PAH DEGRADING CAPABILITY OF MICROBES ISOLATED FROM HYDROCARBON CONTAMINATED SITES

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Abstract

The polyaromatic hydrocarbons (PAH) are occurred in petroleum, petroleum derivatives, or its products and come off from the incomplete combustion of organic matters. These compounds are hardly degradable (persistent) in the environment. Human health risk of PAHs caused by their mutagenic, teratogenic and endocrine distrupting effects. Seven PAH compounds were identified as human carcinogenic by WHO, IARC and US EPA. The aim of our work was to look for microorganisms, which can be applied for biodegradation of PAHs.

Bacteria were selected from the microbial culture collections of SZIU, Department of Environmental Protection and Environmental Safety. These microbes were previously isolated from soil and groundwater samples of Hungarian hydrocarbon (TPH, BTEX, PAHs) contaminated sites. The PAH degrading ability of chosen microorganisms were tested in three steps. In the first step, the method of Chinese researchers (Zhao et al., 2009) was used for screening the collection with three compounds [benzo(a)pyrene, benzo(a)anthracene,

fluoranthene] to select possible degrading strains. In the second step PAH containing shaking cultures were used to verify degrading ability of the selected strains *in vitro*. Strains were paired for increasing degradation rate because, by the results of scientific references, a mixture of strains generally have improved degrading ability. Prior strain pairing tests were performed to analyze the multiplications of selected microbes together in mixed cultures. The third step was a degradation and soil respiration test (OxiTop, WVR International Inc.) with PAH and TPH polluted soil sample from a Hungarian contaminated site. The intensification of respiration and PAH degrading were compared between the control, and inoculed (with strain pairs) ones. Our results could lead to the creation of a PAH degrading inoculum, which can be used in contaminated sites, *in vivo*.

Keywords: polycyclic aromatic hydrocarbons (PAH's), PAH degrading microbes, biodegradation

Összefoglalás

A poliaromás szénhidrogének, mint szennyezőanyagok, kőolajokban, kőolajszármazékokban, illetve termékeiben előforduló, valamint szerves anyagok tökéletlen égése során keletkező vegyületek, amelyek nehezen lebonthatóak, perzisztensek. Környezetvédelmi jelentőségüket növeli, hogy humán egészségügyi hatásaikat tekintve eddig hét PAH vegyületről bizonyosodott be humán karcinogén hatás, továbbá egyeseknek mutagén, teratogén, valamint hormonháztartást zavaró (EDC) hatásaik is ismertek. Munkánk célja olyan mikroszervezetek keresése volt, melyek okszerűen alkalmazhatóak PAH vegyületek biológiai lebontására.

Ennek érdekében a Környezetvédelmi és Környezetbiztonsági Tanszék törzsgyűjteményének felhasználásával olyan mikroba törzseket választottunk ki, amelyeket korábban kőolajszármazékokkal (TPH, BTEX, PAH) szennyezett területekről (talaj-,

talajvízmintákból) izoláltak. A kiválasztott mikroszervezetekkel több lépésben bontási kísérleteket végeztünk, amelyek során degradációs képességüket vizsgáltuk. Az első lépésben kínai kutatók (Zhao et al., 2009) módszerét használtuk három PAH vegyület [benza(a)pirén, benz(a)antracén, fluorantén] esetében, hogy kiválasszuk a potenciálisan bontásra képes törzseket. A második szakaszban rázatott mikroba tenyészetet alkalmaztunk annak érdekében, hogy a törzsek PAH degradációs képességét elemezzük *in vitro*, folyékony közegben. Ehhez törzspárokat állítottunk össze, amelyekkel a kísérletet megelőzően együttszaporítási vizsgálatokat végeztünk, mivel az irodalmi adatok azt bizonyítják, hogy a törzskeverékek általában nagyobb bontási képességgel rendelkeznek PAH vegyületek esetében. A harmadik munkaszakaszban OxiTop talajrespirációs vizsgálatot végeztünk két ismétlésben PAH, és TPH szennyezett kárhelyről származó talajmintán. Eredményeim megalapozzák egy olyan oltóanyag kialakítását, amely PAH vegyületekkel szennyezett kárhelyeken *in vivo* használható.

Introduction

Total Petroleum Hydrocarbons (TPH) are one of the most common group of organic contaminants in the environment, originated from crude oil. These contaminants contain only hydrogen and carbon atoms linked with aliphatic covalent bonds. The group members have 5-40 carbon atoms, which can be divided into two groups. One of these groups has 5-10 carbon atoms, which are VALPH (Volatile Aliphatic Petroleum Hydrocarbons) compounds, and the rest of the group is non-volatile EPH, (Extractable Petroleum Hydrocarbons). The increase of the carbon atom's number the bioavailability, and biodegrability reduce.

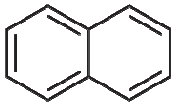

Figure1. Naphtalene

Polycyclic aromatic hydrocarbons, also known as poly-aromatic hydrocarbons (PAHs) which are included in petroleum, petroleum derivatives, or its products and occur during the incomplete combustion of organic matters. Nearly 200 compounds are belonging to this group. PAHs are built up with two, or more benzene rings, where these condensed aromatic hydrocarbon rings have common carbon atoms. The simplest polycyclic aromatic hydrocarbon is the only volatile PAH, naphtalene, which is built up with two benzene rings (Figure 1). Until three benzene rings PAH compounds are generally considered as well biodegradable. Above this number, the degrability, the water solubility, and the bio-avability are reduced. PAHs with more than three benzene rings are often referred in bioremediation literature as high molecular weight PAH's (Kanaly et al., 2000). Some of PAH compounds are belonging to POP (Persistent Organic Pollutant) compounds (http1). These hardly degradable materials in the environment could be accumulated in living organism (bioaccumulation). Human health risk of PAHs, caused by their mutagenic, teratogenic and endocrine distrupting effects (EDC), improve the environmental importance of these compounds. The possible degradation of PAH compounds is catalyzed by the microbial monooxygenase, dioxygenase enzymes.

Different groups of fungus, and bacteria use these proteins to degrade hydrocarbon compounds. Some of these bacteria are belonging to the Genus *Acinetobacter, Achromobacter, Pseudomonas, Bacillus*, *Rhodococcus*, (Anton, 2010; Haritash and Kaushik., 2009; Kim et al., 2007). Furthermore *Pseudomonas*, and *Sphingomonas* species are capable to degrade anthracene, fluoranthene, and benzo(a)anthracene (Mrozik et al., 2003; Schneider et

al., 1996). Other researches have found that 85% of benzo(a)pyrene degraded by *Bacillus subtilis* (Lily et al., 2009). *Mycobacterium*, *Novosphingobium, Arthrobacter* strains were successfully used for degradation of poly-aromatic hydrocarbons (Willumsen et al., 2001; Kallimanis et al., 2009; Yuan et al., 2009). Most published research works have been shown, that microbial consortiums are capable to degrade high molecular weight polyaromatic hydrocarbons (Luan et al 2006, Yu et al 2005).

In our work microorganisms isolated from Hungarian hydrocarbons polluted sites were tested in our PAH degradation experiments. It was assumed that these microbes are able to stay alive in these contaminated matrixes, thus they have possible hydrocarbon degradation potential. These microbes are deposited in international strain collections, furthermore they are members of two different strain culture collections (SAFEREMED, INOSAFE) in the SZIU, Department of Environmental Protection and Environmental Safety (Table 1). The strain collections are made up of non-pathogenic, identified and taxonomical classified species. The degradation profiles of these microorganisms are well known, as well as tolerance against heavy metals. The biodegrading ability was verified in contaminated sites.

Table 1. The members of the strain collection

SAFEREMED	AK-35	*Rhodococcus erythropolis*
	AK-36	*Rhodococcus globerulus*
	AK-37	*Rhodococcus pyridinivorans*
	AK-38	*Rhodococcus gordoniae*
	AK-40	*Rhodococcus rhodochrous*
	AK-44	*Rhodococcus aetherivorans*
	CHB-15p	*Rhodococcus pyridinivorans*
	CHB-20p	*Chryseobacterium hungaricum*
	NCP-3	*Pseudomonas putida*
	NCAIMB-1494	*Pseudomonas putida*
INNOSAFE	TBF2/20.2	*Olivibacter oleidegradans*
	ZFM 23.1	*Rhodococcus erythropolis*
	S-8	*Rhodococcus ruber*
	OM-7.2	*Rhodococcus erythropolis*
	SZM5/4.2	*Pseudomonas mandelii*

The aim of our work, was to seek microorganisms from this two strain collections which can be applied for degrading high molecular weight polyaromatic hydrocarbons, such as benzo(a)pyrene, benzo(a)anthracene, and fluoranthene. Furthermore pairing tests were made for consortial use of these degrading bacteria.

Methods and Material

The TPH and PAH degradation capability of the members of these two strain collections was examined on compounds by an own improved experimental system. The steps of our system are demonstrated on the figure below (Figure 2.). As follows the figure is described in details.

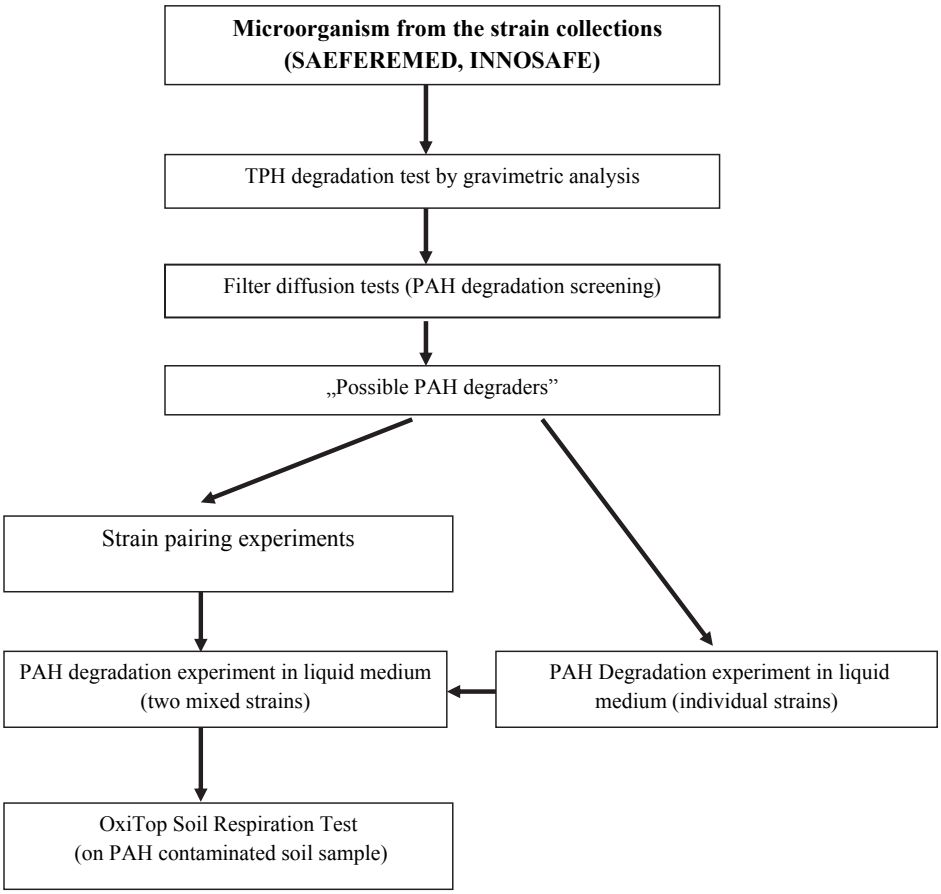

Figure 2. The elaborated experimental system for screening of degradation of PAH compounds

Gravimetric analysis

Individual bacteria were transferred from the collection to nutrient agar slant. After incubation time (24h) the prepared microbe colonies were washed into 50 ml liquid medium (LB medium) with sterile glass stick. The inoculated Erlenmeyer flasks were being shaken during 72 hours at 28°C with170 rpm. From these shaked cultures 5-5 ml of the suspension was placed into 100ml OIR-III liquid medium, [5.0 g $(NH_4)_2SO_4$; 1 g K_2HPO_4; 0.5 g KH_2PO_4; 0.5

g $MgSO_4 \times 7H_2O$; 0.2 g $CaCl_2 \times 6H_2O$, 0.01 g $FeSO_4 \times 7H_2O$, 1000 cm^3 distilled water, sterilization: 121 °C, 1 bar, 15 minutes]. 2 ml of a mixture of diesel and crude oil (approximately 1.7 g) was used as single carbon source that were put into the medium. Next to the inoculated samples the same gas oil – crude oil mixture filled OIR-III medium was applied with no bacterium suspension as control. The flasks were being incubated during 120 hours at 28°C and shaked at 170 rpm.

After the incubation the contents of the Erlenmeyer flasks were poured into glass shaker funnel. It was washed with organic solvents (petrol ether, chloroform) and the rest oil was separated. The oil quantity of the inoculated and the control samples were compared and the degradation percentage was described.

Filter Diffusion Test (PAH degradation screening)

The primary aim of this experiment was the selection of possible PAH degrading microbes from the strain collections. Chinese researchers (Zhao B. et al 2009) method was developed for this test. Degrading capability of the bacterial strains with three PAH compounds [benzo(a)pyrene (BaP), benzo(a)anthracene) (BaA), fluoranthene (Flu)] was screened.

As a first step four Millipore filters (d=2.5 cm) with 0.45 µm pore (MERCK Millipore cat. no. HAWP02500) were put onto OIR-III (1.5 v/v% agar) mineral medium (in Petri-dishes) with sterile clips. Three PAHs in 5000 ppm concentration were prepared in stock solutions, dissolved in diethyl-ether. 100 µl of the prepared PAH stock solutions were streaked onto the filter by sterile glass sticks. The fourth was the control treated by only diethyl-ether. After 5-minute drying in exhaust cabinet the solvent evaporated and then 100 µl of the bacterial suspension was streaked onto the four filters. Previously the individual bacterial suspensions had been inoculated on LB agar (10 g tryptone; 5 g yeast extract, 10 g NaCl; 18 g bacteriological agar; 1000 cm^3 distilled water). The suspension of the inoculum

was set to OD_{600}=1 in the experiment. If bacterial colonies were formed on the filters that treated with the three different kind of PAHs that noted as “probably PAH degrading” strain.

Strain pairing tests

24-hour, pure cultures of individual bacteria from the strain collections were transferred on native agar medium slant. After the incubation (28°C), the prepared microbes colonies were being inoculated into 50 ml liquid medium (LB medium) with sterile glass stick. The inoculated Erlenmeyer flasks were being shaken during 72 hours at 28°C and 170 rpm. The suspension of the inoculum was set to $OD_{600=}$0,6 in every case. 2.5 ml of this suspension was injected into LB liquid medium then we determined the number of living cells with pour plate technique in every 24 hours. The Petri-dishes were incubated at 28°C and after 96–120 hours. The number of colonies were counted on the dishes by the presence of the different morphology.

PAH-degradation in liquid medium

Based on the screening analysis (PAH Filter Diffusion tests) another experimental system was started where those strains were examined in liquid medium which could form colonies on the filters. The incubation time was 21 days at 28°C and 170 rpm. In this case we used only individual strains to analyse the degradation. After the 14- and 21-day incubation times the samples were sent to analytical examination to an accredited Hungarian analitical laboratory (Wessling Hungary Kft.).

In the next section, two microbes in a mixture were shaken in liquid medium in order to analyse their PAH-degradation capability in liquor. Pairs of strains were selected by the results of above mentioned strain pairing tests. Stock solutions from the three PAH compounds, benzo(a)pyrene, benzo(a)anthracene and fluoranthene in 15 mg/ml concentration

was prepared. In this case 300 mg from each compound have been dissolved in 20 ml acetone. Then 500 µl from each stock solutions were filled into 300 ml sterile Erlenmeyer flasks under exhaust cabinet. After the evaporation of the solvent it was poured up with 45 ml Bushnell-Haas medium (0.2 g $MgSO_4x7H_2O$; 0.02 g $CaCl_2x2H_2O$; 1 g KH_2PO_4; 1 g K_2HPO_4; 1 g NH_4NO_3; 0.05 g $FeCl_3$; 1000 cm^3 distilled water), that contained Tween80 detergent in 1 v/v%. 2.5 ml of each suspensions of the strain-pairs were injected into 45 ml Bushnell-Haas liquid medium contained all the three PAH compounds. Thus the PAH-concentration of the 50 ml medium has been 450 ppm (150 ppm of each Flu, BaP, BaA).

The incubation period was 14 days at 28°C and 170 rpm. Bushnell-Haas liquid medium contained only PAH compounds was used as control in equivalent concentration without any bacterium suspension.

Soil respiration and PAH degradation tests in OxiTop system

These experiments were carried out in OxiTop pots that are able to track respiration of soil.

The OxiTop is able to count the emission of CO_2. Since in the closed system the CO_2 derived from the soil respiration is consolidated in NaOH solution. As a result of this, micro-vacuum arises in the closed system and the registered reduction of pressure could show the activation of bacterial respiration of soil samples. In the closed system the changes of pressure is noticed by the head part of the instrument without disturbing the sample.

The soil sample was originated from a Hungarian hydrocarbon contaminated area (near Szank). The texture of the hydrocarbon compounds (TPH, PAH) contaminated soil was sand. 150 g of the soil samples were inoculated by the mixture of two strains. Strain pairs were selected by the results of strain pairing tests. Strains from 24-hour agar slants were put into 50 ml TGE-5 liquid medium (tryptone: 5.0 g; yeast extract: 2.5 g; glucose: 5.0 g; 1000 ml distilled water) with sterile glass stick. The inoculated medium was incubated during 48 hours

at room temperature and mixed with 150 rpm. After this shaking period the cell density was set to OD_{600}=0.6 with sterile liquid TGE-5 medium. The homogenized, sandy, contaminated soil sample was inoculated with 2.5 ml of both strain suspensions separately. This OxiTop pots had two repetitions. The incubation period was 14 days. In this experiment AK-35 + AK-38 (*R. erythropolis* + *R. gordoniae*), AK-38 + NCP-3 (*R. gordoniae* + *Pseudomonas putida*) and CHB-15p + AK 40 (*R. pyridinivorans* + *R. rhodochrous*) strain pairs were applied. We had three repetitions and a control without any treatment.

Results

The results will be presented by the above shown steps of matherials and methods. The partition of sub-chapters is following the own prepared system of experiments (Figure 2.)

Gravimetric analysis, Filter Diffusion tests

As it can be seen in Table 2., the hydrocarbon degrading capacity of AK-35 *(Rhodococcus erythropolis*), AK-38 *(Rhodococcus gordoniae*), AK-40 *(Rhodococcus rhodochrous*), AK-44 (*Rhodococcus aetherivorans*), TBF2/20.2 *(Olivibacter oleidegradans)* are up to 50% by the gravimetric analysis. By the results of filter diffusion tests AK-35, AK-37 (*Rhodococcus pyridinivorans)*, AK-40, AK-44, and CHB-15p *(Rhodococcus pyridinivorans)* were able to grow on benzo(a)pyrene, benzo(a)anthracene, and fluoranthene treated filters from the microbial collection SAFEREMED. Only one microbe (TBF2/20.2) from the strain collection INNOSAFE was able to form colonies on the PAHs containing filters.

Table 2. The hydrocarbon (TPH) degrading capacities of tested strains and their growth on filters treated with PAH compounds (Filter Diffusion tests)
*: Average of 3 measurements
BaP: Benzo(a)pyrene, BaA: Benzo(a)anthracene, Flu: Fluoranthene

	Strains	Species	TPH Gravimetric analysis *	Filter Diffusion tests		
			Degrading capacity (%)	BaP	BaA	Flu
SAFEREMED	AK-35	*Rhodococcus erythropolis*	**65.55**	+	+	+
	AK-36	*Rhodococcus globerulus*	35.57	-	-	-
	AK-37	*Rhodococcus pyridinivorans*	26.96	+	+	+
	AK-38	*Rhodococcus gordoniae*	**73.30**	-	-	-
	AK-40	*Rhodococcus rhodochrous*	**69.25**	+	+	+
	AK-44	*Rhodococcus aetherivorans*	**53.40**	+	+	+
	CHB-20p	*Chryseobacterium hungaricum*	n.d			
	CHB-15p	*Rhodococcus pyridinivorans*	n.d	+	+	+
	NCP-3	*Pseudomonas putida*	22.11	-	-	-
	NCAIMB-1494	*Pseudomonas putida*	n.d	-	-	-
	DSM 7226	*Brevundimonas vesicularis*	n.d	-	-	-
INNOSAFE	TBF2/20.2	*Olivibacter oleidegradans*	**58.33**	+	+	-
	ZFM 23.1	*Rhodococcus erythropolis*	37.19	-	-	-
	S-8	*Rhodococcus ruber*	19.82	-	-	-
	OM-7.2	*Rhodococcus erythropolis*	37.80	-	-	-
	SZM5/4.2	*Pseudomonas mandelii*	n.d			

Strain pairing experiments

Until the end of our work 6 strain pairs were examined and just AK-35 + AK-38, AK-40 + CHB-15p, as well as AK-38 +NCP-3 strain pairs, were able to multiply, in mixed cultures with high cell numbers within 72 hours (Table 3.). Based on these results the degrading capacity of strain pairs on PAH compounds in liquid medium was examined in OxiTop soil respiration system.

Table 3. Results of strain pairing experiments after 72 hours (Cologny Forming Unit/ml)

Examined strain pairs	CFU/ml after 72hrs
AK-35 + AK-38 (*R. erythopolis* +*R. gordoniae)*	10^{9} \ 10^{8}
AK-40 + CHB-15p (*R. pyridinivorans* + *R. rhodochrous*)	10^{9} \ 10^{9}
AK-38 + NCP-3 (*R. gordoniae* + *Pseudomonas putida*)	10^{8} \ 10^{9}
TBF2/20.2 + AK-35 (*O. oleidegradans* + *R. erythopolis)*	10^{5} \ 10^{9}
TBF2/20.2 + AK-40 *(O. oleidegradans* + *R. pyridinivorans)*	10^{8} \ 0
TBF2/20.2 + AK-44 *(O. oleidegradans* + *R. aetherivorans)*	10^{9} \ 0

PAH degradation in liquid medium

By the results of this experiment AK-37, AK-40 and AK-44 were able to decrease the amount of PAH compounds individually, between 11–15% (Table 3.). The highest amount was 15% (AK-44) on fluoranthene. The strain pair AK-35 + AK-38 degraded the tested PAH compounds between 6–22%, in highest extent (above 20%) was in case of benzo(a)pyrene. The incubation was 14 days with individual strains and strain pairs, too. AK-35 was not able to degrade benzo(a)pyrene individually, but it could in AK-35 + AK38 strain pair.

Table 4. Results of PAH degradation in liquid medium with individual strains, and strain pair

	Average of degradation %		
Strains	**Flu**	**BaA**	**BaP**
AK-35	2.3	0.0	0.6
AK-37	7.8	0.9	**11.6**
AK-40	**12.2**	0.7	5.5
AK-44	**15.5**	1.1	**12.2**
CHB-15p	2.5	0.0	0.2
TBF2/20.2	0.8	0.0	0.0
AK-35 + AK-38	6.4	**14.2**	**22.1**

OxiTop soil respiration test

Based on the results of the strain pairing tests, a contaminated soil sample, originated from PAH and TPH polluted site, was inoculated with mixed bacterial cultures and their degradation ability was tested. The reason why these strains were used, that because they were able to grow together in appropriate living cell numbers, furthermore previous examinations showed that each strains had good TPH-degradation capability and AK-35 + AK38 had also PAH-degradation capability. An untreated (not inoculated) control was applied to compare degradation rates.

Table 5. Results of OxiTop respiration tests.

	Control average	AK-38 + NCP-3	CHB-15p + AK-40	AK-35 + AK-38
		Quantity of PAHs compare to Control (%)		
Sum of PAHs without naphtalene	**100%**	**75,5**	**81,5**	**63,8**

The results can be seen in Table 5. The quantity of PAH compounds in the contaminated soil was 299.6 mg/kg. After 14 days the strain pair AK-38 + NCP-3 degraded PAH compounds nearly 24%, CHB-15p + AK-40 18%, and the AK-35 + AK-38 was able to degraded poliaromatic compounds in highest quantity, 36%.

The respiration activity of the strain pair AK-35+AK-38 inoculated soil (RTK AK I., RTK AK II.) compared to the non inoculated control (Control 1, Control 2) can be seen in Figure 3. In the case of Controls soil samples the respiration values are low. By the end of the 14-days respiration test, the pressure decreased in the control samples to 12 hPa by the average of them. As the reason, the low values are caused by the presence of the sole indigenous microbial population. Compared to this the respiration activity of the inoculated soil samples were more intensive, and related with this the decrease of the pressure was more significant. On the 6th days the oxygen amount has been reduced because of the aerobic biological activity. Thus the OxiTop systems were opened once during the time of experiment and soil sample was rehomogenized.

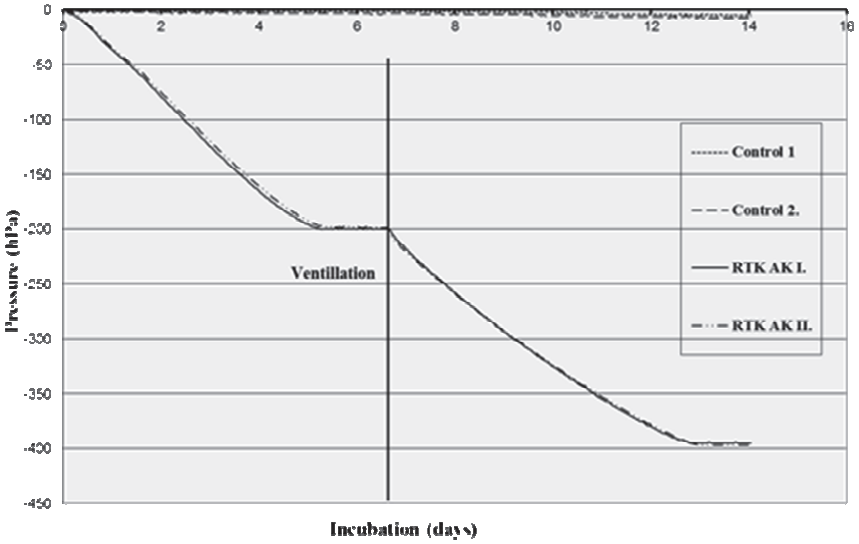

Figure 3. Respiration activities (pressure decrease) of two repetitives of AK-35+AK-38 strain pairs and controls

Discussion

The capacity of two microbial culture collection (SAFEREMED and INNOSAFE) was examined for degrading total petroleum hydrocarbons (TPH), and polycyclic aromatic hydrocarbons (PAHs). In the first step the capacity of collection strains for hydrocarbon degradation was examined with gravimetrical analysis. In the experiments hydrocarbon degrading capacity of AK-35 *(Rhodococcus erythropolis*), AK-38 *(Rhodococcus gordoniae*), AK-40 *(Rhodococcus rhodochrous*), AK-44 (*Rhodococcus aetherivorans*), TBF2/20.2 *(Olivibacter oleidegradans)* was up to 50%. These strains were selected based on their growth on PAH compounds, in filter diffusion tests. AK-35 (*Rhodococcus erythropolis)*, AK-37 (*Rhodococcus pyridinivorans)*, AK-40 (*Rhodococcus rhodochrous*), AK-44 *(Rhodococcus aetherivorans)*, CHB-15p (*Rhodococcus pyridinivorans)* and TBF2/20.2 (*Olivibacter oleidegradans*) were able to forming colonies on benzo(a)pyrene, benzo(a)anthracene, and fluoranthene diffused Millipore filters which were put

on LB solid agar in Petri dishes. PAH degradation was tested with individual strains, based on the results of filter diffusion tests, in liquid medium as well. Individually AK-37, AK-40, and AK-44 strains were able to decrease the amount of fluoranthene between 7,8-15.5% and 5.5–12% on benzo(a)pyrene comparing to the uninoculated control. The strain pair AK-35+AK-38 was degraded benzo(a)pyrene in 22%. Based on these results OxiTop soil respiration tests were made with hydrocarbon contaminated soil sample in two replicates parallel to verify degrading ability. It was found, that the strain pair AK-38 + NCP-3 was able to decrease the amount of ∑PAHs (without naftalene) in 24% and CHB-15p + AK-40 was in 18% compearing to the untreated control. The AK-35 + AK-38 strain pair has reduced with 36% the amount of the same polyaromatic pollutants in the examined soil sample. The incubation period was only 14 days, however some of scientific references advise 30-40 days incubation period for degrading PAH compounds (Bishnoi et al., 2009., Kanaly et al 2000). Thus it is possible, that degradation efficiency of strain pairs can be higher in longer term experiments.

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