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Degradation of Salicylic Acid by Free and Immobilized Cells of *Pseudomonas* sp. Strain NGK1

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Abstract A *Pseudomonas* sp. strain NGK1 (NCIM 5120) capable of utilizing salicylate was immobilized in alginate and polyurethane foam (PUF). The degradation rate of salicylate by freely suspended cells was compared with the degradation rate by immobilized cells. In an initial 20 and 40 mM salicylate, free cells (2×10^{11} cfu ml^{-1}) degraded to 16 and 14 mM, alginate-entrapped cells degraded to 18 and 26 mM, and PUF-entrapped cells degraded to 20 and 32 mM salicylate, respectively, in batch cultures. The alginate- and PUF-entrapped cells were used in repeated batch and continuous culture systems. The efficiency of both the immobilized systems for the degradation of salicylate was compared. It has been observed that the PUF-entrapped cells could be reused for more than 20 cycles whereas alginate-entrapped cells could be reused for a maximum of only 12 cycles, after which a decrease in degradation rate was observed with the initial 20 and 40 mM salicylate. The continuous degradation of salicylate by freely suspended cells showed a negligible degradation rate of salicylate when compared with immobilized cells. With the immobilized cells in both alginate and polyurethane foam, the degradation rate increased with an increase in the dilution rate up to 2 h^{-1} for 20 mM, and 1.5 h^{-1} for 40 mM salicylate. The results revealed that PUF-entrapped cells were more efficient for the degradation of salicylate than alginate-entrapped cells and freely suspended cells.

Key words: Degradation, immobilization, salicylate, polyurethane foam, alginate, *Pseudomonas* sp. strain NGK1

The most important contribution of microorganisms to the environment is the degradation/mineralization of different organic compounds, many of which have been identified as pollutants. Salicylate is a key intermediate in the catabolism of aromatic compounds by various microorganisms commonly occurring in the environment [13]. There are several reports on the degradation of salicylate by various microorganisms using free cells [8, 21]. However, the degradation of salicylate by immobilized cells has not yet been investigated.

Immobilized cells have been used extensively for the production of useful chemicals and the degradation of wastewater pollutants [6]. Cell immobilization is one of the most attractive methods in avoiding problems inherent in the use of free bacteria because it allows ease of handling and cell separation. The main advantages in the use of immobilized whole microorganisms are their higher operational stability, ease of use in continuous reactors, high-cell density, and ability to scaleup [10]. Many microorganisms have been immobilized by entrapment and adsorption methods. The potential for using immobilized cells in industrial processes is considerable [3, 5]. Cells at different stages (viable, resting, dead) have been successfully entrapped in various matrices and their ability to degrade the various organic compounds has been studied [4, 14-16, 18, 20]. Bioremediation using immobilized cells has been widely investigated for numerous toxic chemicals such as phenol [2], pyridine [12], dimethylphthalate [18], naphthalene [14, 15], and 2-methylnaphthalene [19]. In the present study, the degradation of salicylate by freely suspended and immobilized cells of *Pseudomonas* sp. strain NGK1 in alginate and polyurethane foam (PUF) has been investigated. The rates of degradation of salicylate by immobilized cells in these matrices were compared with that of freely suspended cells in batches, repeated batches, and continuous degradations. Furthermore, the immobilized cells were reused for the efficient degradation of salicylate in shaken cultures. The impact of the dilution rate in a continuous system on the degradation of salicylate has also been studied.

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MATERIALS AND METHODS

Chemicals

Salicylate was procured from Central Drug House, Chemicals, Mumbai, India; sodium alginate was obtained from S.D. Fine Chemicals, Mumbai, India. Polyurethane foam was purchased from a local supplier, M/s. Udayagiri Industries, Gulbarga, India. The foam was elastic and its low density (16 $kg \cdot m^{-3}$) provided a large surface area. All other chemicals used in this study were of analytical grade.

Microorganism

A *Pseudomonas* sp. strain NGK1 (NCIM 5120) was originally isolated in this laboratory by the naphthalene enrichment culture method [13]. This strain is capable of utilizing salicylate as the sole source of carbon and energy. It was used for our studies and maintained on the slants of a salicylate mineral salts medium solidified with 2% (w/v) agar.

Medium

Two different media were used in this study. The medium used for the precultivation of *Pseudomonas* sp. strain NGK1 was a mineral salts medium containing ($g \cdot l^{-1}$) K_2HPO_4, 6.3; KH_2PO_4, 1.83; $(NH_4)_2NO_3$, 1.0; $MgSO_4$, 0.1; $MnSO_4 \cdot 7H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.1; $FeSO_4$, 0.1, and $NaMoO_3$, 0.005. The pH was adjusted to 7.0 and the medium was supplemented with 0.1% (w/v) salicylate. The medium used for the fermentation studies contained ($g \cdot l^{-1}$) K_2HPO_4, 0.38; $MgSO_4 \cdot 7H_2O$, 0.1; KNO_3, 1.0, and $FeSO_4$, 0.05. The pH was adjusted to 7.0 and sodium salicylate at different concentrations (20 and 40 mM) was added after sterilization of the medium.

Immobilization

Pseudomonas sp. strain NGK1 was grown in the mineral salts medium containing 0.1% (w/v) salicylate as the sole source of carbon and energy. The cells were harvested during the mid-logarithmic growth phase by centrifugation at 5,000 ×g for 10 min at 5°C and washed twice with potassium phosphate buffer (50 mM of pH 7). The washed cells were then immobilized in PUF and alginate.

Immobilization of Cells in Alginate

The alginate-entrapment of the cells was performed according to the method of Bettemann and Rehm [2]. Alginate (2% w/v) was dissolved in boiling water and autoclaved at 121°C for 15 min. A 50-ml bacterial cell suspension (20 g wet weight/50 ml sterilized alginate solution) was added to 200 ml sterilized alginate solution (2% w/v) and mixed by stirring on a magnetic stirrer. This alginate-cell mixture was extruded drop by drop into a cold, sterile 0.2 M $CaCl_2$ solution. Gel beads of approximately 2 mm diameter were obtained. The beads were hardened by resuspending in a fresh $CaCl_2$ solution for 2 h with gentle agitation. Finally, these beads were washed with distilled water and used for experimentation. 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 [14].

Immobilization of Cells in Polyurethane Foam

The PUF-immobilization was carried out by the method of Hall and Rao [9]. The PUF was cut into approximately 4 mm cubes, washed twice with distilled water, and dried. The freshly grown bacterial cell suspension (100 ml, 6×10^{10} $cfu \cdot ml^{-1}$) was added to a 500-ml conical flask containing sterilized foam cubes (4 g). The content of the flask were mixed by stirring on a magnetic stirrer for 2 h. The flask was kept on a rotary shaker for 1 h at 120 rpm and left undisturbed for another 2 h. The medium was removed and foam cubes containing the immobilized bacteria were washed with saline. The decanted bacterial suspension and the saline wash were combined and the bacterial population in the mixture was counted by the spread-plate method.

Design of the Reactor for the Continuous Degradation of Salicylate

A cylindrical glass column (4×50 cm, volume 650 ml) with outlet facilities at 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 22 cm. The column was attached to a reservoir of the salicylate mineral salts medium, and kept on a magnetic stirrer for proper mixing of the salicylate in the medium. The medium was then continuously fed into the column with the help of a peristaltic pump (Miclins, India) through a side arm near the bottom of the column. During the experiments, the dynamic flow of oxygen was maintained at 1 bar (10^5 Pa) throughout the entire system, through the bottom of the column. The effluent was continuously removed from the side arm situated just above the packed bed.

Fermentation Conditions

Batch Fermentations. Batch fermentations were performed with both freely suspended cells and immobilized cells in these matrices. With the freely suspended cell culture, exponentially growing cells (2×10^{13} cfu 100 ml^{-1}) were added to 250-ml conical flasks containing 100 ml of salicylate (20 and 40 mM) mineral salts medium. The fermentation process was carried out at room temperature (~34°C) on a rotary shaker at 150 rpm for 12, 24, 36, 48, 60, and 72 h incubation periods. Samples from the culture broth were withdrawn under sterile conditions at the above-indicated time intervals for the analysis of residual salicylate.

Batch fermentations with the immobilized cells were performed by taking 6 g wet beads of alginate-entrapped cells (2×10^{12} cfu·6 g^{-1}) and 4 g of PUF-entrapped cubes (1.2×10^{12} cfu·4 g^{-1}) into the 250-ml conical flasks containing 100 ml of mineral salts medium, at two different concentrations of salicylate (20 and 40 mM). The flasks were incubated on a rotary shaker under identical fermentation conditions as shown above. The control experiments were carried out in parallel for the above-indicated salicylate loadings.

Repeated Batch Fermentations. For establishing the long-period stability of salicylate degradation by immobilized cells in both the matrices, repeated batch fermentations were carried out. After each cycle of incubation (72 h/cycle), the used medium was decanted and beads were washed with sterile water and transferred into a fresh sterile mineral salts medium containing salicylate. The fermentation process was carried out as described above and the residual salicylate in the spent medium was analyzed.

Continuous Fermentations. The continuous fermentations were carried out in a packed bed reactor. The degradation of salicylate (20 and 40 mM) was performed by the supplementation of salicylate-mineral salts medium into the reactor at different dilution rates. The reactor was packed with 150 g wet alginate-entrapped beads (3.2×10^{11} cfu·g^{-1}) and 50 g PUF-entrapped foam cubes (3×10^{11} cfu·g^{-1}) to a height of 22 cm with a working volume of 100 ml. For free cells in the continuous degradation studies, the cells (2×10^{11} cfu·ml^{-1}) were suspended in a fermentation medium and this cell suspension was placed in the reactor. The effluents were analyzed for the degradation of salicylate [1] by free and immobilized cells, for each set of experiments.

RESULTS

Degradation of Salicylate by Freely Suspended and Immobilized *Pseudomonas* sp. Strain NGK1 in Batch Cultures

The degradation of 20 and 40 mM of salicylate was carried out both by freely suspended cells and cells immobilized in sodium alginate and PUF. The results obtained with 20 and 40 mM salicylate in batch cultures of free cells, and of alginate- and PUF-entrapped cells are given in Fig. 1. It is evident that the freely suspended cells degraded 16 mM of salicylate after 72 h of incubation from an initial 20 mM of salicylate. With the same cell population, only 14 mM of salicylate was degraded when the initial concentration of salicylate was increased to 40 mM. No further degradation of salicylate was observed even after 6 days of incubation. However, there was no significant change in the degradation rate of salicylate when the cell population was doubled (data not shown). The cells entrapped in alginate degraded

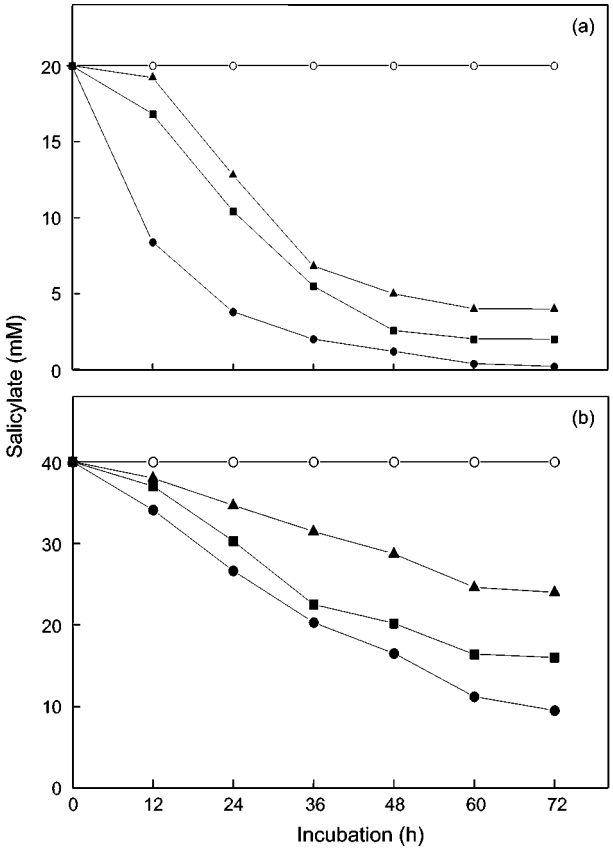

Fig. 1. Degradation of salicylate in batches with (a) 20 mM and (b) 40 mM.
(○), (▲), (■), and (●) are uninoculated control, freely suspended cells (2×10^{11} cfu·ml^{-1}), alginate (3.2×10^{11} cfu·g^{-1}), and polyurethane foam (3×10^{11} cfu·g^{-1}) immobilized cell systems, respectively.

18 and 26 mM of salicylate from an initial 20 and 40 mM salicylate load, respectively, whereas, the results obtained from PUF-immobilized cells revealed that the initial 20 mM of salicylate was completely degraded after 72 h of incubation, and a maximum of 32 mM of salicylate was degraded from an initial 40 mM of salicylate load (Figs. 1a and 1b).

Semi-Continuous Degradation of Salicylate by Immobilized *Pseudomonas* sp. Strain NGK1

The repetitive degradation of salicylate by cells entrapped in alginate and PUF was carried out using two different salicylate loadings (20 and 40 mM) for 72 h of incubation. The results of these studies are shown in Fig. 2. It was observed that, for an initial 20 mM of salicylate, the alginate-entrapped cells could be reused only for a maximum of 12 cycles, after which a slight decrease in the degradation rate was observed (Fig. 2a). In contrast, the PUF-entrapped cells could be reused for more than 20 cycles without losing their salicylate degrading capacity. When the initial concentration of salicylate was increased to 40 mM, the immobilized cells could also be reused, but with a

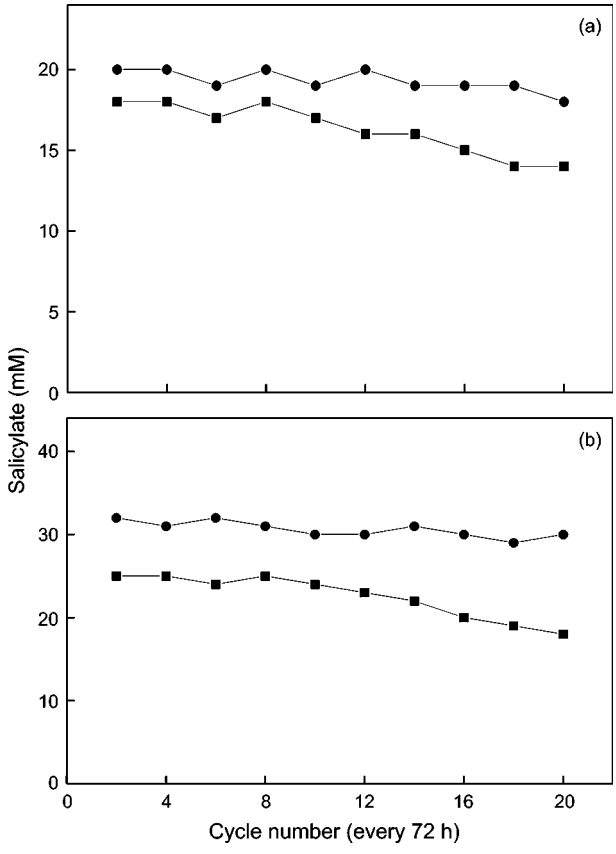

Fig. 2. Semi-continuous degradation of salicylate at 20 mM (a) and 40 mM (b).
(■) and (●) are alginate (3.2×10^{11} cfu·g^{-1}) and polyurethane foam (3×10^{11} cfu·g^{-1}) immobilized cell systems, respectively.

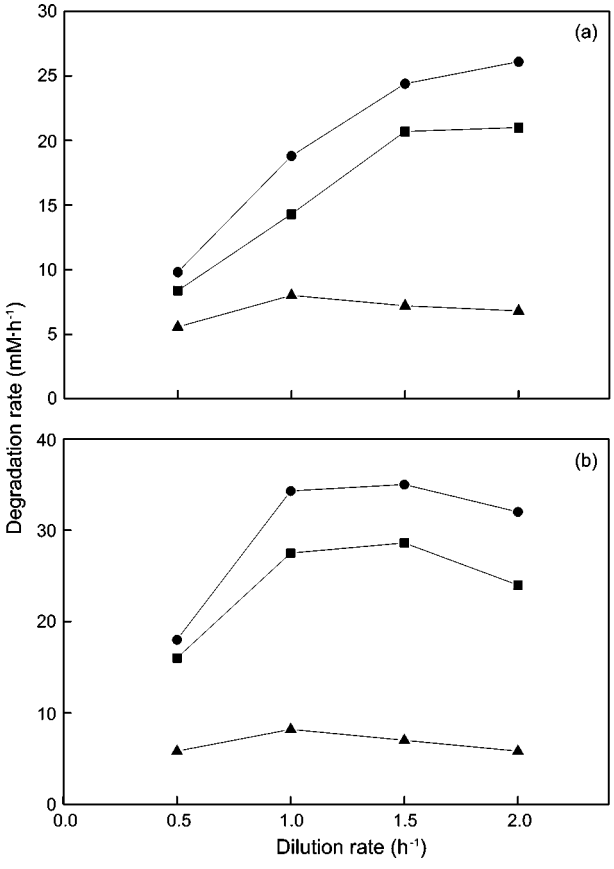

Fig. 3. Continuous degradation of salicylate at 20 mM (a) and 40 mM (b) in a packed bed reactor.
(▲), (■), and (●) are freely suspended cells (2×10^{11} cfu·ml^{-1}), alginate (3.2×10^{11} cfu·g^{-1}), and polyurethane foam (3×10^{11} cfu·g^{-1}) immobilized cell systems, respectively.

decreased salicylate degradation rate (Fig. 2b). However, the degradation rate of salicylate by alginate-entrapped cells was shown to decline after 9 cycles, although PUF-entrapped cells still showed an enhanced degradation capacity. Based on the above results, it is clear that the PUF-entrapped cells are more efficiently degrading compared to the alginate-entrapped cells. The results further demonstrate that the lower amounts of salicylate (20 mM) can be fed at a much higher frequency than higher amounts of salicylate (40 mM).

Continuous Degradation of Salicylate by Freely Suspended and Immobilized *Pseudomonas* sp. Strain NGK1

The continuous degradation of salicylate in a packed bed reactor by both freely suspended cells, and cells immobilized in alginate and PUF, was studied. The results are presented in Fig. 3. The impact of the dilution rate on the rate of degradation of salicylate in both alginate and PUF immobilized systems was compared, by allowing salicylate (20 and 40 mM) to pass through the packed bed reactor at different dilution rates (0.5, 1, 1.5, and 2 h^{-1}).

The results of an initial 20 mM salicylate in a continuous system by freely suspended cells showed that an increase in the dilution rate up to 1 h^{-1} enhanced the degradation rate, and at higher dilution rates (2 h^{-1}), the degradation rate was decreased. In contrast, the cells immobilized in alginate and PUF showed that the degradation of salicylate increased with an increase in the dilution rate up to 1.5 h^{-1}, and even at dilution rate 2 h^{-1}, there was no significant decrease in the degradation rate of salicylate (Fig. 3a). When the initial concentration of salicylate increased to 40 mM, the degradation rate by freely suspended cells showed no significant increase with an increase in the dilution rate from 0.5 to 1 h^{-1}, and at the dilution rate above 1 h^{-1}, the degradation rate was significantly decreased. In contrast, the immobilized cells showed an increased degradation rate with an increase in the dilution rate from 1.0 h^{-1} to 1.5 h^{-1}, while a decrease in the degradation rate at dilution rate 2 h^{-1} was observed in alginate-entrapped cells. However, the PUF-immobilized cells, at a dilution rate of 2 h^{-1}, still showed a higher degradation rate than the alginate-entrapped cells (Fig. 3b).

DISCUSSION

The degradation rates of salicylate caused by freely suspended cells of *Pseudomonas* sp. strain NGK1, and by cells immobilized in alginate and PUF, were compared. In batch cultures, the freely suspended cells degraded salicylate only at the lower initial loading (20 mM). The degradation rate decreased at a higher initial salicylate loading (40 mM). The data obtained from the degradation of salicylate by immobilized cells of both the matrices in the batch cultures suggest that the rate of degradation of salicylate was much higher than that of freely suspended cells, even at higher loadings (40 mM). However, the fact that the rate of degradation of salicylate was initially high in PUF-entrapped cells (Fig. 1a) might be attributed to the free diffusion of the substrate into the foam cubes. The increased degradation by immobilized cells was probably due to the accelerated reaction rates caused by the high local cell density or on the immobilized matrix. Such observations have been reported in the degradation of other chemicals and in the production of fuels and chemicals [9]. Immobilization may also provide a kind of membrane stabilization, which is assumed to be responsible for the protection of cells and better degradation rates in immobilized cells [15].

The results of semi-continuous degradation revealed that the PUF-entrapped cells retained their salicylate degrading ability for a longer period (60 days) than the alginate-entrapped cells. The enhanced degradation of salicylate by PUF-immobilization of cells is probably due to porosity, mechanical strength, hydrophobicity, and hydrophilicity of PUFs. The storage stability and microbial activity of immobilized cells in PUFs are known to be occasionally better than those cells immobilized in alginate [7]. However, the alginate-entrapped cells showed a lower degradability of salicylate with an increased cycle number even though there was lower (10^4 cfu/ml) cell leakage. The mechanical instability of alginate-entrapped beads finally led to cell leakage from these beads, thus decreasing the degradation rate with the increased cycle number [20]. This may be due to a restriction of diffusion of the substrate inside the beads.

The results of continuous degradation of salicylate in a packed-bed reactor suggest that the increase in the dilution rate increased the degradation rate of salicylate, but only when it was kept below 1.5 h^{-1} for 20 mM of salicylate and 1 h^{-1} for 40 mM of salicylate, and beyond these ranges the degradation rate decreased (Figs. 3a and 3b). This saturation in a response curve was also encountered by Lakhwala *et al.* [11], who attributed the effect to an increase in oxygen demand by a cell population that had increased in size. The immobilized system is much better to degrade salicylate than the freely suspended cells at dilution rates above 1 h^{-1}. Superiority of the immobilized cell systems is more pronounced with the higher dilution rate. This is probably based on the total number of viable cells available for bioreaction compared to freely suspended cells. The decrease in the degradation rate of free cells at higher dilution rates was probably due to the formation of flocs at these higher dilution rates, and the washout of unflocculated cells contributing to a loss of degradative capability. Similar observations were reported for the degradation of 2-methylnaphthalene by this bacterium [19], and for the degradation of phenol by immobilized *Pseudomonas putida* [17].

The present study indicated that a more efficient and long-term degradation of salicylate at high concentrations was achieved by immobilized *Pseudomonas* sp. strain NGK1 in both the matrices tested when compared to freely suspended cells. Further, the PUF-immobilized cells had a higher degradation rate than alginate-entrapped cells. The operational stability and longevity of cells immobilized in PUF was significantly better than alginate. The biodegradation of organic molecules with immobilized bacterial strains in PUF is an extremely versatile approach that can be used in the detoxification of different pollutants for much longer periods of time than with nonimmobilized microorganisms.

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