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APPLICATION OF *PSEUDOMONAS PUTIDA* AND *RHODOCOCCUS SP.* BY BIODEGRADATION OF PAH(S), PCB(S) AND NEL SOIL SAMPLES FROM THE HAZARDOUS WASTE DUMP IN POZĎÁTKY (CZECH REPUBLIC)

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Key words: hazardous waste disposal, bacteriae, biodegradation

Abstract

The objective of the project was a laboratory check of biodegradation of soil samples contaminated by polyaromatic - PAH(s), polychlorinated biphenyls - PCB(s) and gasoline range organic plus diesel range organic reported in text as NEL from the hazardous waste dump in the Pozďátky locality. For the laboratory check, pure bacterial cultures of *Rhodococcus sp.* and *Pseudomonas putida* have been used. It is apparent from the laboratory experiments results that after one-month bacterial leaching, applying the bacterium of *Rhodococcus sp.* there is a 83 % removal of NEL, a 79 % removal of PAH(s) and a 14 % removal of PCB(s). Applying a pure culture of *Pseudomonas putida* there is a 87 % removal of NEL, a 81 % removal of PAH(s) and a 14 % removal of PCB(s).

Ključne riječi: odlaganje opasnog otpada, bakterije, biorazgradivost

Sažetak

Cilj projekta bilo je laboratorijsko istraživanje biorazgradivosti uzoraka tala zagađenih s poliaromatskim ugljikovodicima (PAH), polikloriranim bifenilima (PCB) i organskim spojevima kao što su benzin i dizel obuhvaćeni u radu skraćenicom (NEL) s odlagališta opasnog otpada na lokalitetu Pozd'átky. Za laboratorijska ispitivanja korištene su čiste kulture bakterija *Rhodococcus sp.* i *Pseudomonas putida*. Iz rezultata laboratorijskih ispitivanja vidljivo je da je nakon mjesec dana (ispiranja) djelovanja bakterija *Rhodococcus sp.* odstranjeno 83% NEL, 79% PAH i 14% PCB. Primjenom čiste kulture bakterija *Pseudomonas putida* odstranjeno je 87% NEL, 81% PAH i 14% PCB.

INTRODUCTION

Human activities lead to the contamination of our planet by organic and inorganic pollutants. The pollution is spreading and it represents a real threat to a healthy development of mankind, animals and plants. One of the most questionable is the group of persistent – exceptionally resistant substances which have been produced by man in significant amounts in the course of last 50 years in the form of herbicides, pesticides, insecticides, as well as they are present in many industrial products (e.g. capacitor, transformer or hydraulic charges).

For degradation of classical biogenic compounds, which have been present in the environment for millions of years, microorganisms have developed special mechanisms. Xenobiotics, i.e. synthetic substances produced by man, have been more abundant in the environment only for a few decades. Nevertheless, microorganisms are able to use some of these compounds as the only sources of carbon and energy (Brenner, 2003). The fate and direct removal of extraneous substances from the environment mainly depends on their metabolism intermediated by enzyme systems of organisms forming trophic (food) chains.

Biotechnological processes include mining, dressing and processing methods, during which post reaching the desired qualitative changes of raw materials and refuse, they make use of microorganisms or products of their metabolic activity. Basically, nature develops these systems on her own, and unconsciously people have been using their products since the beginning of their history. Biotechnologies embrace mainly microbiological, biochemical and chemical knowledge.

Biodegradation of hazardous harmful substances in the environment embody significant prospective methods, when complex and ecologically unsound pollutants are decomposed into simpler substances (sound ones) by the action of microorganisms. The principle of biodegradation technologies is an optimization of nutrient ratios (to support the growth of selected microorganisms able to degrade the target contaminants) and an application of suitably selected isolated microorganism strains with relevant degradation abilities.

Previously suggested decontamination technologies were often very costly and severe to the environment. There is though a tendency to propose and apply procedures that are not only cheaper but also more natural. One of the

prospective ways is a biological decontamination of the environment by means of microorganisms and plants (Páca, 2003).

Currently, biodegradation technologies, biological methods to remove a variety of pollutions are being improved in an intense way. Biodegradation technologies first proved practical on a wider scale during an oil spill of Exxon Valdez tanker. Since than they have been used more frequently in the majority of countries.

In the Czech Republic, this trend began to develop after 1989. These are mainly biodegradation technologies designed for the decontamination of soil and water contaminated by oil substances and their derivates, for the decontamination of coal tar-phenol pollutions and last but not least for the decontamination of persistent organic pollutants (xenobiotics). Since 2000 a number of companies dealing with this issue have been active in the Czech Republic. Already now, some of them reach very good results and they make use of technologies on a worldwide level. For example, in the Czech Republic hundreds of tons of contaminated soils by oil substances are annually treated by the technologies of biological improvement in many decontamination sites.

The application of biological improvement technologies should be preferred to physical and chemical methods as in principal they remove only the share of contamination in question. Moreover, in some cases, these methods harm the environment even more than the very pollution. The advantage of biodegradation of contaminated soils by oil substances is the fact that in the course of microbial degradation no waste materials polluting the environment are formed (the final products are carbon dioxide and water in the last stage).

One of the problems during the application of decontamination technologies based on biological methods in the CR are insufficient valid general rules and missing specific legislation in the aspect of removal of old ecological burdens. At present, biotechnologies fall under the State Health Institute of the CR and the State Hygiene Stations in terms of occupational safety and public health protection.

In the course of application of biodegradation methods it is necessary to keep in mind that it is a complex process. Their success or failure depend on the following factors: chemical (types of contaminant, pH medium, concentration of macro and microbiogenic elements, water content, chemical composition of the contaminated material, chemical composition and concentration of suitable nutrient solutions, etc.), microbiological (degradation activity of microorganisms), physical (temperature, water solubility, sorption onto solid particles).

Without external interference, the speed of a natural biodegradation process in the improved localities is very low. An appropriate improvement method may increase the speed of biological processes several times.

Ways of biodegradation of aromatic and polyaromatic hydrocarbons

Degradation of organic substances by means of microorganisms makes part of the natural carbon cycle in the nature. The process of biodegradation is based on the abilities of microflora to use the present harmful substances as a source of carbon and energy for their own growth. The ability of microorganisms to degrade hydrocarbons has been known since 1895, when a growth of yeast fungus on paraffin was described.

Later, the ability of bacteria to use methane as a source of carbon was discovered, and finally in 1969 Davis et. al. in (Žebrák, 1997) proved that there are bacteria degrading virtually all component parts of crude oil. Prokaryotes (bacteria and cyanobacteria) as well as eukaryotes (fungi including yeast fungus, algae and protists) assert one selves in these cases. In addition, archaebactera play an important role in many cases.

More than 200 microorganism species capable of hydrocarbon degradation have been identified. They follow in the order of importance: heterotrophic bacteria, fungi, aerobic bacteria, actinomycetales, phototrophes and oligotrophic bacteria. The most applied bacteria fall under the genders of *Pseudomonas, Arthrobacter, Acinetobacter, Flavobacterium, Alcaligenes, Micrococcus* and *Corynebacterium* (Masák, 1992). Intense research in this area confirms that besides bacteria, other microorganisms, including fungi and algae, can be used.

The ability of biodegradation is given by enzymatic make-up of the individual bacterial genders. The enzymatic spectrum of a given cell is given by its genetic potential. A part of genes coding enzymes utilisable for biodegradation are found in the DNA of plasmids. Plasmids usually carry complement information which is not vital for the cell under normal circumstances and whose loss is not lethal to the cell. However, sometimes it can be an advantage in certain special conditions. In that respect, catabolic plasmids are very important as they permit their cells to use non-traditional sources of energy.

Basically, biochemical processes during which the decay of hydrocarbons occur, can be divided into two fundamental groups, i.e. anaerobic processes taking place with the access of oxygen and anaerobic processes taking place without the access of oxygen. As the anaerobic degradation is very slow, mainly aerobic processes are used in practice.

Benzene and non-substituted polyaromatic hydrocarbons, hereinafter referred to as PAH(s), have a high negative resonance energy, and therefore they are thermodynamically very stable, which reflects in their chemical properties. In practice, usually 16 polycyclic aromatic hydrocarbons are monitored, which are on the list of priority pollutants of US EPA. PAH(s) range among persistent organic pollutants. Their molecules are formed by two or more condensed benzene nuclei. Bonding of

other substituents (e.g. halogen-, sulfo-, amino-, nitro-) onto the benzene nucleus, the reactivity of the nucleus resonance structure towards oxygen falls considerably and the circle becomes more resistant to opening (Holoubek, 2000).

An overview of microorganisms degrading poly-aromatic hydrocarbons: *Aeromonas, Alcaligenes, Bacillus, Beijerinckia, Corynebacteria, Cyanobacter, Falvobacterium, Micrococcus, Mycobacterium, Nocardia, Vibrio, Pseudomonas, Rhodococcus.*

Characteristics of *Pseudomonas* bacteria

Pseudomonas bacteria are gram-negative, chemoorganotrophic, aerobe obligate, aerobically respiratory metabolisms. Some species are facultative chemolithotrophic. They are straight or curved rods. Their dimensions range between 0.5 and 1.0 μm x 1.5 – 4.0 μm. They move by one or more polar-located flagella. They are arranged mainly individually or in small clusters or chains. They grow under strict aerobic conditions in common substrates, on which they form irregularly large colonies producing water-soluble exopigment (pyocyanine and fluoresceine), which diffuse into the atmosphere and dyes it yellow or blue-green. Older cultures dye dark brown. The temperature range of their growth is 0 – 42 °C; the optimum temperature is 35 °C. The enzymatic activity is dependent on ecological conditions out of which the individual genders were isolated. They make use of some sugars, out of which they form acids, but not gas. Many genders oxidize glucose into gluconic acid, 2-keto-gluconic and other acids. The majority of the studies genders reduce nitrates down to nitrites. They live saprophytically in soil and water. There appears a high affinity with the *Vibrio* and *Xantomonas* genders. In total, there are approximately 29 genders. Figure 1 shows *Pseudomonas putida* bacteria.

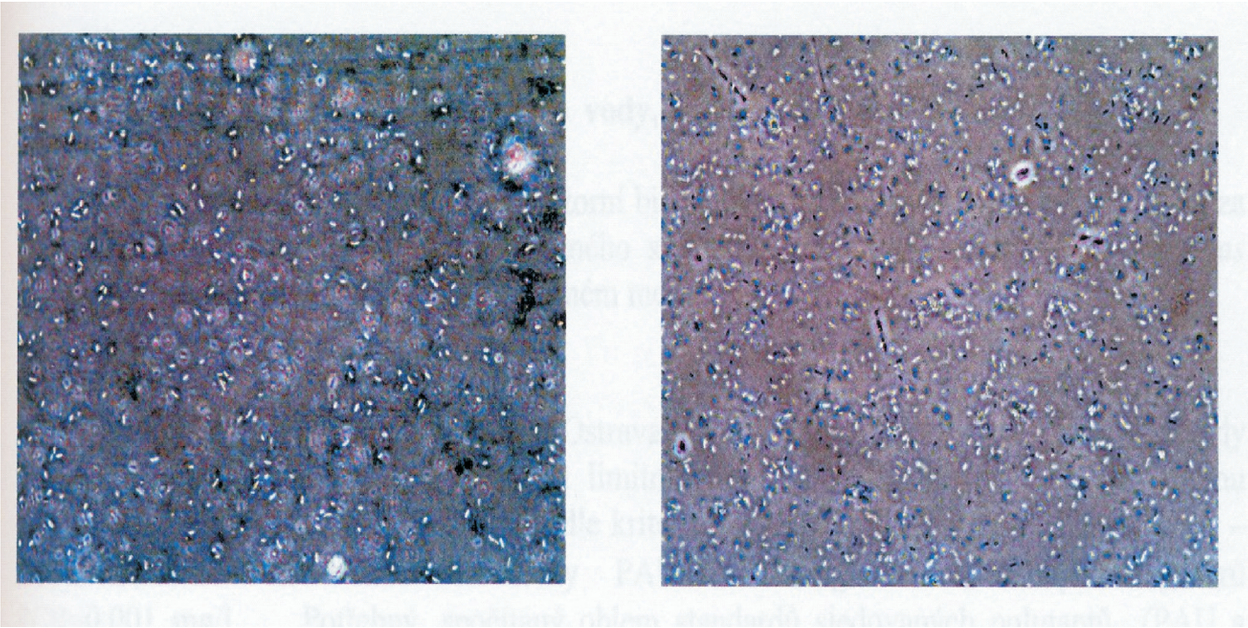

Figure 1 Pseudomonas putida

Slika 1 Pseudomonas putida

Characteristics of *Rhodococcus* bacteria

These are gram-negative, chemoorganotrophic, aerobe obligate, aerobically respiratory metabolisms. The cells are of a spheroidal shape, the average size of the cells fluctuate between 0.5 and 3.5 μm; they appear individually or two and more cells aggregate into irregular clusters, sometimes tetrads or bundles. They grow under aerobic conditions in common substrates, under the optimum temperature of 25 – 35 °C. On the substrates they form shiny colonies with the dimensions of 2 – 4 μm. Many colonies precipitate pigments of various colours (pink, yellow, orange). In the nature, they occur as saprophytes. Figure 2 shows *Rhodococcus sp.* bacteria.

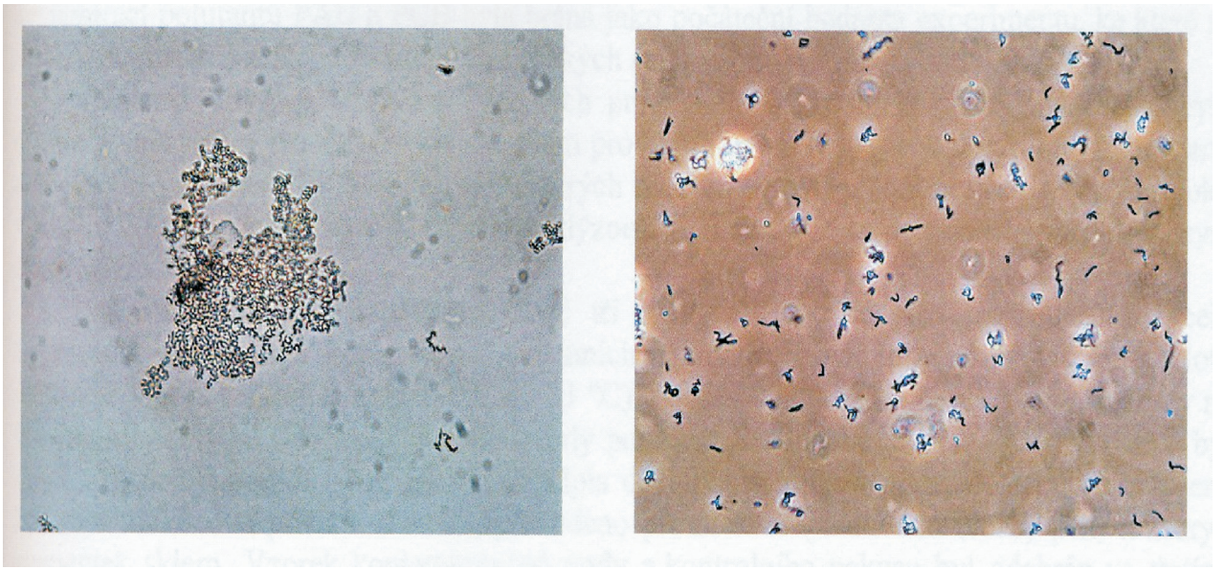

Figure 2 Rhodococcus sp.

Slika 2 Rhodococcus sp.

Methods of the experimental work

The experimental biodegradation of the selected harmful substances – PAH(s), PCB(s) and NEL - took place with a soil sample from the hazardous waste dump and municipal refuse dump near Pozďátky, Třebíč district. It was carried out by means selected pure cultures of *Pseudomonas putida* and *Rhodococcus sp.* bacteria. These microorganism cultures were acquired from the Czech collection of microorganisms with the Natural Science Faculty at the Masaryk University in Brno.

In the course of the cultivation works the following media were made use of:

- liquid medium M1 (Beef extract Broth, peptone, NaCl, distilled water, pH 7,2),
- liquid medium M96 (Mineral Medium with Vitamins, Media, Bacteria, pp. 123),
- liquid medium M65 (Mineral Medium for Chemolitotrophic Growth H-3, Media, Bacteria, pp. 120).

The check of bacteria viability and an approximate determination of their number is done by means of a microscope. For this task we used the Carl Zeiss Jena "Amplival" microscope and Cyrus I cell with a raster for reading the number of bacteria. The enlargement ratio of the microscope ranged from 400 (reading the number of bacteria) up to 1000-fold enlargement (observation of bacteria viability).

Post treatment, the mineralogical composition of the sample was determined by an X-ray diffraction analysis in the laboratory of the Institute of Geological Engineering at VŠB – TU Ostrava. The measurement was carried out using a modernized, fully-automated diffractometer URD-6 (Rich. Seifert-FPM, SRN). With the given sample, the following phases were identified: quartz, microcline, plagioclase, amphibole chlorite, biotite, illite – montmorillonite.

The determination of NEL, PAH(s) and PCB(s) was carried out in an accredited laboratory for fuels, waste and water of VÚHU, a.s. Most.

In total, the laboratory experiment lasted four weeks. 100 g of soil, 100 ml of bacterial solution and 500 ml of substrate were inserted into 1-litre containers which were following closed. Aeration was secured by means of aquarium pumps. The required volume was gradually filled with distilled water. Samples for analyses were taken after one and four weeks.

The results of bacterial biodegradation

It is apparent from the results of bacterial leaching using a pure bacterial culture of *Rhodococcus sp.* that after one-month leaching it is possible to remove 83 % of NEL, 79 % of PAH(s) and 14 % of PCB(s) from the sample. The results are shown in Table 1. In the table Σ PAH(s) represents 15 individual polyaromatic hydrocarbons. It is apparent that the application of this bacterial culture is suitable for the degradation of PAU and NEL. However, the degradation of PCB is very low.

Table 1 The course of degradation of the selected harmful substances by means of *Rhodococcus sp.* bacteria (values stated in mg/kg solid)

	NEL	**Σ PAH(s)**	**Σ 7 congeners PCB(s)**
Input	513	11.05	0.07
One week	445	5.75	0.06
Four weeks	85	2.36	0.06

The results of bacterial leaching applying a pure bacterial culture of *Pseudomonas putida* imply that after one-month leaching it is possible to remove 87 % of NEL, 81 % of PAH(s) and 14 % of PCB(s) from the sample. The results are displayed in Table 2. It is apparent from the acquired results that the application of this bacterial culture is suitable for the degradation of NEL and PAH(s), but the degradation of PCB(s) is low.

Table 2 The course of degradation of the selected harmful substances by means of *Pseudomonas putida* bacteria (values stated in mg/kg solid)

	NEL	**Σ PAH(s)**	**Σ 7 congeners PCB(s)**
Input	513	11.05	0.07
One week	435	6.20	0.07
Four weeks	66	2.11	0.06

The results imply that for the given sample are both bacterial cultures suitable.

CONCLUSION

The objective of the project was a laboratory check of biodegradation of NEL, PAH(s) and PCB(s) with a soil sample from the Pozďátky locality. The acquired results show that for the given sample are both clean bacterial cultures very suitable.

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