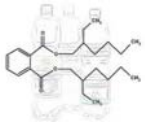
PHTHALATE ESTERS AS A TARGET OF BIOREMEDIATION TECHNOLOGIES

Martina Siglová[1], Jiří Mikeš[1], Miroslav Minařík[2]
1 EPS, s.r.o. Přílepská 1692, 252 63 Roztoky, Czech Republic; vyvoj@epssro.cz
2 EPS, s.r.o. V Pastouškách 205, 686 04 Kunovice, Czech Republic; www.epssro.cz



Introduction

Phthalates are a family of chemicals that are produced in the millions of tons annually worldwide, and are principal component of many diverse products that consumers come into daily contact with at home, at work, and in hospitals. They include, among other items, products made of softened polyvinyl chloride plastic (PVC, substances added to plastics to increase their flexibility), cosmetics and other personal care goods, pesticides, building material, lubricants and adhesives. Phthalates are released into the environment not only by manufacturers and it also escapes from finished products (Pašková, 2003).

Worldwide ecosystem contamination and direct contact with phthalates-containing products result in virtually ubiquitous human exposures with serious damage to health (comparable with well known adverse effects of PCBs).

Phthalates are classified as endocrine disruptors. Studies have linked endocrine disruptors to adverse biological effects in animals (Fig. 1 and 2), giving rise to concerns that low-level exposure might cause similar effects in human beings (WHO, 2002).

Among other effects phthalates are detrimental to fetus and child evolution, male reproducing system, viability of sperms etc. They are also suspected carcinogens (Yuan, 2002). Those facts led to the first legal limitations of some phthalates in toys or baby-care products in EU. But limit concentrations for example in environment or food are still missing.

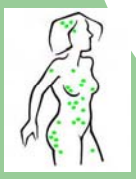


Fig. 1 The normal testis, left, comes from a healthy male rat. Tissues center and right come from a rat exposed to DEHP while its reproductive tract was maturing. The center tissue is a small testis filled with fluid. The corresponding tissue from the other side of the same animal exhibits no visible testis or sperm-storing epididymis. Another DEHP-exposed animal from the same set of EPA tests developed a "testis" that was nothing but a sack of blood. (Joseph Ostby / EPA)

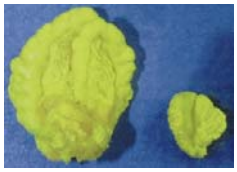


Fig. 2 Seminal vesicle from unexposed adult rat (left) weighs 1,700 milligrams, or more than 7 times as much as the one from the animal exposed to DEHP during development (right). Males have a pair of these pouches, which secrete fluid to carry sperm. (Joseph Ostby / EPA)

Material and methods

Strains from contaminated matrix were isolated and preselected on agar plates (Bacteriological agar No. 1, Oxoid) with phthalates (DMP, DEP). Precultivations of selected strains were carried out on shaker (90 rpm, 20 °C) in Nutrient broth. Biodegradation potential was evaluated via cell growth analysis performed in Bioscreen C analyzer (Labsystem, Finland). Basic salt medium (K_2HPO_4, K_2HPO_4, Mg_2Cl, $(NH_4)_2SO_4$) with marker substrates (DMP, DEP, glucose; 100 mg.l^{-1} each) as the sole/combined carbon source was used for this test. Confirmation tests on promising strains were performed in Erlenmeyer flasks cultivated on rotary shaker, with Basic salt medium and phthalates as a sole carbon source. Parallel respiration activity tests were carried out as well.

Microorganisms and culture conditions

Bacterial strains (*Pseudomonas fluorescens, Comamonas acidovorans, Pseudomonas putida, Pseudomonas veronii, Acinetobacter* sp., *Pseudomonas stutzeri, Rhodococcus erythropolis* CCM 8596, *Gordonia terrae*) and eukaryotic strains (*Saccharomyces cerevisiae, Fusarium proliferatum, Rhodotorula mucilaginosa*) were chosen from company collection of microorganisms as representatives of the allochthonous microflora. Unidentified bacterial isolates from contaminated soil and groundwater, which proved their ability to growth on phthalates (DMP-dimethyl phthalate, DEP-diethyl phthalate) on agar plates were chosen as representatives of autochthonous microflora. The strains differ from each other by morphology of colonies and some biochemical activities.

Cultivation conditions were following:

Agar plates: Cultivation medium - Nutrient agar or Bacteriological agar No.1; Temperature - 20 °C; Cultivation time - 4 days

Precultivation before Bioscreen analysis or flasks experiment: Cultivation medium - Nutrient broth; Temperature - 20 °C; Shaking - 120 rpm; Cultivation time - 2 days

Bioscreen analysis and flasks experiments: Cultivation medium - Basic salt medium; Temperature - 20 °C or 15 °C; Shaking - 120 rpm; Cultivation time - 14 days; Carbon sources - DEP (100 mg.l^{-1}), DBP (100 mg.l^{-1}), DEP + DBP (100 mg.l^{-1} + 100 mg.l^{-1}), DEP + glucose (100 mg.l^{-1} + 100 mg.l^{-1}), DBP + glucose (100 mg.l^{-1} + 100 mg.l^{-1}), glucose (100 mg.l^{-1})

Experiments and Results

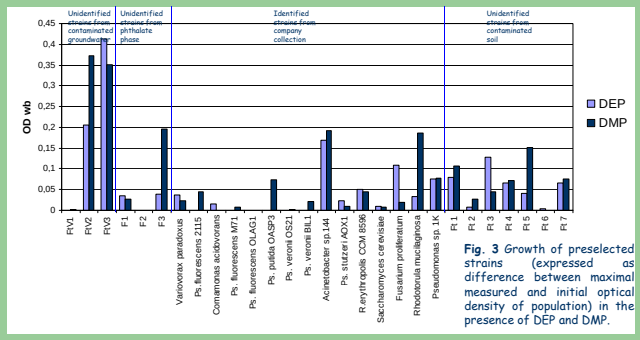


Fig. 3 Growth of preselected strains (expressed as difference between maximal measured and initial optical density of population) in the presence of DEP and DMP.

In the European Union, possible effects of five of the most widely used phthalates (DEHP-diethylhexylphthalte, DBP-dibutylphthalate, DINP-di-isononylphthalte, DIDP-di-isododecylphthalate, and BBP-butylbenzylphthalate) are in the process of being reviewed by the European Chemicals Bureau (ECB).

Characteristics of phthalate esters have lately raised a need of limiting the risks. Basically there are two technological approaches available:

Physical-chemical remediation methods, which are used mainly on sites massive contaminated.

Bioremediation technologies are more suitable and profitable, especially for phthalate contamination, which usually fulfils the definition of diffusion contamination, i.e. phthalates concentration is low but with the serious impact on the environment.

From many studies it is known that leading role in the environmental fate of phthalate esters in water plays biodegradation. Other physical-chemical reactions like oxidation, hydrolysis, photolysis or evaporation have no important impact on the phthalates concentration decrease (Staples, 1997).

Examples of chosen strains growth activity on dimethyl phthalate (DMP), diethyl phthalate (DEP), glucose (Glu) and their combinations:

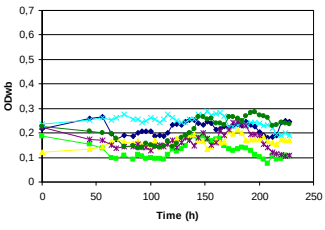


Fig. 2 Time dependence of the *Pseudomonas stutzeri* growth on chosen sources of carbon and energy.

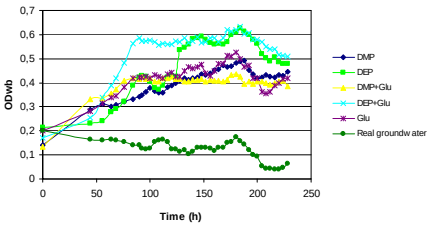


Fig. 3 Time **dependence** of the unidentified strain FtV3 (obtained from groundwater) growth on chosen sources of carbon and energy.

The principle of the tests was a laboratory simulation of bioremediation processes that could take place on the contaminated area under real conditions and finding the microbial strain/consortium suitable for technological applications.

Company EPS Ltd. has started research programme solving phthalate esters biodegradation. Both isolation of suitable microbial strains from the natural matrix long term contaminated by phthalates and physiological adaptation of the strains from the company collection of microorganisms and from the Czech Collection of Microorganisms (CCM) are exploited.

Experiments were designed as a sequence of micro and small-scale cultivations of above named microbial strains in various media. Taxons were tested either individually or in mixtures of 2 – 5 taxons.

During the initial phase of experiments the reproduction activities of autochthonous and allochthonous microflora were observed. Starting screening which was carried out in the micro-well reader Bioscreen C paid attention on the choice of suitable microflora. Two bacterial populations (*Acinetobacter* sp., *Rhodococcus erythropolis*) and unidentified bacterial mixture of autochthonous strains, which were able to growth on tested phthalates as a sole carbon source, were chosen for further set of experiments, which should give us more detailed data on biodegradation rates and metabolic pathways. If these taxons confirm their suitability for intended use, they will create a basis of bioremediation technology (both *in situ* and *ex situ*) applicable on sites contaminated with phthalates. As phthalates are not widely treated as some other pollutants we believe that our technology will be an alternative of choice for authorities dealing with pthhalates pollution.

Final result of our work should be a set of aerobic phthalic acid esters (PAE) degrading microbial strains and knowledge of their behaviour under both stress and environmental condition. A set of taxons has already been selected and are being screened with intension to find taxons which are able to rapidly degrade PAE with shorter alkyl-chains such as diethyl phthalate (DEP) or dimethyl phthalate (DMP). Selected strains will constitute a basic tool for newly developed bioremediation technology.

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