Studies on Neomycin Production Using Immobilized Cells of *S marinensis* NUV-5 in Various Reactor Configurations: A Technical Note

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Bandi Srinivasulu,[1] Poluri Ellaiah,[1] and Kunamneni Adinarayana[1]

[1]Pharmaceutical Biotechnology Division, Department of Pharmaceutical Sciences, Andhra University, Visakhapatnam-530 003, India

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INTRODUCTION

Most of the available reports on antibiotic production with immobilized cells are confined to shake flask studies, and only a few reports are available on the bioreactor studies. Successful operation of a 3-phase fluidized bed reactor using immobilized *Penicillium urticae* with 35% higher patulin antibiotic production was reported by Berk et al.[1] Sarra et al[2] showed continuous production of antibiotic by *Streptomyces livigans* in a 3-phase fluidized bed reactor with self-aggregated immobilized cells for longer periods of time. In addition, reports are also available on nutritional influence on effective continuous production of bacitracin, patulin, and penicillin by various organisms.[3-5] Park et al[6] have demonstrated the enhanced production of neomycin in an airlift bioreactor by partial immobilization of *Streptomyces fradiae* on cellulose-immobilized beads and by reducing the high viscosity of fermentation broth. The success achieved by other investigators on continuous production of different antibiotics prompted us to conduct studies on continuous production of neomycin.

The purpose of the present investigation was to study the neomycin production in various reactor configurations and selection of a suitable reactor for neomycin production with immobilized cells. For the selection of a suitable reactor, stirred tank, packed bed, fluidized bed, and airlift reactors were employed.

Corresponding Author: Poluri Ellaiah, Pharmaceutical Biotechnology Division, Department of Pharmaceutical Sciences, Andhra University, Visakhapatnam-530 003, India. Phone: +91-891-2505159; Fax: +91-891-2755547; Email: adikunamneni@rediffmail.com

MATERIALS AND METHODS

Materials

All the chemicals and medium constituents used in this study were procured from Hi-Media (Mumbai, India). Sodium alginate was procured from Loba Chemie (Mumbai, India).

Culture

A mutant strain of *Streptomyces marinensis* NUV 5, producer of neomycin, was used in the present study. The strain was isolated from seawater of Bay of Bengal, Visakhapatnam, India.[7] It was maintained on jowar starch agar slants at 4°C and subcultured at every 4 weeks. A test organism, *Staphylococcus epedermidis* National Collection of Industrial Microorganisms (NCIM) 2493 was used for the microbiological assay of neomycin.

Inoculum Preparation

The organism was grown on jowar starch agar slants at 30°C for 7 days for complete sporulation. Five milliliters of sterile water was added to the slant and the spores were scraped and transferred into a 250-mL Erlenmeyer flask containing 50 mL of inoculum medium. The inoculum medium was composed of soluble starch 2.5% (wt/vol), corn steep liquor 1.0%, $(NH)_2 SO_4$ 0.5% (wt/vol), NaCl 0.5% (wt/vol), $CaCO_3$ 0.5% (wt/vol) and pH 7.5. The flasks were incubated at 30°C in shaker incubator (at 220 rpm) for 48 hours. The microorganism cells were harvested and washed with sterile saline solution and the cells were resuspended in 25 mL sterile saline solution. This cell suspension was used as inoculum for immobilization as well as for free cell fermentations.

Immobilization of Cells in Calcium Alginate

Cells were immobilized using sodium alginate by the ionotrophic method.[8] The cell suspension (0.3% cells on dry cell basis) was thoroughly mixed with 2% alginate slurry, and the mixture was extruded into 0.2M $CaCl_2$ solution to form spherical beads using peristaltic pump through small orifice. The beads thus formed were cured for 2 hours by incubating in 0.2M $CaCl_2$ solution. The beads were thoroughly washed with sterile distilled water, preserved in saline (0.9% NaCl wt/vol solution), and stored in saline solution at 4°C for further use. The diameter of the randomly selected known number of beads was calculated using Vernier calipers, and the mean value was taken. Bead diameter ranged from 3.23 to 3.25 mm with the average diameter being 3.24 mm. All operations were carried out under aseptic conditions.

Fermentation with Free Cells and Immobilized Cells

The cells/beads equivalent to 0.06% dry cell weight (DCW) were added to the reactor with production medium. The immobilized beads were activated in the production medium for 72 hours at 30°C, and the broth was drained. The reactor was fed with fresh production medium and the fermentation was continued for 6 days. Periodically (every 24 hours) samples were withdrawn and evaluated for cell mass and neomycin titer. The following medium was used for production strategies. The production medium was composed of maltose, 4% (wt/vol); sodium glutamate, 1.2% (wt/vol); K_2HPO_4, 0.010% (wt/vol); $MgSO_4.7H_2O$, 0.05% (wt/vol); $CaCl_2$. $2H_2O$, 0.01% (wt/vol); $FeSO_4.7H_2O$, 0.005% (wt/vol); $ZnSO_4.7H_2O$, 0.0005% (wt/vol) with pH 8.0.

Bioreactor Configurations

Stirred Tank Reactor

A 2-L stirred tank reactor (STR) (Biostat A, B. Braun Biotech International, Germany) was used in the present study. The schematic diagram is shown in **Figure 1**. The reactor was operated with 1.5 L working volume at 30°C; aeration, 1.5 L/min; and agitation, 300 rpm.

Packed Bed and Fluidized Bed Reactors

A schematic diagram of a packed bed reactor (PBR) and a fluidized bed reactor (FBR) is shown in **Figure 1**. The reactors were made up of good quality Corning glass (National Scientific Glass Works, Hyderabad, India). The dimensions of the reactors were 2.4 cm internal diameter, 39 cm total height, and 36.5 cm working height, with working volume of 125 mL. The reactors were designed and fabricated at National Scientific Glass Works (Hyderabad, India). The dimensions of PBR and FBR were identical except for aeration provision in FBR. The fermentations were conducted in the reactor with 125 mL of production medium and with aeration of 0.5 L/min in FBR.

Airlift Reactor

A schematic diagram of the ALR, used in the present study, is shown in **Figure 1**. The reactor was made of glass, 37 cm in height and 4.5 cm in diameter, containing a concentric draft tube of 21 cm in height and 1.5 cm in diameter. The working volume of the reactor was 450 mL. The air sparger was located at the bottom of the draft tube. The reactors were designed and fabricated at National Scientific Glass Works. Filtered and humidified sterile air was introduced at the rate of 1.8 L/min. The liquid medium was fed into the reactor with the help of a peristaltic pump. The reactor outlet was provided with a screen to prevent the elimination of immobilized beads from the reactor.

Analytical Methods

The neomycin content was quantitatively determined by microbiological assay using *Staphylococcus epedermidis* NCIM 2493 as test organism.[9,10] The standard neomycin sulfate (Shanghai Pharmaceutical Industry Corporation, China) was used to construct the calibration curve. All the experiments were conducted in triplicate and the mean of the 3, with SD, is represented.

The cells leaked from the gel matrix were collected by centrifugation at 3000 rpm for 10 minutes and dried at 105°C for 3 hours. The amount of the entrapped cells in alginate was determined as follows: the specified quantity of alginate beads was taken and washed with distilled water; they were dissolved in 2% wt/vol sodium hexametaphosphate; and the cell mass was collected by centrifugation and dried.[11]

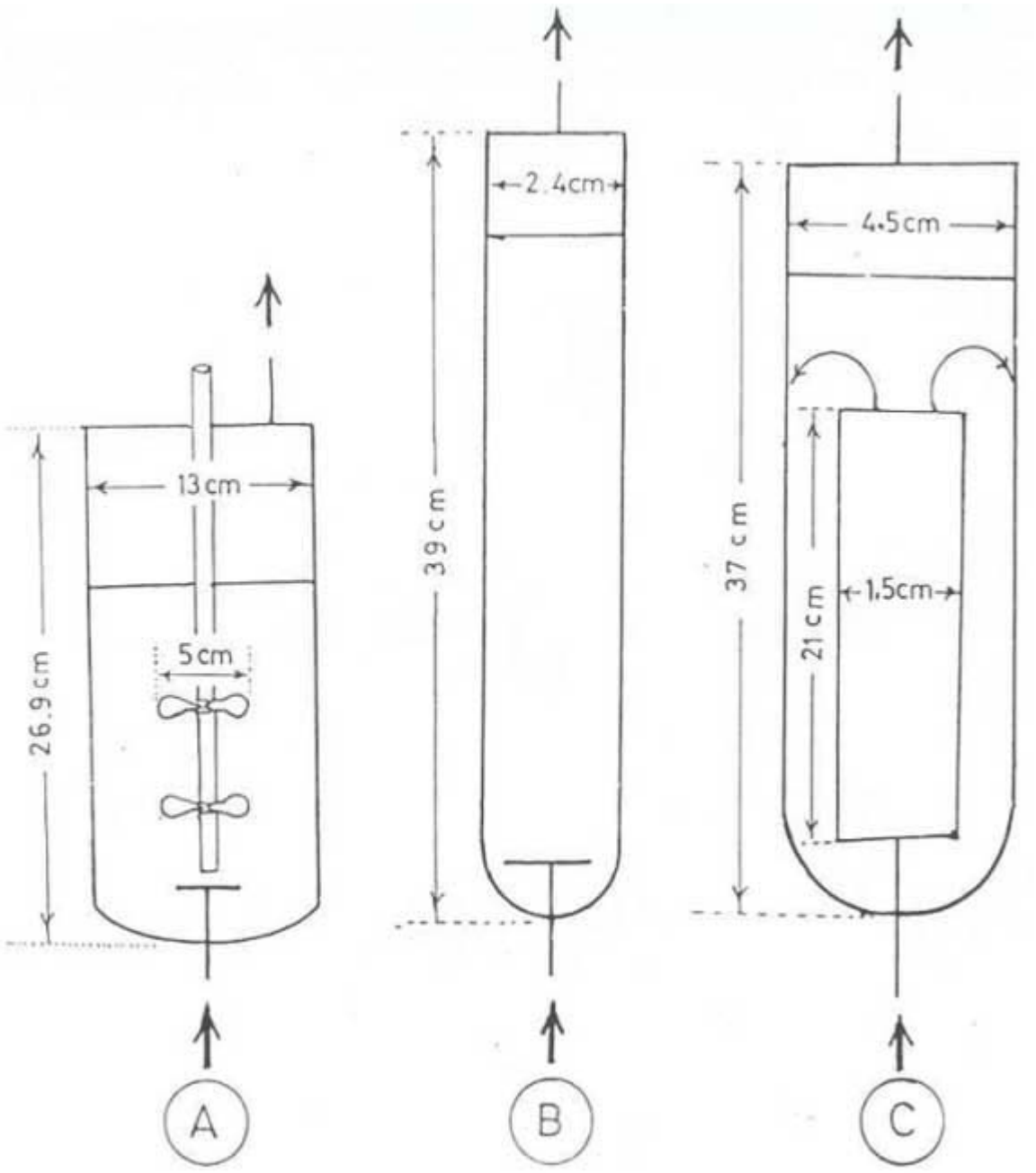

Figure 1. Geometries and dimensions of (A) Stirred tank reactor (STR), (B) Packed bed reactor (PBR) and Fluidized bed reactor (FBR), and (C) Airlift reactor (ALR).

RESULTS AND DISCUSSION

Neomycin Production with S marinensis NUV-5 in Stirred Tank Reactor

The neomycin and cell mass production profile of free and immobilized cells of *S marinensis* NUV-5 were investigated in an STR. The fermentation was conducted for 168 hours. The cell mass and neomycin titers were determined during the fermentation cycle and the results are shown in **Figures 2** and **3**.

The results showed that neomycin production started by 24 hours and reached a maximum titer (7135 mg/L) by 96 hours with immobilized cells, while the maximum titer (7315 mg/L) was obtained at 144 hours with free cells. However, the operation of STR with immobilized cells for 144 hours resulted in the disintegration

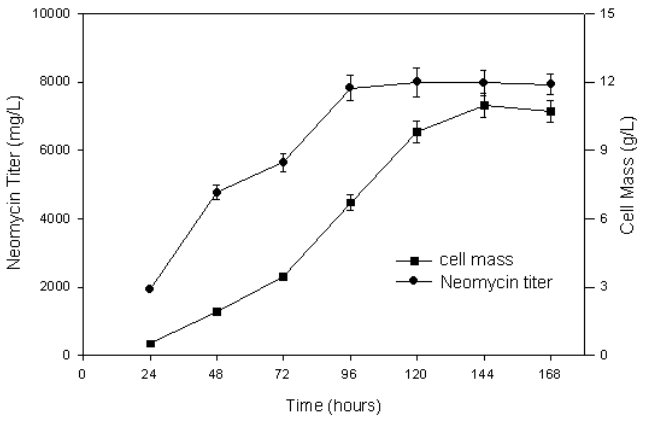

Figure 2. Time course profiles of cell mass and neomycin production with free cells of *S marinensis* NUV-5 in stirred tank reactor.

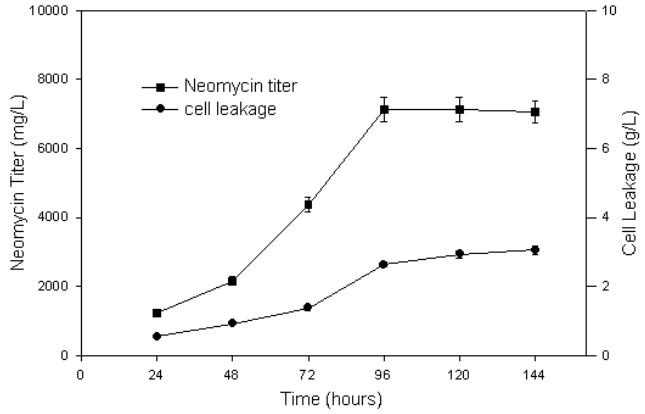

Figure 3. Time course profiles of cell mass and neomycin production with immobilized cells of *S marinensis* NUV-5 I in stirred tank reactor.

of beads. This may due to mechanical shear leading to the breakage of alginate beads.

Neomycin Production with Immobilized Cells in Packed Bed Reactor

The neomycin production profiles of immobilized cells of *S marinensis* NUV-5 in PBR were studied. The fermentation was carried out for 144 hours in batch mode. The neomycin titers were assayed during the fermentation cycle, and the results are shown in **Figure 4**. The results indicated poor neomycin titer (1353 mg/L) with immobilized cells in PBR.

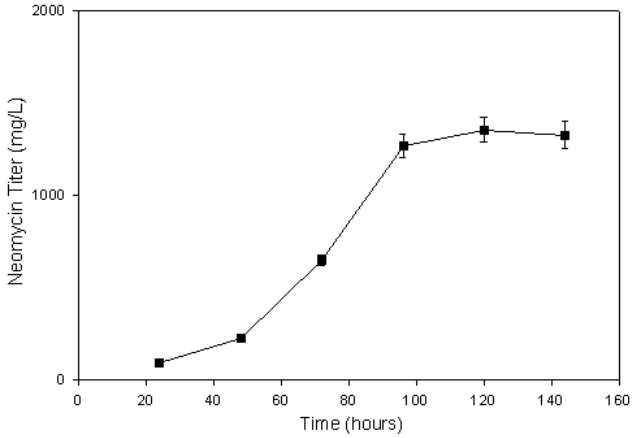

Figure 4. Time course profiles of neomycin production with free cells of *S marinensis* NUV-5 in packed bed reactor.

Neomycin Production with Immobilized Cells in Fluidized Bed Reactor

The neomycin production aspects of immobilized cells of *S marinensis* NUV-5 in FBR were evaluated. The fermentation was run for 144 hours. The neomycin titers and cell mass leakage were determined, and the results are shown in **Figure 5**.

From the results, it was observed that the neomycin production started at 24 hours fermentation and reached a maximum level by 96 hours (6923 mg/L). On further incubation, there was no appreciable change in neomycin titer. The leakage of cell mass was gradually increased during the fermentation time. At 144 hours fermentation, the disintegration began due to high-pressure drops in the reactor, and defluidization was observed due to leakage of cell mass. Neomycin titer was slightly lower as compared with the immobilized cells in STR.

Production of Neomycin with Immobilized Cells of S marinensis NUV-5 in An Airlift Reactor

The neomycin and cell mass production patterns were investigated in an ALR with immobilized cells. The fermentation was carried out for 144 hours. Neomycin titer and cell mass production were determined, and the results are shown in **Figure 6**. From the data, it was observed that neomycin production started at 24 hours fermentation and reached a maximum titer (7435 mg/L) by 96 hours; upon further incubation, no improvement in neomycin production was noticed. It may be inferred that neomycin production in ALR was

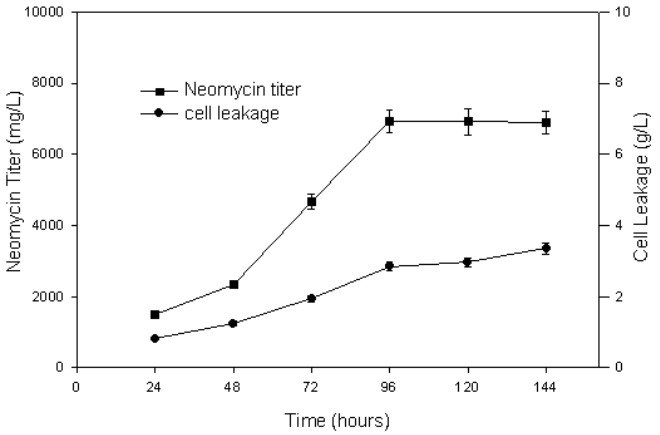

Figure 5. Time course profiles of cell mass and neomycin production with free cells of *S marinensis* NUV-5 in fluidized bed reactor.

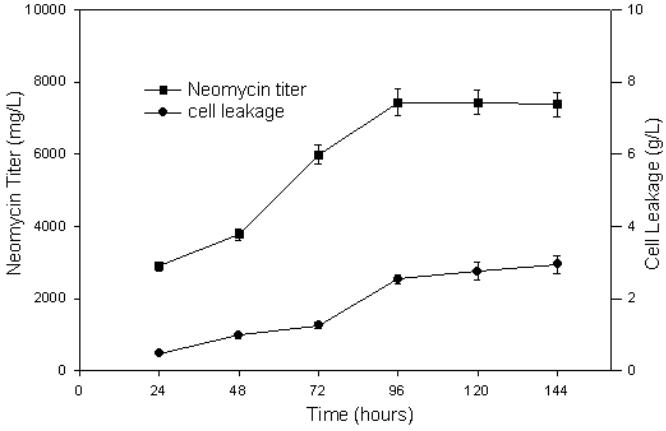

Figure 6. Time course profiles of cell mass and neomycin production with free cells of *S marinensis* NUV-5 in airlift reactor.

Table 1. Comparison of Neomycin Production with Immobilized Cells of *S marinensis* NUV-5 in Calcium Alginate in Various Reactor Configurations*

Type of Reactor	Type of Cells	Optimum Production Time (h)	Mechanical Agitation	Mixing	Neomycin Titer (mg/L)
Stirred tank	*FC*	144	Yes	Mechanical	7315 ± 139
Stirred tank	IMC	96	Yes	Mechanical	7135 ± 131
Packed bed	IMC	120	No	No	1353 ± 46
Fluidized bed	IMC	96	No	Pneumatic	6923 ± 124
Airlift	IMC	96	No	Pneumatic	7435 ± 152

*FC indicates free cells; IMC, immobilized cells.

higher as compared with the other reactors. The leakage of cell mass increased with fermentation time.

Comparison of Neomycin Production with Immobilized Cells in Various Reactor Configurations

A comparison of neomycin production with immobilized cells in various reactors was made. The data are represented in **Table 1**. The data showed that higher neomycin production (7435 mg/L) with immobilized cells was obtained in ALR as compared with immobilized cells in other reactors. Poor neomycin titer was noticed in PBR, possibly due to low oxygen availability to the microorganism.

Though the neomycin titer with immobilized cells in STR was comparable with that of free cells, the immobilized beads' disintegration started at 120 hours fermentation. The fermentations in FBR resulted in comparable neomycin titers with that of immobilized cells in STR and slightly lower titers compared with the immobilized cells in ALR. At the end of fermentation, the reactor was defluidized due to heavy cell growth in the reactor and could not be operated to continue the fermentation. Moreover, the beads started disintegrating after 120 hours fermentation because of attrition problems. These problems could be because of high-pressure drops in the reactor. The fermentation of immobilized cells in ALR resulted in high neomycin production over other reactors. This may be due to increased aeration and diffusion rates in ALR.

CONCLUSION

Stirred tank, fluidized bed, and airlift reactors produced similar neomycin activity with immobilized cells. Packed bed reactor clearly under performed, probably because of insufficient aeration or mixing. Neomycin

production using immobilized cells in fermentors requires good mixing and aeration.

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