Application of indigenous microorganisms in bioremediation of soil contaminated by fuel oil

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The present study was undertaken to evaluate the efficacy of introduced indigenous bacterial isolates for bioremediation of fuel oil contaminated soil. For this purpose five hydrocarbon-degrading indigenous bacterial isolates were screened from petroleum oil contaminated soil and repeatedly used for inoculation of fuel oil contaminated soil. The total petroleum hydrocarbons (TPH) content was determined by the gravimetric method. Hydrocarbon fractions (alkanes, aromatics, asphaltenes and resins) present in TPH were obtained by silica gel column chromatography. The study showed that some of the introduced bacterial isolates effectively adapted to the contaminated soil. The more efficient degraders were *Pseudomonas* and *Acinetobacter* isolates. Our results indicated that disappearance of TPH from inoculated soil samples depended on general soil impurity, the term of bacterial treatment and individual microorganism efficacy.

Key words: soil bioremediation, bacterial isolates, fuel oil hydrocarbons

INTRODUCTION

Biodegradation is a natural process carried out by soil and aquatic microorganisms – mostly bacteria and fungi. Certain bacterial strains have demonstrated the ability to break down or transform chemical substrates present in petroleum products. Biodegradation of hydrocarbons by natural populations of microorganisms is one of the primary ways by which crude oil is eliminated from contamined sites. Indeed, bacterial strains able to degrade many of the compounds present in oil are known to be ubiquitous in nature. For this reason, bioremediation has been considered a potentially useful tool in the cleaning of oil spills and the treatment of oil residues. Nevertheless, the presence of hydrocarbon-degrading strains in sites contaminated with oil, or oil residues do not guarantee that oil components will be metabolized.

Biodegradation of petroleum hydrocarbons in soil can be limited by many factors, including nutrient availability (usually N), bioavailability of pollutant, bacterial biomass (both total and hydrocarbon degraders) and the toxicity of the pollutant for the microorganisms degrading the pollutants [1].

The persistence of residual levels of petroleum hydrocarbons in bioremediation soil is therefore believed to be a consequence of the limited availability of contaminants for the microorganisms and not the inability of the microorganisms to degrade these compounds [2]. The availability can be limited by physical processes, through the presence of the contaminant in a separate organic phase or crystals [3]. Diffusion limitation in soil aggregates can result in slow desorbtion and reduce degradation over time [4].

Microbiological processes can reduce hydrocarbon concentrations in soil to a level that no longer poses an unacceptable risk to the environment or to human health.

From an environmental perspective, bacteria which grow on hydrocarbons and mineralize them may be especially useful for soil bioremediation. Many microbial strains, each capable of degrading a specific petroleum compound, are available commercially for soil bioremediation [5]. The reintroduction after enrichment of indigenous microorganisms isolated from a contaminated site helps to overcome this problem [6, 7]. The selected indigenous bacterial consortium has been shown to assist in bioremediation and has an advantage of being resistant to variations in the natural environment.

The aim of the current study was to evaluate the efficacy of introduced bacterial isolates for bioremediation of fuel oil contaminated soil for the

purpose of screening the active microorganisms for creation of biopreparates.

MATERIALS AND METHODS

Bacterial isolates. Some bacterial isolates that could degrade fuel oil were developed in minimal salt medium containing (g/l): NH_4NO_3 – 1.0, $MgCl_2$ – 0.1, KH_2PO_4 – 3.0, K_2HPO_4 – 7.0, $CaCO_3$ – 1.0 with oily sludge from the samples of fuel oil contaminated soil. Soil samples were collected from the Kiškėnai soil decontamination enterprise (Klaipėda region). Five indigenous bacterial isolates were selected for the present study. Bacterial isolates were screened on minimal salt medium, using oily-sludge as the sole carbon and energy source. These five isolates were characterised by standard morphological, physiological and biochemical techniques: two isolates were identified as *Pseudomonas* sp., two as *Alcaligenes* sp., the last was identified as *Acinetobacter* sp. [8].

Extraction of total petroleum hydrocarbons (TPH) from fuel oil contaminated soil. The TPH content was determined by the gravimetric method. 10 g of soil was four times extracted with 50 ml CH_3Cl. The residual TPH obtained after Soxhlet extraction was dried over 20 g of Na_2SO_4 and concentrated by evaporation at 60 °C to constant weight [9].

Fractionation of TPH. Hydrocarbon fractions present in TPH were obtained by silica gel column chromatography following the technique described elsewhere [9]. After four successive elusions of 100 mg fuel oil with 100 ml hexane, 100 ml benzene, 100 ml chloroform and 100 ml mixture of chloroform and ethanol (1:1), four fractions (alkanes, aromatics, asphaltenes and resins) were obtained. Each assay was performed in triplicate.

Biodegradation assays. In order to evaluate hydrocarbon biodegradation, bottles containing 500 g of unsterilized fuel oil contamined soil were inoculated with a separate pure bacterial inoculum (1%) and with a bacterial population mixture (1%) where each isolate made the equal part of inoculum, and grown at room temperature (14–18 °C). Each bacterial inoculum had 10^{10} cells / 1 ml physiological solution. Bottles containing non-inoculated, unsterilized soil were used as control. Triplicate cultures and controls were sampled after 1, 2, 4 and 6 months of growth to analyse the degradation.

Detection of antibacterial activity. A diffusion assay was used for this purpose [10]. For the agar-well diffusion assay, an overnight culture of the indicator isolate was used to inoculate a meat peptone agar medium (MPA) at 30 °C and poured into Petri dishes. After solidification, wells 5 mm in diameter were cut and 50 µl of culture supernatant fluid showing antibacterial activity was added to each well. The supernatant was obtained by growing the inhibitory producer isolate overnight on MPA at 30 °C. Cells were then removed by washoff with 0.9% NaCl solution. The supernatant placed in the wells was allowed to diffuse into the agar for 24–48 h at 30 °C and examined for inhibition. Each assay was performed in triplicate.

RESULTS AND DISCUSSION

The aim of the present investigation was to evaluate the efficacy of bacterial isolates introduced to stimulate the bioremediation of fuel oil contaminated soil where the indigenous population of hydrocarbon-degrading bacteria was low. It has been reported that when the population of indigenous microorganisms capable of degrading the target contaminant is less than 10^5 cells/g of soil, no significant bioremediation will occur [6, 11].

The initial population of bacteria in fuel oil contaminated soil was found to be 10^3 cells/ g soil. This level suggested that a bacterial consortium needed to be added.

The level of TPH contamination in soil samples at different time periods in the course of the study is shown in Fig. 1. The concentration of TPH in contaminated soil samples was 47 to 49 g/kg. As is shown in Fig. 1, TPH degradation in soil samples inoculated with pure bacterial culture was quite fast during the first two months of incubation, except for *Alcaligenes* sp. 1MK and bacterial mixture. The decrease of TPH from inoculated samples after 4 months was slower and in some cases the TPH was higher than in previous ones. This observation is in agreement with those of Barbeau et al., Mishra et al., who reported that disappearance of TPH from treated plots was faster during the first 3 months of inoculation and slowed down later [6, 12].

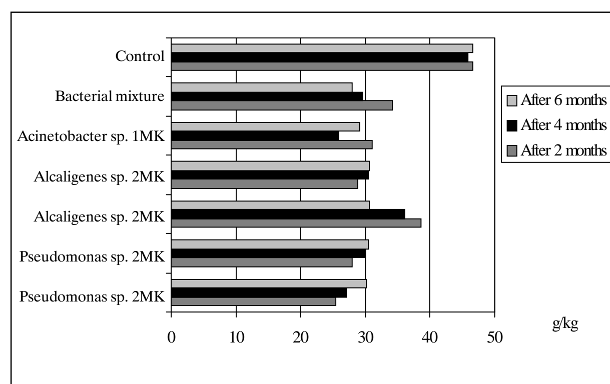

Fig. 1. The level of total petroleum hydrocarbons (TPH) in fuel oil contaminated soil (g/kg) during 6 months by addition of pure bacterial isolates and artificially mixed bacterial population

Table 1. **Inhibitory activity of some bacterial isolates (inhibition zones, mm)**

Indicator isolate	Test isolate (supernatant)				
	Pseudomonas sp. 1MK	*Pseudomonas* sp. 2MK	*Acinetobacter* sp. 1KM	*Alcaligenes* sp. 1MK	*Alcaligenes* sp. 2MK
Pseudomonas sp .1 MK	–	0	0	0	0
Pseudomonas sp. 2 MK	0	–	0	0	0
Acinetobacter sp. 1 MK	0	0	–	0	0
Alcaligenes sp. 1 MK	2	1	0	–	0
Alcaligenes sp. 2 MK	7	6	1	0	–

Five bacterial strains were tested for antimicrobial activity (Table 1). Bacteriocins of the bacteria demonstrate a inhibitory activity, which is directed principally against certain other bacteria. It is very important for determination of bacterium interrelationships and their reciprocity, especially in artificial associations. Two *Alcaligenes* isolates tested for antimicrobial activity at a high level against the other isolate did not show any inhibitory activity, while these isolates were sensitive to other three isolates. The implication is that the before-mentioned isolates are partly repressed. So their relation to bacterial association is dispensable and sometimes defensible. Confirmative results are presented in Fig. 1. These results showed that the more efficient degraders were *Pseudomonas* and *Acinetobacter* isolates.

The levels of TPH contamination in soil also play a major role during bioremediation. After a short period of intensive microbial pollutant conversion, the degradation nearly stopped for a high residual soil contamination. The removal of various fractions of TPH by a mixture of bacteria is shown in Fig. 2 and 3. One can see that biodegradation processes in less polluted soil (25.18 g/kg) were 1.6 times faster than in more polluted soil during the first month of incubation. In soil samples where the concentration of TPH was 46.77 g/kg, a total of 55.7% of alkane, 37.5% of aromatic, 46.3% of asphaltene fractions were removed within the first month. However, a total of resins in soil was higher and increased from 7.1 to 11.07 g/kg soil. The increase reached more than 54%. It seems that substrate-dependent causes (low water solubility, slow diffusion, adsorption, inhomogeneous pollutant distribution) are mainly responsible for the adsorption of pollutants in the soil and their biodegradability, because hydrocarbons can bind strongly to humic and other soil substances. In the soil samples where TPH concentration in control was 25.18 g/kg (Fig. 3), the removal of alkane, aromatic fractions was higher than in the soil with TPH 47 g/kg. A total of 44.8% of alkane, 83.3% of aromatic, 50.6% of asphaltene fractions were removed. But the content of resins after one month increased and was 3.8 times higher than in control, possibly because asphaltene and resins are but slowly degraded by microorganisms and therefore are known as high molecular weight hydrocarbons. Although some of asphaltenes are degraded by some microbial species, their degradation products can be bound to resins and make a new

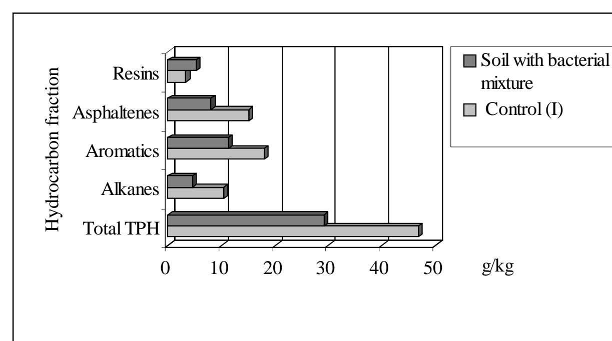

Fig. 2. The removal of various fractions of total petroleum hydrocarbon in fuel oil contaminated soil (46.44 g/kg) within 6 months by addition of artificially mixed bacterial population

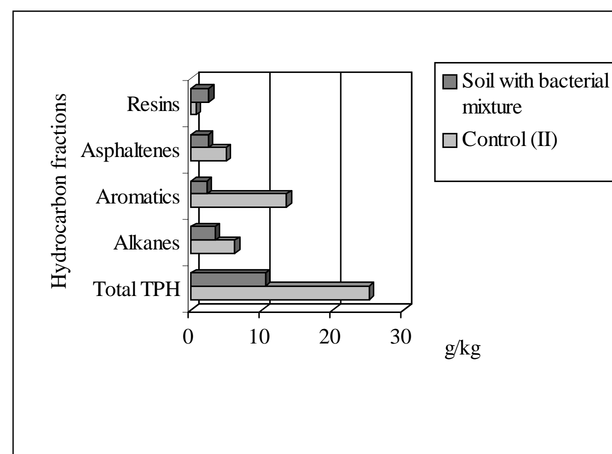

Fig. 3. Removal of various fractions of total petroleum hydrocarbons (TPH) in fuel oil contaminated soil (25.18 g/kg) within 6 months by addition of artificially mixed bacterial population

asphaltene [13]. Resins and asphaltenes are similar in many ways. Both groups are thought to be composed of condensed aromatic nuclei, which may carry alkyl and alicyclic systems containing heteroatoms such as nitrogen, sulfur and oxygen. Polar, higher molecular weight aromatics may fall in the resin or asphaltene fraction. It is now recognized that the heaviest fraction is formed by aromatic compounds with π–π interactions, undergo acid-based interactions, and self-associate through hydrogen bounding [14–16]. Consequently, the content of resins can constantly increase. The resin fraction showed that the highest biodegradation efficacy falls to the first two months. Subsequently the content of resins in the soil can increase due to the fact that resins gradually accumulate.

The study showed that some of introduced bacterial isolates effectively adapted to the contaminated soil. The removal of various TPH fractions from fuel oil contaminated soil is shown in Table 2. Various bioremediation strategies are available at present, but the use of indigenous microorganisms with the adaptation potential has proven to be one of the most powerful tools [17, 18]. The most promising isolate in our study was *Acinetobacter* sp. 1MK (Table 2). Isolates of this genus are reported in some recent studies [6, 12, 19]. The introduced strains of *Acinetobacter* sp. were found to be stable and active after 1 year at the bioremediation site. This might be due to the maintanance of suitable soil conditions, including moisture level, nutrients and aeration. Under our conditions, bacteria remain active for 3 months at longest. As is shown in Table 2, the decrease of alkanes, aromatics from fuel oil contaminated soil inoculated with *Acinetobacter* sp. was much higher than in soil samples inoculated with pure cultures of the *Pseudomonas* sp. The higher removal of asphaltene fraction correlated with a higher content of resins in the soil. These facts suggest that the biopreparation can be viable for the fuel oil contaminated soil bioremediation.

The results showed that both indigenous microbial populations in fuel oil contaminated soil were able to feed on every hydrocarbon fraction and that the application of the bioremediation technology could be a very useful tool for the treatment of hydrocarbon fractions present in waste. However, in order to increase the rate of hydrocarbon bioremediation, it will be necessary to obtain additional information with the purpose to select the most appropriate way of application of active microbial strains.

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Table 2. **Removal of various fractions of total petroleum hydrocarbons (TPH) from fuel oil contaminated soil (g/kg) inoculated with pure bacterial culture**

Variants	Alkanes (g/kg)	Aromatics (g/kg)	Asphaltenes (g/kg)	Resins (g/kg)	Total (g/kg)
Control	6.14 ± 0.3	13.47 ± 1.3	4.92 ± 0.2	0.65 ± 0.1	25.18 ± 0.6
Pseudomonas sp. 1 MK	5.94 ± 0.6	1.67 v 0.3	3.05 ± 0.1	2.79 ± 1.1	13.45 ± 0.8
Pseudomonas sp. 2 MK	4.19 ± 0.5	1.19 ± 0.2	3.62 ± 0.4	2.17 ± 0.5	11.17 ± 0.7
Acinetobacter sp. 1 MK	2.79 ± 0.2	1.17 ± 0.4	3.93 ± 1.2	1.88 ± 0.3	9.77 ± 0.5

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VIETINIŲ MIKROORGANIZMŲ PANAUDOJIMAS MAZUTU UŽTERŠTO GRUNTO BIOREMEDIACIJAI

S a n t r a u k a

Darbo tikslas – įvertinti iš mazutu užteršto grunto išskirtų vietinių mikroorganizmų efektyvumą juos pakartotinai introdukuojant į mazutu užterštą gruntą. Atrinkti penki bakterijų izoliatai, skirtingu aktyvumu naudoję mazuto frakcijas. Šių junginių biodegradacija tirta gravimetriniu ir skysčių chromatografijos metodais. Rezultatai parodė, kad grunto bioremediacijos procesas priklausė nuo jo užterštumo lygio, mikroorganizmų introdukcijos laiko bei bakterijų specifiškumo. Per kelis mėnesius mazutu užterštame ir grynomis bakterijų kultūromis bei jų mišiniais inokuliuotame grunte sumažėjo alkanų ir aromatinių junginių frakcijos, tačiau tiriamuose pavyzdžiuose padidėjo dervų kiekis. Geriausiai mazuto frakcijų biotransformacija vyko grunto pavyzdžiuose, inokuliuotuose *Acinetobacter* ir *Pseudomonas* genčių izoliatais.