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Production of Salicylic Acid from Naphthalene by Immobilized *Pseudomonas* sp. Strain NGK1

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Abstract The *Pseudomonas* sp. strain NGK1 (NCIM 5120) was immobilized in calcium alginate, agar, and polyacrylamide gel matrices. The salicylic acid-producing capacity of freely suspended cells was compared with immobilized cells in batches with a shake culture and continuous culture system in a packed bed reactor. Freely suspended cells (4×10^{10} cfu/ml) produced 12 mM of salicylic acid, whereas cells immobilized in calcium alginate (1.8×10^{11} cfu/g beads), agar (1.8×10^{11} cfu/g beads), and polyacrylamide (1.6×10^{11} cfu/g beads) produced 15, 11, and 16 mM of salicylic acid, respectively, from naphthalene at an initial concentration of 25 mM. The continuous production of salicylic acid from naphthalene was investigated in a continuous packed bed reactor with two different cell populations. The longevity of the salicylic acid-producing activity of the immobilized cells from naphthalene was also studied in semicontinuous fermentations. The immobilized cells could be reused 18, 13, and more than 20 times without losing salicylic acid-producing activity in calcium alginate-, agar-, and polyacrylamide-entrapped cells, respectively. The study reveals a more efficient utilization of naphthalene and salicylic acid production by the immobilized *Pseudomonas* sp. strain NGK1 as compared to the free cells.

Key words: *Pseudomonas* sp., salicylic acid, naphthalene, immobilization, fermentation

In recent years, there has been intensive research and development in the immobilization of microbial cells for their use as biocatalysts. The immobilization of microorganisms offers an easy to handle biocatalyst, which has great advantages in several processes [5, 7, 8, 11, 15, 18, 21, 22]. Although there are various methods of immobilization, the cell-entrapment technique has been most widely accepted [2, 5, 9, 11, 19, 20]. The cells at different stages have been successfully entrapped in various matrices, and potential use of immobilized cells in industrial processes is regarded as valuable applications [3, 5, 6, 14, 20, 21].

Salicylic acid is used as a precursor for the synthesis of substituted salicylates, which have therapeutic values. Salicylic acid has been isolated as an intermediate from naphthalene-utilizing microorganisms [1, 23, 24]. Subsequent investigations have focussed on the elucidation of the metabolic pathways of naphthalene in various microorganisms [10, 12, 13, 16]. Salicylic acid is produced petrochemically and apparently there is no source and economical method currently available to synthesize salicylic acid. The microbial production of salicylic acid from naphthalene has a substantial value for specific chemical industries.

The *Pseudomonas* sp. strain NGK1 was isolated by the enrichment culture method [16]. Salicylic acid can be produced continuously from naphthalene by this bacterium without the addition of any expensive cofactors. This paper examines a possibility of producing salicylic acid from naphthalene with microbial cells. In addition, the rates of production of salicylic acid from naphthalene by the freely suspended cells of the *Pseudomonas* sp. strain NGK1 are compared with those of the immobilized cells in alginate, agar, and polyacrylamide.

Materials and Methods

Microorganism and Culture Conditions

The *Pseudomonas* sp. strain NGK1 (NCIM 5120) used in this investigation was isolated from biological wastewater treatment effluent by the enrichment culture method [16, 17]. The culture was maintained on a Luria-Bertani (LB) agar medium. The *Pseudomonas* sp. strain NGK1 was grown in a mineral salts medium with the following compositions (g/l): K_2HPO_4, 0.15; $MgSO_4 \cdot 7H_2O$, 0.2; NH_4Cl, 1.0; $FeCl_3$, 0.05, and $CaCl_2 \cdot 2H_2O$, 0.2. The pH of the medium was adjusted to 7.0. Naphthalene was dissolved in a minimum

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amount of dimethylformamide (DMF) and the solution was forced into the mineral salts medium (containing Tween-80 at approximately 80× critical micelle concentration) with a syringe. This medium was then twice subjected to ultrasonication (ultrasonicator Vibra-cell model 375, U.S.A.) for 1 min. The final naphthalene-mineral salts medium appeared milky.

Authentic salicylic acid was procured from Aldrich Chemical Co. (U.S.A.). Naphthalene was purchased from Standard Drug Fine Chemicals (India). Sodium alginate was obtained from British Drug House (India). Agar was procured from Qualigens (India). Acrylamide and *N,N,N',N'*-tetramethylethylenediamine were purchased from Fluka (India). All other chemicals used in this study were of analytical grade.

Analytical Methods

Analyses of salicylic acid and naphthalene were carried out using HPLC and UV spectrophotometric methods. The spent medium was extracted with ether and the residue was dissolved in methanol and analyzed by a reverse-phase high performance liquid chromatography (Shimadzu HPLC, Japan) with a UV detector (wavelength 276 nm for naphthalene and 234 nm for salicylic acid). A Shimpack CLC-C8 (M) Octadecylsilane (ODS) column (4.6×15 cm) was employed with methanol/water (9:1) at a flow rate of 1 ml/min. Salicylic acid and naphthalene were also quantified in the spent medium using a spectrophotometer (UV/Visible spectrophotometer, Shimadzu model 160A, Japan).

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

Immobilization

The naphthalene-grown *Pseudomonas* sp. strain NGK1 cells were harvested during the mid-logarithmic growth phase by centrifugation at 5,000 ×g for 10 min. The cells were immobilized in different matrices: alginate, agar, and polyacrylamide [17].

The alginate-entrapment of the cells was performed according to the method of Betteman and Rehm [3]. The agar-entrapment procedure was carried out according to the method described by Nilson *et al.* [19]. The polyacrylamide-entrapment procedure was carried out according to the methods of Chibata *et al.* [8] and Starostina *et al.* [22].

Design of Reactor for Continuous Production of Salicylic Acid

The 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 30 cm. The column was attached to a reservoir of the naphthalene/mineral salts medium, kept on a magnetic stirrer for proper mixing of the naphthalene in the medium. The medium was then fed into the column continuously with help of a peristaltic pump (Miclins, India) through a side-arm at 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 from an oxygen cylinder. The effluent was continuously removed from the side-arm situated just above the packed bed (30 cm height).

Batch Fermentations

Batch fermentations for the production of salicylic acid were performed in different matrices using both freely suspended cells and immobilized cells. For freely suspended cell culture, exponentially growing cells of *Pseudomonas* sp. were added to 250 ml Erlenmayer flasks containing the mineral salts medium with different cell concentrations (4×10^{10} cfu/ml and 8×10^{10} cfu/ml) and various amounts of naphthalene (25, 50, and 70 mM). 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 analyses of the salicylic acid and naphthalene.

For the immobilized cells, 25 g wet beads (approx. 150 beads) of the respective entrapment matrices were added to a 250 ml conical flask containing 100 ml of the mineral salts medium with various amounts of naphthalene indicated. The initial cell populations were in the range of 1.8×10^{11} cfu/g beads in alginate and agar, and 1.6×10^{11} cfu/g gel beads in the polyacrylamide entrapment. Like the freely suspended cell culture, the entrapped cell cultures in all the matrices were incubated at room temperature on a rotary shaker under identical fermentation conditions. The control experiments for the evaporation of naphthalene were carried out in a sterile medium at the above mentioned naphthalene loadings. The evaporation of naphthalene from the sterile controls was found to be about 0.15 mM/day, which was not used to correct the utilization of naphthalene and production of salicylic acid by this bacterium.

Semicontinuous Batch Fermentations

To determine the long term stability of the immobilized cells of the *Pseudomonas* sp. strain NGK1 in different matrices for salicylic acid production, repeated batch fermentations were carried out. After every 2-3 days during the incubation period, the spent medium was decanted and the beads were washed with water and

transferred into a fresh mineral salts medium containing naphthalene. The fermentation process was carried out under the identical fermentation conditions.

Continuous Fermentations

Continuous fermentations for the production of salicylic acid from naphthalene with two different cell populations (2×10^{11} and 5×10^{10} cfu/g) were performed in a packed bed reactor. The reactor was packed with 250 g of the 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 to 1 bar, also guaranteeing good mixing in the culture medium. The fermentation process was carried out with a continuous supply of the medium with different concentrations of naphthalene at various flow rates. The amounts of salicylic acid and the residual naphthalene were monitored in the effluent for each set of experiments.

Results

Growth Behavior and Utilization of Naphthalene

The *Pseudomonas* sp. strain NGK1 was grown in a mineral salts medium containing NH_4Cl as a source of nitrogen and supplemented with naphthalene (7.8 mM) as the sole carbon and energy source. The maximum growth of the bacterium was observed after 48 h of incubation. The specific growth rate of the bacterium during its exponential growth was calculated to be 0.32/h. The naphthalene utilization by this bacterium was accompanied by a concomitant decrease in the pH of the culture medium from pH 7 to 3.8 [16]. The initial viable cell population of 2×10^5 cfu/ml reached 1.6×10^7 cfu/ml at 24 h of incubation and a maximum of 1×10^8 cfu/ml at 48 h of incubation. Although there was an appearance of turbidity at 48 h of incubation, the culturable cell population fell to 2.8×10^4 cfu/ml at 72 h. The color of the culture medium turned to bright yellow during the growth of the bacterium. The utilization of naphthalene by this bacterium was also observed. A maximum of 6.25 mM naphthalene was utilized by the bacterium from a total of 7.8 mM naphthalene after 60 h incubation. Furthermore, the naphthalene utilization by the bacterium was accompanied by the concomitant production of salicylic acid in the culture medium (Fig. 1).

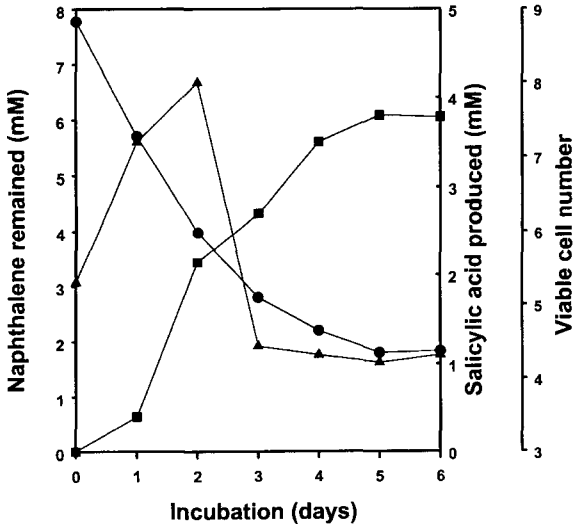

Fig. 1. Utilization of naphthalene and production of salicylic acid by *Pseudomonas* sp. strain NGK1.
Naphthalene utilization (●), salicylic acid production (■), and viable cell number in log (cfu/ml) (▲).

Production of Salicylic Acid by Freely Suspended and Immobilized *Pseudomonas* sp. Strain NGK1

Batch fermentations for salicylic acid production were carried out using both freely suspended cells and cells entrapped in the above matrices. The results on the production of salicylic acid from naphthalene by freely suspended cells are presented in Fig. 2A. It was shown that about 12 and 14 mM of salicylic acid was produced by freely suspended cells (4×10^{10} cfu/ml) from an initial 25 and 50 mM of naphthalene, respectively, after 3 days of incubation. A conversion of 51, 30, and 20% of naphthalene to salicylic acid was produced by the bacterium from an initial 25, 50, and 75 mM of naphthalene, respectively. The salicylic acid formed remained in the culture medium even after 15 days of incubation. The biotransformation of naphthalene to salicylic acid was accompanied with a decrease in the pH of the medium to below 4 [16]. The viable cell population in this system fell to 5×10^9 cfu/ml and 1×10^8 cfu/ml after 6 and 12 days of incubation, respectively, with 50 mM of naphthalene in the medium.

Batch fermentations for salicylic acid production from naphthalene by immobilized cells of *Pseudomonas* sp. in different matrices were performed as batch cultures with shake flasks. The results of these studies are shown in Figs. 2B, 2C, and 2D. It was evident that the alginate-entrapped cells produced 13 mM, agar-entrapped cells 11 mM, and polyacrylamide entrapped cells 15 mM salicylic acid after 3-4 days of incubation, respectively, from initial 25 mM naphthalene loadings. The salicylic acid formed was utilized after 8, 6, and 7 days of incubation by alginate-, agar-, and polyacrylamide-entrapped cells, respectively. When the initial concentration of naphthalene was increased to 50 mM, it was observed that a maximum of 22 and 16 mM of salicylic acid was produced from the initial 50 mM naphthalene by the alginate- and agar-entrapped cells after 4 and 3 days of incubation, respectively, whereas the polyacrylamide-entrapped cells produced 24 mM of salicylic

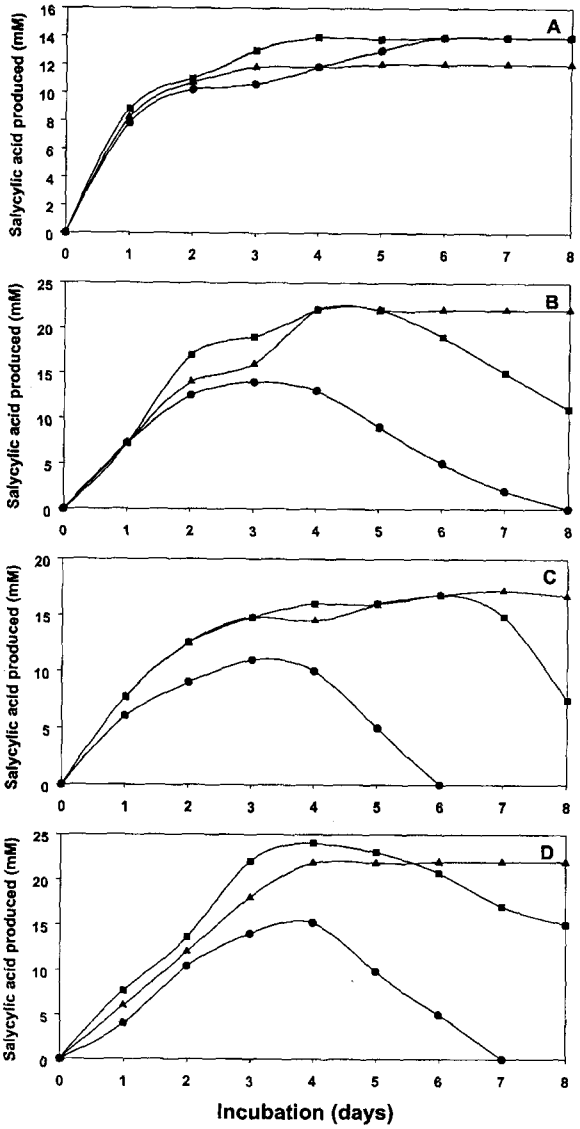

Fig. 2. Production of salicylic acid from different initial naphthalene concentrations

Freely suspended cells (A) and immobilized cells of *Pseudomonas* sp. strain NGK1 immobilized in alginate (B), agar (C), and polyacrylamide (D) in batch culture fermentations containing 25 mM naphthalene (●), 50 mM (■), and 75 mM (▲).

acid after 4 days. Furthermore, when the initial naphthalene concentration was increased to 75 mM, a maximum of about 24 mM of salicylic acid was accumulated in the medium of polyacrylamide-entrapped cells after 4 days of incubation. It was observed that about 52% and 44% biotransformations of naphthalene to salicylic acid occurred with alginate- and agar-entrapped cells, respectively, and 60% with the polyacrylamide-immobilized cells from an initial 25 mM of naphthalene. The rate of production of salicylic acid was slightly higher in the polyacrylamide-entrapped cells when compared to the alginate- and agar-entrapped cells.

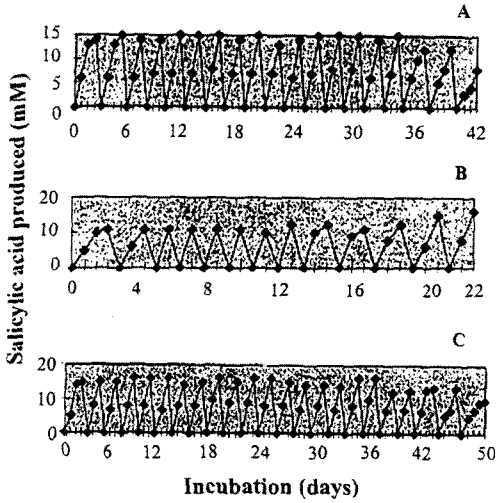

Fig. 3. Semicontinuous production of salicylic acid from naphthalene (25 mM) by cells (1.2×10^{11} cfu/g beads) immobilized in alginate (A), agar (B), and polyacrylamide (C).

Semicontinuous Production of Salicylic Acid by Immobilized *Pseudomonas* sp. Strain NGK1

The semicontinuous production of salicylic acid from naphthalene by immobilized cells was carried out at two different initial naphthalene loadings (25 and 50 mM). It was observed that about 15, 11, and 16 mM of salicylic acid was produced from 25 mM of naphthalene by the cells immobilized in alginate (Fig. 3A), agar (Fig. 3B), and polyacrylamide (Fig. 3C), respectively. When the initial naphthalene concentration was raised to 50 mM, it was observed that 23, 16, and 24 mM of salicylic acid was produced by the alginate-, agar-, and polyacrylamide-entrapped cells, respectively (data not shown). It was also observed that for an initial 25 mM of naphthalene, the alginate-entrapped cells could be reused 18 times, the agar-entrapped cells 12 times, and the polyacrylamide-entrapped cells 23 times without losing their salicylic acid producing activity. Furthermore, about 0.2% cell leakage (10^4 cfu/ml) was observed from the agar-entrapped cell system after 7 transfers, from the alginate-entrapped cell system (10^4 cfu/ml) after 15 transfers, and about 0.1% from the polyacrylamide-entrapped cell system (10^2- 10^3 cfu/ml) after 19 transfers. The stable initial cell population was maintained during repeated fed-batch fermentations with 25 mM of naphthalene by the agar-entrapped cell system for about 22 days, by the alginate-entrapped cell system for about 42 days, and by the polyacrylamide cell system for 50 days.

Continuous Production of Salicylic Acid by Immobilized *Pseudomonas* sp. Strain NGK1

The impact of residence time on the performance of the immobilized cells in a packed-bed reactor during continuous operation for the production of salicylic acid from

naphthalene was carried out with two different cell populations (2×10^{11} cfu/g and 5×10^{10} cfu/g beads) at different flow rates (100, 125, 150, 175, and 200 ml/h). When the continuous packed bed reactor was operated with a cell population of 2×10^{11} cfu/g beads, it was observed that at 20 mM of initial naphthalene, 0.2, 0.3, 1, 1.5, and 2 mM of salicylic acid was produced by the alginate-entrapped cells at flow rates of 100. 125, 150, 175, and 200 ml/h, and the agar entrapped-cells produced 0.1, 0.2, 0.5, 0.9, and 1.4 mM of salicylic acid. In contrast, the polyacrylamide-entrapped cells produced 0.1, 0.2, 0.6, 1.2, and 1.6 mM of salicylic acid at the same flow rates as mentioned above. With a 40 mM initial naphthalene concentration, the alginate-entrapped cells produced 0.3, 0.5, 1.3, 2, and 3 mM of salicylic acid, and the agar-entrapped cells produced 0.1, 0.2, 0.6, 1.1, and 1.8 mM of salicylic acid. In contrast, the polyacrylamide-entrapped cells produced 0.1, 0.3, 0.8, 1.5, and 2.2 mM of salicylic acid at the same flow rates as mentioned above. At a 60 mM naphthalene loading, the alginate-entrapped cells produced 0.5, 0.9, 1.8, 4, and 6.8 mM of salicylic acid, the agar-entrapped cells produced 0.3, 0.4, 0.9, 1.8, and 3.3 mM of salicylic acid, and the polyacrylamide-entrapped cells produced 0.4, 0.7, 1.2, 3, and 4.5 mM of salicylic acid at the same flow rates as mentioned above. From 80 mM of initial naphthalene, the alginate-entapped cells produced 1, 1.8, 3, 4.8, and 7.6 mM of salicylic acid, the agar-entrapped cells produced 0.5, 1.0, 1.8, 3.6, and 4.8 mM of salicylic acid, and the polyacrylamide-entrapped cells produced 1, 1.4, 2.8, 4, and 6.5 mM of salicylic acid at flow rates of 100, 125, 150, 175, and 200 ml/h, respectively. These results are represented in Figs. 4A1, 4B1, and 4C1.

When the continuous packed-bed reactor was operated with a cell population of 5×10^{10} cfu/g beads, the alginate-, agar-, and polyacrylamide-entrapped cells completely utilized 20 and 40 mM of naphthalene. The alginate-entrapped cells produced 11 and 16 mM of salicylic acid, the agar-entrapped cells produced 7 and 11 mM, and the polyacrylamide-entrapped cells produced 11 and 15 mM of salicylic acid from initial 20 and 40 mM of naphthalene, respectively, at a flow rate of 200 ml/h. With 60 and 80 mM of initial naphthalene, the alginate-entrapped cells produced 18 mM of salicylic acid at a flow rate of 200 ml/h. With 60 and 80 mM of naphthalene, the agar-entrapped cells produced 13 mM of salicylic acid at a flow rate of 200 ml/h. The polyacrylamide-entrapped cells at the above mentioned initial naphthalene loadings produced 18 mM of salicylic acid at a flow rate of 200 ml/h (Figs. 4A2, 4B2, and 4C2).

DISCUSSION

It is evident from the above results that the *Pseudomonas* sp. strain NGK1 has an ability to produce salicylic acid in a mineral salts medium when supplemented with naphthalene. The results from the batch fermentation studies indicate that the immobilized cells showed higher abilities to produce salicylic acid (2-3 times) when compared to the freely suspended cells (Fig. 2). It was also observed that the polyacrylamide-entrapped cells produced higher amounts

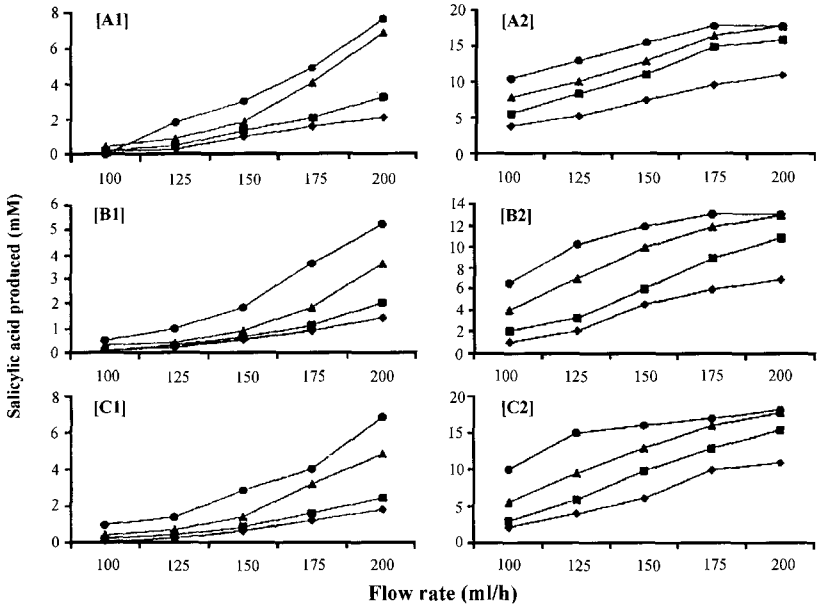

Fig. 4. Continuous production of salicylic acid.
Naphthalene at 20 (◆), 40 (■), 60 (▲), and 80 mM (●) by cells (2×10^{11} cfu/g beads; A1, B1, and C1) and (5×10^{10} cfu/g beads;A2, B2, and C2) of *Pseudomonas* sp. strain NGK1 immobilized in alginate (A), agar (B), and polyacrylamide (C) in a packed-bed reactor under the various flow rates.

of salicylic acid (15 mM) than the alginate- or agar-entrapped cells. Furthermore, semicontinuous fermentation studies showed that the entrapped cells could be reused for the production of salicylic acid without losing their activity (Fig. 3). Thus, the production of salicylic acid could be enhanced with a decreased cell population and increased flow rates (Figs. 4A2 and 4C2 and 4C2) in the continuous packed-bed reactor.

The increased production of salicylic acid by the immobilized cells may be due to membrane stabilization and increased permeability. This may be responsible for the protection of cells and better utilization. Such observations were made by Hall and Rao [14] in the production of fuels and chemicals by immobilized cells. Bisping and Rehm [4] reported the multistep reactions with immobilized cells in the production of alcohol and glycerol. Although polyacrylamide is toxic to some microorganisms, it has been frequently used for entrapment studies [9, 17]. It was observed that, during the fermentation process, about 0.2% of the cells were released from the agar gel beads into the medium. This may be due to the high porosity of the agar gel. Accordingly, in all matrices tested, the present study reveals a more efficient utilization of naphthalene and salicylic acid production by the immobilized *Pseudomonas* sp. strain NGK1 as compared to the free cells.

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