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BIOAUGMENTATION OF SOIL CONTAMINATED BY FUEL OIL RESIDUE

Strains of microorganisms actively degrading fuel oil were used as inoculants in the process of biological treatment of contaminated soil. Inoculants were applied to the soil in the form of free bacterial cells suspension and in the form of immobilizates on ash wood shavings. The study showed that biodegradation process was faster in the case of inoculation with immobilized bacterial cells. Based on bioindication study it was observed that some harmful metabolites were produced during the biodegradation process.

1. INTRODUCTION

Fuels, oil and other petroleum derivatives are very important in contemporary energy management. Their extensive use over past few decades has led to their widespread release into the environment. They contaminate soils and both surface and ground waters. Pollution of environment with these compounds is particularly dangerous because of their detrimental effects on living organisms. As petroleum and its products are used by some microorganisms as food substrates, the biodegradation of these compounds appears to be very important in their removal from the environment. Moreover, the treatment of contaminated soils by means of microorganisms is usually considered as primary means of soil reclamation. This offers many advantages in comparison to the conventional practices of excavation and removal of soil to landfill sites or combustion which is associated with the possibility of formation of highly toxic products. Bioremediation seems to be a very economically competitive and environmentally friendly remediation technology.

When biodegradation of fuel oil starts, at first easily degradable compounds are used by microorganisms as a source of carbon and energy. More recalcitrant compounds usually remain unchanged for a long time. Cycloalkanes, aromatic hydrocarbons and PAHs are often hardly degradable. They are considered as very dangerous

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fuel oil residue and some of them are toxic or mutagenic and carcinogenic. It is also important to avoid the formation of metabolites having such properties during biodegradation of fuel oil residue (WATKINSON [5]). After bioremediation, the soil should not contain any toxic or mutagenic and carcinogenic substances.

Immobilized microbial enzymes, organelles and cells have been used in a variety of scientific and industrial applications. Immobilization is a general term that describes many different forms of cell attachment or entrapment. These different forms comprise flocculation, adsorption on surfaces, covalent bond between a cell and carrier, cross-linking of cells, encapsulation in a polymer-gel and entrapment in a matrix.

Immobilization technology has been used extensively in commercial bioreactor fermentations. The results from bioreactor tests have proved that encapsulated cells have advantages over free cells under various conditions. For example, an intensified activity in metabolite production, protection from toxic substances and increased plasmid stability of encapsulated cells compared to free cells have been observed.

The use of immobilized cells has also been investigated as an alternate technology for environmental applications. For example, the use of immobilized cells in agriculture, biocontrol, pesticide application and also phenol, pentachlorophenol, *p*-cresol and some PAH's biodegradation in contaminated soil or ground water have been examined. Most of the investigations were carried out on laboratory scale and the applications of immobilized cells in an open environment have yet to be realized (CASSIDY et al. [1]).

The aim of this paper was to compare adaptation and performances of the inocula introduced into soil contaminated with fuel oil residue as free-cell suspensions and as immobilized bacterial cells in order to determine the possibility of application of such an immobilization to *in situ* treatment of sites contaminated with fuel oil.

2. MATERIALS AND METHODS

2.1. ISOLATION AND IDENTIFICATION

Bacterial strains used as inoculants were isolated from the soil highly contaminated by petroleum and from the effluents from field lysimeters containing such a soil. Soil extracts were made using 0.9% NaCl, and pure bacterial strains were isolated on nutrient agar plates. They were then tested several times during 3 months for their individual ability to degrade fuel oil. Fuel oil served as sole carbon and energy source and was added in the concentration of 0.5% (v/v) to a mineral medium containing (per liter of demineralized water): 1.56 g of KH_2PO_4, 2.13 g of Na_2HPO_4, 0.5 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7\ H_2O$, 0.02 g of $CaCl_2 \cdot 2\ H_2O$, 1 ml of a trace-element solution (per liter of demineralized water): 2.7 g of $FeCl_3 \cdot 6\ H_2O$, 0.1 g of

H_3BO_3, 0.1 g of $ZnSO_4 \cdot 7\ H_2O$, 0.05 g of $Co(NO_3)_2 \cdot 6\ H_2O$, 0.005 g of $CuSO_4 \cdot 5\ H_2O$, 0.005 g of $MnCl_2 \cdot 6\ H_2O$.

The bacterial strains selected for their high capacity to use fuel oil as a sole source of carbon and energy were identified based on their morphological, physiological and biochemical features. The isolated strains were all strictly aerobic, psychrophilic, catalase- and oxidase-positive and showed a negative reaction in the MR test. They gave different responses in tests of liquefaction of gelatin, digestion of casein, hydrogen sulphide production, β-galactosidase activity, Voges–Proskauer reaction, carbohydrate fermentation and nitrate reduction. According to Bergey's identification manual they were identified as members of the following genera: *Pseudomonas* (3), *Bacillus* (1), and *Arthrobacter* (1).

2.2. SOIL

The soil used in this experiment was taken from one of the field lysimeters used in other experiment (KOŁWZAN and STEININGER [3]). The surface layer of soil in lysimeters (to the depth of 15 cm) was contaminated with fuel oil up to the final oil concentration of 9.5% dry matter. Ammonium nitrate, ammonium phosphate and potassium phosphate were added to the soil in order to provide the proper ratio of nitrogen and phosphorus to carbon, C:N:P = 10:1:0.1. The lysimeters were then inoculated with different bacterial strains.

In this experiment soil from a lysimeter inoculated with *a Pseudomonas* sp. (3) strain four months before was used. The concentration of fuel oil in soil at the beginning of the experiment was 1400 mg/kg of dry matter.

2.3. FUEL OIL

The fuel oil used for soil contamination was a pure fuel oil fraction from petroleum refining (obtained from Mazowieckie Zakłady Rafineryjne i Petrochemiczne). It was a mixture of C_8–C_{25} hydrocarbons (especially C_{10}–C_{20}). N-alkanes, isoalkanes and naphthenes were predominating (about 70% by weight). Other fuel oil compounds were different aromatic hydrocarbons (some PAHs were present).

2.4. IMMOBILIZATION PROCEDURE

The carrier choice in the case of soil inoculation does not seem to be very easy. Every anthropogenic substance should be avoided as a potential xenobiotic. Natural mineral carriers may affect the ratio of elements in the soil. Natural organic carriers such as shavings, shells, etc., seem the best. In this group, such a substance must be chosen that does not contain too much resins and that is easily degraded by microor-

ganisms, but not too fast – the degradation of carrier instead of fuel oil biodegradation should not take place.

In this immobilisation assays, ash wood shavings were used as carriers for immobilized bacterial cells. Before the immobilization the wood shavings were just once washed with phosphate buffer of pH = 7.

Based on the results from previous degradation assays, it was decided that for this experiment 3 pairs of pure bacterial strains and one single strain would be chosen. Bacterial strains used for inoculation were grown on nutrient agar plates. The cultures were washed with phosphate buffer (50 cm^3). Each bacterial suspension was twice centrifuged and cells were resuspended in phosphate buffer (500 cm^3). Immobilisation was performed in a special system. The bacterial suspension was recycled through containers, each containing 50 g of ash wood shavings at a flow ratio of 500 ml/30 min during 20 hours. The peristaltic pump worked for 30 minutes per hour.

2.5. EXPERIMENTAL SET-UP

Inoculation experiments and native biodegradation experiments were performed in aerated bioreactors filled up with 1250 g of soil contaminated with fuel oil in the concentration of 1400 mg/kg of dry matter. Six aerated bioreactors were inoculated and one was left as a control of the bioremediation process, where biodegradation was carried out by the indigenous soil microflora. Three reactors were inoculated with free bacterial cells in suspension (pairs of strains: E12 – *Pseudomonas* sp. E1 and *Pseudomonas* sp. E2; J5 – *Bacillus* sp. $J5_1$ and *Pseudomonas* sp. $J5_2$; single strain: *Arthrobacter* sp. 45) and three reactors with immobilizates (E12I, J5I, 45I, respectively). The initial number of bacteria in bioreactors was circa $6 \cdot 10^5$ colony-forming units (CFU)/g of soil.

The experiment stand was protected from precipitations and solar radiation, but not from temperature fluctuations. The soil moisture was held on the level of 12–15%. The bioreactors content was mixed every two days. During the whole experiment (6 weeks), air temperature and soil pH were controlled.

Temperature was kept on the level of the thermic optimum characteristic of the strains tested and its variations were very low. The pH variations did not exceed 0.7 for one bioreactor. Soil samples for microbiological and biochemical analyses were taken at regular intervals throughout the whole experiment (every 7 days).

2.6. ANALYTICAL METHODS

Quantitative estimation of fuel oil removal at the end of the experiment was performed by means of mass spectrometer connected with gas chromatograph GS-MS Shimadzu (QP 2000, GC-14A) on SPB-5 column after hexane extraction.

Dehydrogenase activity of soil was determined based on the TTC method. To 6 g of soil, 60 mg of $CaCO_3$, 1 ml of 3% TTC solution, 2.5 cm^3 of distilled water were added. Each sample was incubated at 37 °C for 24 hours. Then 10 cm^3 of acetone were added, the sample was shaken, and after 5 minutes of sedimentation in the dark, the supernatant was filtered. TF concentration was monitored using a spectrophotometer (Shimadzu UV-1202) at 485 nm.

For bioindication analysis the Ames method was used. The tests were carried out with two strains, *Salmonella typhimurium* TA 98 and TA 100 (obtained from the Ames Laboratory, Department of Biochemistry, University of California, USA), with microsomal fraction S-9 and without this fraction. The whole assay was carried out minutely according to the author's indications and the mutagenic activity ratios were calculated in order to determine the mutagenic and carcinogenic properties of soil after the biodegradation process (MARON and AMES [4]). The samples for bioindication were prepared by extracting the remaining oil and its metabolites from 25 g of soil with dichloromethane and methanol (continuous extraction for 6 hours in Soxhlet apparatus) (BROWN et al. [2]).

3. RESULTS

Experiment of the bioremediation of soil contaminated by fuel oil residue was carried out in seven aerated bioreactors. Three reactors were inoculated with free bacterial cells in suspension, three reactors with immobilizates and one left as a control of the bioremediation process, where biodegradation was carried out by the indigenous soil microflora. Electron micrographs were performed after immobilization of bacterial cells.

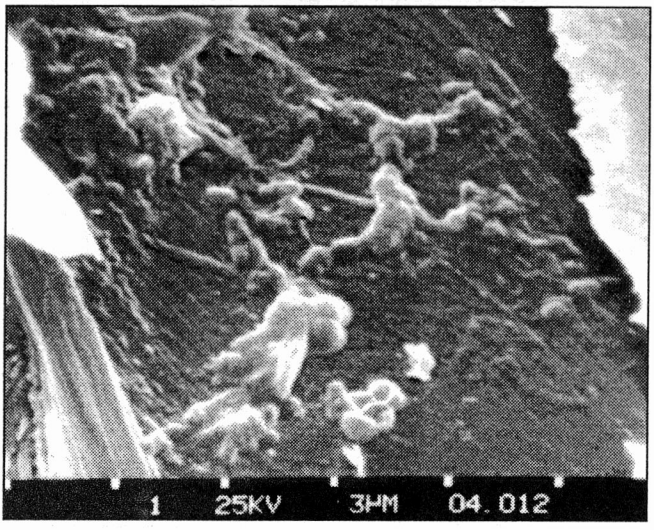

Fig. 1. E12 mixture (*Pseudomonas* sp. E1 and *Pseudomonas* sp. E2) on the surface of ash wood shavings

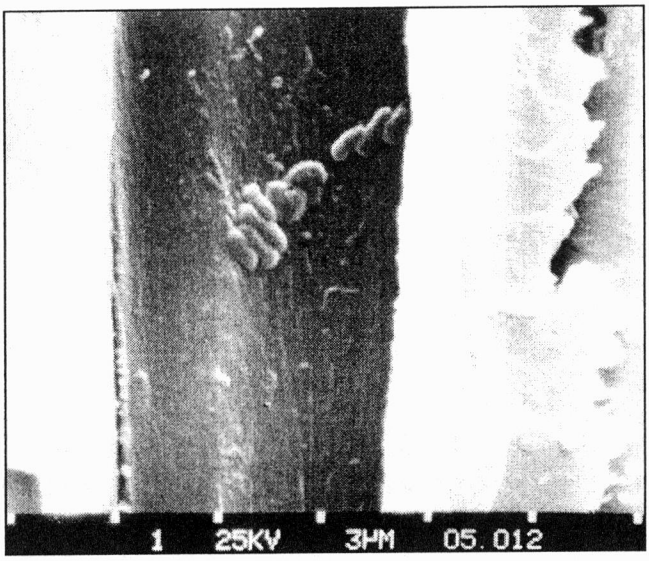

Fig. 2. J5 mixture (*Bacillus* sp. $J5_1$ and *Pseudomonas* sp. $J5_2$) on the surface of ash wood shavings

Table 1

Dehydrogenase activity of soil during fuel oil degradation

Time (days)	Dehydrogenase activity of soil (mg TF/g soil)						
	E12	E12-I	J5	J5-I	45	45-I	Control
0	0.169	0.169	0.169	0.169	0.169	0.169	0.169
7	0.351	0.264	0.224	0.286	0.262	0.377	0.212
14	0.884	0.821	0.947	0.836	0.862	0.774	0.260
21	1.258	0.991	0.965	0.986	0.699	0.946	0.269
28	1.136	1.230	0.891	1.136	0.986	0.775	0.254
35	0.662	0.574	0.677	0.612	0.736	0.566	0.369
42	0.570	0.587	0.390	0.533	0.430	0.279	0.231

Table 2

Removal of fuel oil from contaminated soil

Bacterial mixtures	E12	E12-I	J5	J5-I	45	45-I	Control
Fuel oil concentration at the end (mg/kg soil)	140.00	3.22	85.40	79.52	194.46	121.80	653.52
Fuel oil removal from soil (%)	90.00	99.77	93.90	94.32	86.11	91.30	53.32

Dehydrogenase activity of soil was monitored throughout the whole experiment. Substrate removal and bioindication analysis were performed at the end of the experiment. The dehydrogenase activity increased considerably in the second week of the experiment, reached its maximum in the fourth week and droped thereafter. The highest dehydrogenase activity was measured in the bioreactor inoculated with the pair E12 (*Pseudomonas* sp. E1 and *Pseudomonas* sp. E2), while the lowest in the bioreactor inoculated with *Arthrobacter* sp. 45. Variations in dehydrogenase activity in the control bioreactor were very small compared to inoculated ones (table 1).

Measurements of the fuel oil concentration in soil show that biodegradation was particularly efficient in the inoculated bioreactors. At the beginning of the experiment, the fuel oil concentration in the soil was equal to 1400 mg/kg of dry matter. After six weeks of the experiment, the substrate removal approached 86 to 99%, while in the control bioreactor, where biodegradation was carried out by the indigenous soil microflora, the drop in oil content reached only 53%. The highest oil removal at the end of the experiment was observed in the bioreactor inoculated with the immobilized pair E12 (*Pseudomonas* sp. E1 and *Pseudomonas* sp. E2). Oil content in the soil was reduced in this bioreactor in 99%. Inoculation with *Arthrobacter* sp. 45 was modestly successful. The elimination of fuel oil hydrocarbons was achieved in only 86% (table 2).

The Ames test has demonstrated that during the biodegradation of fuel oil residue some potentially mutagenic and carcinogenic compounds are formed, since many samples have caused an increase in the reversion frequency of one of the test strains used in this experiment. The strain of *Salmonella typhimurium* TA 98 proved the potential mutagenic or carcinogenic properties of soil being subjected to bioremediation carried out by all bacterial mixtures, except for the mixture E12. The Ames test gave also negative results for the control sample. The bioindication tests carried out with the strain TA 100 revealed no mutagenic and carcinogenic activity of the soil extracts tested (table 3).

Table 3

Results of the Ames test

Salmonella typhimurium	Mutagenic activity ratio for different samples						
	E12	E12-I	J5	J5-I	45	45-I	Control
TA 98	1.35	1.14	6.62	8.35	4.82	6.12	6.50
TA 98 + S9	1.90	1.13	4.69	5.84	3.69	4.67	4.12
TA 100	0.88	0.84	1.03	1.16	0.95	1.09	1.09
TA 100 + S9	0.93	1.01	1.09	1.4	0.97	1.14	0.72

At the end of the experiment, degradation of the major part of wood shavings was observed in the reactor inoculated with the mixture E12. Apparently, this carrier can

also be degraded by the strains *Pseudomonas* sp. E1 and *Pseudomonas* sp. E2 when the fuel oil residue is already used.

4. CONCLUSIONS

- Biodegradation was strongly enhanced and the inoculum adaptation occurred faster and easier when instead of free cells the bacteria immobilized in ash wood shavings' carrier were used for the inoculation of the soil contaminated by fuel oil.
- The measurement of dehydrogenase activity of soil is an appropriate method for monitoring the biodegradation of fuel oil residue.
- Constant monitoring of bioremediation process allowing us to minimise the risk associated with harmful and potentially mutagenic and carcinogenic products of fuel oil biodegradation is urgently needed.
- Repeated inoculation of soil allowed almost complete removal of fuel oil residue.
- Microbial treatment of the sites contaminated by fuel oil should not be carried out *in situ* because of the possibility of forming some dangerous metabolites.

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BIOLOGICZNE OCZYSZCZANIE GLEBY SKAŻONEJ OLEJEM NAPĘDOWYM

Szczepy drobnoustrojów aktywnie rozkładające olej napędowy zastosowano jako inokulanty w procesie biologicznego oczyszczania skażonego gruntu. Inokulanty wprowadzono do gleby w postaci zawiesiny bakteryjnej oraz zimmobilizowane na wiórkach jesionu. Badania wykazały, że proces biodegradacji oleju napędowego zachodzi szybciej w przypadku inokulacji mikroorganizmami zimmobilizowanymi. Na podstawie badań bioindykacyjnych stwierdzono, iż podczas biodegradacji tworzyły się niebezpieczne metabolity.