INVESTIGATION OF HYDROCARBON-DEGRADING MICROBIAL COMMUNITIES OF PETROLEUM HYDROCARBON CONTAMINATED SOILS IN HARGHITA COUNTY, ROMANIA

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ABSTRACT: *Microorganisms able to degrade hydrocarbon pollutants (gasoline, and other petroleum derivatives) have been isolated from two contaminated areas (Sândominic and Bălan) by enrichment technique. The hydrocarbon degradative capacity of contaminated sites were determined using "Biometer flasks" (respiratory CO_2 production measurement). Total counts and hydrocarbon degrading counts were determined using the MPN method. Isolated strains were tested for their capacity to degrade petroleum derivatives (such as benzene, toluene, xylene, n-dodecane and naphthalene). Those strains which showed great degradation efficiency in case of all investigated hydrocarbons were identified based upon the sequence analysis of their 16S rDNA. Based on the cumulative CO_2 production curves it is evident that in the contaminated soil samples there is an increased activity of hydrocarbon degrading microorganisms. During our study we isolated 30 bacterial strains which showed fast growth on at least one of the compounds tested. So far we have identified four strains which could be applicable in bioaugmentation processes. Strains R22 and R31 were identified as Rhodococcus erythropolis; R26 as Micrococcus luteus, R27 as Bacillus cereus and strain R12 as Bacillus subtilis.*

KEYWORDS: *petroleum hydrocarbon contamination, bioremediation, hydrocarbon degrading bacteria, 16S rDNA sequences*

1. INTRODUCTION

Petroleum derivatives, usually mixtures of aromatic and aliphatic hydrocarbons, are widely used compounds to power automobiles, heat homes, and fuel other means of energy production. Though they are of intensive public concern due to their ecotoxicological and other deleterious effects on human and environmental health. The ecotoxicity of petroleum hydrocarbons means that they affect negatively the biochemical pathways of organic matter degradation and nutrient cycling. Contamination with petroleum products during storage and transportation is unavoidable. Oil spills results from accidental leaking of aboveground and underground storage tanks, pipelines, spillage during transport, abandoned gasoline sites and industrial processes (Sarkar et al., 2004; Vasudevan and Rajaram, 2001). Petroleum contains hazardous, carcinogenic chemicals such as BTX (benzene, toluene, xylenes) compounds hazardous to the health of plants, animals and humans (Prantera et al., 2002).

Petroleum-contaminated soils can be treated using physical, chemical and biological processes. Physical (condensation, adsorption on solids, absorption in liquids, incineration, disposal in a landfill) and chemical (thermal and catalytic destruction, ozonization etc.) methods are capable of removing a broad spectrum of compounds, but energy consumption and/or consumption of chemicals are disadvantages of these methods. Biological treatment involves the transformation of contaminants into nontoxic compounds based on the metabolic activity of microorganisms. Using microorganisms in destruction of toxic compounds (bioremediation) is a cost-effective method, applicable over large areas and (usually) leads to the complete breakdown of the contaminants, leads to pollutant mineralization to CO_2 and H_2O. In the case of physical-chemical treatments the pollutants are transferred from one environmental compartment to

another (Trindade et al., 2004; Mrozik and Piotrowska-Seget, 2009; Sung-Chang and Young-Sook, 2002; Bento et al., 2004; Sabaté et al., 2003; Sarkar et al., 2004).

Bioremediation is an attractive, cost-effective, environmentally sound method of cleaning up petroleum hydrocarbons using microorganisms. Main strategies for *in situ* bioremediation include natural attenuation, biostimulation and bioaugmentation. Bioremediation of petroleum contaminated soil can be enhanced by stimulation of the indigenous microorganisms, by introducing nutrients (sources of N, P) and oxygen to soil (biostimulation) (Seklemova, 2001) or through inoculation of an enriched microbial consortium (indigenous, allochthonous, or genetically modified strains) into soil (bioaugmentation) (Richard and Vogel, 1999; Barathi and Vasudevan, 2001).

The aim of our research was the investigation of hydrocarbon-degrading microbial communities of petroleum hydrocarbon contaminated sites in Harghita County, and the isolation and characterization of bacteria which are adapted to the pollution and able to degrade the diesel oil compounds. The isolated strains may be applicable to further use in bioaugmentation processes.

2. MATERIALS AND METHODS

2.1. SOIL SAMPLES

Petroleum contaminated soil samples were collected from two hydrocarbon polluted sites – the mine fuel depot, and the Sândominic fuel depot of the Bălan Mine Company – on March 2007 and April 2009. Samples were taken from the top 10 cm of soil with sterile spatula into glass bottles. In case of each contaminated site one soil sample was collected for chemical analysis, another for biological investigations. A non-contaminated one was collected at the same sites as control sample. Samples were stored at 6°C until laboratory investigations.

2.2. RESPIRATORY ACTIVITY

Respiratory activities in petroleum-contaminated soil samples were evaluated based on CO_2 production of microorganisms using Biometer flasks (Bossert and Kosson, 1997). Biometer flask (250 ml) (*fig.1*) is a CO_2 trapping system which contained 50 g soil, 5-10 ml of BBH (Bacto-Buschnell-Hass – 0.2 g $MgSO_4$, 0.02 g $CaCl_2$, 1 g KH_2PO_4, 1 g K_2HPO_4, 1 g NH_4NO_3, 0.05 g $FeCl_3$ x $6H_2O$, 1000 ml distilled water) solution as N and P source and 15 ml 0.1 N KOH solution placed in the lateral tank destinated to absorb the CO_2 produced by microorganisms. The flasks were incubated at 10°C, liquid in the lateral tank was analyzed weekly to calculate the amount of CO_2 absorbed by the KOH solution. This procedure was repeated for up to 28 weeks.

The CO_2 content was analyzed by the addition of 1 ml 2 N $BaCl_2$ solution to 50 ml "KOH-CO_2" (15 ml "KOH-CO_2" + 35 ml CO_2-free distilled water) trapping solution from lateral tank. The volume was supplemented with 3-4 drops of phenolphthalein, was then titrated with 0.05 N HCl solution. The amount of CO_2 released in each flask during microbial respiration was calculated using the equation below:

$$V_{CO_2} = 1.175 \cdot 10^{-5} \cdot C_{HCl} \cdot \left(V_{KOH} - V_{sample}\right)$$

where:

- V_{CO2} – amount of CO_2 produced, ml
- C_{HCl} – Concentration of HCl, mol/L
- V_{KOH} – quantity of HCl used for titration of 15 ml KOH solution, ml
- V_{sample} – quantity of HCl used for titration of 15 ml KOH solution after incubation, ml

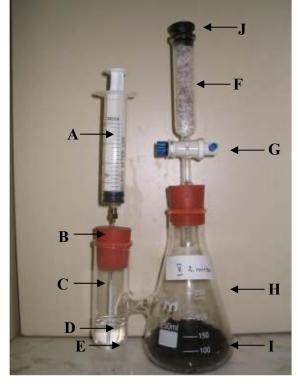After titrations, the amounts of CO_2 for each sample were illustrated in function of time in cumulative curves.

Fig.1. Biometer flask

A. syringe; **B**. rubber with syringe needle; **C**. KOH solution tank; **D**. 0.1 N KOH solution; **E**. polyethylene socket; **F**. Raschig rings and KOH pastilles; **G**. tap; **H**. Erlenmeyer flask (250 cm^3); **I**. soil sample; **J**. closure cap.

2.3. ESTIMATION OF THE NUMBER OF TOTAL CULTIVABLE- AND HYDROCARBON DEGRADING BACTERIA

Total counts (total cultivable bacteria/ml), and hydrocarbon degrader counts were estimated by MPN (Most Probable Number) method (Braddock and Catterall, 1999), using 96 well microtitre plates in case of soil samples collected in March 2007.

Nutrient broth (1 g meat extract, 2 g yeast extract, 5 g peptone, 5 g NaCl, 1000 ml distillated water; pH 7, sterilization at 121 °C, 15 min) was used for determining the number of total cultivable bacteria and BBH solution supplemented with resazurin indicator for hydrocarbon degraders. 3 g of each soil sample was placed into 50 ml Erlenmeyer flasks, vortexed for 10 min. with 27 ml distilled water and serially diluted to 10^{-8} in BBH solution. From each member of dilution 0.3 ml was dispensed into wells of microtitre plate (five replicates) which contained 1 µl of sterile gasoline and incubated at 28°C for 2-4 days.

On basis of the number of positive test tubes the counts of microorganisms were statistically evaluated (Braddock and Catterall, 1999).

2.4.   MICROORGANISMS AND GROWTH CONDITIONS – SCREENING ASSAY

Bacterial strains used in this study were isolated from the hydrocarbon contaminated soils. Strains designated with "R" were isolated from contaminated soil samples collected at March 2007, while others marked with "CSB…, CSG…, BB…, BG…" at April 2009. Gasoline degraders were enriched and screened for their ability to degrade gasoline as sole source of carbon and energy in a mineral salts basal liquid medium (100 ml) (BBH) containing gasoline 1% (v/v) as sole source of carbon and energy. After 2 weeks of incubation at 23°C the enriched cultures, as a second selection, were inoculated onto BBH agar plates. The lid of Petri-dishes contained 250 µl of sterile gasoline as sole source of carbon and energy.

Bacterial isolates that showed the most rapid growth in screenings were inoculated onto nutrient agar plates and stored at 6°C for further investigations.

2.5.   TESTING STRAINS FOR THEIR ABILITY TO DEGRADE INDIVIDUAL BTX COMPOUNDS

Isolates that showed a good growth on BBH agar supplemented with gasoline were tested, i.e. their capacity to degrade benzene, toluene, xylene, n-dodecane and naphthalene was tested. Test solutions contained 50 ml BBH solution, one of the hydrocarbons (1 g/l; as above) and resazurin as a redox indicator and were inoculated with 250 µl of individual isolates ($OD_{600} = 1$). The change in color of resazurin (blue >> pink >> colorless) provided information about the degradation rate. In the case of increased microbiological activity the initial blue color of test solution changed to colorless via pink (*fig.2*). Each test runs were incubated for a week on a rotary shaker at 145 rpm and 28°C.
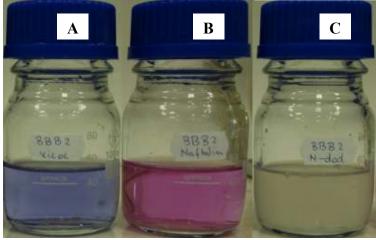

Fig.2. Color change of resazurin indicates the microbiological degradation of petroleum hydrocarbons – no degradation-blue color (A); reduced or slow degradation-pink color (B); increased degradation-colorless (C).

2.6.   IDENTIFICATION OF MICROORGANISMS ON THE BASIS OF THEIR 16S RDNA SEQUENCES

Selected strains were identified using the 16S rDNA gene-based sequence comparison technique. Genomic DNA of strains was extracted with Wizard Genomic DNA Purification Kit (Promega), and used as template in PCR reactions to amplify 16S rDNA gene sequences. 27 forward and 1492 reverse primers were used to amplify the 16S rDNA fragments (Stapleton et al., 1998). The 16S rDNA PCR product was purified with Viogene DNA Purification Kit (Viogene-Biotek Corp.) and used as template for sequencing reactions. The nucleotide sequence determination was performed with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and sequences were analyzed with ABI 310 Genetic Analyzer (Applied Biosystems). The obtained sequences were aligned with the Chromas software http://www.technelysium.com.au), initially against known sequences of the GenBank

database (BLAST tool, NCBI) (Altschul et al., 1997). Sequence similarity over 98% was accepted as species level identification and 95%, respectively, for genus level identification.

3. RESULTS AND DISCUSSION

3.1. RESPIRATORY ACTIVITY

We observed that the indigenous microorganisms of soils produced a gradually decreasing amount of CO_2 over the 28 week period excepting one sample (Bălan contaminated). Soil samples contaminated with hydrocarbons produced significantly more CO_2 than the non-contaminated soils. The amount of produced CO_2 in case of non-contaminated soil samples was 3-4 times lower than in contaminated ones. The non-contaminated soil sample originated from Bălan produced only 1.75 ml of CO_2/ 50 g soil over the period. The reason of reduced soil respiration is due to the soil type, a practically "sterile mass" of spoil, which is poor in nutrients and carbon sources. Non-contaminated soil sample from Sândominic, in comparison to non-contaminated soil sample from Bălan, produced two times more CO_2 (3.89 ml CO_2/ 50 g soil). The Sândominic non-contaminated soil sample was rich in organic and humic materials and in plant residues (*fig.3*).

Among the petroleum contaminated soil samples most CO_2 was produced by the contaminated soil sample from Bălan (20.15 ml CO_2/ 50 g soil). The contaminated soil samples from Sândominic produced only 9.66 ml CO_2/50 g soil (contaminated I - Sândominic) and 10.42 ml CO_2/50 g soil respectively (contaminated II - Sândominic).

The increased amount of CO_2 produced by petroleum derivatives contaminated soil samples is due to the increased quantity of hydrocarbon pollutants which acts as energy and carbon source.

3.2. ESTIMATION OF THE NUMBER OF TOTAL CULTIVABLE- AND HYDROCARBON DEGRADING BACTERIA

The number of heterotrophic- and - among them - hydrocarbon degrading bacteria was estimated in soil samples collected at March 2007. The number of total cultivable- and hydrocarbon degrader bacteria was low (10^3 cells/g soil) in soil samples collected from Bălan. In contrast, in soil samples collected from Sândominic, the number of heterotrophic (10^5-10^7 cells/g soil) - and hydrocarbon degrading bacteria (10^4-10^5 cells/g soil) is higher with two or three orders of magnitude. The difference may be explained with the differences in soil characteristics. While the soil from Bălan is lithosoil, poor in organic matter until, soil samples originated from Sândominic are rich in humic and organic matter (*fig.4*).

It was observed that the number of heterotrophic bacteria in contaminated ($3.8 \cdot 10^3$ cells/g soil) and non-contaminated ($3.4 \cdot 10^3$ cells/g soil) soil samples from Bălan is similar. In the petroleum contaminated soil the number of heterotrophic- and hydrocarbon degrading bacteria is almost identical ($3.5 \cdot 10^3$ cells/g soil), this fact means that in the contaminated soil sample almost all bacteria are capable of utilizing hydrocarbon pollutants as source of carbon and energy. In non-contaminated soil sample the number of hydrocarbon degrading bacteria is $2 \cdot 10^2$ cells/g soil, lower whit one order of magnitude than the number of heterotrophic bacteria $2.5 \cdot 10^3$ cells/g soil (*fig.4*).
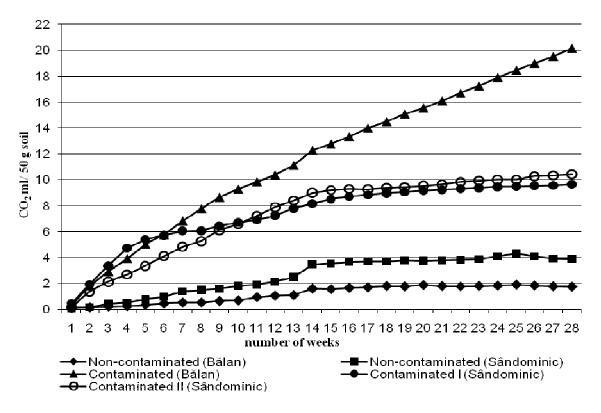
Fig.3. The result of microbial respiratory activities in contaminated and non-contaminated soil samples originated from Bălan and Sândominic.

In non-contaminated soil sample from Sândominic the number of heterotrophic bacteria is higher with two orders of magnitude ($1.7 \cdot 10^7$ cells/g soil), than in the contaminated soil sample ($1.7 \cdot 10^5$ cells/g soil). The reason could be that the organic matter and nutrient content of non-contaminated, lawn-covered soil is suitable for growth of practically all physiological types of bacteria. A large proportion of heterotrophic bacteria are given by the number of hydrocarbon degrading bacteria, because the petroleum contaminants are the sources of energy and carbon.

3.3. TESTING HYDROCARBON DEGRADER STRAINS FOR THEIR ABILITY TO DEGRADE BTX COMPOUNDS

During our investigations 84 hydrocarbon degrading strains were isolated from soil samples originated from Bălan and Sândominic. 30 strains were selected for detailed studies, those which showed fast growth on BBH mineral salt medium supplemented with gasoline as sole source of carbon and energy.

To express the relationship between microbial hydrocarbon degradation efficiency and change in color of resazurin indicator a color scale was established. Samples with no degradation activity (blue color) were marked "–", purple colored samples with reduced microbial degradation activity were marked "±", the pink samples "++", while samples with increased hydrocarbon degradation activity (colorless) were marked "+++".

In table 1. the summarized results of hydrocarbon compound degradation capacity of microorganisms are depicted. It can be observed that the best hydrocarbon degrading isolates were R2, R7, R8, R10, R18, R22, R26, R27, R31, BBB1B. These strains were capable of utilizing all of the hydrocarbons (benzene, toluene, xylene, naphthalene, n-dodecane) tested as sole source of carbon and energy.
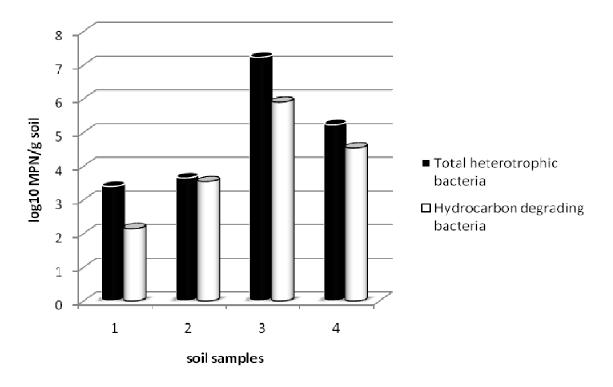
Fig.4. Results of the estimates of total heterotrophic and hydrocarbon degrading bacteria
1. Non-contaminated (Bălan); 2. Contaminated (Bălan)
3. Non-contaminated (Sândominic); 4. Contaminated (Sândominic).

Strains R20, R23, CSGN1 did not degrade any of them, while R35 and R29 isolates slightly degraded xylenes of the hydrocarbons tested. Strains, which were not able to degrade any of tested hydrocarbons, used other degradable constituents of gasoline for their growth and multiplication.

3.4. IDENTIFICATION OF MICROORGANISMS ON THE BASIS OF THEIR 16S RDNA SEQUENCES

Among the 30 hydrocarbon degrading isolates the so far identified 6 strains originated from Sândominic: R5, R7, R12, R22, R26 and R31.

Strains R22 and R31 were identified as representatives of *Rhodococcus erythropolis,* an aerobic, non-sporulating, Gram positive bacterium, which has an important role in biodegradation of petroleum derivatives. *Rhodococcus* strains may be utilized as industrial organisms, primarily for biotransformations and biodegradation of many organic compounds. Members of the genus *Rhodococcus* can be applied in environmental remediation and chemical industries (Bell et al., 1998; van der Geize and Dijkhuizen, 2004; Larkin et al., 2005).

Strain R26 belongs to the species *Micrococcus luteus*, strains R7, R5 were identified as *Bacillus cereus* and strain R12 as *Bacillus subtilis.*

Micrococcus luteus is often isolated from petroleum-contaminated soils, it is able to transform a large scale of bifenyls, aliphatic and aromatic hydrocarbons (Zhuang, 2003).

Bacillus cereus is a Gram positive, facultative aerobic, endospore-forming bacterium, capable of utilizing aromatic hydrocarbons (benzene, phenol) as source of carbon and energy (Banerjee and Ghoshal, 2010).

Cubitto et al. (2004) established that the species *Bacillus subtilis* has a significant ability to degrade different hydrocarbon pollutants.

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Table 1. Hydrocarbon compound degrading efficiency of isolated strains

Nr.	Strain	Origin	Degradation rate				
			benzene	toluene	xylenes	naphthalene	n-dodecane
1	R8	Bălan	+ +	+ +	±	±	+ + +
2	R19	Bălan	±	−	±	+ +	+ ++
3	R15	Bălan	±	±	±	±	±
4	R29	Bălan	−	−	±	−	−
5	BGN2	Bălan	++	±	±	++	+++
6	BBB1B	Bălan	++	++	++	++	+++
7	BBN3	Bălan	±	−	−	±	++
8	BBB2	Bălan	++	−	−	++	+++
9	R2	Sândominic	+ +	±	+ +	+ +	+ + +
10	R3	Sândominic	±	−	−	±	±
11	R5	Sândominic	+ +	++	++	+ +	+++
12	R7	Sândominic	++	++	±	+ +	+++
13	R10	Sândominic	+ +	±	±	+ +	±
14	R11	Sândominic	±	±	±	±	±
15	R12	Sândominic	++	++	±	+ +	+ ++
16	R18	Sândominic	+ +	+ +	+ +	±	+ + +
17	R20	Sândominic	−	−	−	−	−
18	R22	Sândominic	+ +	+ +	+ +	+ +	+ + +
19	R23	Sândominic	−	−	−	−	−
20	R26	Sândominic	++	++	++	±	+ ++
21	R27	Sândominic	+ +	±	±	+ +	+ + +
22	R30	Sândominic	±	±	±	±	±
23	R31	Sândominic	+ +	+ +	+ +	+ +	+ + +
24	R34	Sândominic	−	+ +	−	−	−
25	R35	Sândominic	−	−	±	−	−
26	R39	Sândominic	±	±	±	+ +	±
27	CSBG4	Sândominic	−	±	±	−	−
28	CSGN3	Sândominic	++	−	±	++	++
29	CSGG7	Sândominic	±	±	±	±	++
30	CSGN1	Sândominic	−	−	−	−	−

4. CONCLUSIONS

Isolated hydrocarbon degrading strains have an important role in the self purification processes of petroleum contaminated soils in Sândominic and Bălan.

Based on the cumulative CO_2 production curves it is evident that in the contaminated soil samples there is an increased activity of hydrocarbon degrading microorganisms.

In hydrocarbon-contaminated soil samples from Bălan the number of heterotrophic and hydrocarbon-degrading bacteria was low (10^3 cells/g soil), while in Sândominic soil samples it was higher with 2-3 orders of magnitudes.

From contaminated soil samples 84 strains were isolated, 30 from Bălan and 51 from Sândominic.

Strains R8, BBB1B (Bălan) and strains R2, R7, R10, R18, R22, R26, R27, R31 (Sândominic) were able to utilize all of the hydrocarbons tested: benzene, toluene, xylenes, n-dodecane, naphthalene.

Strains R22 and R31 were identified as *Rhodococcus erythropolis,* strain R26 as *Micrococcus luteus*, strain R27 as *Bacillus cereus* and strain R12 as *Bacillus subtilis.*

Identified strains are suitable for further use in bioaugmentation processes.

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