided valuable suggestions for improving this paper.

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Received: December 8, 2022
Accepted: April 12, 2023
Published online: April 25, 2023