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Investigations on Neomycin Production With Immobilized Cells of *Streptomyces marinensis* Nuv-5 in Calcium Alginate Matrix

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ABSTRACT

The purpose of this investigation was to study the effect of *Streptomyces marinensis* NUV-5 cells immobilized in calcium alginate for the production of neomycin. The effect of various parameters, such as the effect of alginate concentration (1%, 2%, 3%, 4%, and 5% wt/vol), the effect of cation ($CaCl_2$, $BaCl_2$, and $SrCl_2$), the concentration of cation (0.01M, 0.125M, 0.25M, 0.375M, and 0.5M), the curing times (1, 6, 11, 16, and 21 hours), and the diameter of the bead (1.48, 2.16, 3.24, 4.46, and 5.44 mm), on neomycin production and bead stability were studied. The effect of maltose (4%, 3%, 2%, and 1% wt/vol) and sodium glutamate (0.6%, 0.3%, 0.15%, and 0.075% wt/vol) concentration on neomycin production was also studied. Better neomycin production was achieved with optimized parameters, such as alginate at 2% wt/vol, 0.25M $CaCl_2$, 1-hour curing time, and 3.24 mm bead diameter. Effective neomycin production was achieved with 3% wt/vol maltose and 0.6% wt/vol sodium glutamate concentration. The repeated batch fermentations were conducted (every 96 hours) using the optimized alginate beads, employing the production medium with 3% wt/vol maltose and 0.6% wt/vol sodium glutamate along with mineral salts solution. The increase in antibiotic production was observed up to the 5th cycle, and later gradual decrease in antibiotic production was observed. Comparison of the total antibiotic production with free cells and immobilized cells was also done. An enhanced antibiotic productivity of 32% was achieved with immobilized cells over the conventional free-cell fermentation, while 108% more productivity was achieved over the washed free-cell fermentation.

From these results it is concluded that the immobilized cells of *S marinensis* NUV-5 in calcium alginate are more efficient for the production of neomycin with repeated batch fermentation.

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KEYWORDS: neomycin production, *Strepromyces marinensis* NUV-5, immobilized cells

INTRODUCTION

Neomycin is an important aminoglycoside antibiotic, which is effective against gram-positive and gram-negative bacteria, and mycobacteria, and is produced by different species of *Streptomyces* such as *Streptomyces fradiae*,[1] *Streptomyces marinensis*,[2] and other microbial sp.[3] Many workers have studied the factors and chemical composition of media favoring the fermentative production of neomycin by free-cell cultures.[4-7] Also, studies were carried out on solid-state fermentation to produce neomycin using *S marinensis*,[8-10] immobilization of *S marinensis* cells by adsorption technique,[10] and partial immobilization of *S fradiae* on cellulose beads.[11]

The purpose of the present investigation was to study the entrapment of *S marinensis* NUV-5 cells in calcium alginate gel for neomycin production. The effect of alginate concentration, different cations, cation concentration, calcium chloride curing time, bead diameter, the effect of nutrients' strength, and the reusability of the entrapped whole cells for the neomycin production were studied.

MATERIALS AND METHODS

Chemicals

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

Microorganism

A mutant strain of *S marinensis* NUV 5 was used in the present study. It was isolated from seawater of Bay of Bengal, Visakhapatnam, India.[28] It was maintained on jowar starch agar slants at 4°C and subcultured at every 4 weeks.

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% (wt/vol), $(NH)_2SO_4$ 0.5% (wt/vol), NaCl 0.5% (wt/vol), $CaCO_3$ 0.5% (wt/vol); 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

Entrapment of cells in nontoxic alginate is one of the simplest, cheapest, and most frequently used methods of immobilization.[12,13] Sodium alginate and calcium chloride were used to prepare the alginate beads containing the whole cells. Sodium alginate solution (3% wt/vol) was prepared by dissolving sodium alginate in 100 mL hot water. The contents were stirred vigorously for 10 minutes to obtain thick uniform slurry without any undissolved lumps and then sterilized by autoclaving.

Both alginate slurry and cell suspension (equivalent to 0.03 g dry cell weight) were mixed and stirred for 10 minutes to get a uniform mixture. The slurry was taken into a sterile syringe, added drop-wise into 0.2M $CaCl_2$ solution from 5-cm height, and kept for curing at 4°C for 1 hour. The cured beads were washed with sterile water 3 to 4 times. When the beads are not in use, they are preserved in normal saline solution in a refrigerator. All of these operations were carried out aseptically under laminar flow unit.[14]

Fermentations

The immobilized beads/blocks (cells equivalent to 0.03 g dry cell weight) were transferred into 50 mL modified production medium[15] in 250-mL Erlenmeyer flasks. The modified production medium was composed of maltose 4% (wt/vol), sodium glutamate 1.2% (wt/vol), K_2HPO_4 0.01% (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); pH 8.0. The flasks were incubated at 30°C for 96 hours. Five-milliliter quantities of samples were withdrawn and assayed for neomycin titre.

Parameters Investigated for Optimization of Alginate Matrix for Maximum Neomycin Production

To determine the optimum concentration of sodium alginate for antibiotic production, various concentrations of sodium alginate (1%, 2%, 3%, 4%, and 5% wt/vol) were used to prepare the beads with 0.2M $CaCl_2$ solution. These beads were employed for the production of neomycin and the fermentation was conducted for 96 hours at 30°C. The cell mass and neomycin production at different times of fermentation were estimated.

Different cationic solutions such as $BaCl_2$ and $SrCl_2$ in addition to $CaCl_2$ at 0.2M level were used to prepare the alginate beads containing the cells and were used for antibiotic production. The concentration of the cationic solution has a significant effect on the gelling behavior of alginate. For the evaluation of bead stability, the immobilization procedure was carried out with different concentrations of various cationic solutions (0.01, 0.125, 0.25, 0.375, and 0.5 M). The beads were transferred into the production medium and incubated at 30°C for 96 hours. The neomycin production and disintegration time of alginate beads was evaluated.

To study the appropriate conditions for the production of neomycin, the cells immobilized in calcium alginate were prepared with 2% wt/vol alginate and 0.25M calcium chloride solution with varying curing times (1, 6, 11, 16, and 21 hours). The immobilized beads were used for the production of antibiotic, and fermentations were run as described earlier.

Different sizes of needles were used to prepare beads. The diameter of the randomly selected known number of beads was calculated using Vernier calipers, and the mean value was taken. To study the effect of bead diameter on neomycin productivity, different sizes of beads containing equal amounts of biomass were selected, and fermentations were run as described earlier.

The critical concentration of nutrients is the key factor in triggering the biosynthesis of antibiotic and in controlling cell growth, which leads to increased longevity of the biocatalyst. The effect of maltose (4%, 3%, 2%, and 1% wt/vol) and sodium glutamate (0.6%, 0.3%, 0.15%, and 0.075% wt/vol) concentration on neomycin production was studied. For these experiments, immobilized alginate beads were incubated in the production medium for 72 hours for activation of cells, and the broth was decanted from each flask. Fresh production medium with various concentrations of maltose (with 1.2% wt/vol of sodium glutamate) was introduced into each flask. Fermentations were conducted as described earlier, and samples were assayed. In a similar way, the effect of various concentrations of sodium glutamate on the production of neomycin (with 3% wt/vol maltose) was studied. The fermentations were carried out as described earlier.

In repeated batch process with all optimum alginate conditions, after attaining the maximum production of neomycin (96 hours), the medium was replaced with fresh production medium, and the process was repeated for several batches until the beads started disintegrating. The antibiotic titres and cell leakage of each cycle were determined.

Analytical Methods

The neomycin content was quantitatively determined by microbiological assay using *Staphylococcus epedermidis*, National Collection of Industrial Microorganisms (NCIM) 2493, as test organism.[16,17] The standard neomycin sulfate (Shanghai Pharmaceutical Industry Corp, Shanghai, China) was used to construct the calibration curve.

For the determination of bead disintegration time, 20 beads were placed in a 100-mL conical flask containing 20 mL of 0.1M phosphate buffer (pH 7.0) and agitated on a rotary shaker at 140 rpm. The criterion to evaluate the strength of the bead was to determine the time required for the complete disintegration of beads.

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 number of alginate beads were taken and washed with distilled water. They were dissolved in 2% (wt/vol) sodium hexametaphosphate, and then the cell mass was collected by centrifugation and dried.[18]

RESULTS AND DISCUSSION

Among various supporting matrices studied so far for whole-cell immobilization of *S marinensis*, calcium alginate was found to be a better entrapment matrix for neomycin production.[10,19] The effect of several parameters, such as the effect of alginate concentration, the effect of cation, the concentration of cation, and the curing times, on neomycin production and bead stability were studied.

In order to evaluate the stability of calcium alginate beads, various concentrations of alginate (1%, 2%, 3%, 4%, and 5% wt/vol) were used for the immobilization of *S marinensis* NUV-5 cells. The results indicated that the calcium alginate concentration for the immobilization of cells plays a prominent role in the production of neomycin. From the data, it was observed that the antibiotic titre was reduced with increased alginate concentration, which may be due to reduced porosity of the beads limiting the nutrient supply and oxygen diffusion. Alginate at 2% wt/vol was found to be the optimum concentration for formulation of spherical and stable beads with better antibiotic production (**Figure 1**). A similar type of result was reported by Ellaiah et al.[19]

Different cationic solutions such as $BaCl_2$ and $SrCl_2$ in addition to $CaCl_2$ were used to prepare the alginate beads and used for the production of the antibiotic. It was observed from the data that the antibiotic production was found to be higher with calcium alginate beads (**Figure 2**). Whereas, the antibiotic titre was low with the alginate beads prepared with barium and strontium ions, which may be due to reduced porosity.

The strength of the beads was determined by stirring an equal number of beads prepared from different cationic solutions in 0.1M phosphate buffer. The calcium alginate beads disintegrated within 90 minutes, whereas the barium alginate and strontium alginate beads did not disintegrate until 48 hours. It could be inferred that the gel stability was higher with $BaCl_2$ and $SrCl_2$ when compared with $CaCl_2$. However, $CaCl_2$ gave better porous beads, and higher production of antibiotic was observed when compared with other cations. In the subsequent experiments, $CaCl_2$ was used to prepare the beads because of its better porosity, with good antibiotic production and low cost.

The alginate beads were prepared with various concentrations of cation (0.01M, 0.125M, 0.25M, 0.375M, and 0.5M) and used for the production of neomycin. After fermentation, the neomycin titre and disintegration time of beads was determined. The data indicated that the cation has a significant effect on gelling behav-

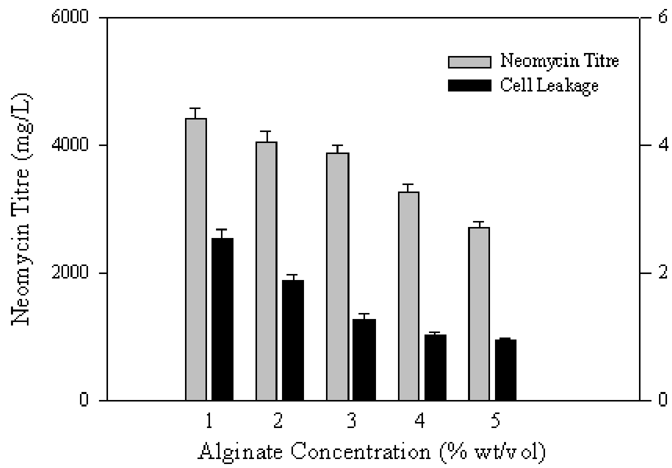

Figure 1. Effect of alginate concentration on neomycin production.

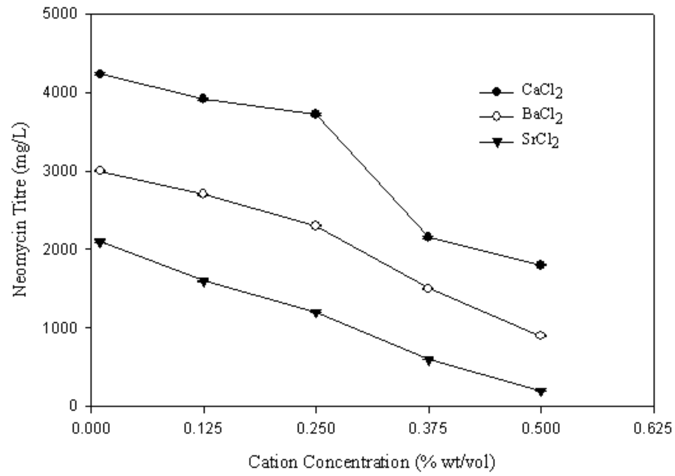

Figure 2. Effect of cation and cation concentration on neomycin production.

ior of alginate (**Figure 2**). When 0.01M solution was used, the gelling was relatively slow and it required more time for curing. A central core was observed inside the bead. At higher concentrations of cation (0.375M) the gelling was instantaneous and a case-hardening effect (gelling outside, leaving slurry as it was inside) was noticed. As the concentration of cation increased, it seems that the microporous structure of the bead was altered. The pore size and number of pores might have decreased.

The beads prepared with 0.01M and 0.125M cation solution were irregular in shape, whereas the beads prepared with 0.25M and higher concentrations of cation were spherical in shape. The beads prepared with 0.01M were found to be better antibiotic producers when compared with the beads prepared with other higher concentrations. However, the beads prepared with 0.01M and 0.125M cation had disintegrated at the end of the first cycle itself. The beads prepared with 0.25M cation were found to be relatively more stable and showed better neomycin production.

The beads prepared with 1-hour curing time were found to be better antibiotic producers than the beads prepared at higher curing times (**Table 1**). The stability of the beads was tested in the 0.1M phosphate buffer as described earlier, and the stability of beads increased with increase of curing time. Increase of curing time resulted in a hard type of beads with less antibiotic production. The results indicated that curing time of 1 hour is optimum for the formation of stable calcium alginate beads and better antibiotic production.

Table 1. Effect of $CaCl_2$ Curing Time on Neomycin Production

Curing Time (hours)	Neomycin Production (mg/L)
1	4210 ± 121
6	3625 ± 106
11	3140 ± 98
16	2550 ± 76
21	925 ± 34

To study the effect of bead size on neomycin production, 5 different sizes of alginate beads (1.48-, 2.16-, 3.24-, 4.46-, and 5.44-mm diameter) were prepared. The results indicated that the smallest-size beads exhibited better neomycin production when compared with large-size beads. This might be due to increased surface area of the bead, which enhances the mass transfer. At the same time, cell leakage was increased with a decrease in bead diameter. The optimum bead diameter was found to be 3.24 mm and was used in the subsequent experiments.

Effective neomycin production (6920 mg/L) was achieved with 3% wt/vol maltose. Further studies with varying concentration of sodium glutamate with 3% wt/vol maltose showed that neomycin production was decreased with decreasing sodium glutamate. The optimum and effective neomycin production was achieved with 0.6% wt/vol sodium glutamate concentration (**Figure 3**). Hence, the production medium with 3% wt/vol maltose and 0.6% wt/vol sodium glutamate

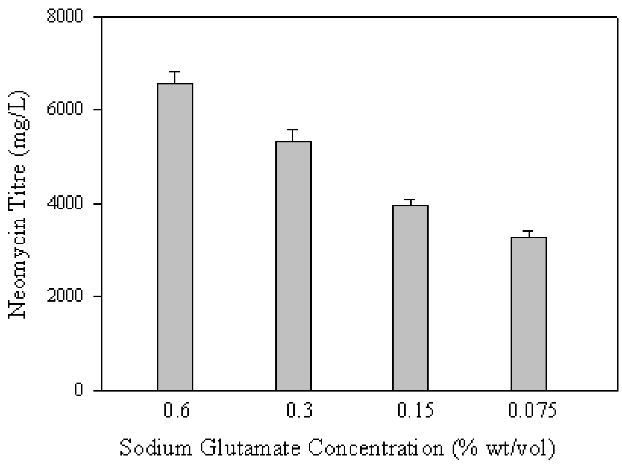

Figure 3. Neomycin production with various concentrations of sodium glutamate.

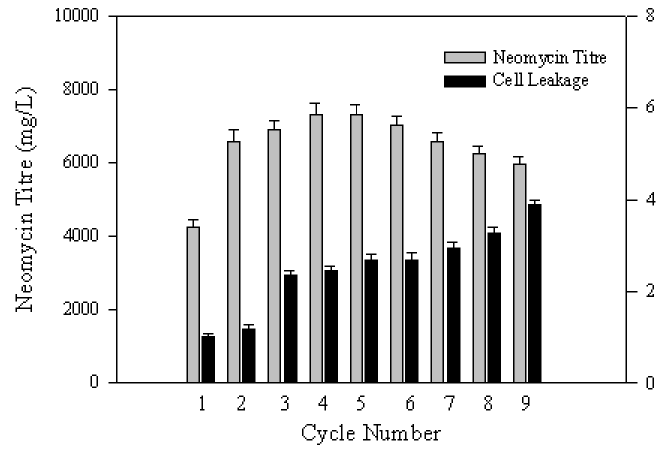

Figure 4. Neomycin production and cell leakage with immobilized cells of *S marinensis* NUV-5 in calcium alginate by repeated batch fermentation.

Table 2. Comparison of Neomycin Production with Free Cells and Immobilized Cells in Shake Flask Culture

Matrix	Fermentation Period for Each Batch	No. of Batches	Total Fermentation Time (*t*)	Total Neomycin Production (U/mL)	Average Neomycin Concentration (U/mL)	Average Specific Volumetric Productivity (mg/L/h)
1 Free Cells (conventional)	6	1	6	43920 ± 824	7325 ± 234	50.9
2 Washed Free Cells	4	3	16	18275 ± 389	6092 ± 178	32.2
3 Immobilized Cells	9	9	36	57770 ± 906	6418 ± 197	67.1

along with mineral salts solution was employed for the subsequent repeated batch experiments.

The repeated batch fermentations were conducted (every 96 hours) using the optimized alginate beads, employing the modified production medium. The fermentation was continued for 36 days until the beads disintegrated. The data showed that the increase in antibiotic production was observed up to the 5th cycle, and later a gradual decrease in antibiotic production was noticed (**Figure 4**). Gradual cell leakage was observed with each batch.

Comparative data on the total antibiotic production with free cells and immobilized cells is shown in **Table 2**. The data indicated that the average specific volumetric productivity was 67.1 mg/L/h with the immobilized cells, whereas it was 50.9 mg/L/h in case of conventional free-cell fermentation and 32.2 mg/L/h with washed free-cell fermentation. An enhanced antibiotic productivity of 32% was achieved with immobilized cells over the conventional free-cell fermentation, while 108% more productivity was achieved over the washed free-cell fermentation.

CONCLUSION

From the results, it is concluded that the immobilized cells of *S marinensis* NUV-5 in calcium alginate are more efficient for the production of neomycin with repeated batch fermentation.

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