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Original Research

Microbiological Diversity and Biotechnological Potential of the Soil Ecosystem of a High-Mountainous Landfill

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Abstract

Strains isolated from high mountain industrial landfills have high biotechnological potential and studying their diversity is relevant. The objective of this study was to investigate the microbial diversity of oil-contaminated soils at a high mountain mine located 4000 meters above sea level, isolated and characterized by hydrocarbon-resistant bacteria and compare the degradation efficiency of two bacterial consortia. The surface layer (0-30 cm) that consists of 10440 mg/kg hydrocarbons were used for the experiment. A bacteria group of the three genera *Pseudomonas, Flavobacterium,* and *Rhodococcus* dominated. Fungi *Aspergillus, Penicillium,* and *Trichoderma* were present in relatively high abundances in the samples. The study shows that the actinomycetes of the *Streptomyces* group of the *Cinereus* section are most sensitive to hydrocarbon contamination. Three superior indigenous bacteria *Rhodococcus rhodococcus N1, Pseudomonas fluorescens W3,* and *Flavobacterium NE2* has been isolated from oil-polluted soil. The consortium composed of bacteria strains *Rhodococcus rhodococcus N1, Pseudomonas fluorescens W3,* and *Flavobacterium NE2* is more effective than a consortium composed of bacteria strains *Rhodococcus rhodococcus N1, Pseudomonas fluorescens W3* hydrocarbon removal efficiency 70% and 22.9%, respectively. These findings provide highly valuable information on the production of bacterial consortium for the remediation of oil-contaminated soil.

Keywords: high-mountainous landfill; oil-contaminated soil; biodiversity; consortium

Introduction

Kumtor, the largest gold mine in Central Asia, is located southeast of the Kyrgyz Republic at an altitude of 4000 m a.s.l. in a partially glaciated permafrost zone at 41º52'N and 78º11'E. Its climate is continental, with an average annual temperature of -8ºC [1]. The Kumtor complex is in close proximity to the active glaciers [2] belonging to the Naryn River basin, which has international importance [3]. As a result of the large-scale activity of the Kumtor gold mine, only 162.9 tons of oiled rags were formed at the landfill site between 2014 and 2016, which relate to dangerous waste of

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classes 3 and 4 [1]. In turn, we are facing the task of remediation and biodiversity conservation of microbial communities of the mining complex's landfill with the aim of their sustainable functioning.

It is common knowledge that among the various technogenic disturbances in nature, oil pollution is one of the most serious and hard to eliminate [4]. Oil and its components (aromatic, naphthenic and paraffinic hydrocarbons) [5] are among the most dangerous pollutants that enter soil and water ecosystems in the processes of extraction, transportation, processing, and storage [6-11]. To accelerate the process of self-cleaning of soils from oil, all natural reserves of the ecosystem are used, including biological ones. Microbiological methods of soil purification are able to supplement various technologies [12, 13] and in certain situations have no analogs [14-17]. Biodiversity, especially the microbiodiversity of both indigenous ecosystems and those prone to anthropogenic pressure, is the key to finding ecological methods for maintaining and restoring disturbed ecosystems [18-20].

The study of the biodiversity of microorganisms and the responses of biota give an idea not only of the state of the biota but also allow us in many cases to predict its development as a whole ecosystem [17, 21]. A soil is the most diverse environment on Earth [22], and many of the local microorganisms have the ability to resist and degrade hydrocarbons of crude oil [23-26]. A number of scientists have established the important role of hydrocarbon oxidizing microorganisms in the processes of biodegradation of various classes of hydrocarbons in oil and oil products [27-31]. Some microorganisms are able to degrade hydrocarbons of crude oil, which is the goal for research aimed at mitigating any possible consequences of soil contamination [32-35]. Oil degenerative strains are usually isolated from polycyclic aromatic hydrocarbon of polluted soils. However, little is known about the ecology and diversity of indigenous populations of these microorganisms in contaminated environments [36].

The aim of our work was to investigate the biodiversity of indigenous microorganisms of soil from hazardous waste landfill of Kumtor mine and to search for a potentially active strain-destructor of oil products.

Material and Methods

Sample Collection

Soil samples were gathered from the hazardous waste landfill of Kumtor mine in September at an air temperature of 5°C, humidity of 45% and pressure of 658.5 mm Hg. Background soil sample was gathered 200 m from the landfill. Soil samples were collected from five random points in each site to a depth of 0-30 cm. The samples were combined, dried, sieved (2 mm mesh), placed in a plastic bag and stored at 4°C until used.

Chemical Analyses

The pH was measured in triplicate using a Horiba B-213 twin pH meter after extraction of soil using one-part solid to 2.5 parts of distilled water.

Estimation of total concentrations of hydrocarbons in soil samples was determined at the accredited laboratory of the State Agency for Environmental Protection and Forestry under the government of the Kyrgyz Republic.

5 g soil samples were mixed with 40 mL of dichloromethane as the extraction solvent. Then hydrocarbon extraction was performed using a Soxhlet extractor. Solvent extract after cleanup and concentration was analyzed using a Shimadzu gas chromatograph (Japan) equipped with a flame ionization detector and a 30-m-long 0.25 mm i.d. (0.25 μm film thickness). Oven temperature was kept at 100ºC for 1 min, then ramped up at 10°C/min to 250ºC and kept at this temperature for 5 min. The injector temperature was 280°C. Carrier gas flow was 31 cm/s.

Hydrocarbons removal efficiency (E%) was measured as the percentage of removal in accordance with the following equation: $E\% = \frac{Ci-Cf}{Ci}100\%$, where C_i and C_f are the initial and final hydrocarbon concentrations.

Microbial Analyses

The number of microorganisms was determined by the plate method. Ten grams 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 plated on appropriate culture media. Czapek media was used for fungal growth, meat-peptone agar (MPA) for bacteria growth and starch-ammonia agar (SAA) for actinomycetes growth. All experiments were performed in triplicate. After incubation at 25 or 30ºC for up to 10 days, the colony forming units (CFU) were counted [37]. In addition, the cultures were determined according to macromorphological types.

Isolation and Detection of Bacteria from Samples

To isolate hydrocarbon-degrading bacteria from hazardous landfill, soil samples were taken on the Voroshilova-Dianova (VD) medium, contained, per liter: NH_4NO_3 1.0 g, K_3HPO_4 1.0 g, K_2HPO_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. 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 rpm and incubated at 30ºC 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 suspended in 100 ml of VD medium with 1% oil. Bacteria were grown for 7 days on the shaker at 200 rpm. The bacteria able to grow on oil contained medium were monitored spectrophotometrically at 590 nm.

The key features in determining the bacterial generic membership were: mobility, bacterial colony characteristics, Gram staining, availability of oxidase and catalase. The shape and mobility of the cells were determined by microscopy of living bacterial preparations. Morphological features were detected on fixed preparations stained with fuchsin or methylene blue according to microbiological standard methods [38].

- Catalase test: One drop of hydrogen peroxide (3 % H_2O_2) solution was placed on the glass slide. A single colony of overnight grown culture was mixed with the H_2O_2 drop. The formation of bubbles after 5 minutes indicated the presence of catalase.
- Oxidase test: Oxidase reagent (1% tetramethyl-p-phenylenediamine dihydrochloride) was prepared, and filter paper was moistened with this reagent. Overnight grown organisms were smeared over the paper with the help of a glass rod or plastic loop or platinum wire. Change in color was observed within 10-20 s. The formation of purple color shows the presence of oxidase.

Biodegradation Experiments

The ability of isolated microorganisms to decompose petroleum products was carried out under laboratory conditions. Approximately 500g of contaminated soil sample (10440 mg/kg) were placed into each container. The following experiments were carried out: Consortium 1 in soil sample inoculating the monoculture of two strains of *Rhodococcus N1 + Pseudomonas W3*; Consortium 2 in soil sample inoculated the monoculture of three strains of *Rhodococcus N1 + Pseudomonas W3 + Flavobacterium NE2*; and Control in soil sample wasn't inoculated strains. Each experiment was tested in triplicate. Cultivation was carried out for 30 days at room temperature +24-28ºC; soil aeration and moisture (60%) were controlled daily.

Results and Discussion

The content of oil products in the contaminated soil sample was 10440 mg/kg, while the background sample contained 320 mg/kg. The reaction of the soil solution affecting the characteristics of the functioning of microorganisms in accordance with the analytical data was neutral pH value = 6.9.

The study of the bacteria microflora showed that the species diversity of bacteria was insignificant. Five species of bacteria of the genera *Pseudomonas, Rhodococcus, Flavobacterium, Bacillus,* and *Nocardia* were identified and among them the percentage of three species of bacteria *Pseudomonas, Rhodococcus,* and *Flavobacterium* dominated (Fig. 1), which confirmed their activity in the destruction of petroleum products and bacterial capability for resistance and adaptability to high contamination levels [39-44]. Similar results were obtained by other researchers of Antarctic soils, where in oil-contaminated soil dominated Proteobacteria, mainly by the genera *Pseudomonas, Rhodococcus, Sphingomonas*, and *Variovorax* [45, 46].

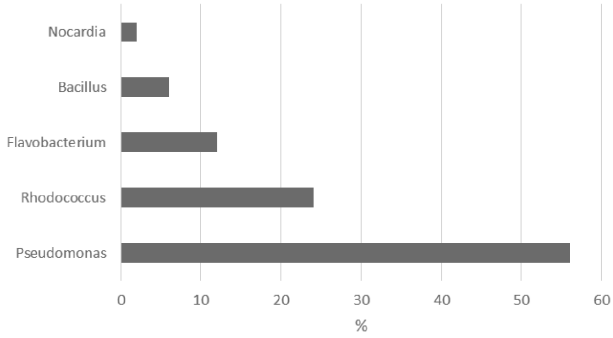

Fig. 1. Percentage expression of bacterial species in test samples.

Representatives of dark-colored fungi predominated over light-colored fungi in contaminated soil, while in the sample of the background soil light-colored forms of fungi predominated (Fig. 2). The greatest count of CFUs of fungi also was contained in the contaminated sample (6 * 10^5 CFU) and the background sample B (2.1 * 10^5 CFU). It should be noted that the biodiversity of micromycetes was minimal, limited only by 5 genera: *Aspergillus, Penicillium, Acremonium, Fusarium*, and *Trichoderma*. Our studies have revealed that oil products have a toxic effect on the soil microbiota, inhibiting its biological diversity and in the dominance of the most resistant species of fungi. The same results were obtained by other researchers [47-52]. Many researchers confirmed that the diversity of the fungal community is dominant among other inhabitants in the oil contamination of soils. Apparently, *Aspergillus* and *Penicillium* are the most common species in high oil-contaminated soils [53-56].

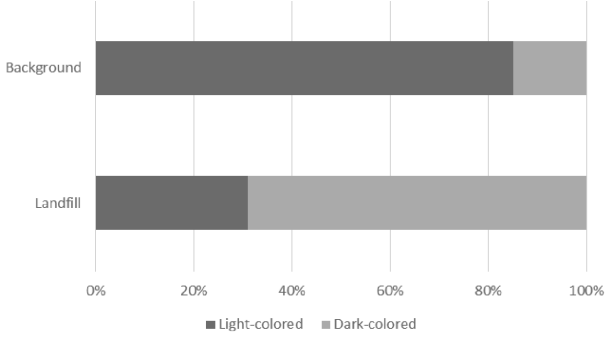

Fig. 2. Abundance of dominant groups of fungi (%).

Table 1. Characteristics of superior strains isolated form contaminated soil.

Strain	Macroscopic characteristics	Microscopic characteristics	Mobility test	Grams staining test	Catalase test	Oxidase test
W3	Smooth edge, mucoid, and milky color	Small bacil	Positive	Negative	Positive	Negative
N1	Smooth edge, mucoid, and milky color	Small cocci	Negative	Positive	Negative	Negative
NE2	Smooth edge, mucoid, and milky color	Small bacil	Negative	Negative	Positive	Positive

Table 2. Content of oil in soils according to the variants of the experiment.

	Initial content of total hydrocarbon, mg/kg	Content of total hydrocarbon after 1 month, mg/kg ± SD	Total hydrocarbon removal efficiency E%
Control	10440	8080±58,6	23
Consortium 1	10440	7343±61,5	30
Consortium 2	10440	3097±36,5	70

SD - standard deviation

The study of actinomycetes showed that this group did not contain a large species diversity, and the group was mainly represented by the *Cinereus* section of the genus *Streptomyces*, in a minor amount. Representatives of other sections could not be singled out for us. Perhaps this group has minimal properties to absorb hard-to-reach hydrocarbons.

In a model laboratory experiment, as a result of screening, more than 70 strains were isolated and three indigenous bacterial strains which showed the greatest oil-consuming activity were identified. Table 1 presents the characteristics of these three isolates. The use of bacterial consortia consisting of isolated natural sources is considered to be more appropriate than using monocultures, as noted by many authors [39, 57-59], which was also confirmed by our studies. So the introduction of consortia based on two strains of microorganisms *Pseudomonas W3* and *Rhodococcus N1* contributed to a 1.3-fold decrease in oil content and the introduction of a consortium of 3 strains of microorganisms *Pseudomonas W3, Rhodococcus N1*, and *Flavobacterium NE2* contributed to a 3.11-fold decrease in oil content compared to control. From the data of Table 2, it follows that the natural process of self-cleaning of soils from oil in the creation of favorable conditions led to the destruction of oil by 23%. In soils with the introduction of consortia 1 and consortia 2, the purification process increased to 30% and 70% (Fig. 3). The most effective was consortium 2. Identified strains of bacteria have the most active properties for the destruction of oil products. The introduction of indigenous strains for further remediation of soil from the landfill is a good prospect, as strains of microorganisms are adapted to the extreme climatic conditions of the Kumtor region.

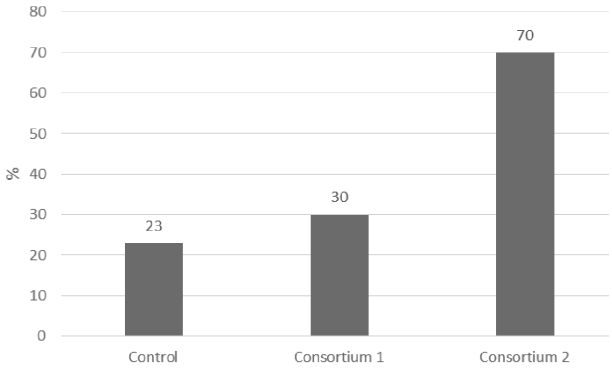

Fig. 3. Hydrocarbon removal efficiency within 30 days.

Conclusions

Our research has shown that despite the climatic conditions of Kumtor, regional microorganisms quite successfully coped with the role of decomposers – oil products in particular. However, the microbiological diversity of the landfill soil is sparse and mainly represented by resistant microorganisms: bacteria of the genera *Pseudomonas*, *Flavobacterium* and *Rhodococcus;* fungi *Aspergillus, Penicillium, Trichoderma*, which indicates the weak stability of the soil ecosystem in relation to anthropogenic pressure. Highly sensitive to the contamination of petroleum products were actinomycetes of the genus Streptomyces of the Cinereus section. Three indigenous bacterial isolates *Pseudomonas W3, Rhodococcus N1* and *Flavobacterium NE2* showed how they work as a consortium. Best hydrocarbon removals were observed

in soils inoculated with three strains of bacteria (consortium 2) 70%, while in the binary consortium (consortium 1) hydrocarbon removals were 30%. We established that, despite the active action of the enterprise, soil microorganisms quite successfully fulfill their ecological functions and carry out the degradation of hydrocarbon products. The study of microorganisms communities of soil hazardous waste landfill of Kumtor mine is a priority in the environmental management of waste. Therefore, obtained results of this work can form the basis for further work on bioremediation.

Conflict of Interest

The authors declare no conflict of interest.

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