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POTENTIAL BACTERIAL SOIL INOCULANT MADE UP BY *RHODOCOCCUS* SP. AND *PSEUDOMONAS* SP. FOR REMEDIATION *IN SITU* OF HYDROCARBON – AND HEAVY METAL POLLUTED SOILS

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ABSTRACT. Since bioremediation is an environmentally friendly, cost effective approach to treat hydrocarbon-contaminated soils, in this study our aim was to develop a bacterial soil inoculant applicable in elimination of aliphatic, simple- and polyaromatic hydrocarbons from soils. To reach our goal two bacterial strains, *Pseudomonas fluorescens* BBN1 and *Rhodococcus qingshengii* BBG1, were selected to investigate their hydrocarbon-degrading abilities at DNA level and in microcosm experiments followed by GC-FID, as well as their ability to tolerate heavy-metal concentrations. Our results revealed that the proposed bacterial soil inoculant is suitable for elimination of aliphatic, monoaromatic and polycyclic aromatic hydrocarbons from soil samples, reaching degradation rates of 95% (n-dodecane), 66% (toluene), 70% (naphthalene) and 67% (phenanthrene) of initial pollutant concentrations within 42 days.

Keywords: *bioaugmentation, in situ bioremediation, PAHs, catA, C23O, alkB, GC-FID*

INTRODUCTION

In recent years microorganisms (bacteria, fungi) have increasingly gained interest in different industrial, biotechnological settings, since they can synthesize compounds (surfactants, amides, polymers etc.), or even they can transform a wide range of chemicals (aliphatic-, mono- and polycyclic aromatic hydrocarbons; chlorinated hydrocarbons; biphenyls etc.) into harmless

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substances. Taking advantage of these beneficial properties, several studies have reported successful use of microorganisms in elimination of organic contaminants from different environments (soil, aquatic ecosystems, sediments etc.). For instance, Ueno et al. (2006) by introduction of *Pseudomonas aeruginosa* strain WatG into diesel-oil-contaminated soil microcosms achieved a higher degradation rate (64% ± 4.2%) of pollutants during the first two weeks, than in only Luria-Bertani medium amended microcosms (49.5% ± 12%) [1]. In addition, Franzetti et al. (2009) reported a surface active compounds (extracellular bioemulsans and cell-bound biosurfactants) producing *Gordonia* sp. strain BS29 which effectively contributed to the removal of crude oil and PAH (polycyclic aromatic hydrocarbon) from soil. Beside bioaugmentation (introduction of microbial strains into contaminated environments in order to enhance the bioremediation process) with single strains, the use of microbial consortia of many hydrocarbon-degrading microorganisms proved to be more effective in remediation approaches, by the fact that intermediates of a catabolic pathway of one strain may be further degraded by other strains [3]. For example, Jacques et al. (2008) studied the capacity of a microbial consortium (*Mycobacterium fortuitum*, *Bacillus cereus*, *Microbacterium* sp., *Gordonia polyisoprenivorans*, *Microbacteriaceae* bacterium and *Fusarium oxysporum*) to degrade and mineralize PAH compounds. They found that the consortium degraded 96-99% of the PAHs in the soil, in 70 days, although the autochthonous soil microbial population showed no remarkable mineralization of these pollutants [4]. Another beneficial effect of the use of microbial consortia would be the observation that the presence of one strain in a mixed-contaminated environment (e.g. heavy-metals and hydrocarbons) may promote the expression of beneficial properties of another strain by e.g. sequestration of heavy-metals. In this context, Roane et al. (2001) used dual-bioaugmentation technique to decontaminate both 2,4-dichlorophenoxyactetic acid (2,4-D) and cadmium (Cd) impacted soil using a bacterial consortium made up by *Pseudomonas* sp. strain H1 and *Ralstonia eutropha* JMP134. The Cd tolerant *Pseudomonas* sp. H1, by intracellular sequestration, was able to decrease the level of soluble toxic Cd ions enhancing thus the degradation of 2,4-D by the less heavy-metal resistant *R. eutropha* [5].

Forasmuch, throughout Romania the number and extension of hydrocarbon polluted zones is increased (50 000 ha in 1999, according to Research Institute for Soil Science and Agro-chemistry) [6] the need of an innovative, efficient remediation technology is indispensable. Taking into account the aforementioned successful use of microorganisms in degradation processes of hydrocarbon pollutants, and the fact that bioremediation is a cheaper and more environmentally friendly approach than the conventional methods (physical and chemical approaches), we are committed to develop a microbial soil inoculant which can be used in decontamination of petroleum hydrocarbon polluted sites even in presence of heavy-metals.

RESULTS AND DISCUSSIONS

The isolation source of the investigated bacterial strains is discussed in detail in our previous work [7]. Seen from that preliminary results this highly hydrocarbon and heavy-metal impacted environment contributed to the evolution of several distinctive species with increased ability to degrade hydrocarbons or/and to resist in presence of heavy-metals. Therefore, to prepare a bacterial soil inoculant which might be applicable in bioremediation of hydrocarbon/ heavy-metal contaminated soils we selected two bacterial strains *R. qingshengii* BBG1 and *P. fluorescens* BBN1 isolated from contaminated soil samples collected from Bălan (Harghita County, Romania).

Testing hydrocarbon-degrading ability of strains revealed that *R. qingshengii* strain BBG1 is able to proliferate in presence of all tested hydrocarbons and shows a remarkable higher degradation activity in test solutions, indicated by the reduced absorbance of the resazurin amended test solution at 610 nm. The highest biodegradation activity of strain BBG1 was observed in test solution amended with n-dodecane, where formation of a biofilm also occurred. Strain BBN1 showed a lowest degradation capability supported by the weak discoloration of the resazurin and hydrocarbon amended test solution (Figure 1), compared to the blank samples.

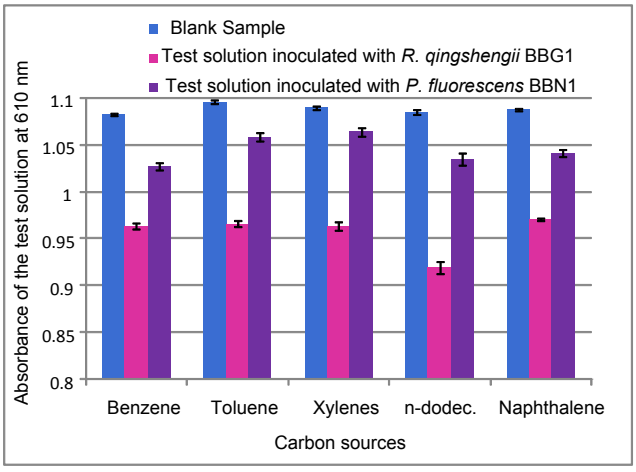

Figure 1. Hydrocarbon degradation potential of investigated strains.

The aliphatic and aromatic hydrocarbon degradation ability of *R. qingshengii* strain BBG1 was also demonstrated by the detection of biodegradative functional *catA* and *alkB* genes. By using RHO-F/R and RalkB-F/R primer pairs polymerase chain reaction yielded amplicons of 530 bp and 595 bp, respectively.

The presence of *C23O* gene, responsible for extradiol cleavage of the aromatic ring, was demonstrated by obtaining a 250 bp sized amplicon in *P. fluorescens* strain BBN1, although in test solution, supplemented with aromatic hydrocarbons, the activity of strain BBN1 was not remarkable (data not shown).

Regarding heavy-metal tolerance, we found that *R. qingshengii* BBG1 and *P. fluorescens* BBN1 have similar resistance against the tested heavy-metals, and are able to tolerate high heavy-metal levels. Since the tolerated Pb^{2+} and Zn^{2+} levels by the two strains are the same, the highest Cu^{2+} resistance was observed at strain BBG1 (Table 4). In the most increased manner the Zn^{2+} was tolerated by both strains.

Table 4. Heavy-metal tolerance of the selected strains

Tested bacterial strains	Maximum Tolerable Concentrations (mM)		
	$Pb(NO_3)_2$	$ZnSO_4 \cdot 7H_2O$	$CuSO_4 \cdot 5H_2O$
P. fluorescens BBN1	3.5	4	2
R. qingshengii BBG1	3.5	4	3

Optimal culturing conditions (pH and temperature) determination revealed that in case of *P. fluorescens* BBN1 different pH values did not play a decisive role in proliferation of bacteria, within the investigated pH interval. The highest cell density was observed after 12 hour of incubation, when the optical density of the inoculated nutrient solution exceeded the value of 0.7 (Figure 2.B.). As it is observable from Figure 2.A. *R. qingshengii* strain BBG1 had a longer adaptation period (*lag* phase), the exponential phase peaked after 18 hour of incubation, when a cell density of almost 0.8 was reached. A pH value of 7.5 seemed to extend the adaptation phase of bacteria.

The influence of different temperatures on the multiplication of investigated strains is presented on Figure 3. (A; B). As it is evident from Figure 3. A. the highest cell densities (absorbance of the inoculated solution at 590 nm ~ 0.8) of *R. qingshengii* BBG1 after 30 hour of incubation were reached in nutrient solutions maintained at 26ºC and 30ºC, respectively. A temperature of 20ºC delayed (~ 8 hours) the exponential phase of the bacterial growth. No or slight proliferation was observed in case of both investigated strains at 10ºC. As in the previous case, the largest increase in cell densities of *P. fluorescens* BBN1 inoculated nutrient solutions were obtained at 26ºC and 30ºC, although a temperature of 20ºC did not remarkably delayed (4 hours) the exponential phase of bacterial growth as in case of strain BBG1.

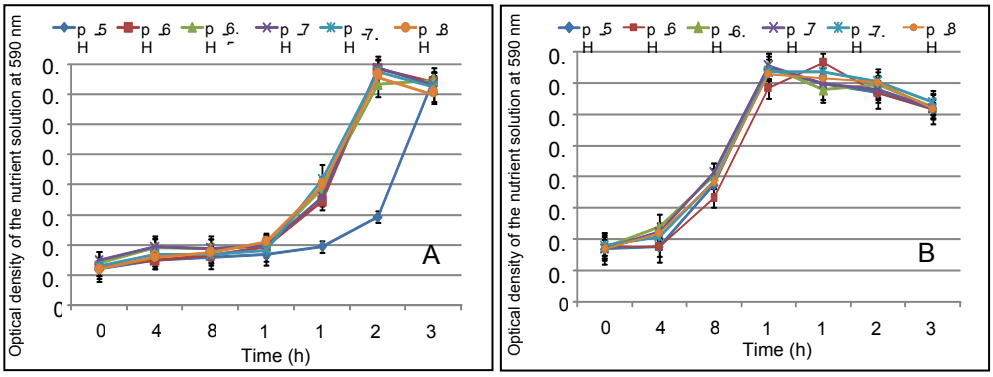

Figure 2. Influence of pH variation on proliferation of (A) *R. qingshengii* BBG1 and (B) *P. fluorescens* BBN1.

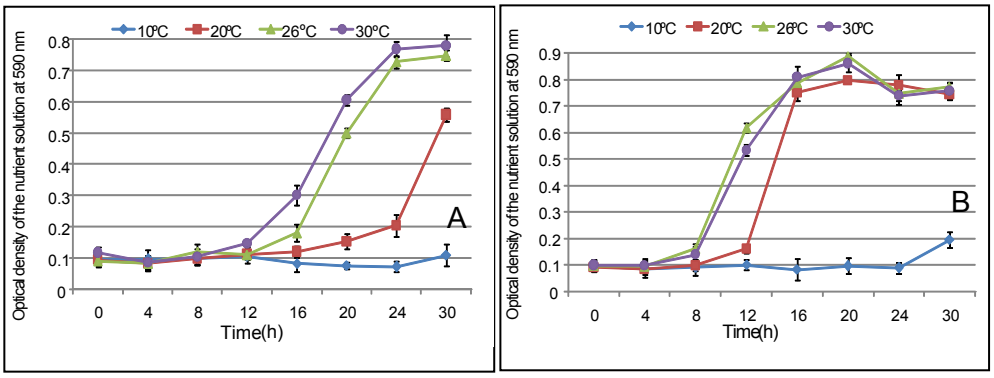

Figure 3. Influence of different temperature values on proliferation of (A) *R. qingshengii* BBG1 and (B) *P. fluorescens* BBN1.

Microcosm experiments revealed that the inoculated bacterial strains were suitable to improve the bioremediation potential of the investigated soil. Developed soil inoculant was able to degrade aliphatic, simple aromatic, as well as polycyclic aromatic hydrocarbons, proven by the results of the gas chromatography measurements. The highest biodegradation rate was observed in case of n-dodecane, when the bioaugmentation contributed to the almost complete elimination of the pollutant from soil (~95% of the initial n-dodecane was degraded). Compared to the degradation activity of indigenous microorganisms the inoculated microcosms showed a remarkably higher degradation potential (B1 and B2 samples degraded only 56%, respectively 40% of the initial n-dodecane concentration) (Figure 4).

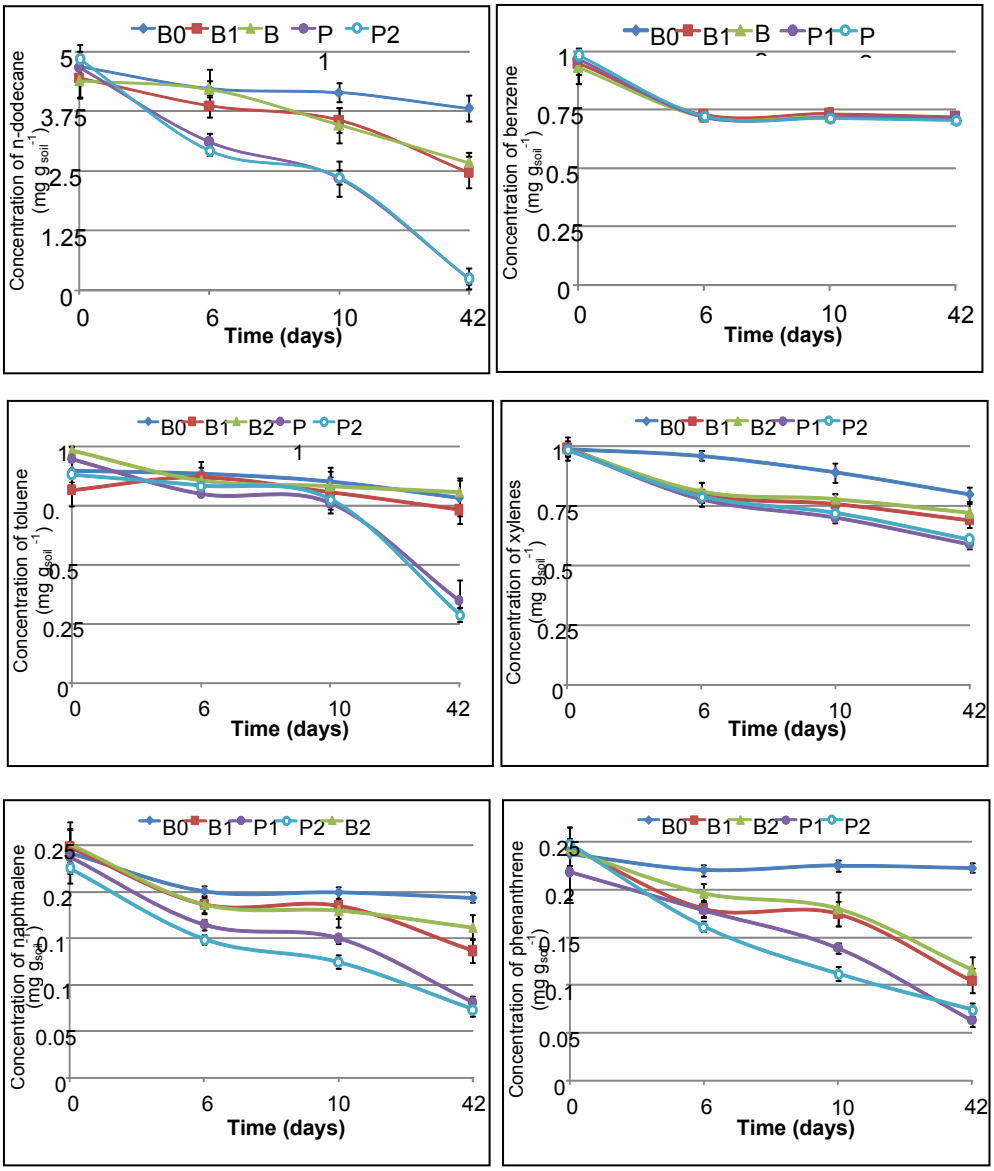

Figure 4. Biodegradation of aliphatic (n-dodecane), simple aromatic (benzene, toluene, xylenes) and polycyclic aromatic hydrocarbons (naphthalene, phenanthrene).

Benzene was the sole hydrocarbon pollutant whose degradation was not observed in none of the microcosms. A slight decrease might be observable among the first two samplings for chemical analysis, but to our opinion this decrease is due to the volatilization of benzene (Figure 4). After 42 days of

incubation a significantly higher toluene degradation was observed in bacterial inoculated soil microcosms compared to other samples. In bioaugmented soil almost 66% of the initial toluene concentration was degraded, in contrast with toluene degradation activity of indigenous microorganisms (soil microcosms without inoculum) where the efficiency of the pollutant degradation was only 35% (B1) and 29% (B2), respectively. Compared to abiotic control slight, unremarkable xylenes degradation was observed in un-sterilized and inoculated soil microcosms, although soil samples with *P. fluorescens* BBN1 and *R. qingshengii* BGG1 bacterial inoculum showed a higher toluene degradation, but the difference is not remarkable (Figure 4). Interestingly, a higher degradation of recalcitrant, hard-biodegradable polycyclic aromatic hydrocarbons (naphthalene, phenanthrene) by the used microbial consortia was also observed. The initial concentration (0.25 g kg^{-1}) of naphthalene was decreased to 0.081 g kg^{-1} in heavy-metal free inoculated soil sample, as well as to 0.074 g kg^{-1} in heavy-metal impacted soil representing a degradation rate of almost 67%. The degradation rate of phenanthrene in inoculum treated and heavy-metal contaminated sample, as well as in heavy-metal free sample was 70% and 68%, respectively. The degradation rate of naphthalene and phenanthrene in un-inoculated soil samples by indigenous bacterial population was 42%, respectively 60% in heavy-metal free samples, and 36%, respectively 65% in heavy-metal amended soils.

Since introduced heavy-metals Cu^{2+}, Pb^{2+}, Zn^{2+} did not influenced negatively the degradation rate of hydrocarbon pollutants, neither in inoculum free soil samples we may assume that the applied concentration of heavy-metals did not affected negatively the bacterial growth and the biodegradation activity.

CONCLUSIONS

A bacterial consortium made up by *Rhodococcus qingshengii* BBG1 and *Pseudomonas fluorescens* BBN1 was developed in order to eliminate hydrocarbon pollutants from contaminated soils.

Due to the wide pH tolerance this bacterial inoculum might be applicable in various soils with different pH.

Since selected strains possess also a remarkable heavy-metal tolerance the developed bacterial inoculant might be used for elimination of hydrocarbons from soil even in presence of heavy-metals.

By using this soil bacterial inoculant 95% of the initial n-dodecane, 66% of toluene, 67% of naphthalene and almost 70% of phenanthrene concentration was degraded within 42 days of microcosm experiment.

EXPERIMENTAL SECTION

Isolation source of hydrocarbonoclastic bacteria

The used hydrocarbon-degrading bacterial species, namely *Rhodococcus qingshengii* strain BBG1 and *Pseudomonas fluorescens* strain BBN1, in this study were obtained after enrichment from hydrocarbon and heavy-metal

contaminated soil collected from Bălan. As described previously, during the enrichment 1 g of contaminated soil sample was added to 99 ml BBH mineral broth medium supplemented with 1% (v/v) diesel fuel. After 2 weeks of incubation (23ºC), as a second selection, the obtained cultures were serially diluted and inoculated onto BBH agar plates. The lid of the Petri-dishes contained 250 µl of sterile diesel-fuel as sole source of carbon and energy [7]. Since this soil contains remarkable amounts of total petroleum hydrocarbons (TPHs – 16 400 mg kg^{-1}), PAHs (73.8 mg kg^{-1}), as well as heavy-metals (Pb^{2+} – 67 mg kg^{-1}, Cu^{2+} – 441 mg kg^{-1}, Zn^{2+} – 298 mg kg^{-1}) we assumed that this ecosystem contributed to the evolution of bacterial species, with increased ability to degrade hydrocarbons and to tolerate the presence of heavy-metals at the same time. The used bacterial strains in this study are deposited in the National Collection of Agricultural and Industrial Microorganisms under the following accession number: NCAIM(P)B001401 for *R. qingshengii* BBG1 and NCAIM(P)001400 for *P. fluorescens* BBN1.

Screening hydrocarbon-degradation capability of strains

Selected bacterial strains *R. qingshengii* BBG1 and *P. fluorescens* BBN1 were tested in order to determine their ability to degrade different hydrocarbons. Tests were done in 50 ml BBH (Bacto-Bushnell-Haas) [8] mineral salts solution supplemented with resazurin indicator (10 mg l^{-1}), and one of the following filter sterilized (0.2 µm) hydrocarbons (1 g l^{-1}) as sole carbon source: n-dodecane, benzene, toluene, xylenes and naphthalene (Sigma-Aldrich, Germany). The obtained test solutions were inoculated with 250 µl strain culture suspensions (OD_{600}=0.5). Degradation ability of strains was determined spectrophotometrically, by measuring the absorbance of the solution at 610 nm (absorbance of the redox indicator resazurin) compared to the absorbance of blank (un-inoculated, negative control) samples. A lower absorbance of the solution at 610 nm represents a higher activity of tested bacteria, and an increased degradation rate of the tested hydrocarbons.

Functional *catA, C23O* and *alkB* gene detection

Functional *catA* and *C23O* genes encode for intradiol *"ortho"* cleavage enzyme catechol 1,2-dioxygenase and extradiol "*meta*" cleavage enzyme catechol 2,3-dioxygenase, respectively. These key enzymes are known to accomplish the first step of catalysis of the aromatic ring fission, the most time consuming process in biodegradation of aromatic compounds [9]. Since *catA* and *C23O* genes catalyze the aromatic ring fission, *alkB* genes are responsible for expression of alkane-1-monooxygenase enzyme, involved in the initial attack of the aliphatic hydrocarbon chain by microbial mono-oxygenation.

To demonstrate the hydrocarbon-degradation potential of selected bacterial isolates at DNA level as well, *catA* and *alkB* functional genes were detected in *R. qingshengii* strain BBG1, as well as *C23O* genes in *P. fluorescens* strain BBN1 by using polymerase chain reaction (PCR) method.

PCR reaction mixture (50 µl) contained 1X DreamTaq^{TM} buffer with $MgCl_2$ (2 mM), 0.2 mM of each of the four dNTPs, 0.3 µM of each primer, 1 U *DreamTaq^{TM} DNA Polymerase* (Fermentas, Lithuania), 1 µl DNA template and molecular-grade water up to 50 µl. The used temperature profile for primer sets presented in Table 1 was the following: initial denaturation for 3 min at 95°C, followed by 32 cycles of denaturation for 0.5 min at 94°C, annealing for 0.5 min at the annealing temperature reported in Table 1, and elongation for 1 min at 72°C. The last step was a final extension for 10 min at 72°C. All amplifications were performed using ABI GeneAmp 2700 thermo cycler system (Applied Biosystems, USA).

Detection of genes was realized under UV light, after electroforetic migration (80V, 40 min) of PCR amplified gene fragments in 1% agarose gel.

Table 1. Used primer pairs for detection of *catA*, *C23O* and *alkB* genes

Primer pair	Primer sequence	Gene	Targeted organism	Annealing temperature (ºC)	Ref.
XYLE1-F XYLE1-R	5'-CCGCCGACCTGATCWSCATG-3' 5'-TCAGGTCAKCACGGTCAKGA-3'	*C23O*	*Pseudomonas* sp.	61.5	[10]
RHO-F RHO-R	5'-GCCGCCACCGACAAGTT-3' 5'-CACCATGAGGTGCAGGTG-3'	*catA*	*Rhodococcus* sp.	56	[11]
RalkB-F RalkB-R	5'-TACTACCGGTACTGCACCTAC-3' 5'-CCGTARTGYTCGAGGTAGTT-3'	*alkB*	*Rhodococcus* sp.	54	[12]

Testing heavy-metal tolerance of strains

Heavy-metal tolerance of strains was tested by using casamino-acid agar plates (casamino-acid 5 g l^{-1}; agar 17 g l^{-1}) containing different concentrations (0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mM) of $Pb(NO_3)_2$, $CuSO_4 \cdot 5H_2O$ and $ZnSO_4 \cdot 7H_2O$ (Merck, Germany). Heavy-metal amended plates were spot inoculated with bacterial cell suspensions (5 µl, OD_{600}=1) and incubated at 30ºC for 2 days [7]. All tests were done in triplicates and the results were evaluated visually for growth against heavy-metal free control cultures.

Determination of the optimal culturing conditions (pH and temperature)

For determining the optimal values of pH for meso-scale cultivation of *R. qingshengii* BBG1 and *P. fluorescens* BBN1 Nutrient Broth liquid media were used with different values of pH (5; 6; 6.5; 7; 7.5; 8). Initial bacterial suspension (OD_{590}=0.5) was obtained in physiological solution (0.9% NaCl) using a bacterial culture of 24 h. Liquid nutrient solutions (20 ml) with different pH values after sterilization were inoculated with 250 µl of previously made bacterial solution. Inoculated mediums were incubated at 28°C and shaken

at 145 rpm for 34 hours. Growing curves of the investigated strains at different pH levels were obtained after measuring (time intervals 0, 4, 8, 12, 16, 20 and 34 h) the optical density of the inoculated nutrient solutions at 590 nm by using a microplate reader type FLUOstar Optima (BGM Labtech).

In order to determine the optimal temperature for cultivation of investigated bacterial strains, bacterial isolates were incubated at the following temperatures: 10, 20, 26 and 30°C. Bacterial inoculated Nutrient Broth solutions were prepared as described in the earliest paragraph, and maintained at different temperatures by shaking at 145 rpm. Evaluation of the results was carried out on basis of the growth curves, obtained after measuring the cell densities of nutrient solutions at 590 nm at different time intervals (0, 4, 8, 12, 16, 20, 24, 30 h).

Microcosm experiment

For determining the beneficial effect of bacterial soil inoculation on the degradation of hydrocarbon-pollutants soil, microcosm experiments were set up (maintained at 26ºC), using argillic brown soil with known pedological characteristics (Table 2) collected from Cozmeni (Harghita County).

Soil samples serving as abiotic controls (sample label B0, negative control), as well as inoculated soil samples (sample labels P1, P2) prior bacterial strain inoculation were first time sterilized at 101ºC for 60 min for three times. As a second sterilization round the aforementioned microcosms were flooded with water and autoclaved again at 101ºC for 60 min. As a final sterilization soil samples were air dried and autoclaved again at 120ºC for 20 min.

Table 2. Pedological characterization of the utilized soil (data provided by Pedological and Agrochemical Research Institute, Harghita County, Romania)

Pedological characteristics								
Gravel (%)	Sand (%)	Silt (%)	Clay (%)	N_T (%)	Humus (%)	pH	C:N	Humidity (%)
24.7	46.1	18.5	10.7	0.108	1.6	6.2	11.6	16.6

The artificially hydrocarbon contaminated soil microcosms contained: n-dodecane (5 g kg^{-1}); benzene, toluene and xylenes (1 g kg^{-1}); naphthalene and phenanthrene (0.25 g kg-1) as hydrocarbon pollutants. In order to investigate the biodegradation potential of strains even in the presence of heavy-metals heavy-metal and hydrocarbon contaminated microcosms were also set up, where the concentration of artificially introduced heavy-metals were as follows: Cu^{2+} (2 mM), Pb^{2+} (3 mM), Zn^{2+} (4 mM).

Table 3. Designation of the soil microcosms

Sample designation	Sample description	Type of contaminants
B0	Abiotic control	n-dodecane, BTEX, PAHs and heavy-metals (Cu^{2+}, Zn^{2+} and Pb^{2+})
B1	Non sterilized, un-inoculated soil sample	n-dodecane, BTEX and PAHs
B2	Non sterilized, un-inoculated soil sample	n-dodecane, BTEX, PAHs and heavy-metals (Cu^{2+}, Zn^{2+} and Pb^{2+})
P1	Sterilized soil inoculated with *R. qingshengii* BBG1 and *P. fluorescens* BBN1	n-dodecane, BTEX and PAHs
P2	Sterilized soil inoculated with *R. qingshengii* BBG1 and *P. fluorescens* BBN1	n-dodecane, BTEX, PAHs and heavy-metals (Cu^{2+}, Zn^{2+} and Pb^{2+})

Soil microcosms were set up in triplicates using 500 ml vials with screw cap containing 100 g soil with known soil characteristics (Table 2). Multi-sterilized abiotic control sample (B0) was contaminated with both heavy-metals and hydrocarbons. To determine the effect of the indigenous microorganisms on biodegradation non-sterilized, un-inoculated soil samples (positive control) were polluted with hydrocarbons (B1) and hydrocarbons/heavy-metals (B2) as well. P1 and P2 soil microcosms were contaminated with hydrocarbons or hydrocarbons/heavy-metals and were inoculated with *R. qingshengii* BBG1 and *P. fluorescens* BBN1.

To prepare the inoculum *R. qingshengii* BBG1 and *P. fluorescens* BBN1 were grown to the late exponential phase on Nutrient Broth solid mediums. Cells were collected with sterile inoculation loop and placed to sterile physiological solution to obtain a bacterial cell suspension OD_{590}=0.5 (approx. 10^8 bacterial cells ml^{-1}). The bacterial suspensions (1-1 ml) were applied in droplets to the soil surface to reach a cell concentration of 10^6 cell g_{soil}^{-1}, and then the soil was thoroughly mixed with sterile spatula

In order to insure the oxygen supply for bacterial activity, microcosms were aerated in every 2 days by mixing in sterile environment. The water content of all microcosms was adjusted to 50% of the maximum water holding capacity of soil using sterile water.

Analysis of hydrocarbons in soil microcosms

To follow the biodegradation rate of hydrocarbons at various intervals, 2 g (dry wt) of soil sample from each microcosm was transferred to 35 ml vials and mixed with 10 ml hexane (Merck, Germany), followed by sonication (50 Hz)

for 50 min in a water bath. 1 µl of extract was analyzed using gas chromatograph (Varian CP 3380; Varian, USA) equipped with flame ionization detector (FID) and CP SIL-5CB WCOT Fused Silica column (25 m x 0.25 mm internal diameter). During the analysis the injector and detector temperatures were maintained at 140ºC and 290ºC, respectively. The temperature program was set to 40ºC for 1 min, increasing at a rate of 25ºC min^{-1} to 140ºC, and finally hold at 140ºC for 10 min.

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