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Microbiological degradation of pentane by immobilized cells of *Arthrobacter* sp.

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Abstract

The increasing production of several plastics such as expanded polystyrene, widely used as packaging and building materials, has caused the release of considerable amounts of pentane employed as an expanding agent. Today many microorganisms are used to degrade hydrocarbons in order to minimize contamination caused by several industrial activities. The aim of our work was to identify a suitable microorganism to degrade pentane. We focused our attention on a strain of *Arthrobacter* sp. which in a shake-flask culture produced 95% degradation of a 10% mixture of pentane in a minimal medium after 42 days of incubation at 20 °C. *Arthrobacter* sp. cells were immobilized on a macroporous polystyrene particle matrix that provides a promising novel support for cell immobilization. The method involved culturing cells with the expanded polystyrene in shake-flasks, followed by *in situ* growth within the column. Scanning electron microscopy analysis showed extensive growth of *Arthrobacter* sp. on the polymeric surface. The immobilized microorganism was able to actively degrade a 10% mixture of pentane, allowing us to obtain a bioconversion yield of 90% after 36 h. Moreover, in repeated-batch operations, immobilized *Arthrobacter* sp. cells were able to maintain 85–95% pentane degradation during a 2 month period. Our results suggest that this type of bioreactor could be used in pentane environmental decontamination.

Introduction

Every year over 100 million tonnes of gaseous organic compounds are emitted world wide into the atmosphere. In the past decade, the effects of these substances on our health and environment became fully understood and more stringent regulations on gaseous emissions were imposed. For this reason, the treatment of off-gases remains a major issue for many industries, which employ a variety of physical, chemical and biological processes. In order to maintain air-borne contaminants within defined, acceptable parameters, various chemical and physical methods are useful for volatile organic compound (VOC) removal, e.g. thermal and catalytic oxidation and adsorption onto activated carbon (with subsequent incineration). Biofiltration is the main biological remediation method used for this purpose and involves passing off-gases through a biologically active porous medium. (Barton et al. 1999). This technology is based on the ability of many microorganisms (generally bacteria and, to a small extent, filamentous fungi and yeasts) to aerobically degrade a variety of organic compounds (Leson & Winer 1991). Biofiltration offers a cost-effective and environmentally friendly alternative to physico-chemical methods. In fact, biological waste air treatments do not release residual toxic products such as nitrogen oxide, which require additional treatment and disposal (Ergas et al. 1994); carbon dioxide production also makes a limited contribution to global warming (Ottengraf 1987).

Nowadays, as the use of fluorocarbon declines, the most important classes of volatile organic pollutants are the hydrocarbons produced by several petrochemical industrial activities. In view of this fact, the abilities of microorganisms to degrade such compounds are particularly relevant and has been widely investigated by many researchers who study and apply new processes to remove these contaminated gasses (Watkinson & Morgan 1990).

The main difficulty in hydrocarbon microbial degradation lies in the sparingly soluble short chain alkane. As microorganisms require relatively high water activity, oxidation reactions take place in the aqueous biofilm surrounding the solid medium. Consequently, of all the factors which generally affect the efficiency of VOC removal, it is the partition coefficients that determine their water solubility (Reij et al. 1995). Theoretically, highly volatile chemicals may be present in relatively low biofilm concentrations resulting in slower degradation kinetics (Swanson & Loehr 1997). However, there is evidence that highly volatile aliphatic compounds such as hexane (Morgenroth et al. 1996) and pentane (Barshter et al. 1993; Davison Barton et al. 1997) can be efficiently removed via biofiltration. We focused our attention on one biological process which may prove useful for pentane biodegradation.

Pentane is a paraffin hydrocarbon which, in theory, has not proved hazardous to human health to date. However, this alkane does contributes to the total amount of VOC in a ratio of 0.2%. The most significant emission of pentane comes from the industrial production of expanded polystyrene (EPS), a polymer which is widely employed in packaging and building materials and which is being increasingly used. This gas is used as a blowing agent in EPS manufacturing and moulding operations and the final product typically contains between 3% and 6% per weight of pentane.

For our biodegradation studies, the following microorganisms were used: *Acinetobacter calcoaceticus* (Marin et al. 1994; Lal & Khanna 1996), *Arthrobacter sp.* (Liu et al. 2001), *Pseudomonas citronellonis* (Bhattacharya et al. 2003), *Pseudomonas oleovorans* (Witholt et al. 1990), *Pseudomonas aeruginosa* (Belhaj et al. 2002), *Hormoconis resinae* (Solana & Gaylarde 1995), *Aspergillus niger* (Volke-Sepulveda et al. 2003) and three bacterial enriched consortia isolated from polluted areas as described by Okeke & Frankeberger (2003).

The aim of our work was to use a preliminary screening to identify a suitable microorganism or microbial consortium which could grow on pentane as a substrate and to produce a prototype biofilter in order to biodegrade this compound.

Experimental

Microorganisms and cultivation

The microorganisms used were *Acinetobacter calcoaceticus* (DSM1139), *Arthrobacter* sp. (DSM312), *Pseudomonas citronellonis* (DSM50332), *Pseudomonas oleovorans* (DSM1045), *Pseudomonas aeruginosa* (DSM1128) *Hormoconis resinae* (DSM 1835); *Aspergillus niger* (ATCC 9642 and 15475) and three consortia of microorganisms isolated from polluted areas. All the microorganisms were grown in shake-flasks in a complete medium (NB, nutrient broth) except for *Hormoconis resinae* which was grown in a medium containing 10 g l^{-1} Malt extract and 3 g l^{-1} Bacto peptone, and the two strains of *Aspergillus niger* which were grown in a Potato dextrose broth 20 g l^{-1}.

Bacterial growth and dry weight estimation

Bacterial density (OD_{600}) was measured to estimate the bacterial growth. Cells were recovered by centrifugation (5000 × g) and the dry weights were obtained gravimetrically after drying at 60 °C overnight (18 h).

Pentane degrading microorganisms

The previously grown bacteria in complete media until a late stationary phase as described above were centrifuged, washed and finally transferred to 500 ml shake-flasks containing 50 ml of a synthetic medium for pentane biodegradation. The composition of the medium was as follows (g l^{-1}): NH_4C1, 1.0; KH_2PO_4, 0.7; K_2HPO_4, 0.7; $MgSO_4{\cdot}7H_2O$, 0.7; NaCl, 0.005; $FeSO_4{\cdot}7H_2O$, 0.002; $ZnSO_4{\cdot}7H_2O$, 0.002; $MnSO_4{\cdot}4H_2O$, 0.001; final pH 6.0.

To prevent pentane evaporation (b.p. 36.1 °C), the shake-flasks containing the different culture

strains were sealed with screw caps and Teflon gaskets. They were then incubated with shaking (80 rpm) in a water bath at a temperature never exceeding 26 °C to avoid evaporation and, at the same time, permit the growth of microorganisms. Due to pentane's low water solubility, the optimal conditions of the hydrocarbon concentration were obtained using volumes of minimum media and pentane in a ratio of 9:1. Under these conditions, we performed the growth of microbial pure culture and consortia.

The isolation of microorganisms consortia from the polluted area were performed by collecting soil samples from 10 different sites that were rich in hydrocarbon wastes. One gram of each soil sample was added to 100 ml of tap water and incubated at 26 °C. Successive subcultures in the presence of a mixture of alkanes and decreasing glucose concentrations were then incubated under the same conditions. Final subcultures were grown with no addition of glucose. The viable biomass of each culture was evaluated by serial dilutions on nutrient agar plates incubated at 26 °C. Afterwards, we isolated three types of bacteria consortia, named Consortium A, B, and C, which are able to grow with pentane as the only carbon source. Such consortia were used in the experiments to remove pentane from the liquid shake flask culture.

Removal of pentane in liquid cultures by microorganisms

In order to identify the microorganism or microbial consortium with the highest biodegradation ability, screening was performed at 26 °C in 500 ml shake-flasks containing 50 ml of the synthetic medium previously described with a supplement of 10% (v/v) pentane as the only carbon source. The pentane biodegradation rate was quantified by gas chromatographic (GC) analysis by monitoring its time dependent depletion after 7, 14, 21 and 42 days, respectively.

Pentane analysis

In all cases the amount of pentane was analysed as follows. The extraction of residual quantities of pentane from the microbial culture media was performed by pouring an equal volume of xylene (5 ml) into each flask which had been previously cooled to 4 °C to prevent pentane evaporation. After separating the two phases, 1 μl aliquots from the organic solvents were withdrawn with a micro syringe and analysed by gas chromatography. A Carlo Erba Mega 5300 gas chromatograph was used and the apparatus was equipped with a Supelcowax 10 capillary column by Supelchem or with a Carbowax 20M by Perkin Elmer. A pentane/xylene mixture was used as a standard at the same v/v ratio as in the samples.

Arthrobacter *sp. cells immobilization method*

Beads of non-sintered Expanded Polystyrene (NS-EPS), sintered EPS (S-EPS), and sintered and fragmented EPS (SF-EPS) were used as immobilization supports. Prior to use, the polymeric beads were sterilized with ethanol 100%, washed twice with sterile H_2O and twice with the medium. A volume of 10 ml of each matrix was placed in 500 ml shake-flasks along with 25 ml of complete medium. One milliliter of *Arthrobacter* sp. starter culture was added and the flasks were incubated at 28 °C for 48 h in a shaker (120 rev min^{-1}). The ability of polyvalent cations to flocculate bacteria was exploited by adding a Ca^{2+} solution of different concentrations at the end of the growth phase for a further 2 h in order to flocculate cells within the EPS structure. After this treatment, which allowed the microorganisms to adhere, the columns were packed by pouring the content of the shake-flasks into an upturned 20 ml syringe barrel with a glass wool plug fitted at the base. A glass wool baffle was fitted on the top of the EPS and non-immobilized cells were washed out by passing through 5 volumes of 50 mM potassium phosphate buffer (pH 7.0). Once the columns were packed, the complete medium was re-circulated through the EPS for 24 h to encourage *in situ* cell growth. The temperature was kept at a constant 28 °C by means of a circulating thermostatic bath. The EPS matrix packed column containing immobilized *Arthrobacter* sp. cells were tested for their efficiency in pentane degradation for 36 h at 28 °C as described below.

Scanning electron microscopy (SEM) analysis

Sample of EPS were taken from the column in order to verify, by means of scanning microscopy analyses, whether the microbial immobilization

had occurred on the polymeric matrix. Analyses were performed on cryogenically fractured surfaces using an SEM Philips XL 20 series microscope at different magnifications. Before the observation, all the surfaces were coated with Au/Pd alloy with SEM coating device (SEM Coating Unit E5150, Polaron equipment Ltd.).

Pentane biodegradation in the packed column

A 25 ml volume of synthetic medium with 10% (v/v) pentane was re-circulated through a column (70 × 22 mm) containing the immobilized microorganism for 36 h by means of a peristaltic pump (flow rate = 30 ml h^{-1}) as described in the schematic diagram of the bioreactor in Figure 1. The experiment was performed in 20 repeated-batch operations during a period of 2 months. Each completed batch process was followed by a 3-volume medium washing cycle. At several intervals time triplicate samples were withdrawn and analysed by GC for pentane content. These studies were carried out at 28 °C; the pH was not controlled during the operation and reached levels of around 5.7–6.2.

Results

Screening of pentane-degrading microorganisms

A growth test was performed to assess the ability of each microbial culture and consortia to use pentane. As previously indicated in the experimental section, microorganisms were grown in a liquid culture in shake-flasks using a synthetic minimum medium containing the hydrocarbon as the one and only carbon and energy source at a concentration of 10% (v/v). The microbial growth, determined as dry biomass, was followed for six weeks and the results are reported in Table 1.

Acinetobacter calcoaceticus, *Arthrobacter* sp. and the two *Aspergillus niger* strains already demonstrated significant growth on a pentane synthetic medium in the first week. However, the three consortia and the other strains request several weeks to adapt before a significant growth yield could be obtained.

The screening tests on the biodegradation ability of each microbial culture and consortia were performed in the same experimental conditions. The pentane degradation rate was followed for six weeks and the results are reported in Table 2.

Of all the single microbial monocultures tested, *Acinetobacter calcoaceticus* and *Arthrobacter* sp. displayed the highest rate of pentane removal (70%

Table 1. Pentane-degrading microorganisms growth estimation

Stain	Microbial dry biomass (mg/l)			
	7 day	14 day	21 day	42 day
Acinetobacter calcoaceticus	68	122	130	146
Arthrobacter sp.	186	245	300	290
Hormoconis resinae	28	45	72	64
Pseudomonas citronellonis	22	67	94	102
Pseudomonas oleovorans	12	27	42	68
Aspergillus niger 9642	102	150	180	202
Aspergillus niger 15475	90	143	196	210
Pseudomonas aeruginosa	15	32	50	74
Consortium A	14	23	31	44
Consortium B	19	24	37	60
Consortium C	20	27	44	69

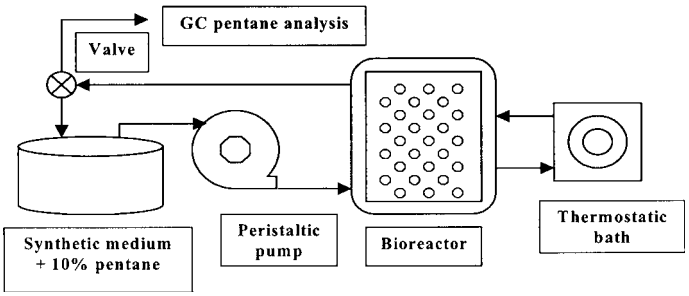

Figure 1. Schematic diagram of the bioreactor.

Table 2. Screening of pentane-degrading microorganisms

Stain	% Pentane degradation			
	7 day	14 day	21 day	42 day
Acinetobacter calcoaceticus	40	52	65	70
Arthrobacter sp.	53	81	85	95
Hormoconis resinae	5	15	20	24
Pseudomonas citronellonis	3	20	29	33
Pseudomonas oleovorans	0	2	4	18
Aspergillus niger 9642	22	30	38	44
Aspergillus niger 15475	10	18	26	37
Pseudomonas aeruginosa	0	5	9	21
Consortium A	0	0	2	14
Consortium B	0	0	1	19
Consortium C	0	0	3	17

and 95%, respectively) in a synthetic medium, after 6 weeks of incubation at 26 °C. With the exception of the *Aspergillus niger* strains and, to a lesser extent, the *Pseudomonas citronellonis*, the pentane degradation yield obtained for the other monocultures and consortia, was lower (14–24%).

Arthrobacter *sp. cells immobilization*

On the basis of our data, *Arthrobacter* sp. proves a better microorganism for degrading pentane compared with *Acinetobacter calcoaceticus* as the growth rate and the degradation percentage were much more efficient. We tried to improve the catabolic process yield by immobilizing *Arthrobacter* cells and building a laboratory scale flow-through bioreactor (Figure 1). Such a system made it possible to obtain a high yield of bioconversion in a relatively short time due to the significant increase in biocatalyst concentration. Immobilized microbial cells also provided better contact with the substrate (Scott 1987).

Macroporous polystyrene particles obtained from sintered and fragmented EPS were used as a solid support for *Arthrobacter* sp. cell immobilization (Figure 2a, b). As described in the materials and methods section, the immobilization process was performed on beads of non-sintered, sintered, and sintered-fragmented EPS using different Ca^{2+} concentrations in order to increase cell flocculation within the EPS structure. The results obtained in pentane degradation tests at 28 °C for 36 h on the different supports are shown in Figure 3.

The best result, i.e. about 90% pentane degradation, was obtained from sintered and fragmented EPS (SF-EPS) treated with a concentration of 0.2 M $CaCl_2$. The immobilizated *Arthrobacter* sp. cells were also examined by electro scanning microscopy analysis shown in Figure 4a–d.

Image observation at four different magnifications highlights extensive bacterial adhesion and growth on the EPS surface and internal cavities.

Pentane degradation by immobilized Arthrobacter *sp. cells in packed colums*

The pentane degradation process was performed by re-circulating a 25 ml volume of the synthetic medium containing 10% (v/v) pentane through a packed immobilized biocatalyst column.

The efficiency of this column was tested by 36 h recirculation of the 10% pentane in a synthetic medium. The results given in Figure 5a show that

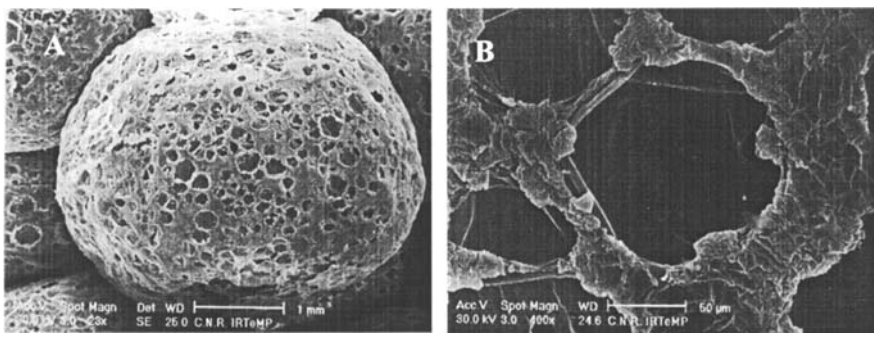

Figure 2. SEM images of expanded polystyrene matrix: (A) 23 × magnification; (B) 400 × magnification.

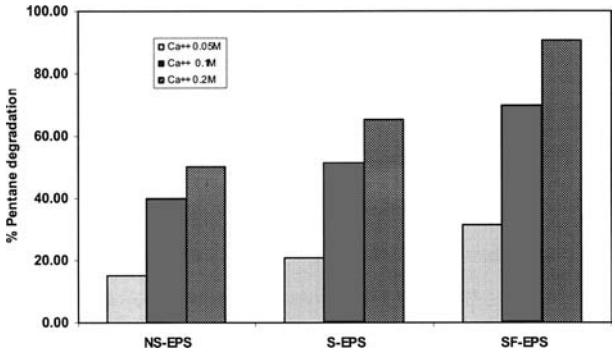

Figure 3. Pentane degradation obtained with *Arthrobacter* sp. cells immobilized on different expanded polystyrene (EPS) matrix. NS-EPS, non-sintered EPS; S-EPS, sintered EPS; SF-EPS, sintered and fragmented EPS in presence of increasing concentration of Ca^{2+}.

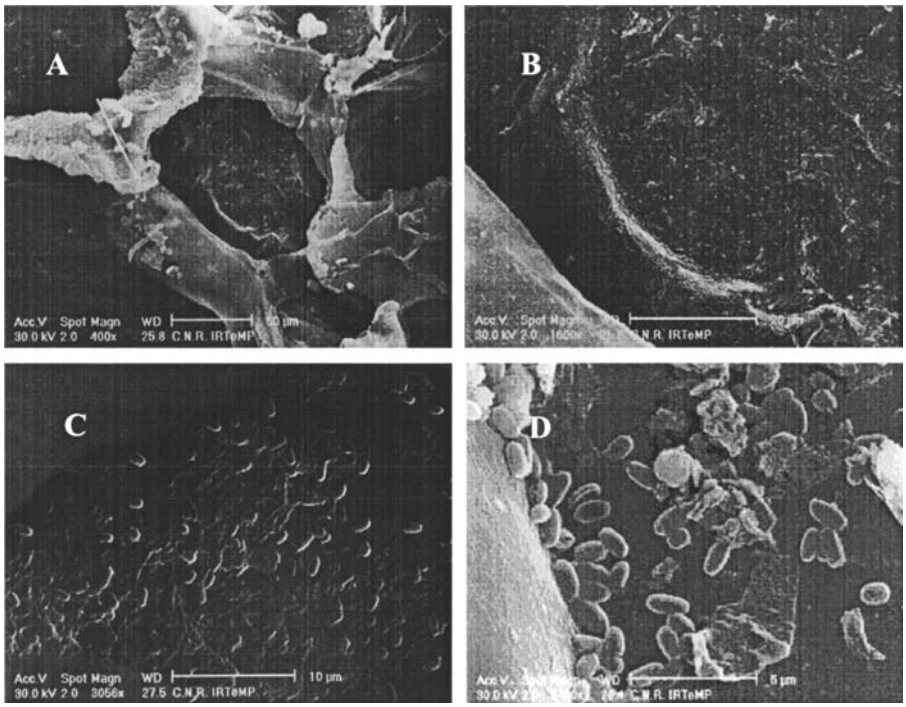

Figure 4. SEM images of immobilized cells of *Arthrobacter* sp. on expanded polystyrene matrix: (A) 400 × magnification; (B) 1600 × magnification; (C) 3056 × magnification; (D) 6400 × magnification.

the immobilized microorganisms are able to actively degrade the pentane and obtain a bioconversion yield of 95%.

Furthermore, during a 2-months period, the immobilized *Arthrobacter* sp. cells were able to maintain a pentane degradation efficiency within 85–95% in 20 repeated-batches of 36 h per operation (Figure 5B).

Discussion

In recent years, many studies have been performed on a great number of strains, microbial consortia and single microorganisms, giving accurate description of their biodegradation capacities. Mixed microbial communities have a powerful biodegradative potential because the genetic information of

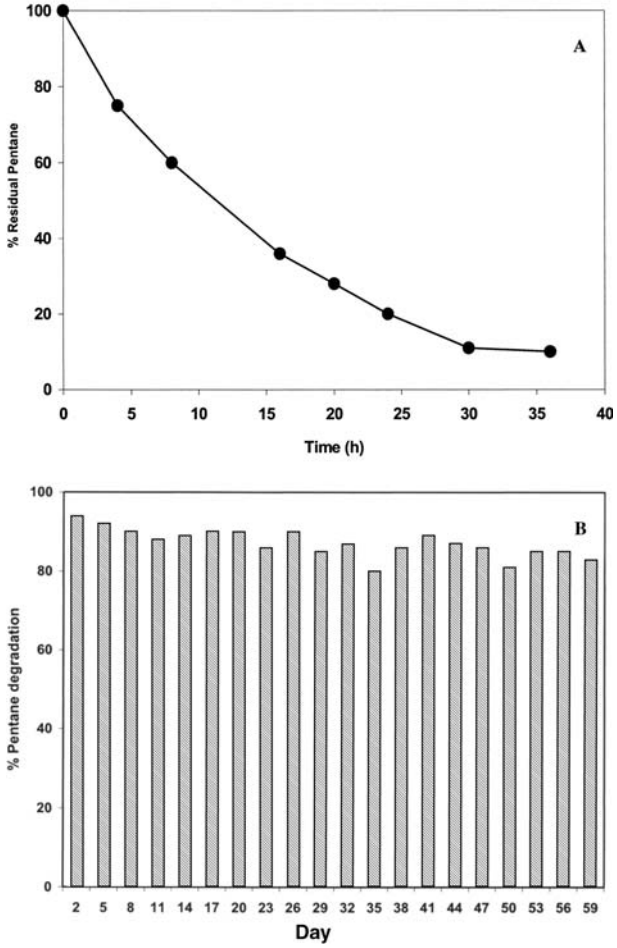

Figure 5. Degradation of pentane by immobilized *Arthrobacter* sp. cells: (A) Degradation ratio of 10% (v/v) pentane by immobilized cells in 36 h test; (B) Percentage of degraded pentane obtained in 20 repeated-batch operations during a period of 2 months.

more than one organism is necessary to degrade the complex mixture of organic compounds present in contaminated areas (MacNaugton et al. 1999; Shen et al. 1998). However, as pentane and other short-chain alkanes ($<C_9$) are toxic to many microorganisms, only the single strains specifically adapted to using such compounds as carbon source are able to degrade them (Fritsche & Hofrichter 2000). For istance, activated sludge cultures which contain microorganisms already adapted to certain contaminants have been used to seed compost biofilters in attempt to reduce acclimatization times (Ergas et al. 1995). Moreover, seeding with specially cultured microbial strains may prove important when treating complex compound such as halogenated organics to ensure degradation (Ottengraf et al. 1983).

In our studies, we reported pentane degradation by bacterial consortia and monocultures. A preliminary screening was performed on some strains indicated in literature as among the best hydrocarbons degraders (Watkinson & Morgan 1990) and on microbial consortia isolated from polluted areas. From our screenings, the best results for pentane degradation were obtained with monocultures rather than with bacterial consortia isolated from polluted areas. *Acinetobacter calcoaceticus* and especially *Arthrobacter* sp. displayed the highest rate of pentane removal. The results are in agreement with the data reported in

literature. In fact, *Arthrobacter* is described as belonging to an important group of aerobic degrading bacteria, i.e. the gram-positive rhodococci and corynebacteria (Atlas & Barta 1998; Holt et al. 1994), which are predominant microorganisms in terrestrial ecosystems polluted with aliphatic and aromatic hydrocarbons.

We improved the pentane degradation process yield by immobilizing *Arthrobacter* cells on a support of macroporous polystirene particles This porous matrix represents a range of low density cellular material (Hainey et al. 1991) which yielded promising results as it possesses high internal phase volumes, large surface areas and high mechanical rigidity, and provides a fully interconnected structure to allow the rapid transfer of nutrients to the cells. The use of this support has already been described for the immobilization of eukaryotic and prokaryotic cells (Hough et al. 1989; Griffiths & Bosley 1993).

The optimization of pentane degradation processes made it possible to ascertain that the best material as a matrix for immobilizing microorganisms is sintered and fragmented EPS. This is not surprising given that the adsorption is facilitated by its rough surface (Kirchner et al. 1987).

We built a laboratory scale flow-through bioreactor using the obtained complex matrix-microorganism. The system was tested for efficiency and stability during two months of repeated-batch operations and demonstrated a high yield of pentane bioconversion (85–95%) throughout the entire period.

To conclude, this study demonstrates substantial degradation of pentane by a bacterial monoculture (*Arthrobacter* sp.) which had never previously been used for this purpose. Immobilized *Arthrobacter* cells offer an attractive treatment option for the removal of pentane from polluted air. Further studies will investigate the best biodegradation conditions and assess the possibility of using such a bioreactor in pentane environmental decontamination.

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