

A New Approach and Tools for Perfecting Phytoremediation Technology

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Abstract: The development of methods for soil remediation for removal of chemical contaminants is a large and challenging problem. A new phytoremediation technology, given in the present work, is based on joint application of natural sorbents, microorganisms and plants. The method aims at prevention of chemical contamination of soils. It has the potential to restore a polluted environment and prevent dissemination of toxic compounds from hotbeds of pollution for a considerable length of time. The presented complex biotechnology is important from the viewpoint of prevention. This technology can be applied to soils contaminated with heavy metals, oil hydrocarbons, explosives, pesticides and other pollutants. The main purpose of the carried out work is the development of a novel approach to the provision of ecological safety. The approach is based on using natural minerals composites which are comprised of natural mineral rocks, microorganism strains with high detoxification abilities and plants-phytoremediators. In this composite material, the function of a sorbent is to uptake and to trap pollutants thus restraining their emission in the environment. The role of the microorganisms is to accomplish the first stage of biodegradation of organic contaminants and then to apply phytoremediation as a unique cleanup strategy. Natural sorbent can be applied during the initial stage of cleaning up of contaminated soil, followed by application of a phytoremediation technology through purposeful planting of selected plants. This results in the total assimilation and complete mineralization of pollutants and/or their intermediates or in their partial transformation by microbes. Following contaminants were used in the present work: explosive – 2,4,6 trinitrotoluene (TNT), oil hydrocarbon (hexadecane) and Wax (long chain hydrocarbons). Special attention was paid to enhancement of soil fertility, which improves microorganism and plant growth conditions and is important for bioremediation processes providing total rehabilitation of soil.

Keywords: Phytoremediation, Bioremediation, Contaminated Soils

1. Introduction

Cleansing of polluted environment, its absolute restoration and long-term protection can be achieved effectively and cheaply via application of phytoremediation technologies [1]. In this case the ecological balance is maintained: molecules of chemical toxicants are totally assimilated by plants and the atoms, forming toxic structure of xenobiotics participating in the natural metabolism as harmless compounds.

Phytoremediation is a unique cleanup strategy. Application of phytoremediation technologies implies the planting of contaminated area with one or more specific, previously selected species of plants having the potential to extract contaminants from the soil [2-7]. The treatment continues by harvesting the plants and composting or incinerating them. To create a truly effective phytoremediation system all components of the system should be thoroughly analyzed. The major constitutive component of such a system is obviously the plant. Plant

selection implies choosing a certain plant species with appropriate characteristics. A survey of the site vegetation should be undertaken in order to determine the species of plants that will grow best on the contaminated site, taking into account the ability of the plants to accumulate and degrade the contaminants.

Phytoremediation has great advantage over other nonbiological and biological technologies. It is envisaged by many private or state companies while projecting and accomplishing remediation or prevention activities.

Some limitations to phytoremediation should be pointed out – very high concentration of pollutants, e.g. accidental oil spills, may significantly reduce plant growth. Therefore, dissemination of toxic compounds from the hotbeds of pollution in the environment (e.g. groundwater) occurs at a higher pace than in case plants provide their assimilation.

The main purpose of the carried out work, is to reduce limitations to phytoremediation, and to develop a new technological approach to the remediation of chemically contaminated soils using "biosorbents" - composites of natural mineral sorbents and microorganism strains with high detoxification abilities. In this composite material, the function of a sorbent is to uptake and to trap pollutants, thus restraining their emission in the environment. The role of the microorganisms is to accomplish the first stage of biodegradation of organic contaminants and then to apply phytoremediation as a unique cleanup strategy. Natural sorbent can be applied during the initial stage of cleaning up of contaminated soil, followed by application of a phytoremediation technology through purposeful planting of selected plants. This results in the total assimilation and complete mineralization of pollutants and/or their intermediates or in their partial transformation by microbes.

By combining specially selected microorganisms having high detoxification abilities with natural minerals having high sorption capacities, a new "biosorbent" technology provides restoration of the polluted environment, and prevents the dissemination of toxic compounds from hotspots of pollution for a considerable period of time. The method can be used to purify and decontaminate soils and water contaminated by a variety of eco-pollutants, including heavy metals, radionuclides, alkanes, aromatic and polycyclic hydrocarbons, pesticides, explosives, chlorinated solvents etc.

The purpose of the present project is to elaborate a new technological approach to chemically contaminated soils, based on simultaneous application of sorption properties of natural minerals and detoxificational abilities of microorganisms and plants.

2. Materials and Methods

2.1. Screening of Bacterial Strains

Bacterial cultures from the Collection of Microorganisms of Durmishidze Institute of Biochemistry and Biotechnology of the Georgian Agricultural University were used to determine

the strains capable of degrading different organic pollutants, particularly crude oil, wax and 2,4,6-trinitrotoluene (TNT).

2.2. Plants

The following plant species were tested as vegetation to be used in purposeful planting during our investigation: maize (*Zea mays*), alfalfa (*Medicago sativa*), mung bean (*Vignaradiata*), ryegrass (*Lolium multiflorum*), soybean (*Glycinemax*), chickling vetch (*Lathyrussativum*), chickpea (*Cicerarietinum*) and chinabean (*Vignasinensis*).

2.3. Investigation of Sorbents

Standard methods were applied to define physical-chemical features (volumetric weight, porosity, displacement, filtration characteristics, oil absorption potential, absorption dependence on temperature, pH, etc.) of rocks [8].

2.4. TPH Determination

The sampling of soil was carried out according to standard methods. The content of total petroleum hydrocarbons (TPH) in the soil samples was measured gravimetrically after Soxhlet extraction as well as by gas chromatography with flame ionization (FID) detection [10-12].

2.5. TNT Determination

TNT content in the samples was determined according to the extinction of colorized TNT in high alkaline medium (pH>12.2) at 447 nm [9].

For determination of residual amounts of TNT, a sample of soil was taken and TNT was extracted by methanol (three times with 300 ml of ethanol on 50 g air-dried soil, mixed during 30 minutes). Extracts were concentrated to a volume of 5 ml on the vacuum-evaporator, then 2 ml of this extract was added to 2 ml of KOH solution (1 M) and after 8 minutes TNT content was determined spectrophotometrically, according to extinction at 447 nm. The amount of TNT determined at the beginning of the experiment was used as the control.

In case of experiments with [^{14}C] TNT, the air-dried soil was weighed (mass of sample 7 kg), 0.5 L of solution of 1.05 g [^{14}C] TNT (specific radioactivity 500 Bq/mg) in diethyl ether was added and was thoroughly mixed. The ether was evaporated during 2 days. After that 10% suspension of microorganisms was added to the samples and was dried to up to 130% of initial weight (according to the standards of soil investigations). Thereafter the soil was placed in special boxes for the experiments and the soybean seeds were sowed in samples (80 seeds on 7 kg of soil).

For determination of radioactivity of soil samples the methanol extract was evaporated until dry residue. The dry residue was dissolved in 5 ml of benzene; 1 ml sample was taken and was measured with the scintillation spectrometer SL-30 Rackbeta, with an efficiency of 95%.

Thin-layer chromatographic (TLC) analysis of methanol extracts from polluted soil samples was carried out. The plates were developed with mixture: benzene: dioxane: acetic acid (90 : 10 : 1). The chromatograms were exposed on X-ray film

during 45 days for revelation of radioactive spots. After exposition the radioactive spots were extracted from chromatograms with benzene and their radioactivities were measured.

3. Results and Discussions

3.1. Sorbents

Application of mineral composites is important in case of all types of contamination, as, on the one hand, it is significant from the viewpoint of prevention, and, on the other hand, it creates favorable conditions for growth of vegetation. After the introduction of the composites into the upper part of the soil and as a result of subsequent watering, the composites become swollen and stimulate growth and development of vegetation at the corresponding stage of phytoremediation.

Three types of mineral rocks were studied for the potential to be used as biosorbent – 2 types of Bentonites and Zeolite. The absorption ability of minerals and their composites for oil and TNT were studied. In composite ratio of minerals for each contaminant was different. Composite content against contaminants was the following: for TNT – B1/Z3 in ratio 1/3, for oil – B1/Z3 in ratio 2/3. It has been stated, content of composites in soil - 20%.

3.2. Soil Contaminated with Crude Oil and Wax

3.2.1. Selection of Microorganisms

Screening of 200 strains of microorganisms was performed to study their abilities to effectively assimilate oil hydrocarbons during growth on media containing crude oil. As a result, optimal growth conditions (content of nutrient medium, pH, temperature) were established, resulting in 20 selected strains. The ability of these selected strains to degrade oil hydrocarbons was determined by their submerged cultivation in nutrient media with crude oil (3%) as the sole carbon source.

The gas-chromatographic analysis has shown that *Pseudomonas* and *Rhodococcus* strains were the most efficient combination of bacterial cultures for oil degradation. Chromatographic analysis also revealed that hydrocarbon fractions up to C_{17} are assimilated completely, while a small amount of heavier hydrocarbons ($> C_{17}$) remain in the incubation medium.

3.2.2. Plants

The following plant species: alfalfa (*Medicago sativa*), chickpea (*Cicer arietinum*), chickling vetch (*Lathyrus sativum*), maize (*Zea mays*), pea (*Pisum sativum*), ryegrass (*Lolium multiflorum*), and soybean (*Glycine max*) were used as phytoremediators.

The model phytoremediation experiments for cleaning the soil artificially contaminated with crude oil were carried out according to the following scheme: the suspension of microorganisms was inoculated in the contaminated soil at the beginning of the experiment. After different incubation periods the plants were sowed in separate samples of soil. The

conditions and details of experiments are presented in legends of Fig. 1.

As shown in Fig.1, bacterial consortia cause effective decreasing of oil content in soil in all model test variants (43-74% of initial contamination). Residual contamination of soil removes after plant using in phytoremediation processes. (55-90% of initial contamination). Most effective phytoremediator in presenting experiment is soybean.

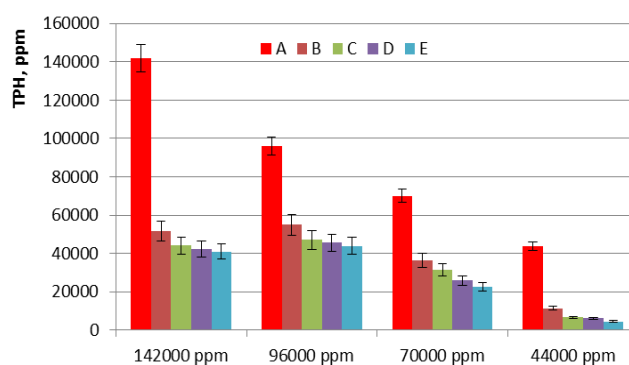


Figure 1. TPH content in soils contaminated with oil hydrocarbons after being treated by bacterial consortium (*Rhodococcus* sp. MO227 and *Pseudomonas* sp. TP 335) and sowing of plants. The suspension of bacteria was inoculated in the soil at the beginning of the experiment. On 31st day of incubation the plants were sowed in separate samples of soil. Initial degree of contamination: 142 000; 96 000; 70 000 and 44 000 ppm of TPH; total time of incubation: 2 months; temperature: 20-2 °C. Sample variants: A – Contaminated soil in the beginning of the experiment. B – Soil treated by bacterial consortium. C – Soil treated by bacterial consortium and sowed with ryegrass. D – Soil treated by bacterial consortium and sowed with alfalfa. E – Soil treated by bacterial consortium and sowed with soybean.

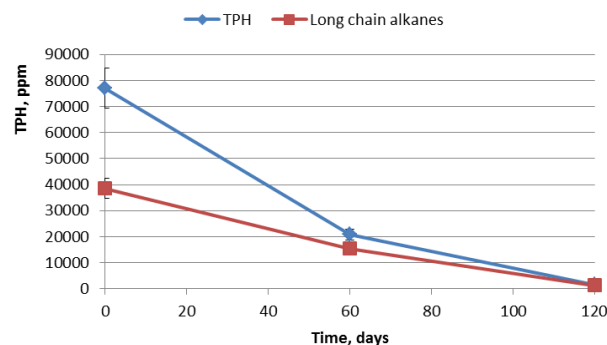


Figure 2. Phytoremediation of soil artificially contaminated with crude oil and wax by using of Alfalfa, bacterial consortium (*Pseudomonas* sp. 6R67 + *Bacillus* spp.). Initial contamination of soil with TPH - 77 000 ppm, duration of experiment – 4 months.

3.3. Soil Contaminated with TNT

3.3.1. Selection of Microorganisms

The screening of microorganisms for their ability to degrade TNT was performed. More than 200 bacterial strains from the Durmishidze Institute of Biochemistry and Biotechnology Collection of Microorganisms and Cultures were tested. These strains were isolated from the soils of different regions of Georgia, including the soil of proving grounds in the territory of Georgia.

The screening was carried out in the solid nutrient media containing different concentrations of TNT (0.01 mM, 0.1

mM and 1.0 mM) as the source of carbon and nitrogen. As a result of the screening 34 strains of genera of *Pseudomonas*, 1 strain of *Bacillus*, 8 strains of *Rhodococcus* and 5 strains of *Mycobacterium* were proven to be characterized by good growth on the solid nutrient medium containing 1 m MTNT, and were subsequently selected. For these strains optimal growth conditions (content of nutrient medium, pH, temperature) were established. In order to increase efficiency of selected strains towards TNT degradation, 7 different bacterial combinations were composed and tested under conditions of submerged cultivation containing 0.5 M of TNT. The cultivation was conducted during 3-7 days on a thermostatic shaker (180-200 rev. per min; at a temperature of 28-30°C) in a modified Czapek agar, containing 0.5 mM TNT. Glucose was added to the activation of strains.

3.3.2. Plants

High tolerance of soybean to TNT while comparing the growth parameters of testing plants should be mentioned. Growth parameters of this plant on the saturated solution of explosive (1.0 mM) decrease only by 5-10%. To summarize the data, it can be concluded that the tolerance of tested 8 plants to TNT decreases according to following orders:

Soybean > Mung bean > Ryegrass > Chickpea > Chickling vetch > Alfalfa > China bean > Maize

3.3.3. Model Experiments

For the purpose testing of selected consortia and plants, was carried out model experiments by using $[1-^{14}\text{C}]$ TNT contaminated soils (conditions of the experiment are given in 2.5). The obtained results are present in Fig.3 and Fig.4.

According to the obtained results, presented in Fig. 3, content of TNT in soil decrease, but radioactivity remaining in the soil. Results of TLC analyze indicates formation of TNT radioactive metabolites in soil (Fig. 4). After a short period of bioremediation (after 8 days) was accomplished purposeful planting of soils. The radioactive metabolites, formed as a result of bacterial transformation of $[1-^{14}\text{C}]$ TNT, were effectively uptake from the soil by the plants. Fig. 4 evidences dynamic decrease of radioactivity and amount of metabolites with different R_f in soil extracts.

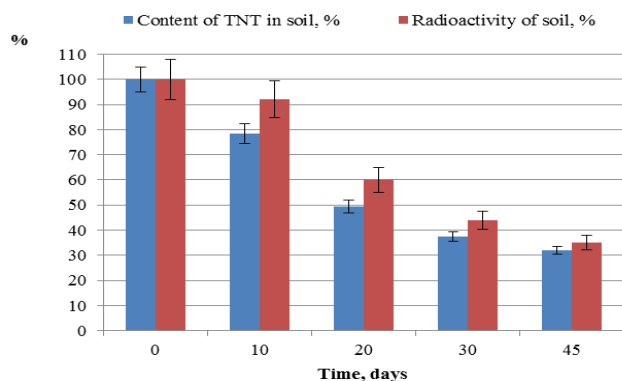


Figure 3. The dynamics of changing of TNT content in soil contaminated with $[1-^{14}\text{C}]$ TNT during cleaning by soybean and bacterial consortium containing strains of *Pseudomonas sp. TNT-44* and *Rhodococcus sp. TNT-74*.

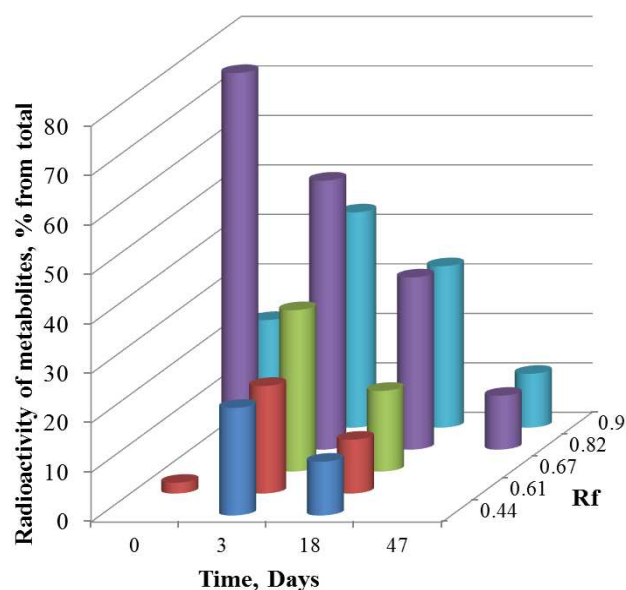


Figure 4. TLC analyses of $[1-^{14}\text{C}]$ TNT metabolites extracted from samples of polluted soil on 3rd, 18th and 47th days of the remediation process

4. Conclusion

1. It has been stated, content of composites for each contaminants: For TNT – B1/Z3 in ratio 1/3, For oil – B1/Z3 in ratio 2/3.
2. According to carried out model experiments was stated:
 - For effective phytoremediation of crude oil contaminated soil, can be used bacterial consortia *Rhodococcus sp. MO227* and *Pseudomonas sp. TP 335* and Soybean.
 - For effective phytoremediation of wax contaminated soil, can be used bacterial consortia *Pseudomonas sp. 6R67* + *Bacillus spp.* and Alfalfa.
 - For effective phytoremediation of TNT contaminated soil, can be used bacterial consortia *Pseudomonas sp. TNT-44* and *Rhodococcus sp. TNT-74* and Soybean.

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