Appl Biochem Biotechnol (2009) 159:155–167
DOI 10.1007/s12010-008-8463-1

Taxonomic Identification and Use of Free and Entrapped Cells of a New *Mycobacterium* sp., Strain Spyr1 for Degradation of Polycyclic Aromatic Hydrocarbons (PAHs)

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Received: 5 August 2008 / Accepted: 2 December 2008 /
Published online: 19 December 2008

Abstract A polycyclic aromatic hydrocarbon (PAH)-degrading bacterial strain Spyr1 was isolated from Greek creosote polluted soil by an enrichment method using pyrene as sole carbon and energy source. Spyr1 was identified as *Mycobacterium* sp. based on 16S rDNA analysis and it was capable of degrading pyrene, fluoranthene, fluorene, anthracene, and acenaphthene. The effect of entrapment in glass beads and alginate/starch mixtures on the survival and pyrene degradation ability of Spyr1 cells in liquid suspensions and soil microcosms was tested and compared with that of freely suspended cells. In general, free cells showed higher degradation of pyrene and other PAH than immobilized cells. However, immobilized cells could better tolerate PAH and they maintained their viability and PAH degradation capability for at least 1 year after storage at 4 °C. Entrapped cells in glass beads exhibited better pyrene biodegradation performance than alginate/starch entrapped cells in liquid suspensions and could be used effectively for at least ten repeated cycles. Alginate/starch entrapped cells exhibited better yields than glass beads entrapped cells for removing pyrene as well as mixtures of PAH in soil microcosms.

Keywords Biodegradation · Entrapment · *Mycobacterium* sp. · Soil bacteria · PAH

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are considered major environmental pollutants due to their toxicity, carcinogenicity, teratogenicity, and recalcitrance [1]. They constitute

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the largest group of released pollutants, and hence, their potential biodegradation is of vital importance.

Microbial activity is the most prominent process of PAH removal [2, 3]. PAH-degrading microorganisms have been widely described [4, 5] including members of the genus *Mycobacterium*, which are considered among the most important representatives of this group [6]. Recent reports on the physiology and ecology of PAH-degrading mycobacteria suggest that strains of the genus *Mycobacterium* are specialists in proliferating in the oligotrophic environment of PAH-polluted soils [7].

Bioaugmentation is a frequently used bioremediation method for the removal of xenobiotic compounds such as PAH from soils. Bioaugmentation involves the addition of laboratory-grown microorganisms in the polluted site, which are capable to degrade the target pollutant [8, 9]. However, indigenous predators, parasites, and toxicants are known to severely restrict biodegradation [10, 11]. Immobilization of cells able to degrade such toxic compounds is a preferable approach for environmental restoration in order to overcome adverse environmental conditions that threaten microbial survival. In addition, the immobilization of cells offers economic benefits of repeated use in continuous and batch cultures, as well as stability advantages over free cells for a variety of applications [12, 13]. Besides, immobilized microbial cells can be handled more easily, because contamination during transport, application, and storage can be avoided [14, 15].

Biodegradation of PAH by immobilized *Mycobacterium* species has not been described so far, although some reports in this field using other bacteria are available [16–18]. This report describes the isolation and taxonomic characterization of a new *Mycobacterium* sp. strain Spyr1 from a Greek creosote polluted site. It is also demonstrated here that free and entrapped cells of Spyr1 were able to degrade a number of low and high molecular weight PAH in liquid suspensions and in soil microcosms.

Materials and Methods

Soil Sampling and Storage

Soil samples were collected aseptically from a creosote polluted site in Epirus, Greece, at 20–40 cm depth. The average temperature of soil was 14 °C. Samples were homogenized, sieved using a 4-mm sieve, and kept in 4 °C before further treatment.

Enrichment and Isolation of the Bacterial Strain

A soil sample (10 g) was suspended in 100 ml minimal medium (M9 MM) containing (per liter) $Na_2HPO_4.2H_2O$ 8.5 g, KH_2PO_4 3.0 g, NaCl 0.5 g, NH_4Cl 1.0 g, $MgSO_4.7H_2O$ 0.49 g, and $CaCl_2$ 0.011 g in a 250-ml conical flask. $MgSO_4.7H_2O$ 1 M and $CaCl_2$ 1 M were sterilized separately. MM also contained trace elements (TE) as follows (per liter): $CuSO_4$ 0.4 g, KI 1.0 mg, $MnSO_4.H_2O$ 4.0 mg, $ZnSO_4.7H_2O$ 4.0 mg, H_3BO_3 5.0 mg, $H_2MoO_4.2H_2O$ 1.6 mg, and $FeCl_3.6H_20$ 2.0 mg. The soil-MM suspension was supplemented with 0.01% (*w*/*v*) pyrene (>97% purity, Fluka) as enrichment substrate and incubated with shaking at 180 rpm in 30 °C for 7 days in the dark. A 10-ml sample was transferred to a new flask and incubated as above. Following several recultivations, an aliquot (100 μl) of serial dilutions from each culture was transferred and spread on solid MM plates, supplemented with pyrene, as the sole carbon source using a spraying technique as described before [19]. After incubation for 7 days, colonies, especially those

forming clear zones on the spray-coated plates, were selected as candidate pyrene-degrading strains. All isolates were stored as liquid cultures containing 15% glycerol at −80 °C.

Taxonomic Identification of the Bacterial Strain

Cell morphology of PAH-degrading bacteria was examined using a microscope equipped with phase-contrast optics. Gram-staining, colony morphology, and the ability to grow at various temperatures were monitored after 24 and 48 h growth on lysogeny broth (LB) or on trypticase soy broth agar. G+C content (%) was determined by the fluorimetric dye-binding method [20] based on the differential binding of Hoechst 33258 (benzidine) and ethidium bromide to A+T and G+C regions of DNA, respectively. Strains *Escherichia coli* DH5a, *Agrobacterium tumefasciens* UBAPF2, and *Pseudomonas aeruginosa* (with a G+C content 52%, 60%, and 67%, respectively) were used as controls. Other standard biochemical tests, performed as described by Smibert and Krieg [21], were as follows: oxidase, catalase and urease activity, nitrate reduction, carbohydrates fermentation, citrate use, gelatin liquefaction, as well as starch, lipid, and casein hydrolysis.

Analysis of 16S rRNA Gene Sequence

For polymerase chain reaction (PCR) amplification of 16S rRNA genes, genomic DNA isolated by AquaPure Genomic DNA Isolation kit (BIO-RAD) was used as template. 16S rDNA sequences were amplified using the primers 16SF 5′-GAGTTTGATCCTGGCTCAG-3′ (9–27 position of *E. coli*) and 16SR 5′-AGAAAGGAGGTGATCCAGCC-3′ (1525–1542 position of *E. coli*) [22]. Hot-start PCR at 95 °C for 5 min was followed by 30 cycles as follows: 94 °C for 1 min, 53 °C for 2 min, 72 °C for 3 min, and a final chain elongation step at 72 °C for 10 min. The amplified fragments were cloned by the TA cloning kit (Invitrogen) and sequenced by Macrogen (Korea). Taxonomic analysis was conducted by the GenBank Basic Local Alignment Search Tool program. For phylogenetic and molecular evolutionary analysis, the MEGA version 3.1 [23] was used. The 16S rRNA gene sequence of strain Spyr1 (~1,300 bp) was aligned with homolog sequences from related taxa using the CLUSTAL W software [24]. The resultant tree topology was evaluated by bootstrap analysis of the neighbor-joining method based on 1,000 resamplings.

Sequence Accession Number The 16S rRNA gene sequence of strain Spyr1 obtained in this study was deposited in the European Molecular Biology Laboratory database (accession number AJ699170).

Carbon Source Utilization

In addition to pyrene, the purified strain was also tested for growth on one of the following compounds at 0.01% (*w*/*v*): naphthalene, acenaphthene, anthracene, fluoranthene, fluorene, and phenanthrene which were added individually as sole carbon sources to liquid MM.

Enzyme Assays

Cells were grown in liquid MM with pyrene as sole carbon source (200 ml cultures as described above) and pelleted by centrifugation. Cells were suspended in 10 mM Tris-HCl pH 7.5 containing 1 mM dithiothreitol, disrupted in a mini Beat Beater (Biospec Products,

Bartlesville, OK, USA) using zirconium beads (0.15 mm diameter) and centrifuged at 12,000×*g* for 30 min. The supernatant was used as the crude cell-free extract in enzyme assays; all performed at 25 °C using a Shimadzu UV-1201 spectrophotometer. 1-Hydroxy-2-naphthoate (1-H-2-N) dioxygenase was estimated spectrophotometrically by measuring the increase of absorbance at 300 nm using 1-hydroxy-2-naphthoate as substrate according to Iwabuchi and Harayama [25]. Salicylate hydroxylase activity was estimated with salicylate as substrate, according to Yamamoto et al. [26] by measuring the decrease of NADH at 340 nm. 2-Carboxybanzaldehyde dehydrogenase was determined with 2-carboxybenzaldehyde as substrate by following the NADH formation as described by Kiyohara and Nagao [27]. Boiled crude extracts of cells were used as negative controls. Catechol 2,3-dioxygenase activity was identified as described by Zukofski et al. [28]. Colonies of cells that express catechol 2,3-dioxygenase become yellow within seconds after spraying with catechol solution (0.5 M), a colorless substrate that is converted by this enzyme to 2-hydroxymuconic semialdehyde.

GC–MS Analyses

For the identification of pyrene intermediates, the cultures containing pyrene were extracted twice with 15 ml dichloromethane to remove the pyrene excess, acidified with HCl to pH 2, and extracted twice with 15 ml ethyl acetate (acidic fraction). The acidic fraction was preconcentrated with a rotary evaporator, added in a screw cap 10 ml vial and evaporated to dryness under a gentle nitrogen stream. Pyrene metabolites were subjected to methylation according to Fiamegos and Stalikas [29] and identified by performing gas chromatography–mass spectrometry (GC–MS) analysis on a Varian 3900 gas chromatographer interfaced with a Varian Saturn 2100T ion trap Mass Spectrometer. A Factor Four VF-5 30 m×0.25 mm×0.25 μm i.d. fused-silica capillary column was used. Helium was the carrier gas at a flow rate of 1 ml/min. Samples were injected in the splitless mode. The GC conditions were as follows: injector temperature, 250 °C; ion trap temperature, 250 °C; initial oven temperature, 50 °C; hold for 2 min then increased to 280 °C at 5 °C/min and held for 20 min at 280 °C. The total program run was 68 min. The mass detector was operated in the electron impact mode at 70 eV and electron multiplier voltage of 1.25 kV and dwell time was 5 ms. The mass fragments of the derivatives were obtained in the full scan mode in the scan range from *m*/*z* 50 to 600. Data were collected and integrated with a personal computer using the Varian Saturn GC/MS Workstation Version 6.40.

Cell Entrapment Matrices

Spyr1 cells grown under shaking in 2,000 ml cultures of M9 MM medium plus pyrene as sole carbon and energy source at 30 °C were harvested by centrifugation (6,000×*g*, 10 min, 4 °C), washed with fresh medium, and resuspended in the same medium (without the carbon source). Two different matrices were used for cell entrapment: macroporous sinter glass Siran^{R} (Schott Glaswerke, Germany) and a mixture of alginate/starch. Both matrices were selected because both were cheap and nontoxic to cells [30, 31]. All glassware and solutions used for cell entrapment were sterilized at 121 °C for 20 min.

Entrapment on Siran^{R} Glass Beads

Porous glass had up to 60% pore volume with surface area about 0.07 m^2/g. The above cell suspension (8 ml, 10^9 cfu/ml) was poured over 4 g of dried glass beads in 50-ml conical

flasks. The mixture was incubated at 30 °C for various time intervals. The beads were rinsed up to ten times by vigorous shaking with the same medium. The number of adhered cells was indirectly estimated by determining viable counts on LB agar plates from cells remaining in the supernatant liquid after rinsing with M9 fresh medium to remove nonadhered cells. The difference between inoculated cells and remaining cells were considered as the cells adhered on the beads. The maximum bacterial cell concentration (4.5×10^8 cfu/g) entrapped in glass beads was obtained in 10 days. After three subsequent rinses, many cells were detached, but, nevertheless, a significant number of cells (0.4×10^8 cfu/g) still remained associated with the glass beads. Beads with entrapped cells after the third rinse were used for all biodegradation experiments. Bacterial enumeration after storage of immobilized cells on glass beads was performed according to Ascon-Cabrera et al. [32]. Glass beads were incubated for 30 min at 25 °C in a phosphate buffer (0.1 M, pH 7) containing ethylene glycol tetraacetic acid 0.001 M, Tween 80 0.1% (*v*/*v*), and sodium dodecyl sulfate 0.1% (*w*/*v*), followed by ultrasonic treatment for 10 min. After rigorous washing, the viable cells released in the solution were determined by plate counting technique.

Entrapment on Alginate/Starch Mixture

The entrapment procedure was carried out according to Sultana et al. [33] with some modifications. A 2% alginate mixture was prepared containing 2% commercially available corn flour (starch) and 10% concentrated culture. Droplets of the mixture were added in a 0.1-M $CaCl_2$ using a 5-ml pipette. The mixture was allowed to stand at room temperature for 1 h. The formed beads were collected by gentle centrifugation and washed once with 0.9% NaCl containing 5% glycerol and stored at 4 °C.

To determine viable counts, the entrapped cells were released from the beads according to the method of Sheu and Marshall [34]. One gram of beads was resuspended in 9 ml of phosphate buffer (0.1 M, pH 7) followed by vigorous shaking (vortex) until gel beads were completely destroyed. The colony-forming units (cfu/g) were determined by plating on LB agar plates.

Biodegradation in Liquid Suspension Microcosms

The biodegradation activity of free and immobilized cells on the removal of pyrene in liquid cultures was investigated in 100-ml conical flasks (in duplicate) on a rotary shaker (180 rpm, 30 °C). Each conical flask contained 30 ml M9 MM supplemented with trace elements and 80 ppm pyrene inoculated with 10^6 cells/ml, either free or entrapped. Dead cells both free and entrapped were used as controls. Samples of 30 ml culture of pyrene were extracted twice, each time with 15 ml dichloromethane (DCM). The organic phase extractions were combined, dried over anhydrous Na_2SO_4, and evaporated. The residue was dissolved in DCM, dried in nitrogen gas, and analyzed by gas chromatography as described elsewhere [35].

Biodegradation in Soil Microcosms

Near-surface nonpolluted ground soil was collected locally. The soil was dried, homogenized, sieved (2 mm), and stored at 4 °C up to 1 week. Sterilized soil microcosms were prepared with 50 g (or 500 g in the case of PAH mixtures) of dry soil in 250-ml beakers (in duplicate) and sterilized by autoclaving at 121 °C for 20 min on three consecutive days. For unsterilized soil samples, only the glass beakers were sterilized. Both

sterilized and unsterilized soil samples were spiked with 100 ppm pyrene or a mixture of PAHs containing pyrene, phenanthrene, and fluoranthene (100 ppm each) in DCM (10 ml DCM/50 g soil) and dried overnight at 30 °C [36]. Each soil was inoculated with either free or immobilized cells and humidity was adjusted up to 60% of water holding capacity. The final concentration of biomass in each bottle was 10^6 cells/g soil. Dead cells, both free and immobilized, were used as controls. All samples were incubated in the dark at 24 °C. To determine the number of cells/gram soil, 10 g of soil were suspended in 100 ml sterile distilled water followed by shaking and ultrasonic treatment (3×1 min); serial dilutions were prepared and spread over LB plates. To measure the pH value, 10 g of soil were suspended in 100 ml 0.1 M KI. In case pH needed correction, CaO was added to soil prior to sterilization. For determination of PAH degradation in soil, 5 g soil was dried with 5 g Na_2SO_4 and extracted with 20 ml of dichloromethane/acetonitrile 3:1 with sonication for 30 min. Various combinations of solvents and time of sonication were applied. The extract was evaporated to dryness under nitrogen stream, redissolved in 1 ml of cyclohexane, and 1 μl was analyzed by GC–flame ionization detector [37].

All experiments were performed in triplicate and the standard error was found to be less than 5%.

Results and Discussion

Isolation and Taxonomic Identification of Strain Spyr1

The new isolate Spyr1 was isolated from the enriched contaminated soil samples essentially on the basis of formation of clear zones on solid M9 MM with sprayed pyrene as the sole carbon source. Strain Spyr1 was found to be a Gram-positive bacterium, with optimum growth on pyrene at 30–37 °C. Standard bacteriological and biochemical methods as described in "Materials and Methods" were applied for its phenotypic characterization. The G+C content of Spyr1 was found to be 67±1.6 mol% (n=4). Analysis of the 16S rRNA gene sequence indicated that strain Spyr1 belongs to the genus *Mycobacterium* (99% identity) with closest known relative *Mycobacterium gilvum* (Fig. 1).

Utilization of Pyrene as Sole Source of Carbon and Energy

Complete degradation of pyrene at concentrations 80 mg/l occurred within 8 days of incubation in the dark (Fig. 2). The extrapolated degradation rate for the growth phase can be averaged to 10 μg ml^{-1} day^{-1}, a value similar to that reported for other *Mycobacterium* species [38, 39]. No significant change in the pyrene concentration was observed in control cultures with heat-killed cells. In addition to pyrene, fluoranthene, fluorene, anthracene, and acenaphthene were also utilized by strain Spyr1 as sole sources of carbon and energy (results not shown here). Additions of vitamins or trace amounts of yeast extract were not required for the growth of Spyr1 on any PAH unlike other *Mycobacterium* sp. [40].

Evidence for the pathway utilized by strain Spyr1 for degradation of pyrene was obtained by the examination of several key enzyme activities in crude extracts after growth of Spyr1 cells on pyrene. Enzyme activities of 1-H-2-N dioxygenase, 2-carboxybenzaldehyde dehydrogenase, and salicylate hydroxylase were detected in cells grown on pyrene, whereas, no 2,3-catechol dioxygenase activity was found in these samples (data not shown).

Following GC–MS analysis, the Spyr1 culture extract was found to contain *o*-phthalic acid and 1-hydroxy-2-naphthoic acid (1-H-2-NA) as intermediates, identified by comparing

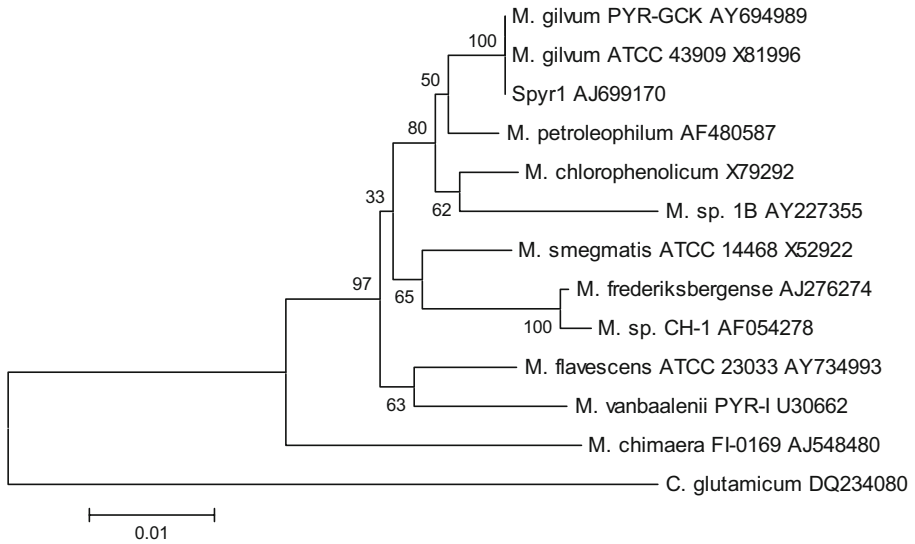

Fig. 1 Phylogenetic location of strain Spyr1 among other *Mycobacterium* species. *Corynebacterium glutamicum* was used as an outgroup. The *scale bar* indicates the number of substitutions per nucleotide position

the GC retention times and mass spectra with those of standards (data not shown). In addition, a third intermediate was detected as 4-phenanthroic acid (*m/z* 236, 205, and 177). An M^+ was observed at *m/z* 236, representing a 14-mass-unit increase over underivatized metabolite, characteristic of single methylation of an organic acid as has been described by Heitkamp et al. [41].

Our results suggest that Spyr1 metabolizes pyrene to 1-H-2-NA which subsequently is degraded via *o*-phthalic acid, a pathway also proposed for other *Mycobacterium* strains [42, 43]. Evidence of this pathway is provided by the identification of (a) 1-H-2-NA, phenanthrene-4-carboxylate, and phthalate as intermediate metabolites detected by GC–MS analysis and (b) detection of 1-H-2-N dioxygenase and 2-carboxybenzaldehyde dehydrogenase activities in pyrene-grown cells. In addition, Spyr1 could grow on 2-carboxybenzaldehyde and phthalate as sole carbon source, both intermediates of pyrene

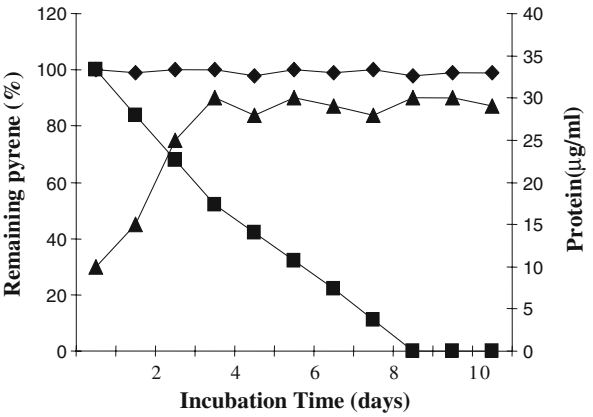

Fig. 2 Pyrene degradation (*closed square*) and growth (*closed triangle*) curves of strain Spyr1 in M9 MM plus pyrene. *Closed diamond*, control with dead cells

degradation by the phthalate pathway [43], whereas no growth was observed on salicylic acid. In parallel with this result, strain Spyr1 lacks 2,3-catechol dioxygenase activity. The detection of salicylate hydroxylase activity may be explained by the low specificity of the enzyme. Pyrene catabolism by strain Spyr1 via phthalate pathway is also confirmed by the total genome sequence of this strain by the Department of Energy Joint Genome Institute (Walnut Creek, CA, USA, unpublished results).

Pyrene Degradation by Immobilized Cells in Liquid Suspensions

The results obtained with pyrene in liquid cultures of Siran and alginate/starch immobilized cells are given in Fig. 3. There was no significant difference between pyrene degradation by free or entrapped in Siran glass beads cells. They both degraded an initial pyrene concentration of 80 μg/ml in batch liquid cultures almost completely within 8 days of incubation (Fig. 3). This concentration is considered relatively high of a single PAH and was selected according to European's directives (http://eu/environment/soil/index_en.htm). To test the time profundity of active Siran immobilized cells, we studied their biodegradation ability in repeated liquid cultures. After each cycle of incubation (8 days/cycle), the beads were washed with sterile medium and transferred into a fresh medium containing pyrene. As shown in Fig. 4, cells entrapment in Siran glass beads maintained their full biodegradation ability for ten consecutive uses in pyrene liquid biodegradation cultures. Estimation of the number of viable cells as described above showed that it remained constant until the last use (data not shown). The maintenance of the capacity of biodegradation for repeated uses suggests that entrapment of Spyr1 cells in glass beads confers significant advantages in liquid waste bioremediations. Nevertheless, recycling of biocatalysts determines the effectiveness of degradation over time, and hence, it is an important factor in industrial bioremediation processes.

In contrast, alginate/starch beads disintegrated within 24 h of incubation under agitation during the biodegradation experiment in liquid suspensions. However, the released cells

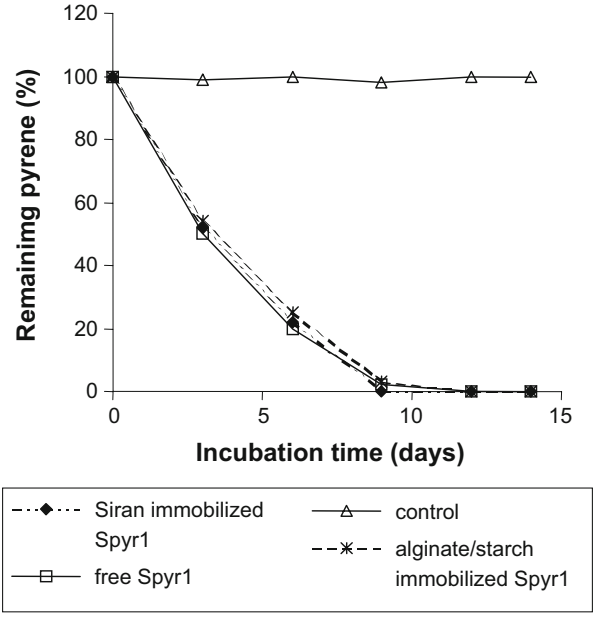

Fig. 3 Biodegradation of pyrene by free and immobilized Spyr1 cells in liquid cultures

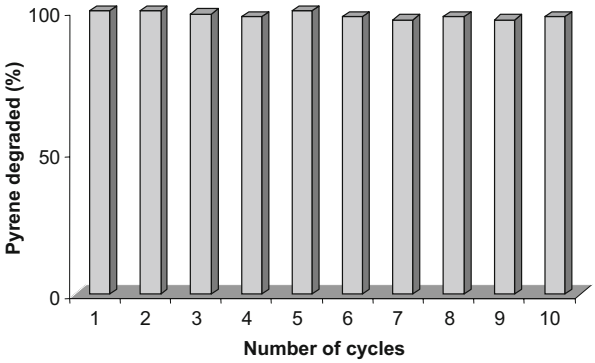

Fig. 4 Reuse of Siran immobilized Spyr1 cells for pyrene degradation in liquid cultures

during the process kept their biodegradation ability (Fig. 3). Disintegration of alginate/starch gel beads during incubation in liquid suspensions probably occurred because chelators, such as phosphate, are present in the medium [44, 45], therefore limiting this particular application in waste-liquid treatments. Similar results have been reported by Rahman et al. [46] while Cassidy et al. [14] reported that *Pseudomonas* sp. cells entrapped in alginate beads could effectively degrade naphthalene without any visual disintegration of the beads.

Both alginate/starch gel and Siran glass beads with entrapped cells of Spyr1 stored at 4 °C were tested for PAH biodegradation and viable counts every month for a period of 1 year. The viable count of alginate/starch gel and Siran glass beads prestorage was $5\pm0.2\times10^6$ and $0.4\pm0.05\times10^8$ cfu/g, respectively, whereas after storage of 1 year was $4.8\pm0.1\times10^6$ and $0.36\pm0.05\times10^8$ cfu/g, respectively, indicating that they maintained their viability over this period of time. In addition, the immobilized beads maintained their PAH degradation capability during the same period (data not shown). Therefore, entrapped Spyr1 cells can be stored without a significant decrease of their PAH-degrading activity, an important parameter in current bioremediation technology. It has been reported before that entrapment preserves the degrading activity of cells by increasing the stability of relevant catabolic enzymes [47].

Biodegradation in Soil Microcosms

The biodegradation of pyrene in sterilized spiked soil microcosms (50 g, 100 ppm pyrene) followed different patterns from that of liquid suspensions (Fig. 5). Pyrene was completely degraded within 14 or 17 days of incubation with free or entrapped cells in alginate/starch gel beads, respectively, whereas only about 58% of pyrene was degraded by glass bead entrapped cells. The biodegradation ability of entrapped cells in unsterilized soil was identical to that of sterilized soil (data not shown).

These results demonstrate the successful performance of alginate/starch entrapped cells for the removal of pyrene and other PAH in soil microcosms, even though the biodegradation rates were not as high as those encountered with free cells. The same or slower degradation rate in soil by entrapped cells compared to free cells has also been reported previously by others [17, 48].

The ability of alginate/starch entrapped cells to degrade mixtures of PAHs was also examined by spiking 500 g of soil with a PAH mixture containing pyrene, phenanthrene, and fluoranthene (100 ppm each). Phenanthrene was degraded completely after 14 days, while pyrene and fluoranthene disappeared after 23 days of incubation with free cells. Degradation by entrapped cells was slower as phenanthrene disappeared from soil within 23 days, whereas at this time, 92% of fluoranthene and 90% of pyrene degraded (Fig. 6).

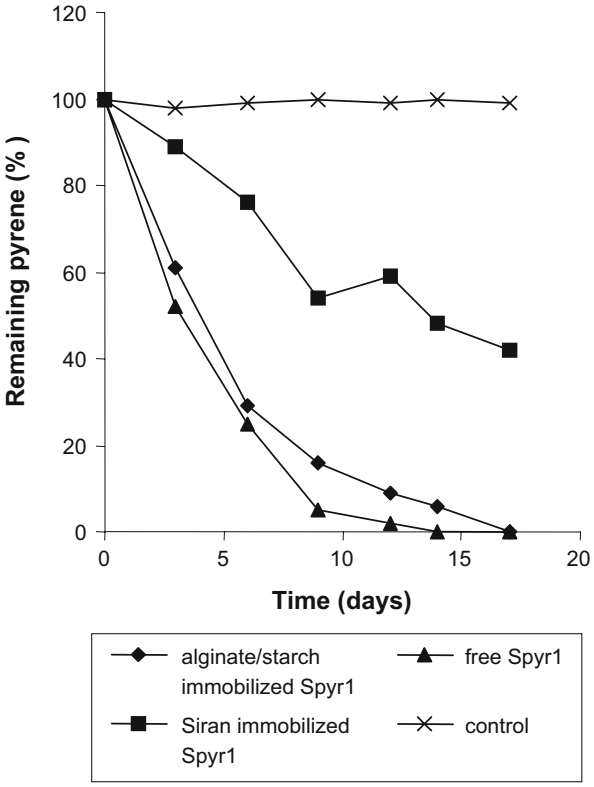

Fig. 5 Biodegradation of pyrene by free and immobilized Spyr1 cells in soil microcosms

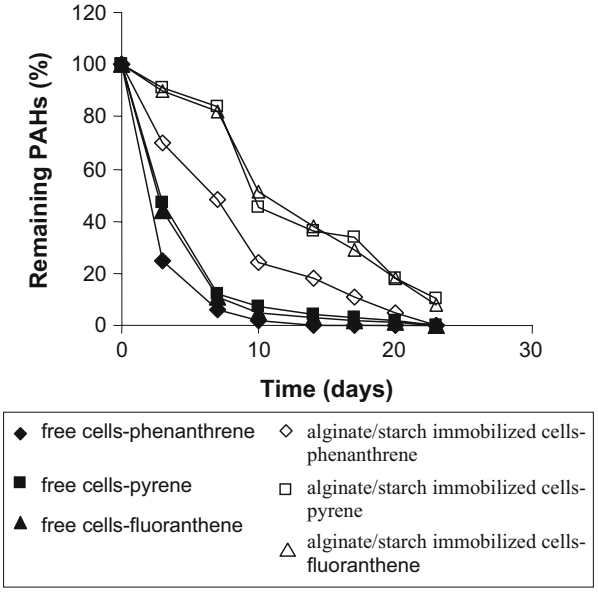

Fig. 6 Biodegradation of PAHs mixture by free (*closed symbols*) and alginate/starch immobilized Spyr1 cells (*open symbols*) in soil microcosms. *Triangles*, phenanthrene; *squares*, pyrene; and *diamond*, fluoranthene

The number of viable entrapped cells/gram soil was maintained stable in sterilized soil during this time, whereas the number of free cells was reduced about 25% and 33% when the soil was spiked with a PAH mixture or pyrene alone, respectively (data not shown). It was not possible to differentiate the inoculated cells from the indigenous ones in nonsterilized soil. The increase of survival of alginate/starch entrapped Spyr1 cells confers a significant advantage for applications in soil remediation. Previous research has also shown that immobilization often decreases the rate of viability loss of the introduced microbial inoculum [14, 17, 47]. PAH-degrading populations in soil are probably not growing, but they are in a pseudostationary phase, where transient growth only replaces decaying cells until the habitat's mass transfer-controlled carrying capacity is reached again [49].

In conclusion, it appears that removal of pyrene (and other PAH) in liquid cultures as well as soil microcosms using free or entrapped Spyr1 cells can be tested as a sustainable processes (see Appendix). The recycling experiments demonstrated that the degradation activity of Siran glass beads immobilized cells was still high after ten cycles, providing an advantage for applications in waste-liquid treatments.

Although alginate/starch immobilized cells had slower biodegradation rates than free cells in the removal of pyrene and other PAH in soil microcosms, the long-term preservation of immobilized Spyr1 cells without decrease of their PAH-degrading activity provides an advantage in soil microcosms bioremediation.

Acknowledgments This work was funded by the Greek Secretariat for Research and Technology (Programme PENED-01ED547). The authors wish to thank Prof. A. Aivasidis from the Department of Environmental Engineering, Faculty of Engineering, Demokritos University of Thrace for providing the macroporous sinter glass (Siran^{R}, Schott Glaswerke).

Appendix

A patent application is pending for the immobilization of petroleum-degrading micro-organisms for wastewater cleanup and soil remediation (Industrial Property Organisation, Application No. 20070100669) [50].

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