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UNCONVENTIONAL YEAST IN THE DEGRADATION OF HYDROCARBONS IN CONTAMINATED SOIL

The influence of *Yarrowia lipolytica* inoculum on biodegradation of hydrocarbons, and changes in microbiota composition in the soil contaminated with petroleum have been investigated. The material under study was contaminated clay soil, containing petroleum-derived substances at approximately 17 000 [mg/kg d. m.]. Microbiological analysis was carried out by the cultivation method and the content of individual hydrocarbons (*n*-aliphatic, BTEX and PAHs) was determined by the GC/MS method. The largest decrease of oil-derived substances, versus the control sample, was recorded at the beginning of the process. During the first 30 days, the yeast inoculation caused most effectively removal of *n*-aliphatic hydrocarbons and PAHs (approximately 80% reduction), however, the content of BTEX increased nearly three times. After 60 days of the process, PAHs concentration further decreased (by 40%), concentration of *n*-aliphatic hydrocarbons decreased a little, however the content of BTEX increased by 10%, compared to the initial concentration. Stimulating the biodegradation process with the yeast inoculum influenced the increase of the bacteria count, mainly Gram-positive, with simultaneous decrease of fungi number.

1. INTRODUCTION

Increased demand for petroleum and petroleum based products causes environment pollution with toxic hydrocarbons. Petroleum-derived compounds are reduced in the soil in consequence of chemical oxidation and photolysis, sorption, precipitation, volatilization and biodegradation [1, 2]. Natural bioremediation processes take place in contaminated soil due to microorganisms abilities to assimilate petroleum-derived hydrocarbons and use them as the source of carbon and energy [3, 4] or in co-metabolism [5]. Recognition of microorganisms capable of degrading petroleum pollutants and their metabolic pathways allowed the use of the activity of selected strains to bioaugmentation of contaminated soil [6, 7]. However, opinions are split on this matter. Karamalidis

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and Bento indicate low efficiency of bioaugmentation with individual strains, which demonstrated biodegradation qualities, compared to spontaneous processes (bioautenuation) [8, 9]. Nevertheless other researchers [10, 11] recorded satisfactory results.

Bacterial inoculates are used most often, however, according to some authors, *Yarrowia lipotylica* yeast can be used to prepare active inocula. The strain is not pathogenic to humans, which makes it useful in bioaugmentation process [12]. It actively stimulates biodegradation of some hydrocarbons and, additionally, improves the soil microbiotic quality [11] and supports rooting of plants [13]. Dead yeast cells add nutritional elements to soil, which increases its fertility, stimulates plants rooting and germinating in the contaminated soil [11, 13]. Many reports have been published on satisfactory biodegradation characteristics of *Y. lipolityca* and its use, both in immobilized [11, 13] and free form [10]. However, Beopoulos et al. [14] report that, so far the mechanism and pathway of petroleum-derived substances biodegradation by *Y. lipolytica* yeast have not been explained.

The aim of the present research was the assessment of the influence of *Y. lipolytica* yeast inoculate on biodegradation of hydrocarbons in the soil contaminated with petroleum-derived substances and studying of dynamics of the soil microbiota changes.

2. EXPERIMENTAL

The material under investigation was clay soil contaminated with petroleum-derived substances, samples at the contaminated spot and stored in natural conditions for 2 months. It was in the form of sticky black mass of a specific odor and pH 8, with the content of petroleum-derived substances approximately 17 000 $mg \cdot kg^{-1}$ DM. The soil was used to prepare model pot experiments.

The *Yarrowia lipolytica* A101 yeast strain used for bioaugmentation was obtained from the collection of cultures at Wrocław University of Environmental and Life Sciences (Poland). The preparation of inoculum involved culture of yeast on slants on YPG medium during 48 hours at 28 °C and rinsing of cells with Tween (0.05 vol. %). The suspension of *Y. lipolytica* was standardized in Thomas chamber to density $2 \cdot 10^7$ CFU cm^{-3} and used as inoculum.

The pot tests were set up in three replications. Each pot was filled with 1 kg of soil. Next, 10 cm^3 of the *Y. lipolytica* yeast suspension was added and the soil was thoroughly mixed (S + Y). Soil without inoculum was a control sample (C), to which 10 cm^3 of sterile water with Tween (0.05% v/v) was added. The soil was incubated in open pots at room temperature 22±2 °C), over a period of 60 days. Humidity was maintained at the level of 60% and water was added every 2 days. The weight of the pots was controlled using scales.

To control the process of removing pollutants from the soil, a 10 g soil sample was collected for analysis from each pot to determine the rate of the chemical and microbiological changes. The samples were collected before the experiment (start), and after

6 hours, 30 and 60 days. Bioremediation efficiency was assessed versus the soil without inoculate (control).

Microbiological methods. Qualitative and quantitative determination of the microbial populations was carried out using standardized plate count agar method. From each pot test 10 g samples of soil were taken and suspended in 90 cm^3 of physiological saline. Five 0.1 cm^3 portions of the suspension were placed on Petri dishes filled with the following media:

- fungi in medium Czapek with glucose [15],
- yeast in medium YPG with chloramphenicol (0.1 $g \cdot dm^{-3}$) [16],
- bacteria in Nutrient LAB Agar™ (BIOCORP),
- actinomycetes in Starch Casein Nitrate Agar (Difco),
- microorganisms capable to grow in medium containing hexadecane as the only source of carbon [11].

Bacterial and yeast counts were made at 25 °C after 5 days of incubation and after 10 days for other investigated microbial populations. The counts of the microorganisms were expressed in $CFU \cdot g^{-1}$ DM±standard deviation (SD). Various bacterial colonies identified under microscope were divided into morphological groups using Gram's staining method. Filamentous fungi were determined by their morphological characteristics using selected taxonomic monographs [12, 17].

Chemical analyses. pH was determined in 1 M KCl by potentiometric method using ELPO N-512 pH-meter. Dry matter was determined by weight during maintenance over anhydrous sodium sulfate at room temperature. Samples for chromatographic determinations were mixed, weighed and dried at room temperature over anhydrous sodium sulfate. Investigated compounds were extracted in an automatic fexIKA® extractor (IKA Werke). Dichloromethane (POCH GC grade) was used for monoaromatic hydrocarbons (BTEX) extraction. Polycyclic aromatic hydrocarbons (PAHs) were extracted using dichloromethane and hexane in proportion 1:9 (v/v) while for alkanes – *n*-hexane (POCH GC grade) was applied. Samples were condensed and purified with activated aluminum oxide before analysis.

Qualitative and quantitative analysis of the produced extracts was carried out by the gas chromatography using a SHIMADZU GC 17A gas chromatograph coupled to MS--QP5000 mass detector. The conditions for the chromatographic analyses were as follows:

BTEX: VF5-ms capillary column 30 m×0.25 mm×0.25 μm; carrier gas (He) flow rate: 1cm^3/min, injection port operating temperature 250 °C, detector temperature: 280 °C; temperature program 30-5/5/170-6; MS detector voltage from 1.2 to 1.4 kV.

PAH: VF5-ms capillary column 30 m×0.25 mm×0.25 μm; carrier gas (He) flow rate: 1 cm^3/min, injection port operating temperature 300 °C, detector temperature 310 °C; temperature program 80-8/10/270-12/300-12; MS detector voltage from 1.2 to 1.4 kV.

n-Aliphatic hydrocarbons: VF1-ms capillary column 30 m×0.53 mm×1.50 μm; carrier gas (He) flow rate: 3 cm^3min, injection port operating temperature: 300 °C, detector temperature: 325 °C; temperature program 100-3/12/320–12; MS detector voltage from 1.2 to 1.4 kV

The amount of petrochemical substances in soil extracts was determined by integrating cumulative peaks covering the boiling temperature range of 126–522 °C. Aliphatic hydrocarbons C_8–C_{40} were determined. From the monoaromatic hydrocarbons group, the following were determined: benzene (B), toluene (T), *m*- + *p*-xylene (*m* + *p*-x), *o*-xylene (*o*-x), styrene (Sty) and isopropylbenzene (Izo-pb). In the polycyclic aromatic hydrocarbons group (PAH) the following 16 compounds recommended for monitoring by the US. EPA were determined: naphtalene (Na), acenaphtylene (Ayl), acenaphten (Aen), fluorene (Flu), fenantrene (Fen), anthracene (Ant), fluorantene (Fla), pyrene (Pur), benzo[a]anthracene (BaA), chryzene (Chr), benzo[b,k]fluorantene (BbF, BkF), benzo[a]pyrene (BaP), dibenzo[a,h]anthracene (DhA), indeno[1,2,3-c,d]pyrene (IcP), benzo[g,h,i]perylene (BgP).

3. RESULTS

Bioaugmentation of soil contaminated with petroleum-derived substances with the use of *Y. lipolytica* yeast biomass had varied impact on development of soil microbiota and the biodegradation of hydrocarbons. A dominating group were prokaryotic microorganisms, and their quantity was growing during the process in the soil inoculated with yeast biomass (Fig. 1a, c). Contrary, the quantity of filamentous fungi with respect to the control sample showed a decreasing tendency (Fig. 1b), and *Trichoderma*, *Acremonium* genus as well as *Zygomycetes* class fungi dominated. The quantity of yeast in the inoculated soil was lower than that in the control sample (Fig. 1d), only after 30 days of the experiment, a tenfold increase was recorded. The number of microorganisms metabolizing hexadecane was similar to the level in the control sample and increased almost 10 times after 30 days of the experiment in inoculated soil, which may be related to breeding of *Y. lipolytica* yeast, originating from the applied inoculant (Fig. 1e).

Stimulation of the bioremediation process with yeast inoculation influenced not only on quantity but also the quality of bacteria. The number of gram-positive bacteria increased in comparison to that of gram-negative ones (Table 1), which may signify the soil purification process.

Microbiological changes in the inoculated soil were accompanied with changes of pH and chemical composition of the analyzed samples. In the presence of inoculum, pH in soil considerably decreased in comparison to that in the control sample and remained on the level of 6.8–6.5 (Fig. 2), which may reflect metabolic activity of microorganisms causing acidification of environment.

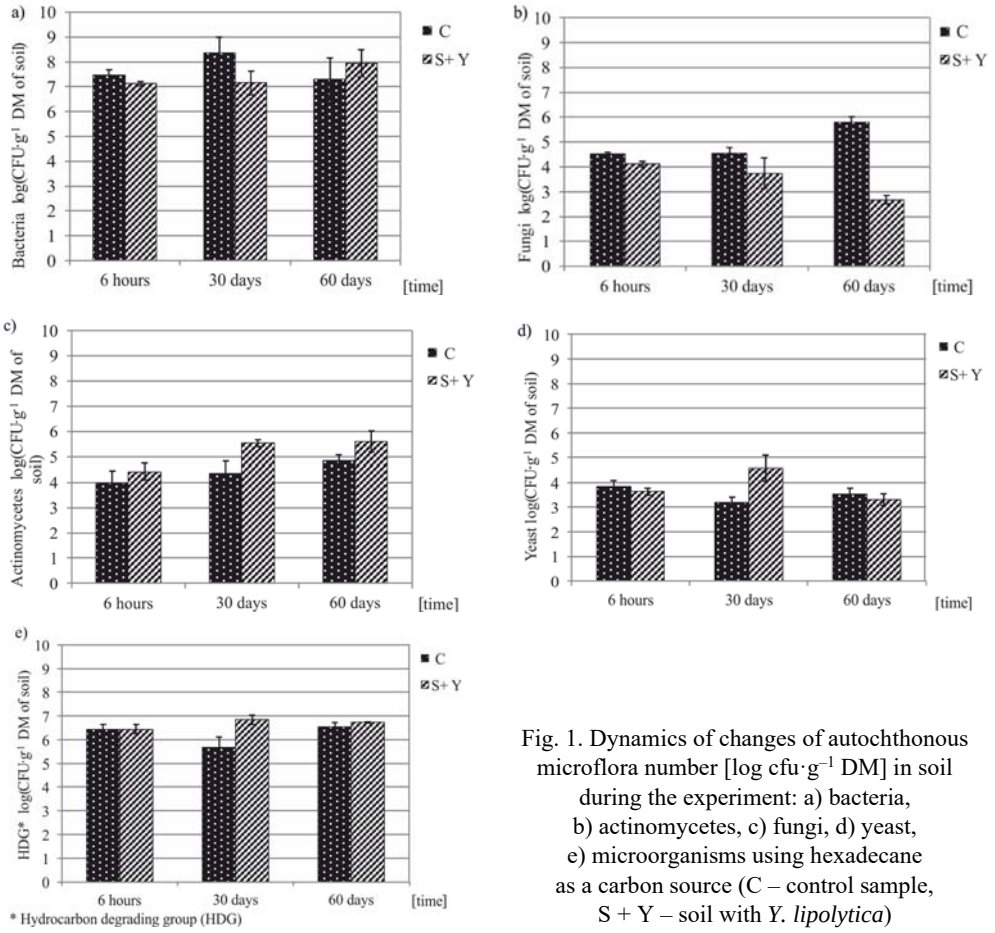

Fig. 1. Dynamics of changes of autochthonous microflora number [log cfu·g^{-1} DM] in soil during the experiment: a) bacteria, b) actinomycetes, c) fungi, d) yeast, e) microorganisms using hexadecane as a carbon source (C – control sample, S + Y – soil with *Y. lipolytica*)

Table 1

Part of gram-positive and gram-negative bacteria in total population during the experiment [%]

Bacteria	6 h		30 days		60 days	
	C	S + Y	C	S + Y	C	S + Y
Gram-positive	16.4	16.0	38.9	43.2	29.0	49.4
Gram-negative	83.6	84.0	61.1	56.8	71.0	50.6

After inoculation of the contaminated soil, efficiency of remediation increased (Fig. 3). In the initial period (after 6 hours), the pollutant biodegradation level was 20% in comparison to the initial value and the value in the control sample. After 30 days, concentration of petroleum-derived substances in inoculated soil as well as in the control sample were on the same level, but lower than those in the initial samples. However,

after 60 days of the experiment, the total content of petroleum-derived substances increased in inoculated soil and simultaneously decreased in the control sample (Fig. 3). This phenomenon may signify local desorption and diffusion of hydrocarbons from the soil, because simultaneous changes in composition and concentration of individual groups of hydrocarbons were observed.

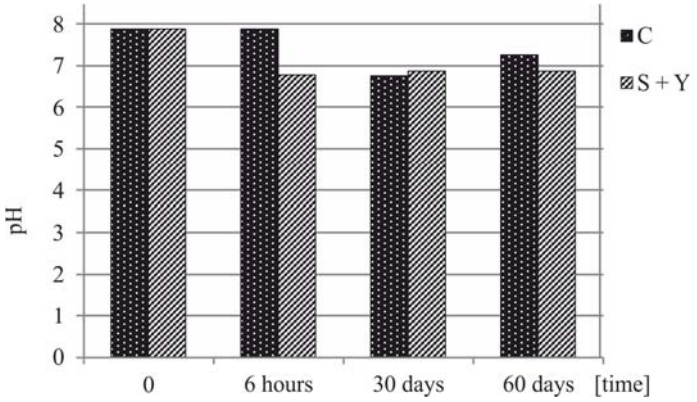

Fig. 2. pH of soil samples polluted with petrochemicals during the experiment after use of hydrogen peroxide (C – control sample; S + Y – soil with *Y. lipolytica*)

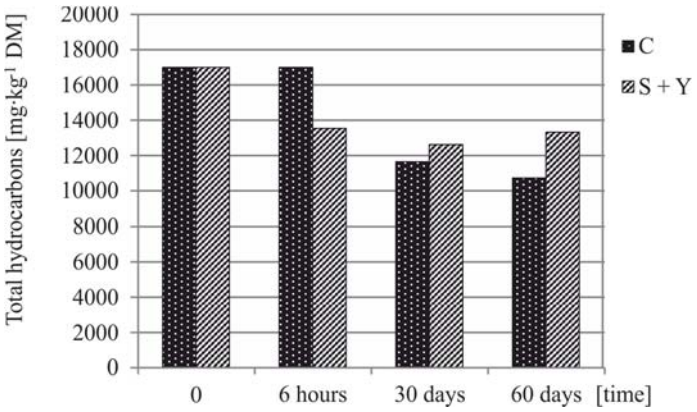

Fig. 3. Total content of petrochemicals [in contaminated soil after biodegradation (C – control sample; S + Y – soil with *Y. lipolytica*)

Total content of *n*-aliphatic hydrocarbons in inoculated soil was lower in comparison to that in the control sample and initial concentration. The largest, approximately 20%, reduction was observed in hydrocarbons C_{15}–C_{19} and C_{24}–C_{30}. However, after 60 days, a small increase of concentration of these hydrocarbons was detected, both in the control sample and inoculated soil (Fig. 4).

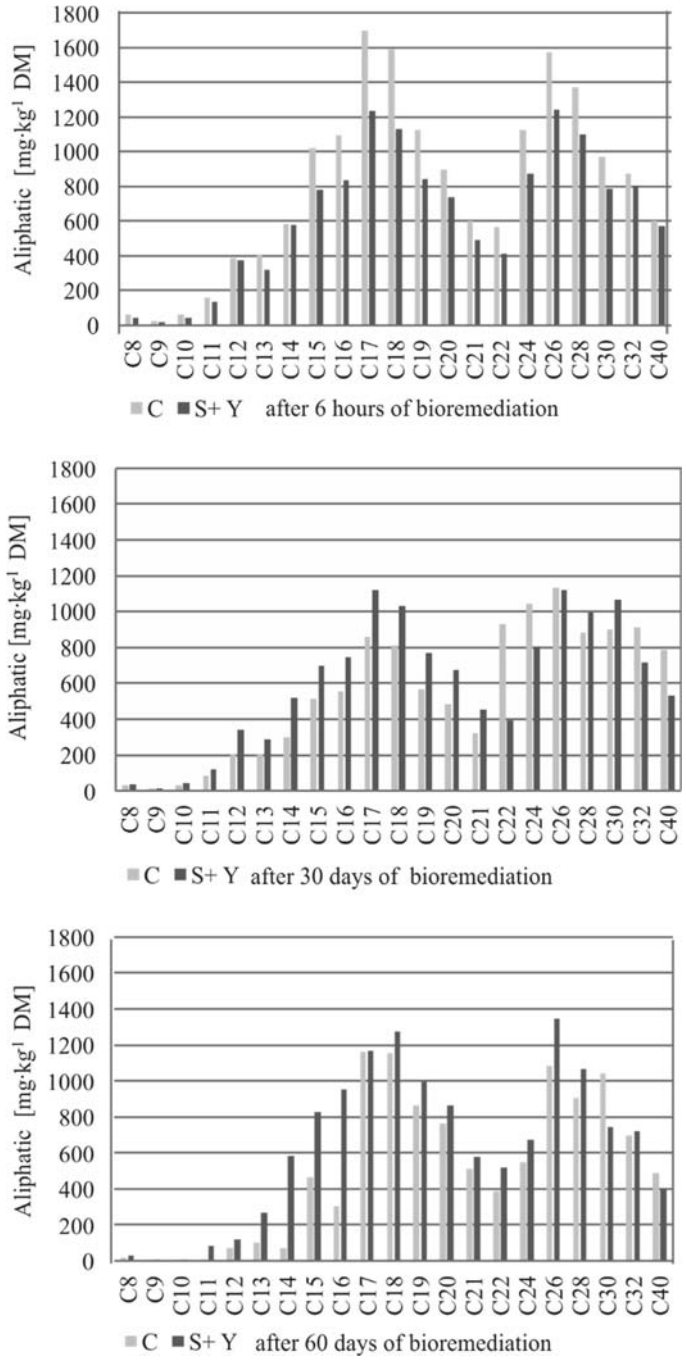

Fig. 4. Aliphatic hydrocarbon content in soil during the experiment (C – control sample; S + Y – soil with *Y. lipolytica*)

A threefold increase of BTEX content was observed after 1 month of bioremediation in every medium, followed by a decrease during next month (in inoculated and control soil). However, the cumulative content of BTEX after 60 days of bioremediation was slightly higher than the initial concentration and their content in the control sample (Table 2). This was caused by increase by several times of benzene content both in inoculated soil and the control sample, most probably stimulated by breaking the side chains in monoaromatic compounds. After 30 days of bioremediation, a decrease of the content of other compounds such as toluene, xylene, styrene and isopropylbenzene was recorded in every pot. In the case of toluene, its content decreased after 60 days in inoculated sample, whereas it increased in the control sample (Table 2).

Table 2

BTEX content in soil during the experiment [mg·kg^{-1} DM]

Hydrocarbon	S + Y				C			
	Start	6 h	30 days	60 days	Start	6 h	30 days	60 days
Benzene	0.7	0.7	34.8	9.6	0.7	0.6	37.1	4.0
Toluene	1.3	1.3	0.5	0.5	1.3	1.6	0.3	0.9
Ethylbenzene	0.3	0.3	0.3	0.2	0.3	0.3	0.6	0.1
m-, *p*-Xylene	1.1	1.1	0.6	0.6	1.1	1.2	0.3	0.2
o-Xylene	1.4	1.4	0.3	0.6	1.4	1.6	0.3	0.2
Styrene	1.4	1.4	0.4	0.7	1.4	1.4	0.2	0.1
Isopropylbenzene	4.1	4.1	1.2	1.6	4.1	5.0	0.4	0.5
Total	10.3	10.3	38.1	13.8	10.3	11.7	39.2	6.2

C – control sample, S + Y – soil with *Y. lipolytica*.

The content of polycyclic aromatic hydrocarbons (PAHs), similarly as that of aliphatic compounds, decreased by approximately 30% 6 hours after inoculation. The most intense reduction was observed during the first 30 days of the process duration and amounted to approximately 80% of the initial content. Total content of PAHs in inoculated soil was slightly lower in comparison to that in the control sample at the end of process.

The content of individual compounds decreased after 30 days of the process duration, however the concentrations of naphtalene (N), acenaphtalene (Ac), acenaphtylene (Acl), fluorene (F) i fenantrene (Fen) were higher than those in the control soil. In that period, the content of other PAHs, including pyrene (P) and benzo[a]pyrene (BaP) was lower than those in the inoculated soil. After 60 days, the quantity of most PAHs with the content of more than 4 aromatic cycles considerably decreased in inoculated soil in comparison to the control. The largest decrease was recorded for the content of acenaphtalene (Ac), acenaphtylene (Acl) and dibenzo[a,h]anthracene. The content of benzo[a]anthracene (BaA), benzo[b]fluorantene (BbF) and dibenzo[a,h]anthracene increased after 60 days of incubation (Table 3).

Table 3

PAHs content in soil [mg·kg^{-1} DM] (C – control sample; S + Y – soil with *Y. lipolytica*)

Hydrocarbon	S + Y				C			
	time 0	6 hours	30 days	60 days	time 0	6 hours	30 days	60 days
Naphthalene	19.43	19.43	9.04	11.01	19.43	23.98	3.70	3.65
Acenaphthylene	11.67	11.67	4.08	0.50	11.67	13.82	2.22	2.90
Acenaphthene	40.36	40.36	12.62	0.31	40.36	42.89	4.87	12.12
Fluorene	6.03	6.03	1.40	0.64	6.03	5.23	0.87	1.07
Phenanthrene	9.01	9.01	2.00	7.08	9.01	10.17	1.34	2.77
Anthracene	12.68	12.68	2.98	12.40	12.68	13.61	1.99	5.27
Fluoranthene	8.34	8.34	1.83	11.68	8.34	23.53	1.26	4.73
Pyrene	1.25	1.25	0.18	0.59	1.25	1.31	0.53	0.57
Benzo(a)anthracene	0.75	0.75	0.86	2.78	0.75	2.09	4.63	17.05
Chrysene	8.23	8.23	1.68	0.71	8.23	11.59	1.07	0.53
Benzo(b)fluoranthene	13.56	13.56	1.83	1.07	13.56	15.57	2.44	6.48
Benzo(k)fluoranthene	0.74	0.74	2.04	2.07	0.74	5.75	2.28	1.32
Benzo(a)pyrene	1.48	1.48	1.00	0.67	1.48	4.97	1.77	0.45
Indeno[1,2,3-c,d]pyrene	0.66	0.66	0.62	1.87	0.66	27.75	4.00	1.24
Dibenzoanthracene	4.79	4.79	0.55	0.96	4.79	1.36	3.76	2.46
Benzopyrene	1.27	1.27	0.91	0.60	1.27	1.84	0.53	0.37
Total	140.25	140.25	43.62	54.94	140.25	205.46	37.26	62.98

C – control sample, S + Y – soil with *Y. lipolytica*.

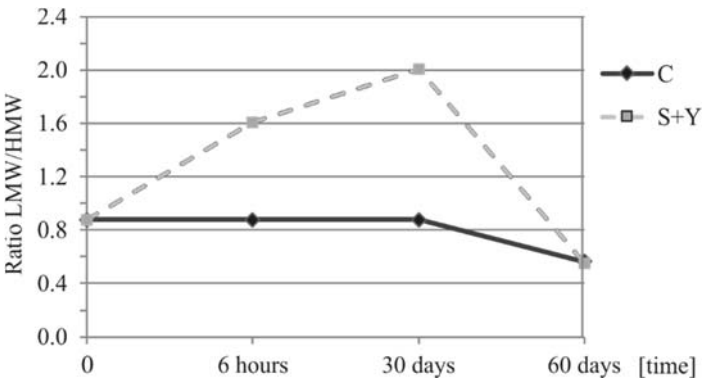

Fig. 5. Ratios of low molecular weight to high molecular weight polyaromatic hydrocarbons during the experiment (C – control sample; S + Y – soil with *Y. lipolytica*)

Efficiency of PAHs biodegradation was higher in the soil inoculated with yeast than that in the control sample. Also the PAHs biodegradation route was different, expressed as the ratio of the content of 2–3-ring hydrocarbons (LMW) to 4–6-ring hydrocarbons (HMW). After the first 30 days of the process, this ratio was higher in the sample inoculated than that in the control sample, which points to intensive degradation of PAHs of

high molecular weight to PAHs with small molecular weight. Then this ratio decreased and in 60 day of the process was the same as that in the control sample, which was probably caused by dimerization of 2–3-ring compounds, with simultaneous creation of large molecule compounds (Fig. 5).

4. DISCUSSION

Biodegradation of petroleum-derived substances in environment can be a spontaneous process, however, various stimulation techniques have been applied for their enhancement, in particular with the use of microbiological inoculants. The use of such stimulations on half-technical and technical scale requires prior laboratory research, because each biostimulation has a different influence on degradation activity of microbiota and, on the direction of transformations of petroleum-derived substances, particularly in highly contaminated soils.

Local desorption and diffusion from different soil layers, causing the increase of contamination, can be additional problem [3, 4, 11]. The use of *Y. lipolytica* yeast inoculum may change the soil microbiota [3, 4]. The ratio of Gram-positive to Gram-negative bacteria may be recognized as a microbiological index of the degree of contamination with petroleum-derived substances, as well as bioaugmentation efficiency [3, 18]. Therefore, it can be assumed that the process of biodegradation supported with *Y. lipolytica* inoculation stimulates removal of pollutants from soil because the quantity of gram-positive bacteria increases with respect to gram-negative ones.

Inoculation by yeast stabilized acidity of the contaminated soil, which directly influenced the path of biodegradation of petroleum-derived compounds [19]. The reaction remained on a stable level (pH ca. 6.8) after an initial decrease. The decrease in pH could be caused by both intermediate and final acidifying products of hydrocarbon decomposition [20].

The inoculate *Y. lipolytica* supported biodegradation process of both *n*-aliphatic hydrocarbons and PAHs, however, high concentration of hydrocarbons in contaminated soil restricted yeast growth and caused decrease of biodegradation degree of this group of hydrocarbons. This phenomenon was also observed by Bento et al. [9]. That is why additional inoculation seems justified during bioremediation process and because the specialized microbial consortia are most effective in a short time after inoculation of the contaminated soil. Efficiency of PAHs biodegradation was higher in the soil inoculated with yeast than in the control sample. However, Jacques et al. [21] suggest that only part of PAHs is completely mineralized and even half of the content may be modified into other metabolites. PAHs turn into complexes which are absorbed and precipitated in soil environment, particularly in the presence of humic acids [1, 2]. PAHs with the lowest number of carbon rings are removed most effectively [1], and decomposition of four- and five-ring hydrocarbons is connected to co-metabolism of their analogues with

the lowest number of rings [20]. However, in the soil inoculated with *Y. lipolytica*, initially a fast decomposition of PAHs with high molecular weight (4–6 rings) to PAHs with low molecular weight (2–3 rings) was recorded. The biodegradation path of PAHs was different than that in the control soil, because high molecular weight hydrocarbons prevailed during the process. The ratios of LMW/HMW higher than 1 pointed to pollution from the petrogenic sources, however microbiological processes may influence this ratios [22].

During the process, the content of the most cancerogenic hydrocarbons such as benzo(a)pyrene and dibenzo[a,h]anthracene decreased in comparison to their content in spontaneous processes. Benzo(a)pyrene is the basic index of soil cancerogenicity [23], but the values of relative cancerogenicity factors for other PAHs have also been defined. The data show that dibenzo[a,h]anthracene is five times more cancerogenic than benzo(a)pyrene [24].

Monoaromatic hydrocarbons BTEX were the least affected by biodegradation in the presence of *Y. lipolytica* inoculum. The increase of benzene content and fluctuation of concentrations of certain BTEX compounds during bioremediation can be correlated with their disintegration into benzene and *n*-aliphatic compounds.

Petrol contaminated soil bioaugmentation with non-conventional *Y. lipolytica* yeast supports biodegradation of PAHs with higher cycle number (thus less bioaccessible) and engendered important reduction of the most cancerogenic hydrocarbons.

5. CONCLUSIONS

- *Y. lipolytica* yeast inoculum caused changes in the population of individual groups of autochthonic microbiota in the soil heavily contaminated with petroleum-derived substances.
- Biodegradation efficiency in various groups of petroleum-derived compounds varied and depended on chemical composition of a given compound. Aliphatic and PAH hydrocarbons group was most susceptible to biological disintegration.
- *Y. lipolytica* was most effective within 6 hours after inoculation of the contaminated soil. Next, the effectiveness decreased and was lower in comparison to natural biodegradation processes.
- The content of benzo(a)pyrene decreased two times during bioremediation and concentration of more cancerogenic benzo[b,k]fluoranthene decreased 5 times.

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