



Revista CENIC. Ciencias Biológicas

ISSN: 0253-5688

editorial.cenic@cnic.edu.cu

Centro Nacional de Investigaciones Científicas
Cuba

Belviso, Simona; Bardi, Laura; Fabbian, Raffaella; Marzona, Mario
Bioremediation of Phenanthrene Polluted Soils
Revista CENIC. Ciencias Biológicas, vol. 36, 2005
Centro Nacional de Investigaciones Científicas
Ciudad de La Habana, Cuba

Available in: <http://www.redalyc.org/articulo.oa?id=181220525071>

- How to cite
- Complete issue
- More information about this article
- Journal's homepage in redalyc.org

redalyc.org

Scientific Information System
Network of Scientific Journals from Latin America, the Caribbean, Spain and Portugal
Non-profit academic project, developed under the open access initiative

Bioremediation of Phenanthrene Polluted Soils

Simona Belviso¹, Laura Bardi², Raffaella Fabbian³, Mario Marzona¹

¹Dip. Chimica Generale e Organica Applicata, Università di Torino, C. Massimo D'Azeglio 48 10136 Turin, Italy. Tel. (39) 011 6707597, fax: (39) 011 6707581, e-mail: mario.marzona@unito.it.

²C.R.A. Istituto Sperimentale per la Nutrizione delle Piante, Via Pianezza 115, 10151 Turin, Italy.

³Biosearch Ambiente Srl, Via Livorno 70, 10144 Turin, Italy.

ABSTRACT: The best metabolic conditions for the biodegradation of hydrocarbons by a selected microbial consortium from a natural soil were investigated in order to improve the efficiency of bioremediation processes for cleaning of polluted waters and soils. Phenanthrene is one of the most recalcitrant pollutants to microbial decomposition and most difficult to remove. A microbial consortium was selected from a non polluted soil using a selective medium containing phenanthrene as a sole carbon source; DNA was extracted from the purified strains and the 16S rDNA was PCR-amplified and sequenced for the characterization. In order to improve the bioavailability of phenanthrene α -, β -, γ -, cyclodextrin were tested as coadjuvants. The degradation kinetics were carried out in presence of α , β , γ -, cyclodextrin in an aqueous phase at 28°C and 37°C; in presence of β -cyclodextrin in slurry phase at 37°C and in solid phase at room temperature. Phenanthrene concentration was detected by HPLC. The degradation of phenanthrene depends on the type of cyclodextrins and on temperature. In aqueous phase, the combined effect of temperature and addition of cyclodextrin improves the degradation of phenanthrene, and β -cyclodextrin is the best coadjuvant when combined to a temperature of 37°C. In slurry phase the degradation rate was lower than in solid phase, indicating a more important role of air availability than of temperature in biodegradation process. The microbial consortium was characterized. In conclusion bioaugmentation of indigenous microbial consortia, increase of bioavailability by β -cyclodextrin, high environmental temperature and good aeration of soil result in a significant decrease (50% in 35 days) of phenanthrene in polluted soils.

RESUMEN: Las mejores condiciones metabólicas para la biodegradación de hidrocarburos por un consorcio microbiano seleccionado de un suelo natural fueron investigadas para mejorar la eficacia de los procesos de la bioremediación para la limpieza de aguas y de suelos contaminados. El fenantreno es uno de los contaminantes más recalcitrantes a la descomposición microbiana y el más difícil de eliminar. Un consorcio microbiano fue seleccionado de un suelo no contaminado usando un medio selectivo que contenía el fenantreno como única fuente de carbono; El ADN fue extraído de las colonias microbianas purificadas y el ADNr 16S fue PCR-amplificado y ordenado para la caracterización. Con objeto de mejorar la biodisponibilidad del fenantreno, las α , β , γ -, ciclodextrina fueron probadas como coadyuvantes. Las cinéticas de degradación fueron realizadas: en presencia de α -, β -, γ -, ciclodextrina en fase acuosa a 28°C y 37°C; en presencia de β -ciclodextrina en fase fangosa a 37°C y en fase sólida a temperatura ambiente. La concentración del fenantreno fue detectada por HPLC. La degradación del fenantreno depende del tipo de ciclodextrinas y de la temperatura. En fase acuosa, el efecto combinado de la temperatura y la adición de ciclodextrina mejora la degradación del fenantreno, y la β -ciclodextrina es el mejor coadyuvante cuando está combinado a una temperatura de 37°C. En fase fangosa, la tasa de degradación fue más baja que en la fase sólida, indicando un papel más importante de la disponibilidad del aire que de la temperatura en el proceso de biodegradación. El consorcio microbiano fue caracterizado. En conclusión, la bioaugmentación de consorcios microbianos autóctonos, el aumento de la biodisponibilidad por β -ciclodextrina, la alta temperatura ambiental y la buena aireación del suelo dan lugar a una disminución significativa (el 50% en 35 días) del fenantreno en suelos contaminados.

Keywords: Bioremediation, Phenanthrene, Polluted soils, Cyclodextrins.

Palabras clave: Bioremediación, Fenantreno, Suelos contaminados, Ciclodextrina.

INTRODUCTION

Hydrocarbons are among the most widespread pollutants of soil. The remediation of polluted soil can be achieved by chemical, physical or biological treatments [1,2]. Many bioremediation processes have been developed to remove the contaminants. In particular in situ bioremediation is a suitable technology that avoids great physical-chemical alterations of the site and allows the complete pollutant degradation [1,2,3,4,5]. One

main factor that influences the extent of pollutants biodegradation is their bioavailability [5,6]. Hydrocarbons bioavailability is very poor; in fact they are hydrophobic and pass very slowly into the aqueous phase liquid in which they are metabolized by microorganisms [6,7]. Moreover in the soil they are strongly adsorbed to clay or humus fraction [6,8]. Microbial strain, temperature, soil texture and composition could also greatly influence the degradation process. Phenanthrene is one of the most recalcitrant PAHs (polycyclic aromatic hydrocarbons). This class of components are widely spread as pollutants of soils and waters. They are the result of incomplete combustion of organic matter like domestic waste, coal and wood waste products, petroleum and gas refining by-products other than natural sources as volcanic eruptions and wood fires [9]. Many PAHs like phenanthrene and naphthalene have been used for the synthesis of organic compounds such as pesticides, fungicides, dyes and moth-killers [10]. PAHs are toxic, mutagenic and/or carcinogenic [11]. Phenanthrene is known as photosensitizing for human skin and potent inhibitor of gap junctional intercellular communication [12]. The complete biodegradation of hydrocarbons lead to final non-toxic products like CO₂, water and cellular biomass [13]. The ability of different microorganisms to degrade phenanthrene has been tested: *Phanaerochaete chrysosporium* oxidises the molecule in C9 and C10 [14]; marine cyanobacteria like *Agmellum quadruplicatum* PR-6 and *Synechococcus* oxidise phenanthrene by a monooxygenase [15]. The addition of surfactants can enhance pollutants bioavailability increasing water solubility [16]; as a consequence the eluviation through the soil can also be increased, but due to high adsorption power of soil this risk is not significant [17]. On the other hand surfactants are frequently toxic and poorly biocompatible; sometimes they are also pollutant compounds. α -, β -, and γ -cyclodextrins are natural cyclic oligosaccharides formed by 6, 7 or 8 α -1,4-linked glucose units respectively [18]. Since they have toroidal hydrophobic cavities with a hydrophilic shell, they are water soluble and form inclusion complexes with hydrophobic molecules of a size compatible with their hydrophobic core. Moreover they are not toxic and easy biodegradable [19].

The aim of this work was to investigate the effect of the addition of the cyclodextrin, as bioavailability enhancer, on the microbial degradation of organic pollutants and of the temperature, in water and soils. α -, β - and γ -cyclodextrins were tested in biodegradation assays of phenanthrene in an aqueous phase at 28°C and 37°C. β -cyclodextrin in a slurry phase at 37°C, and β -cyclodextrin in solid phase at room temperature. The influence of the soil texture effect on biodegradation rate was also studied using a simplified model (dodecane as pollutant). The half-reaction degradation time was determined on three different soils using a degrading microbial consortium selected from a polluted soil. Two different microbial consortia were used and compared: a microbial consortium selected from a polluted soil and a microbial consortium isolated from a natural grass-covered soil in order to take into account the Italian laws that prevent the use of allochthonous degradative strains. Microbial strains isolated from this consortium were characterized by molecular analysis.

MATERIALS AND METHODS

1. Biodegradation of phenanthrene on liquid phase.

Microorganisms and growth conditions. The microbial consortium was selected from a natural, grass-covered soil. 1 g of soil was used as inoculum in flasks containing 200 ml Bushnell-Haas Broth (BHB) with 250 ppm phenanthrene as the sole energy and carbon source. The flasks were incubated at 28°C in an orbital shaker at 150 rpm in the dark for 15 days, then used as inoculum for the biodegradation assays. Single colonies from soil and from liquid microcosms at the end of incubation time were obtained by plating cell suspension on Tryptic Soy Agar (TSA). The purified strains were then plated on BHA and added with phenanthrene to ascertain their capability to grow with phenanthrene as the sole energy and carbon source.

Biodegradation assays. Microcosms were prepared in 250 ml flasks and inoculated with 1 ml enrichment culture cell suspension. 50 ml BHB with 1000 ppm phenanthrene were added with 500 mg α -, β - or γ -cyclodextrin and sterilized at 120°C x 20'; after the inoculum they were incubated in a Dubnoff shaker at 150 rpm, at 28°C or 37°C for 26 days. Blank assays were carried out without inoculum, and with inoculum but without cyclodextrins.

2. Biodegradation of dodecane and phenanthrene on solid phase.

Dodecane

Microbial inoculum. Microbial colonies were isolated from a petroleum-polluted soil by dispersing a sterile water suspension of soil in MMA (Mineral Medium Agar: 0.8 g/l K₂HPO₄, 0.2 g/l KH₂PO₄, 0.05 g/l CaSO₄·2H₂O, 0.5 g/l MgSO₄·7H₂O, 0.09 g/l FeSO₄·7H₂O, 1 g/l (NH₄)₂SO₄, 15 g/l agar) with 4% v/v dodecane as the sole carbon source in Petri dishes, and incubating them at 28°C for 5 days. A random pool of selected colonies was transferred to 200 ml of LMM (Liquid Mineral Medium: as MMA, without agar) with 4% v/v dodecane in a conical flask and incubated on an oscillatory shaker at 140 rpm for 5 days at 28°C. This culture was used as inoculum for the degradation kinetic assays.

Biodegradation kinetics. Three mineral soils with different textures were examined: a sand (sand 97.6%, silt 1.6%, clay 0.8%), a loamy sand (sand 80.4%, silt 17.8%, clay 1.8%) and a clay (sand 3.9%, silt 60.3%, clay 35.8%). Organic matter was absent. 700 g of each soil, previously dried in an oven at 120°C for 7 h, were posed to a depth of 10 cm depth in an impermeable, open box. 470 µl dodecane and 1% (w/w) water-melted β-cyclodextrin were added, then 100 ml of microbial culture were spread as inoculum and 200 ml of mineral growth medium (LMM) were added. Daily, water was balanced and soil homogeneity and aeration were maintained by vigorous and accurate mixing. The temperature was kept at 20°C. The tests were performed by monitoring the dodecane decrease, in the presence and absence of β-cyclodextrin and with the three different soil textures (sand, loamy sand and clay).

Phenanthrene

Microorganisms and growth conditions. As in biodegradation of phenanthrene on liquid phase.

Biodegradation assays. Microcosms were prepared in 250 ml flasks and inoculated with 1 ml enrichment culture cell suspension. 50 g of unsteril soil was added with 1000 ppm phenanthrene, 25% water and 500 mg β-cyclodextrin; it was balanced to C:N:P=100:15:1 with urea and DAP, then inoculated and incubated in static conditions at room temperature for 90 days. Blank assays were carried out without inoculum, and with inoculum but without cyclodextrins; blank soils were sterilised (under fluent vapour for 30', for three times with intervals of two days of incubation at 28°C). All microcosms were incubated in the dark and added with sterile water to restore the initial weight three times a week.

3. Biodegradation of phenanthrene on slurry phase.

Microorganisms and growth conditions. As in biodegradation of phenanthrene on liquid phase.

Biodegradation assays. Microcosms were prepared in 250 ml flasks and inoculated with 1 ml enrichment culture cell suspension. 50 g of unsteril soil was added with 1000 ppm phenanthrene, 80% water and 500 mg β-cyclodextrin; it was balanced to C:N:P=100:15:1 with urea and DAP, then inoculated and incubated in an Infors shaker at 150 rpm at 37°C for 80 days. Blank assays were carried out without inoculum, and with inoculum but without cyclodextrins; blank soils were sterilised (under fluent vapour for 30', for three times with intervals of two days of incubation at 28°C). All microcosms were incubated in the dark and added with sterile water to restore the initial weight three times a week.

Pollutants analysis

Phenanthrene. The phenanthrene was extracted in 2 x 15 ml (aqueous phase) or 3 x 50 ml (slurry and solid phase) ethyl acetate; 500 ppm naphthalene was added as internal standard during the extraction. The extract was diluted, then analysed by HPLC Jasco PU-980 equipped with Jasco UV-2075 Plus UV/V detector in a Supelcosil LC18 15 cm x 4.6 mm column. Operating conditions: elution with acetonitrile:water 60:40, flux 2 ml/min, λ 254 nm. The residual phenanthrene concentration was analysed at the end of degradation process and expressed as percent respect to the value obtained from the analysis of sterile test flasks incubated without microbial inoculum.

Dodecane. Dodecane was extracted by adding 6 ml of water to 15 g of soil sample and shaking with three successive aliquots of a total volume of 30 ml of toluene. The mixture was centrifuged at 4000 rpm for 5 min and the organic phase was transferred to a fresh tube. 100 µl of a standard solution of 1% w/v naphthalene in toluene were added to 1 ml of extract. The samples were analysed by a gas chromatograph GC HP 5890 series II equipped with a flame ionization detector on a HP1 Cross-Linked Methyl Silicone capillary column, 15 m long and 0.32 mm i.d., film thickness 1.0 µm. The operating conditions were: temperature from 100 to 250°C at 20°C/min; pressure 13 psi; injection volume 4 µl. Results were elaborated with excel and modelled as a pseudo first order kinetic.

Isolation of pure cultures of microorganisms able to degrade and tolerate phenanthrene.

1 g of natural grass-covered soil was added to 9 ml physiological solution (NaCl 9‰). The suspension was opportunely diluted and plated at different concentrations on Tryptic Soy Agar (TSA). Plates were incubated at 37°C for 15 days in the dark. Bacterial species were then grown on liquid TSB medium, while fungal species were grown on MALT-agar medium.

Molecular characterization of pure strains

DNA from microbial isolates was extracted and purified as previously described [20]. Primers ITS1 and ITS4 [21] were used to amplify a region of rDNA including ITS1 and ITS2 and the 5.8S rDNA gene. PCR amplifications were performed in a total volume of 50 µl by mixing 2 µl of the template DNA with 0.2 mM concentrations of each primer, 1 µM concentrations of each deoxynucleoside triphosphate, 1.5 mM MgCl₂ and 1U of Taq DNA polymerase in 1X PCR Buffer (Fermentas). These reactions were subjected to an initial denaturation of 3 min at 94°C, followed by 25 cycles of 30 sec at 94°C, 30 sec at 55°C, and 1min at 72°C, with a final extension of 5 min at 72°C in a BIO-RAD Gene Cyclyer thermal cyclyer. Genomic DNA from bacterial isolates was extracted with a glass-beads method. PCR amplifications of the Small-subunit (SSU) rRNA genes were performed with the same reaction mixture described for fungi but using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and

1492R (5'-GGTTACCTTGTTACGACTT-3'). For strain isolated from the consortium, sequence determination was performed by using internal 16S rRNA primers 27F, 531R (5'-CGGCCGCGGCTGCTGGCACGTA-3'), COM1 (5'-CAGCAGCCGCGGTAATAC-3') and 1378R (5'-CGGTGTGTACAAGCCCCGGGAACG -3'). The protocol for amplification consisted of an initial denaturation of 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 50°C, and 90 sec at 72°C, with a final extension of 5 min at 72°C. PCR products were checked by agarose gel electrophoresis and were purified with the GenElute PCR Clean-up Kit (SIGMA). Sequence determination was performed using the automated sequencer model ABI PRISM 3100 (Applied Biosystems). For each strain consensus sequence from Forward and Reverse was obtained with ChromasPro version 1.3. Database searches were conducted by using the BLAST program [22] with the GenBank database. 16S rDNA sequences retrieved from the database were aligned by using ClustalX software, version 1.81.

Results and discussion

In figure 1 is reported the phenanthrene degradation in absence of cyclodextrin and in presence of α -, β - and γ - cyclodextrins at different temperatures. Cyclodextrins improve phenanthrene degradation both at 28°C and 37°C. In presence of β -cyclodextrin the consortium degrades the highest amount of pollutant.

The temperature effect on phenanthrene biodegradation is reported in figure 2. The amount of residual phenanthrene is the smallest at 37°C at day 24. This effect is probably related to the mesophylic nature of selected strains. Temperature effect on phenanthrene biodegradation seems to be more effective than the bioavailability increase.

Table 1 shows the degradation time of dodecane on sand, loamy sand and clay, obtained using a microbial consortium isolated from a petroleum-polluted soil. Pollutant degradation is modelable as a pseudo first order reaction. The half-reaction time ($T_{1/2}$) on sand was 76.9 h without β -cyclodextrin and 45.4 h with β -cyclodextrin. The half-reaction time varied from 101.7 h without β -cyclodextrin to 67.2 h with β -cyclodextrin on loamy sand. The half-reaction time on clay was reduced from 199.3 h without β -cyclodextrin to 151.4 h with β -cyclodextrin. The degradation time gain due to β -cyclodextrin was 40.9% in sand, 33.9% in loamy sand and 24.2% in clay. The positive effect of β -cyclodextrin decreases from sand to clay. As expected, biodegradation is slower in clay, where the fine texture hinders the diffusion of the oxygen required for the aerobic metabolism. Moreover, fine-textured soils have more total pore spaces than sandy soils; these spaces are mostly micropores and the resultant adsorption is very strong. An intrinsically biodegradable compound that diffused into soil micro- and nano-pores is then shielded toward microbial attacks and is only very slowly degraded. This could explain the minor effect of β -cyclodextrin in clay, although its solubilization effect on pollutant does not change. Also in this case the addition of β -cyclodextrin improves the pollutant biodegradation.

Since slurries are used to carry out ex situ treatments on polluted soils, the biodegradation of phenanthrene was studied also in slurry phase. Figure 3 shows the amount of degraded phenanthrene on slurry and solid phase with or without β -cyclodextrin, used as better bioavailability coadjuvant (figure 1, table 1). The presence of cyclodextrin increases the degradation both in solid and in slurry phase, proving the crucial role of the bioavailability in the phenanthrene bioremediation. From figure 3 results that the phenanthrene biodegradation is higher in solid phase than in slurry phase. The better results obtained on solid phase could be explained by the higher oxygen availability respect to the slurry phase, in which soil macropores are filled with water. Soil oxygenation is as important as the temperature increase. Biodegradation of phenanthrene was higher at 37°C and with β -cyclodextrin in slurry phase, even if not at the same extent as in aqueous phase assays.

Since the existing Italian laws do not allow the use of allochthon microorganisms or of exogenous compounds in the "in situ" bioremediation processes, we looked for an autochthon source of strains and selected a microbial consortium from a natural, grass-covered soil in order to degrade phenanthrene on liquid, solid and slurry phase. The selected consortium was composed by a number of microbial strains that were purified and analysed according to 16S and ITS sequences. The detected strains were *Streptomyces* spp., *Bacillus* spp., *Aspergillus* spp., *Penicillium restrictum* spp and *Achromobacter xylosoxidans*. *Penicillium* spp., *Aspergillus* spp. e *Streptomyces* spp. are already known for their capability to degrade PAHs [23, 24, 25]. The presence of *Bacillus* sp. can be considered very interesting for the degradative microbial consortium as these microorganisms can produce bioemulsifier compounds [26], so they could contribute both directly and indirectly to PAH degradation, by increasing bioavailability also to other degrading strains. According to these results the bioaugmentation of indigenous microorganisms significantly improves the biodegradation rate even in the case of a recent pollution or of a non polluted soil. The use of β -cyclodextrin, high temperatures and aeration of soil improve the overall process.

CONCLUSIONS

The bioavailability of phenanthrene is a fundamental factor for its biodegradation both in soil and in water. The positive effect of coadjuvants is influenced by the soil texture, but in any case the degradation time gain is always high. A temperature increase gives rise to a better degradation, in particular in water, probably due to a higher inclusion complex solubility. β -cyclodextrin is a suitable bioavailability coadjuvant and it is safe for the environment. In situ bioremediation strategies in soils can be considered as more effective than ex situ processes carried out in slurry phase. Degradative autochthon microbial consortia selected from natural soils can be used for effective phenanthrene biodegradation, even in the case of a recent pollution event. Then the use of allochthon degrading microbial strains is not strictly required. According to our study 50% of phenanthrene can be removed in 35 days. This means that even in case of a recent pollution event the indigenous microorganisms of a soil can be effective in bioremediation process. Suitable technical interventions aimed to stimulate the microbial activity and to increase bioavailability of hydrophobic xenobiotic pollutants are advantageous.

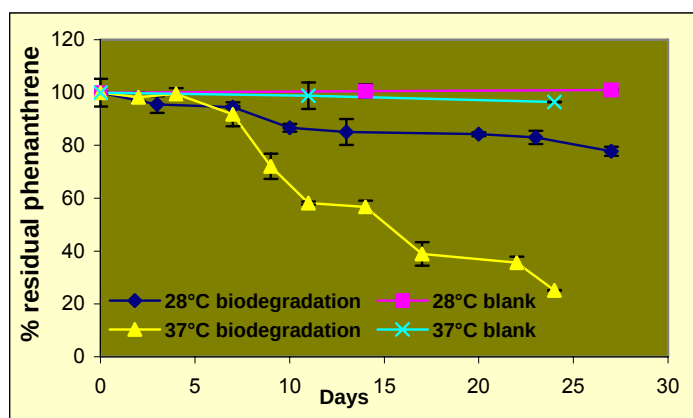


Fig. 1. Cyclodextrin effects on phenanthrene biodegradation.

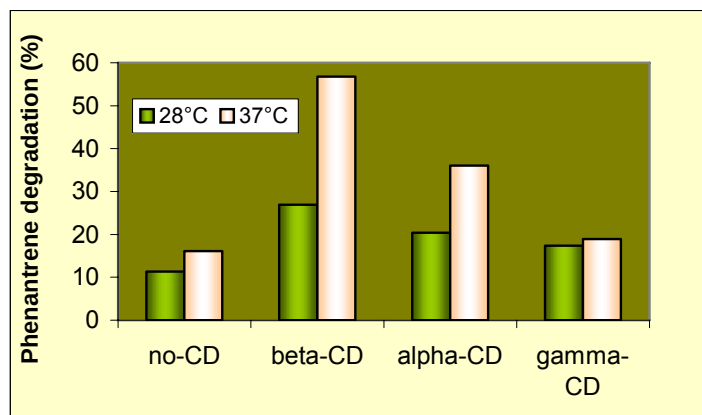


Fig. 2. Temperature effect on phenanthrene biodegradation.

	Sand	Loamy sand	Clay
Full degradation time without β -cyclodextrin	76.9	101.7	199.3
Degradation time with β -cyclodextrin	45.4	67.2	151.4
Degradation time gain (due to cyclodextrin)	40.9%	33.9%	24.2%
Required time	1	1.48	3.33

Tab. 1. Degradation time on sand, loamy sand and clay (hours).

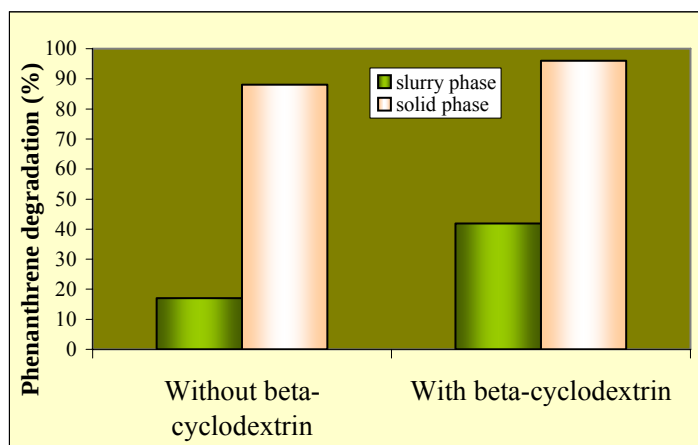


Fig. 3. Phenanthrene biodegradation in slurry and solid phase.

REFERENCES

1. Hincee R.E. In situ bioremediation: practices and challenges. In: Biotechnology for soil emediation. Scientific bases and pratical applications. R. Serra. C.I.P.A. S.r.l., Milan, Italy, 17-20, 1998.
2. Otten A., Alphenaar A., Pijls C., Spuij F., De Wit H. In situ soil remediation. In: Soil & enviroment. Kluwer Academic Publishers, Dordrecht, The Netherlands, 9-12, 1997.
3. Holliger, C., Gaspard, S., Glod, G., Heijman, C., Schumacher, W., Schwarzenbach, R.P., Vazquez, F. Contaminated environments in the subsurface and bioremediation: organic contaminants. **FEMS Microbiol. Rev.**, **20**, 517-523, 1997.
4. Guerin, T.F. Bioremediation of phenols and polycyclic aromatic hydrocarbons in creosote contaminated soil using ex situ landtreatment. **J. Hazard Mater**, **65**, 305-315, 1999.
5. Bouwer, E.J., Zhang, W., Wilson, L.P., Durant, N.D. Biotreatment of PAH-contaminated soils/sediments. **Ann. N. Y. Acad. Sci.**, **829**, 103-117, 1997.
6. Brusseau, M. L. The impact of physical, chemical and biological factors on biodegradation: implications for in situ bioremediation. In: Biotechnology for soil remediation. Scientific bases and practical applications. R. Serra. C.I.P.A. S.r.l., Milan, Italy, 81-98, 1998.

7. De Jonge H., Freijer J. I., Verstraten J. M., Westerveld J., Van der Wielen F. W. M. (1997). Relation between bioavailability and fuel oil hydrocarbon composition in contaminated soils. *Environ. Sci. Technol.*, 31, 771-775.
8. Tabak, H.H., Govind R. (1997). Bioavailability and biodegradation kinetics protocol for organic pollutant compounds to achieve environmentally acceptable endpoints during bioremediation. *Ann. N. Y. Acad. Sci.*, 829, 36-61.
9. Finlayson-Pitts B.J. and Pitts, J.N., Jr (1997). Tropospheric air pollution: ozone, airborne toxics, polycyclic aromatic hydrocarbons and particles. *Science* 276: 1045-1052.
10. Shennan, J.L. (1984). Hydrocarbons as substrates in industrial fermentation. In *Petroleum Microbiology Atlas*. Macmillan R.M.ed. pp. 643-683.
11. Mastrangelo, G. (1997). Polycyclic aromatic hydrocarbons and cancer in man. *Environ. Health Perspect.* 104: 1166-1170.
12. Weis L.M. (1998). Bay and baylike regions of polycyclic aromatic hydrocarbons were potent inhibitors of gap junctional intercellular communication. *Environ. Health Perspect.* 106, 17-22.
13. Atlas, R.M.(1995). Petroleum Biodegradation and oil spill bioremediation. *Marine Pollution Bulletin* 31, 178-182.
14. Hammel K.E., Gai Z.G., Green B. & Moen M.A. (1992). Oxidative degradation of phenanthrene by the ligninolytic fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 58: 1831-1838.
15. Dagley S. (1971). Catabolism of aromatic compounds by microorganisms. *Adv. Microbiol. Physiol.* 6:1-76.
16. Barkay, T., Navon-Venezia S., Ron E.Z., Rosenberg E. (1999). Enhancement of solubilization and biodegradation of polyaromatic hydrocarbons by the bioemulsifier alasan. *Appl. Environ. Microbiol.*, 6, 2697-2702.
17. S. Steffan, L. Bardi, M. Marzona (2001). Biodegradation of Hydrocarbons in polluted soils using β -cyclodextrin as a coadjuvant. *Biolog. J. Armenia, Special issue: Cyclodextrins*, 218-225.
18. Szejtli J. (1988). Cyclodextrin technology. Kluwer Academic Publishers, Dordrecht, The Netherlands, 1-393.
19. Schwartz A., and Bar R. (1995). Cyclodextrin-enhanced degradation of toluene and p-toluic acid by *Pseudomonas putida*. *Appl. Environ. Microbiol.*, 61, 2727-2731.
20. Henrion, B., Chevalier, G. & Martin, F. (1994). Typing truffle species by PCR amplification of the ribosomal DNA spacers. *Mycological Research* 98: 37-43.
21. White, T. J., T. Bruns, S. Lee, and J. Taylor. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315-322. In M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), *PCR protocols. A guide to methods and applications*. Academic Press, San Diego, Calif.
22. Altschul, X., F. Stephen, L. Thomas, X. Madden, A. Alejandro, X. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman. (1997.) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25:3389-3402.
23. Zheng Z., Obbard J.P. (2003). Oxidation of polycyclic aromatic hydrocarbons by fungal isolates from an oil contaminated refinery soil. *Env. Sci. Poll. Res. Int* 10(3): 173-176.
24. Colombo J.C., Cabello M., Arambarri A.M (1995). Biodegradation of aliphatic and aromatic hydrocarbons by natural soil microflora and pure cultures of imperfect and ligninolytic fungi. *Env. Poll.* 94(3): 355-362.
25. Sorkhoh N.A. et al. (1995). Establishment of oil-degrading bacteria associated with cyanobacteria in oil-polluted soil. *J. Appl. Bacter.* 78(2): 194-199.
26. Cubitto M.A. et al. (2004). Effects of *Bacillus subtilis* O9 biosurfactant on bioremediation of crude oil polluted soils. *Biodegradation* 15(5): 281-287.