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Degradation of naphthalene by cells of *Pseudomonas* sp. strain NGK 1 immobilized in alginate, agar and polyacrylamide

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Abstract A *Pseudomonas* sp. strain NGK 1 (NCIM 5120) was immobilized in various matrices, namely, alginate, agar (1.8×10^{11} cfu g^{-1} beads) and polyacrylamide (1.6×10^{11} cfu g^{-1} beads). The degradation of naphthalene was studied, by freely suspended cells (4×10^{10} cfu ml^{-1}) and immobilized cells in batches, with shaken culture and continuous degradation in a packed-bed reactor. Free cells brought about the complete degradation of 25 mmol naphthalene after 3 days of incubation, whereas, a maximum of 30 mmol naphthalene was degraded by the bacteria after 3–4 days of incubation with 50 mmol and 75 mmol naphthalene, and no further degradation was observed even after 15 days of incubation. Alginate-entrapped cells had degraded 25 mmol naphthalene after 3.5 days of incubation, whereas agar- and polyacrylamide-entrapped cells took 2.5 days; 50 mmol naphthalene was completely degraded by the immobilized cells after 6–7 days of incubation. Maximum amounts of 55 mmol, 70 mmol and 67 mmol naphthalene were degraded, from an initial 75 mmol naphthalene, by the alginate-, agar- and polyacrylamide-entrapped cells after 15 days of incubation. When the cell concentrations were doubled, 25 mmol and 50 mmol naphthalene were degraded after 2 and 5.5 days of incubation by the immobilized cells. Complete degradation of 75 mmol naphthalene occurred after 10 days incubation with agar- and polyacrylamide-entrapped cells, whereas only 60 mmol naphthalene was degraded by alginate-entrapped cells after 15 days of incubation. Further, with 25 mmol naphthalene, alginate-, agar- and polyacrylamide-entrapped cells (1.8×10^{11} cfu g^{-1} beads) could be reused 18, 12 and 23 times respectively. During continuous degradation in a packed-bed reactor, 80 mmol naphthalene 100 ml^{-1} h^{-1} was degraded by alginate- and polyacrylamide-entrapped cells whereas 80 mmol naphthalene 125 ml^{-1} h^{-1} was degraded by agar-entrapped cells.

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Introduction

Polycyclic aromatic hydrocarbons in nature are environmental pollutants, owing to their inferred recalcitrance to microbial degradation and potential toxicity to higher organisms (Gundlach et al. 1983; Vandermeulen 1981). Naphthalene is considered to be a primary irritant and the US Environmental Protection Agency (EPA) has classified it as a "priority toxic pollutant" (US EPA 1980a, b). Exposure to naphthalene has been shown to cause a decrease in haemoglobin concentration and inhibit oxygen consumption in various organisms (Darville and Wilhm 1984; Strubble and Harmon 1983). Exposure to naphthalene has been implicated in haemolytic anaemia in people with glucose-6-phosphate dehydrogenase deficiency and in newborn infants (Sitting 1985). Naphthalene and its methyl derivatives are considered some of the most acutely toxic compounds (Anderson et al. 1974). Naphthalene, being the simplest homologue in the polycyclic series, has received considerable interest. Several reports are available on the degradation of naphthalene by various microorganisms with free cells (Gibson and Subramanian 1984; Kuhm et al. 1991; Grund et al. 1992; Eaton and Chapman 1992; Manohar and Karegoudar 1995, 1996; Atlas and Cerniglia 1995; Ashok and Saxena 1995). However, bioremediation of naphthalene using immobilized cells has not been investigated. Many microorganisms have been immobilized by entrapment methods. The potential of using immobilized cells in industrial processes is regarded as a valuable application (Cheetam 1980; Bisping and Rehm 1988). Cells at different stages (viable, resting, dead etc.) have been successfully entrapped in various matrices (Mattiasson 1983; Brodelius and Vandamme 1987; Trevors et al. 1992). Immobilized viable cells stay alive for a long time in the immobilized stage when the conditions are optimal. Bioremediation using

immobilized cells has been widely investigated for numerous toxic chemicals such as phenol (Bettmann and Rehm 1984, 1985), pentachlorophenol (O'Reilly and Crawford 1989), 4-chlorophenol (Westmeier and Rehm 1985, 1987; Balfanz and Rehm 1991; Menke and Rehm 1992). 2,4-dichlorophenoxyacetic acid (Kochar and Kahlon 1995), pyridine (Lee et al. 1994), 3-chloroaniline (Ferschl et al. 1991) and chlorobenzoates (Sahasrabudhe et al. 1988).

In the present study, biodegradation of naphthalene using immobilized *Pseudomonas* strain NGK 1 in various matrices has been investigated. The rate of degradation of naphthalene by immobilized cells in various matrices is compared with that by freely suspended cells, with regard to naphthalene-degrading capacity at various initial naphthalene concentrations. The longevity of the naphthalene-degrading activity by immobilized cells is also studied.

Materials and methods

Chemicals

Naphthalene was purchased from S D Fine Chemicals. Sodium alginate was obtained from BDH, India. Agar-agar was procured from Qualigens, India. Acrylamide, bisacrylamide and *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine were purchased from Fluka. All other chemicals used in this study were of analytical grade.

Microorganism

A naphthalene-degrading *Pseudomonas* sp. strain NGK 1 (NCIM 5120) was isolated from biological waste-water-treatment effluent in our laboratory. This pure strain was maintained on the slants of naphthalene mineral salts medium solidified with 2% (w/v) agar (Manohar and Karegoudar 1995).

Medium

Three different media were used in this study. The medium contained (g l^{-1}) K_2HPO_4, 0.38, $MgSO_4 \cdot 7H_2O$ 0.2, NH_4Cl 1.0, $FeCl_3$ 0.05, yeast extract 1.0 and sodium salicylate 0.25; the pH was adjusted to 7 and the medium was supplemented with naphthalene (0.1% w/v). This medium was used for precultivation of the microorganism, for both freely suspended and immobilized cells.

For fermentation studies, the mineral salts medium contained (g l^{-1}) K_2HPO_4 0.38, $MgSO_4 \cdot 7H_2O$ 0.2, NH_4Cl 1.0 and $FeCl_3$ 0.05. The pH was adjusted to 7. This medium was used for free-cell studies and cells immobilized in agar and polyacrylamide. For alginate-entrapped cells, the fermentation medium contained (g l^{-1}) K_2HPO_4 0.15, $MgSO_4 \cdot 7H_2O$ 0.2, NH_4Cl 1.0, $FeCl_3$ 0.05 and $CaCl_2$ 0.2. The pH of the medium was adjusted to 7. Naphthalene at various concentrations (20–80 mmol) was used as the sole carbon source.

Mode of naphthalene supplementation

Naphthalene at various concentrations was dissolved in the minimum amount of *N*,*N*′-dimethylformamide, and the solution was forced into the mineral salts medium (containing Tween-80 at approximately 80 × critical micelle concentration) with the help of a syringe. This medium was subjected to ultrasonication (ultrasonicator Vibra-cell model 175, USA) twice for 1 min. The final naphthalene/mineral salts medium appeared milky.

Immobilization

Pseudomonas sp. strain NGK 1 (NCIM 5120) was grown in the mineral salts medium containing naphthalene (0.1% w/v) as sole source of carbon and energy. Cells were harvested during the mid-logarithmic growth phase by centrifugation at 5000 *g* for 10 min at 15 °C. The cells were immobilized in different matrices: alginate, agar and polyacrylamide.

Alginate entrapment of cells

The alginate entrapment of cells was performed according to the method of Bettemann and Rehm (1984). Alginate (4% w/v) was dissolved in boiling water and autoclaved at 121 °C for 15 min. A 50-ml bacterial cell suspension (cells 15 g wet weight/50 ml alginate solution) was added to 200 ml sterilized alginate solution, and mixed by stirring on a magnetic stirrer. This alginate/cell mixture (with stirring) was extruded drop by drop into a cold, sterile 0.2 M $CaCl_2$ solution through a burette connected to a tapered pipette tip, by blowing air from the other end of the burette. Gel beads of approximately 2 mm diameter were obtained. The beads were hardened by resuspending into a fresh $CaCl_2$ solution for 2 h with gentle agitation. Finally these beads were washed with distilled water and used for experimentation.

Agar entrapment of cells

The procedure was carried out according to the method described by Nilson et al. (1983). A 4% agar solution (w/v in physiological saline) was homogenized by boiling, sterilized at 121 °C for 15 min and cooled to 50 °C. The bacterial cell suspension (15 g wet weight cells in 50 ml saline) was added to 200 ml agar solution, mixed by stirring on a magnetic stirrer. The beads were prepared as described for alginate entrapment but with paraffin oil as the immobilizing phase instead of $CaCl_2$. The agar beads of 2 mm diameter thus obtained were washed with saline and distilled water.

Polyacrylamide entrapment of cells

The entrapment procedure was carried out according to the methods of Chibata et al. (1978) and Starostina et al. (1987) Bacterial cells (15 g wet weight in saline) were preincubated with $MgSO_4$ to a final concentration of 1 mol. For polymerization, acrylamide monomer solutions (37.5 g acrylamide/2 g bisacrylamide) were dissolved in 200 ml saline, then the above bacterial cell suspension was mixed with acrylamide solution by stirring on a magnetic stirrer. The polymerization was performed in six aliquots in a 2000-ml beaker. Heat evolved during polymerization was eliminated by using an ice bath. The polymer sheet was cut and ground to give fine granules of 1 mm diameter. These were then washed with 70% ethanol and with sterilized water.

Scanning electron microscopy

For scanning electron microscopy, the beads were cut and fixed in a solution of glutaraldehyde (2% w/v) for 2 h. The specimens were then washed with saline, dehydrated in a water/ethanol series (30%, 50%, 70%, 80%, 90%, 94% and 96%, 1 h for each step) and dried to the critical point in a sputter-coating process. The particles were covered with a gold layer in an argon medium. The scanning electron microscopy observations were made in a Phillips scanning electron microscope (model 515).

Design of the reactor for continuous degradation

A cylindrical glass column (4 × 50 cm, volume 650 ml) with outlet facilities every 5 cm was used. The bottom of the column was packed with a circular foam pad (4 cm diameter) followed by a porous glass frit. The reactor was then packed with the respective immobilized-cell matrix to a height of 30 cm. The column was attached to a reservoir of naphthalene/mineral salts medium, kept on a magnetic stirrer for proper mixing of naphthalene in the medium. The medium was then fed into the column continuously with the help of a peristaltic pump (Miclins India) through a side-arm present at the bottom of the column. The dynamic flow of oxygen was maintained at 1 bar (10^5 Pa) throughout the entire system, through the bottom of the column, from an oxygen cylinder during the experiments. The effluent was continuously removed from the side-arm situated just above the packed bed (30 cm height).

Fermentation conditions

Batch fermentations

The batch fermentations were performed for both freely suspended cells and immobilized cells in various matrices. For freely suspended cell culture, exponentially growing cells were added to 250-ml conical flasks containing 100 ml mineral salts medium adjusted different initial cell concentrations (4×10^{10} cfu ml^{-1} and 8×10^{10} cfu ml^{-1}) and various amounts of naphthalene (25, 50 and 75 mmol). The fermentation process was carried out at room temperature (approx. 30 °C) on a rotary shaker at 150 rpm for the desired incubation period. Samples of the culture broth were taken at the indicated times for the analysis of naphthalene.

For immobilized cells, 25 g wet beads (approx. 1500 beads) of the respective entrapment matrices, were added to a 250-ml conical flask containing 100 ml mineral salts medium with various amounts of naphthalene as indicated. The initial cell concentrations were in the range 1.8×10^{11} cfu g^{-1} beads and 3.6×10^{11} cfu g^{-1} beads in alginate and agar, and 1.6×10^{11} and 3.2×10^{11} cfu g^{-1} gel beads in polyacrylamide entrapment. Like the freely suspended cell culture, the entrapped cultures in all the matrices were incubated at room temperature (approx. 30 °C) on a rotary shaker under identical fermentation conditions. The control experiments for the evaporation of naphthalene were carried out in sterile medium at the above-indicated naphthalene loadings. The evaporation of naphthalene from sterile controls was found to be about 0.15 mmol day^{-1}, which was not used to correct the degradation of naphthalene by this bacterium.

Repeated batch fermentations

For establishing the long-term stability of naphthalene degradation by immobilized cells in the respective matrices, repeated batch fermentations were carried out. After every incubation period (2–3 days), the used medium was decanted and beads were washed with water and transferred into a fresh mineral salts medium containing naphthalene. The fermentation process was carried out under identical fermentation conditions.

Continuous fermentations

The continuous fermentations were carried out in a packed-bed reactor. The reactor was packed with 250 g bacteria-entrapping gel beads to a height of 30 cm with a working volume of 140 ml for alginate- and agar-entrapped cells and 120 ml for polyacrylamide-entrapped cells. The continuous flow of oxygen was adjusted 1 bar, also guaranteeing good mixing in the culture medium. The fermentation process was carried out by the continuous supply of medium with various concentrations of naphthalene at different flow rates. The residual naphthalene was monitored in the effluent for each set of experiments.

Analytical methods

Analysis of naphthalene was carried out by HPLC and UV spectrophotometric methods. The spent medium was extracted with ether and the residue was dissolved in methanol and analysed by reversed-phase chromatography on a Shimadzu HPLC model 7161 chromatograph with a UV detector (wavelength 276 nm). A Shim pack CLC-C_8 (M) Octadecylsilane (ODS) column (4.6 × 15 cm) was employed with methanol/water (9:1) at a flow rate of 1 ml min^{-1}. Naphthalene was also quantified in the spent medium by using a UV/visible light spectrophotometer (Shimadzu model 160 A).

The content of the cells entrapped in the alginate beads was measured by dissolving the gel beads in 10 ml sodium pyrophosphate (1% w/v) followed by serial dilution and plating on nutrient agar plates using a Lapiz bacteriological colony counter. The content of cells entrapped in agar beads was measured by dissolving the gel beads at 90 °C for 3 min and reading the absorbance of the gel solution at 660 nm.

Results

The bacterium, *Pseudomonas* sp. strain NGK 1 (NCIM 5120) was isolated from a biological waste-water effluent, capable of utilizing naphthalene as the sole source of carbon and energy. This strain was immobilized in alginate, agar and polyacrylamide. The scanning electron microscopic observations (Fig. 1) revealed that, after 40 days of cultivation, there was very low cell leakage for alginate-entrapped cells, whereas there was considerable leakage of cells from the agar. However, no such leakage of cells was observed for polyacrylamide entrapment, its high cell-retention capacity making polyacrylamide an effective matrix.

Degradation of naphthalene by freely suspended and immobilized *Pseudomonas*

The batch fermentations of naphthalene were carried out both with freely suspended cells and with cells entrapped in the above matrices. The results on the degradation of naphthalene by freely suspended cells are given in Fig. 2A. It was shown that an initial 25 mmol naphthalene (with a cell concentration 4×10^{10} cfu ml^{-1}) was completely degraded within 3 days of incubation. With the same cell concentration, only 30 mmol naphthalene was degraded after 3 days of incubation of an initial 50 mmol naphthalene. No further degradation of naphthalene was observed even at 15 days of incubation. Even when the initial amount of naphthalene was increased to 75 mmol, only 30 mmol was degraded after 4 days of incubation and no further degradation was observed even after 15 days of incubation with the same cell concentration. However, there was no significant change in the degradation rate of naphthalene when the cell concentration was doubled. It was also observed that there was no lag phase in the initial degradation of naphthalene.

The results obtained with immobilized cells in batch cultures are given in Fig. 2B,C and D. It is evident that

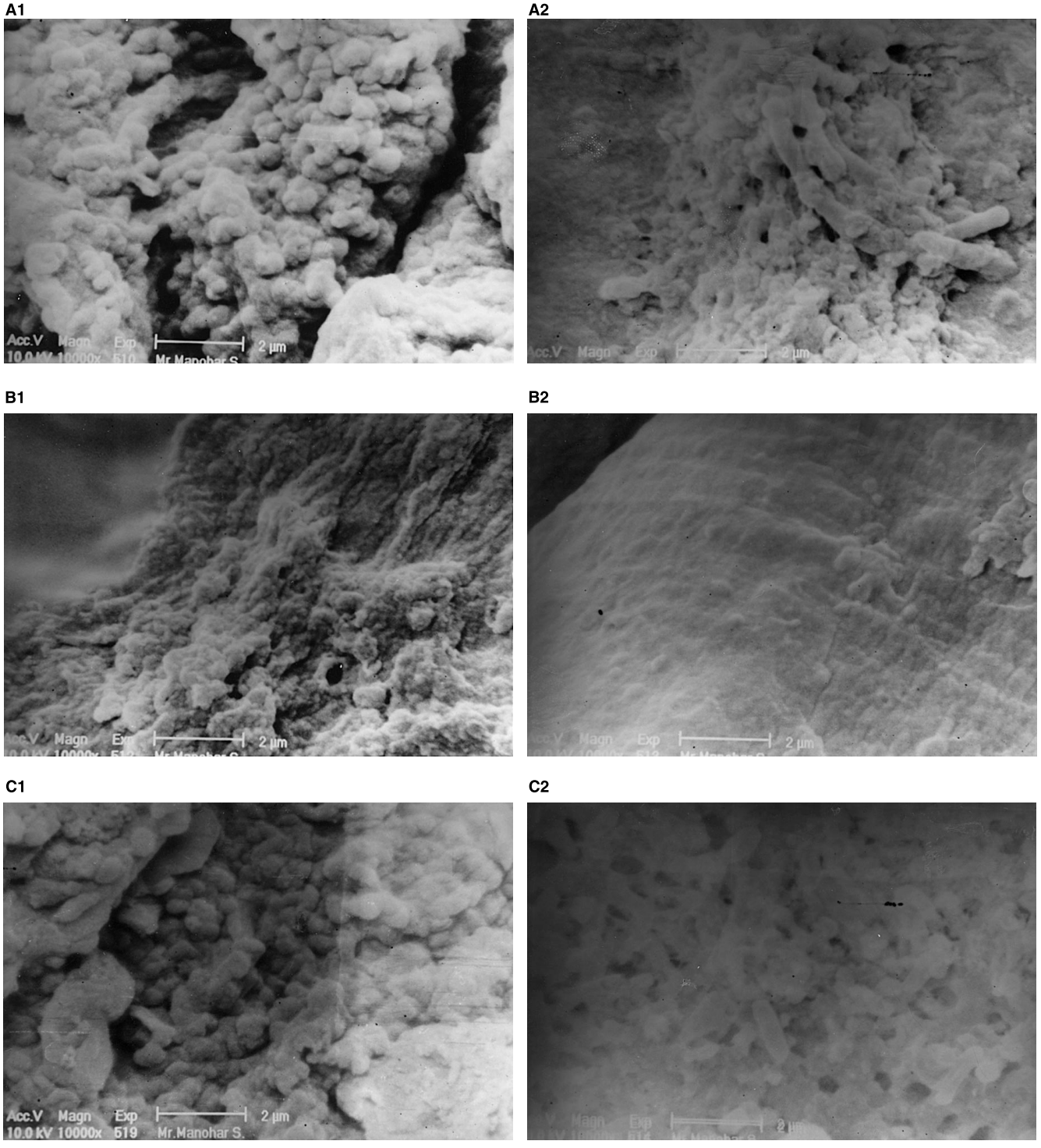

Fig. 1A–C Scanning electron micrographs of *Pseudomonas* sp. strain NGK 1 (NCIM 5120) immobilized in alginate (**A**), agar (**B**) or polyacrylamide (**C**). **A1**, **B1**, **C1** Freshly immobilized cells, **A2**, **B2**, **C2** immobilized cells after 40 days of cultivation

the initial 25 mmol naphthalene was completely degraded after 3.5 days of incubation with alginate-entrapped cells (1.8×10^{11} cfu g^{-1} beads), whereas the same amount of naphthalene was degraded after 2.5 days of incubation when the cells were entrapped in agar or polyacrylamide gels. At a 50-mmol initial naphthalene load, the complete degradation of naphthalene was observed after 6–7 days incubation with alginate-, agar- and polyacrylamide-entrapped cells. Further, it was observed that, when the initial loading of naphthalene increased to 75 mmol, nearly 67–70 mmol was degraded by cells entrapped in agar or polyacrylamide, and only about 60 mmol naphthalene

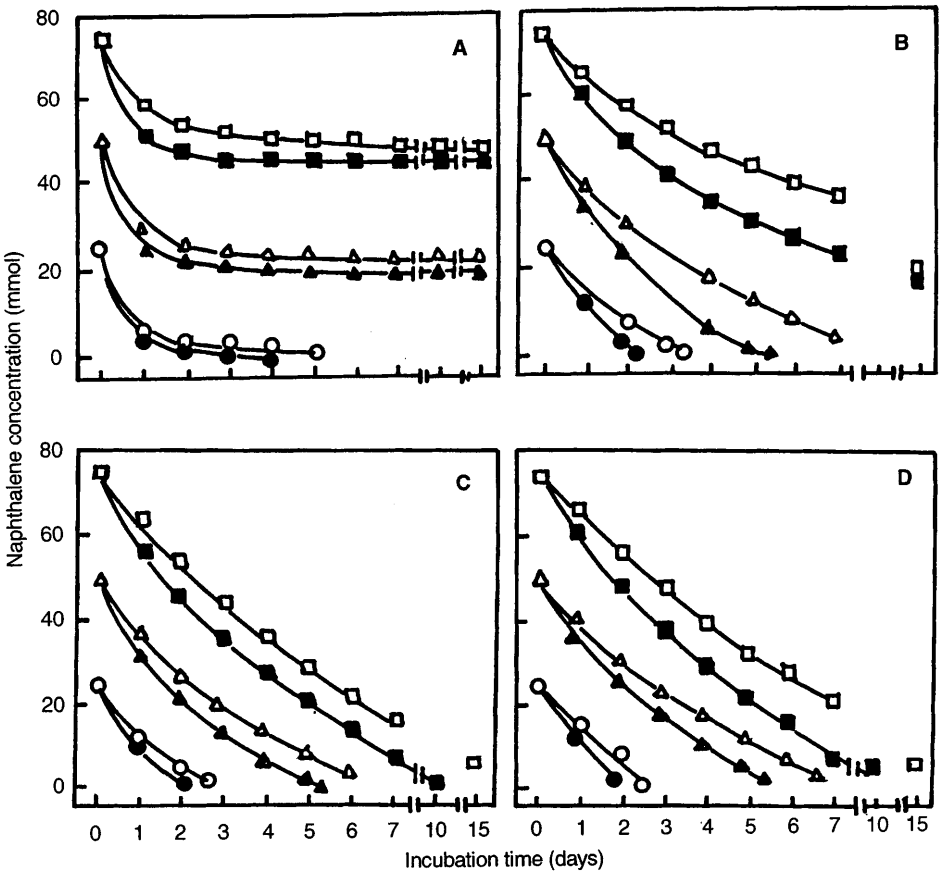

Fig. 2A–D Degradation of naphthalene in batches with (**A**) shaken cultures of freely suspended cells or (**B–D**) cells immobilized in alginate (**B**), agar (**C**) or polyacrylamide (**D**). □ △ ○ 1.8×10^{11} cfu g^{-1} beads in immobilized systems and 4×10^{10} cfu ml^{-1} in cell-free systems, ■ ▲ ● 3.6×10^{11} cfu g^{-1} beads in immobilized systems and 8×10^{10} cfu ml^{-1} in cell-free systems

was degraded by the alginate-entrapped cells. When the cell concentrations were doubled, immobilized cells in all the matrices degraded 25 mmol naphthalene after 2–2.5 days of incubation. An initial 50 mmol naphthalene was completely degraded after 5.5 days of incubation with the immobilized bacteria. However, at 75 mmol naphthalene, alginate-entrapped cells degraded a maximum of 60 mmol after 15 days of incubation, whereas the complete degradation of 75 mmol naphthalene was shown by agar- and polyacrylamide-entrapped cells after 10 days of incubation.

Semicontinuous degradation of naphthalene by immobilized *Pseudomonas*

The repetitive degradation of naphthalene by immobilized cells was carried out at two different naphthalene loadings (25 mmol and 50 mmol). The results of these studies are given in Fig. 3. It was observed that, for an initial 25 mmol naphthalene, the alginate-entrapped cells could be reused 18 times, agar-entrapped cells 12 times and polyacrylamide-entrapped cells 23 times without losing their naphthalene-degrading activity during the culture period tested. However, when the initial amount of naphthalene was increased to 50 mmol, the immobilized cells could also be reused but with a decreased naphthalene degradation rate. The results further demonstrated that small amounts of naphthalene (25 mmol) could be fed at much higher frequency than larger amounts of naphthalene (50 mmol). The balance of naphthalene degradation showed that high-frequency loading of small amounts of naphthalene produced double the degradation of a low-frequency loading of large amounts of naphthalene.

Continuous degradation of naphthalene by immobilized *Pseudomonas*

The impact of residence time on the performance of the immobilized cells in a packed-bed reactor on continuous operation (Fig. 4) was determined by allowing the naphthalene to pass through the packed-bed reactor at different flow rates (100, 125, 150, 175 and 200 ml h^{-1}).

The results showed the complete utilization of naphthalene at concentrations of 20 mmol and 40 mmol by the immobilized bacteria (1.8×10^{11} cfu g^{-1} beads) in all the matrices at the above flow rates. A 60-mmol quantity of naphthalene was completely degraded by the immobilized cells in all the matrices at flow rates of 100, 125 and 150 ml h^{-1}. For the same amounts of naphthalene, at flow rates of 175 ml h^{-1} and 200 ml h^{-1}, alginate-entrapped cells degraded 56 mmol and 49 mmol, agar-entrapped cells degraded 60 mmol and 58 mmol

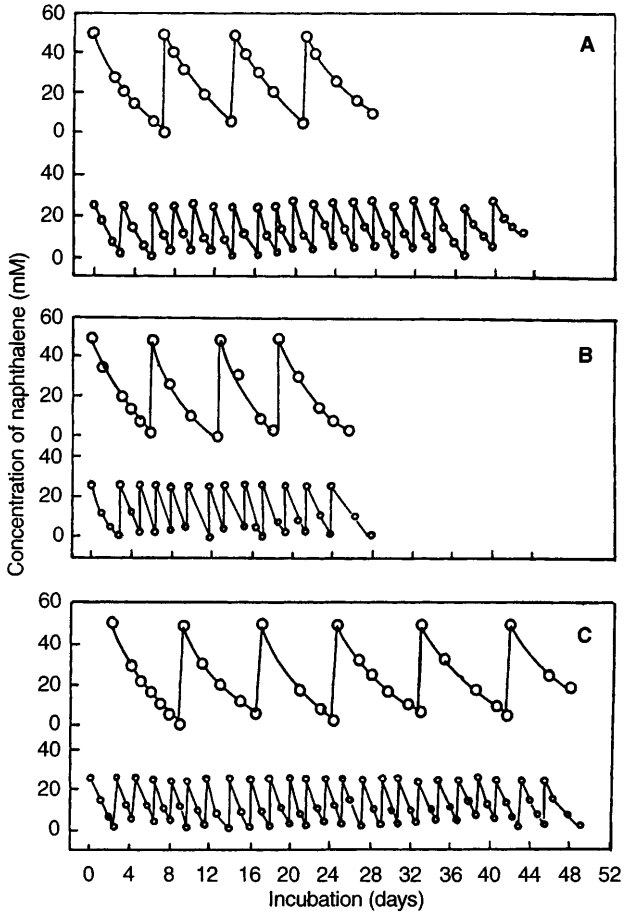

Fig. 3A–C Semicontinuous degradation of naphthalene by cells immobilized in alginate (**A**), agar (**B**) or polyacrylamide (**C**). Naphthalene loading: 25 mmol and 50 mmol; cell concentrations: 1.8×10^{11} cfu g^{-1} beads

and polyacrylamide-entrapped cells degraded 59 mmol and 54 mmol naphthalene respectively. When the concentration of naphthalene was increased to 80 mmol, alginate-entrapped cells degraded 80, 79, 66, 58 and 50 mmol naphthalene at flow rates of 100 ml, 125 ml, 150 ml, 175 ml and 200 ml h^{-1}. Agar-entrapped cells degraded 80 mmol naphthalene at flow rates of 100 ml h^{-1} and 125 ml h^{-1}, whereas 79 mmol, 71 mmol and 63 mmol naphthalene were degraded by the agar-entrapped cells, at flow rates of 150 ml, 175 ml and 200 ml h^{-1} respectively from an initial 80 mmol naphthalene. Polyacrylamide-entrapped cells degraded 80 mmol naphthalene at a flow rate of 100 ml h^{-1}. From 80 mmol naphthalene polyacrylamide-entrapped cells degraded 78 mmol, 72 mmol, 64 mmol and 58 mmol at flow rates of 125 ml, 150 ml, 175 ml and 200 ml h^{-1} respectively. It was also observed that for an initial 40 mmol naphthalene load with a flow rate of 150 ml h^{-1} and for 60 mmol naphthalene with a flow rate of 100 ml h^{-1}, the packed-bed reactor was efficiently operated continuously for 45 days with alginate-, 40 days with agar- and for more than 60 days with polyacrylamide-entrapped cells.

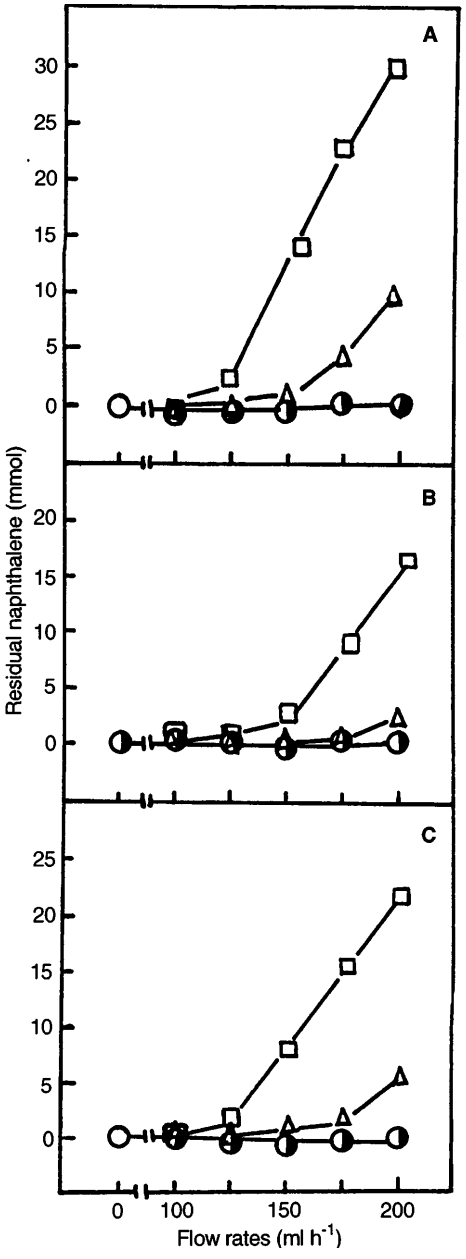

Fig. 4A–C Continuous degradation of naphthalene by immobilized cells in a packed-bed reactor at flow rates of 100 ml, 125 ml, 150 ml, 175 ml and 200 ml h^{-1}. Alginate- (**A**), agar- (**B**) and polyacrylamide-entrapped cells (**C**). ○, ●, △, □ Residual naphthalene (mmol) from initial loadings of 20, 40, 60 and 80 mmol respectively

However, a release of cells from alginate and agar beads was observed during fermentation studies.

Discussion

The degradation of naphthalene by freely suspended cells and *Pseudomonas* sp. strain NGK 1 (NCIM 5120) immobilized in alginate, agar and polyacrylamide was

compared. In freely suspended cell cultures, the results indicate that there was no significant change in the degradation of naphthalene with differing cell concentrations. The complete degradation of naphthalene was observed only at a lower initial loadings (25 mmol) irrespective of the cell concentrations. Free cells degrade naphthalene without a lag period. The data obtained from the degradation of naphthalene by immobilized cells in all the matrices with batch cultures suggest that the rate of degradation of naphthalene even at higher loadings (50 mmol and 75 mmol) was much higher than that with freely suspended cells. This may be due to a kind of membrane stabilization, which is assumed to be responsible for the cell protection and better degradation rates in the entrapped cells. However, the fact that the rate of degradation of naphthalene was initially low in all the matrices may be attributed to the slow diffusion of the compound into the gel beads. The results obtained from the continuous fermentation studies suggest that the rate of degradation of naphthalene was effective even at high concentrations. The complete degradation of 60 mmol naphthalene 150 ml^{-1} h^{-1} was achieved by the bacteria entrapped in alginate and polyacrylamide gel beads, and the same amount of naphthalene 175 ml^{-1} h^{-1} was degraded by agar-entrapped cells. Alginate- and polyacrylamide-entrapped cells brought about the complete degradation of 80 mmol naphthalene 100 ml^{-1} h^{-1}. Whereas 80 mmol naphthalene 125 ml^{-1} h^{-1} was degraded by agar-entrapped cells. The complete degradation of 50 mmol naphthalene occurred after 6 days of incubation with immobilized cells in batch cultures. Alginate-, agar- and polyacrylamide-entrapped cells in batch cultures with 25 mmol naphthalene could be reused 18, 12 and 23 times respectively. Immobilized cells in a packed-bed reactor degraded naphthalene continuously at rates of 40 mmol 150 ml^{-1} h^{-1} and 60 mmol 100 ml^{-1} h^{-1} for 45 days with alginate, 40 days with agar and more than 60 days with polyacrylamide. The very slow degradation of naphthalene in batch cultures may be due to the limited air supply. It was observed that, during the fermentation process, some cells were released from the agar gel beads into the medium, because of the high porosity of the agar gel. Although polyacrylamide is toxic to some microorganisms, it has been used frequently for cell-entrapment studies.

The study reveals the more efficient degradation of naphthalene by immobilized *Pseudomonas* strain NGK 1 in all the matrices tested when compared to free cells. This culture is also capable of degrading other polycyclic aromatic hydrocarbons, namely anthracene and phenanthrene (data not shown). Thus there is a potential for the development of microbial technology for the treatment of effluents containing aromatic hydrocarbons.

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