Comparative Studies of Air Lift and Fluidized Bed Reactors for Streptomycin Production by Immobilized Cells of *Streptomyces bikiniensis* ATCC 11062

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Production of streptomycin by free and immobilized cells of *Streptomyces bikiniensis* ATCC 11062 by AIR LIFT (ALR) and FLUIDIZED BED REACTORS (FBR) were studied. This paper refers to the application of calcium alginate cross-linked with gluteraldehyde immobilized cells as biocatalyst and repeated batch method for 17 days. ALR and FBR with φ = 20 % of beads and an air flow of 1.3 L min^{-1} with glucose as carbon and peptone and meat extract as nitrogen source are operated at different dilutions. It is observed from the previous studies that antibiotic production improved in calcium alginate crosslinked with gluteraldehyde over calcium alginate alone and hence the same system was used in the present study. The increased production of streptomycin in immobilized cells was observed when compared with that in free cells. The enhanced performance of FBR was evident when compared with that of ALR at the same given optimum conditions. Accordingly the carbohydrate utilization was faster in FBR than in STR and ALR. The amount of streptomycin produced was 33.33 % more in FBR than in ALR.

Keywords:
Air Lift Reactor (ALR); Fluidised Bed Reactor (FBR); Immobilized cells; Streptomycin production; *Streptomyces bikinienses* ATCC11062

Introduction

A drive towards "Process intensification" is leading to the search for novel reactor configurations that can enhance process selectivity and productivity. Improved configurations of reactors such as AIR-LIFT REACTOR (ALR), FLUIDIZED BED REACTOR (FBR) and HYBRID REACTORS with improved production are emerging. The process with immobilized microorganisms seems to have several advantages over fermentation with free cells. The immobilization processes can lead to increased activity and stability of microbial cells. Continuous processes are generally more desirable for industry, as they are theoretically more efficient and cost effective. Production of antibiotics is one of the most important areas in the field of microbial biotechnology. The conventional method of production is mainly in STIRRED TANK REACTOR (STR) with free cell cultures. Since antibiotics are secondary metabolites, product formation is mainly achieved during the idiophase, i.e in the stationary phase[1] and therefore fermentation periods need to be extended. It can be difficult to produce some antibiotics in a non-growth associated continuous fermentation process with free cells.

Bioprocessing strategies are important for improved production of antibiotics. The processes with immobilized microorganisms can have several advantages over fermentation with free microbial cells. They can work as effective biocatalysts in repeated and continuous production systems, mainly due to their characteristics of self generation and proliferation. Several attempts have been made to immobilize various microbial species on different support matrices for antibiotic production.[2] The most extensively studied cell immobilization is the entrapment in polymer matrices such as agar, alginate, carrageenan and gelatin.[3] Among different methods employed, calcium alginate entrapment has shown considerable promise in antibiotic production. The application of alginate for the purpose of whole cell immobilization was first reported in 1975.[4] The cell immobilization system effectively decouples the growth of the microbe to the product synthesis, since it is a secondary metabolite and hence could be operated independent of specific growth rate.[5] This can enable the production of antibiotics in a continuous mode.

Most of the reports on antibiotic production are confined to shake flask level, however, a few have attempted different types of bioreactors. The above said mixed flow type reactors (Batch STR's) can have severe limitations e.g. the build up of solid deposits on vessel walls (Filamentous growth, solid substrates, polymeric metabolites) cell damage by high shear – forces in mechanically stirred tanks and foaming.[6] New sophisticated bioreactor designs

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with unique performance characteristics will play a vital role in the economic manufacture of useful biotechnolgical products from natural and genetically modified cell systems of microbial, mammalian, and plant origin.[7]

The use of non mechanically agitated bioreactors such as ALR and FBR may contribute to reducing the process cost, since both types of vessels are considered to have a lower power requirement for comparable values of the mechanically agitated vessels. The ALR is one of the most promising device for the gas-liquid mass transfer for biosynthesis. The production of penicillin V, cephalosporin C and tetracycline in STIRRED TANK and AIR-LIFT TOWER LOOP REACTORS was performed and compared.[8] Earlier workers have tried ALR to improve the oxygenation of the culture.[9] The main feature that distinguishes the FBR from ALR is difference in mode of aeration.

The FBR mode of operation is advantageous over other types of batch STR's in terms of better solid and liquid mixing, higher oxygen transfer, easier CO_2 removal, easier cell regeneration, and a more uniform cell population.[10] The inherent shear effects on the immobilized cell particles caused by bubble motion and liquid flow restrict the operation of a fluidised bed reactor. *Morikawa* et al.[11] produced bacitracin using cells of *Bacillus sp.* entrapped within polyacrylamide gel particles in a three phase fluidized bed reactor. In another study, which was conducted in a three phase FBR using immobilised cells was operated continuously for 360 h with 35 % higher antibiotic production.[12]

Production of streptomycin using *Streptomyces bikiniensis* ATCC 11062 has been carried out in the present study, as it is one of the important broad spectrum aminoglycoside antibiotic and highly effective in the growing stage of *Mycobacterium tuberculosis*, which causes tuberculosis and also used in the treatment of plague. The investigation on the production of streptomycin by ALR and FBR was undertaken in order to assess the potential of such reactors for antibiotic production, using immobilized cells by repeated batch operation. The objectives of the study are to investigate the performance of ALR and FBR and compare it with conventional STR in terms of yield and productivity so that they can be operated in continuous mode.

Materials and Methods

Organism and Culture Conditions

Streptomyces bikiniensis ATCC 11062 was employed throughout this study. It was maintained on agar slopes using Actinomycetes Agar Medium (Hi Media, Bombay). The growth medium contained (in gL^{-1}); glucose, 2; peptone, 5; beaf extract, 5; NaCl, 5; K_2HPO_4, 1; it was used at pH -7. The production medium used was as follows (in g L^{-1}); glucose, 30; peptone, 5; meat extract, 5; NaCl, 10; $CaCO_3$, 2; K_2HPO_4, 1; $MgSO_4$, 5; $ZnSO_4$, 0.05. Freshly prepared agar slant was used to inoculate 50 ml of growth media and kept on shaker for 24 h at (170 rpm) at 28 °C. 100 ml of 48-h-old culture was used to prepare immobilized beads of calcium alginate. 20 % of beads were used throughout this study. The medium was adjusted to pH 7.0 with NaOH/HCl prior to sterilization.

Preparation of cell suspension

To a well-sporulated 7 days old slant, 5 ml of sterile distilled water was added and spores were removed by scrapping. The spore suspension was added to 250 ml Erlenmeyer flask containing 45 ml of growth medium and incubated on a rotary shaker at 170 rpm at 28 °C ± 2 °C. After 72 h of incubation, the culture broth was centrifuged at 5000 rpm and the cell pellets were washed with 0.9 % NaCl solution. This cell suspension was used as inoculum for further experiments and also for the immobilization of whole cells.

Immobilization method

Immobilization of 72 h old biomass pellet collected from 100 ml broth was done using 3 % sodium alginate and 1 mol L^{-1} $CaCl_2$ solution and the solution was added drop wise using a 0.1 ml micropipette to calcium chloride solution at 28 °C as described by earlier workers.[13] The immobilized beads are stored in refrigerator for stabilization overnight. The beads are washed twice with sterile distilled water and stored in physiological saline in refrigerator for further use.

Culture studies

The immobilized beads stored in refrigerator were washed twice with sterile distilled water under laminar flow and charged into sterilized bioreactors of STR, ALR and FBR at w = 20 %. The same beads served as inoculum during repeated batch fermentation for 17 days. If there was any contamination appearing in the middle of the fermentation, the experiment was discontinued and repeated, again starting from biomass preparation and immobilization procedure. Initial cycle was lasted for 5 days followed by 4 days subsequently for 3 more cycles. As the antibiotic production depleted along with carbohydrate utilized by the microbes, the medium was decanted carefully every 4 days in laminar hood, and new sterile medium was poured into the reactors, and monitored the antibiotic produc-

tion for 4 cycles for 17 days. Repeated experiments were carried out for the reproducibility of results.

Analytical methods

Samples were drawn in eppendorf tubes everyday under sterile conditions for antibiotic assay, carbohydrate estimation, and pH measurement. Streptomycin concentration was determined by Agar plate diffusion assay using *E. coli* as test organism along with standard streptomycin disc (10 μg, Himedia) in every plate.[14] Total carbohydrates were estimated by Anthrone method.[15] Biomass in terms of dry cell weight (DCW) was estimated in 2 ml of sample after centrifugation at 8 000 rpm for 10 min. The cell pellets were resuspended in distilled water and filtered on preweighed, dried Whatman No.1 filter paper discs. The cells were dried at 75 °C for 24 h and the dry cell mass was determined.

Design and operation of reactors

STIRRED TANK REACTOR (STR) of 1 L capacity was used with 22 cm height and 10 cm inner diameter. Medium was introduced from the top of the reactor and air was introduced with the help of air pump through air filter (0.2 μm). The working volume of the reactor was 600 ml. Production medium containing 120 g of immobilized beads (20 %) were used throughout the experiment for all types of bioreactors. Both ALR and FBR were made of glass tubing with 55 cm height and 42 mm inner diameter with total volume of 850 mL. The air sparger was located at the bottom of the draft tube in case of air-lift reactor. In FBR, the conical bottom of the reactor space is filled with steel beads of 2 mm diameter and the draft tube from the top was embedded within the steel beads. This kind of arrangement was applied to eliminate the use of additional air sparger. It also assists for breaking of the larger air bubbles into fine air bubbles for smooth fluidisation and to increase the rate of mass transfer. Samples were drawn through the sample port with a disposable syringe for the estimation of biomass, antibiotic, and carbohydrates. Schematic diagram of ALR and FBR were shown in figure 1.

Results and discussion

Calcium alginate cross-linked with gluteraldehyde immobilised cells were used through out this study. This system showed to be stable and gave good yields based on our shake flask studies for repeated use of beads. The working volume of the reactor was 1 L with 20 % of inoculum (beads). The diameter of the beads were in the range of 2.0-2.5 mm. The optimum airflow for streptomycin production was 1.3 L min^{-1}. ALR and FBR were operated with 3 % calcium alginate immobilized cells as repeated batch for four cycles. First cycle was operated for 5 days, whereas the other 3 cycles were operated for 4 days each for about 17 days. The results of antibiotic titer, specific concentration and carbohydrates utilization were studied and used to compare ALR with FBR. For comparison, typical results obtained in STR under similar conditions were included and compared. In general, there was not much change in pH during fermentation from initial pH 7 to 6.5 during fermentation. Foam was controlled by adding antifoam agent (Sigma) and airflow was monitored by Rotameter.

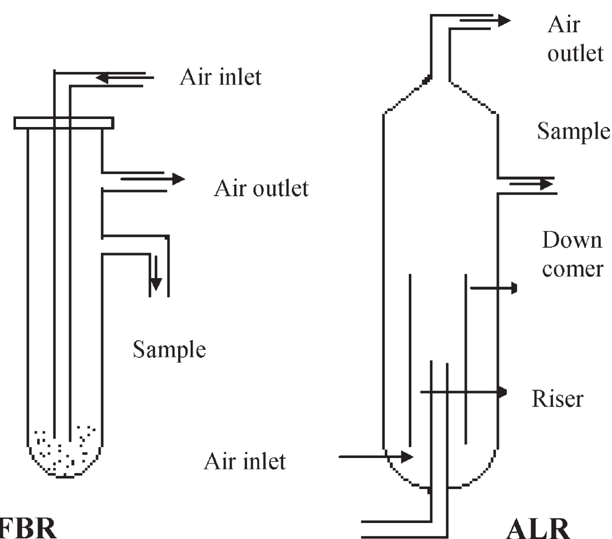


Fig. 1 – *Schematic Diagram of Fluidized Bed Reactor (FBR) and Air-Lift Reactor (ALR) showing air flow pattern*

Schematic diagrams of ALR and FBR with airflow and fluidization pattern are shown in Figure 1. Air inlet and outlet and sample ports are also shown in the same figure. Figure 2 shows comparison of free and immobilized cells in shake flask and immobilised cells in STR under identical conditions. The average antibiotic yield in STR was 348.8 μg

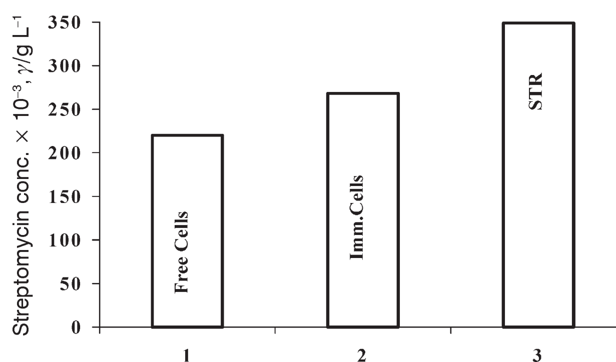


Fig. 2 – *Comparative studies of Streptomycin production by free and immobilized cells in shake flasks and immobilized cells in STR*

mL^{-1} when compared with shake flask with 260 μg mL^{-1} during 4-5 day period, as this was due to good aeration and agitation.[16]

Figure 3 shows the amount of biomass during 5-day fermentation, where there was more biomass in immobilized system. Maximum antibiotic production was recorded during 5-6 day period in free cells, whereas in immmobilized system it was for 2-3 day period. This was due to the fact that they act as catalyst, and that the cells enter into secondary metabolic stage resulting in product formation.

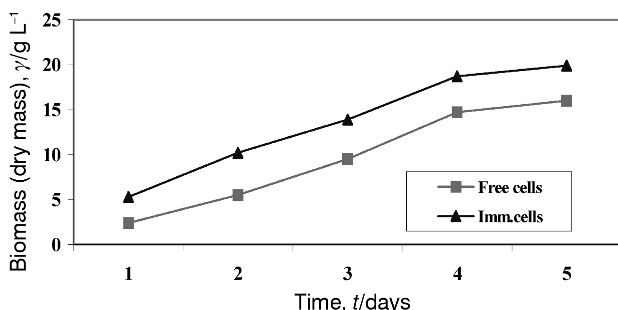


Fig. 3 – *Dry mass of biomass g l^{-1} of free and immobilized cells in shake flasks during streptomycin production*

Figure 4 shows repeated batch operation of, both, ALR and FBR by replacing the medium with half strength media every four days for 17 days. The antibiotic titre drops by fourth day and the media replacement results in high productivity during 3rd day (highest specific concentration 500-800 μg mL^{-1}) in both the reactors. Our earlier studies with 1/4 concentration of media constituents gave lesser yields of antibiotic titre and hence half strength media was used throughout the experimentation based on shake flask studies. The use of full strength media throughout the fermentation also gave the same amount of antibiotic but with more viscosity (Results not shown). This was due to biomass accumulation resulting in primary metabolism due to excess availability of nutrients. The streptomycin production started during 24 h and reached maximum during 4th day. In ALR the maximum amount was 495 μg mL^{-1} when compared with FBR where maximum amount recorded was 880-920 μg mL^{-1} during 3rd and 4th cycle. This was because FBR's maintain high oxygen mass transfer rates as against ALR,[17] wherein the reactor contains aerated (draft tube) and non-aerated zones (down comer).

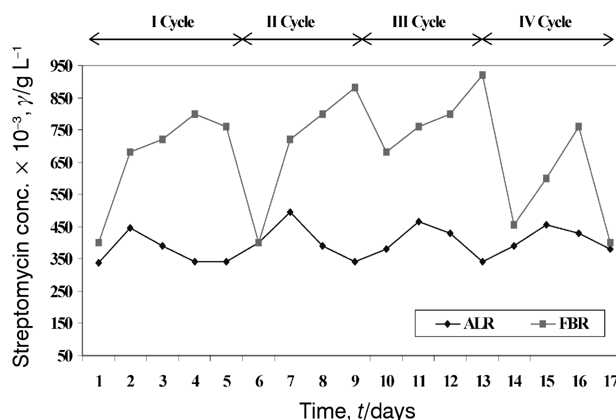


Fig. 4 – *Comparison of ALR and FBR with reference to Streptomycin production by Repeated Batch fermentation with replacing the media for every 4 days*

Figure 5 shows carbohydrate concentration during the fermentation. In general the concentration of carbohydrate during the first few days of fermentation decreased slowly, as the cells enter primary metabolic state by building up biomass. From second cycle on wards there was noticed sudden drop in slopes during 24 h, wherein the antibiotic production starts subsequent to primary metabolism. By supplying required amount of nutrients the cells can be maintained (half strength) at secondary metabolic state to function as catalyst for the production of antibiotic for longer periods. In case of secondary metabolite production such as antibiotics, the primary metabolism and product formation occur at completely separate times. The product is not derived from catabolism, but from amphibolic pathways. In this type of fermentation, primary metabolism functions are primarily accomplished by substrate consumption and growth. Afterwards the product is formed by the reactions of introductory metabolism.[18]

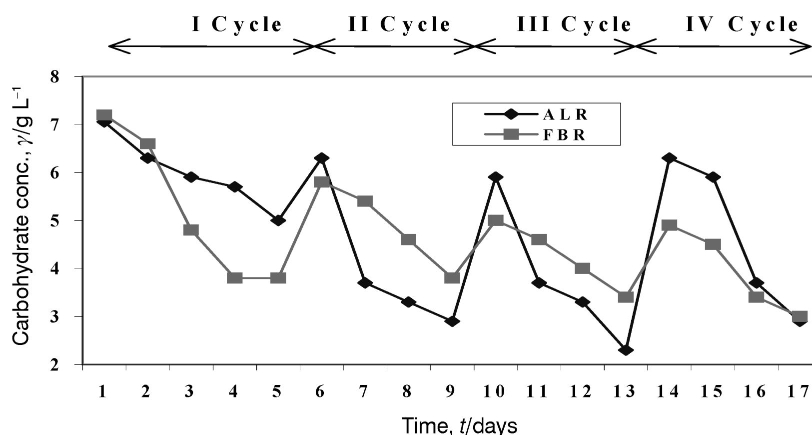
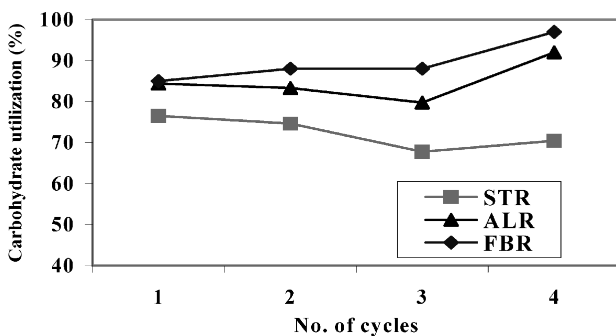
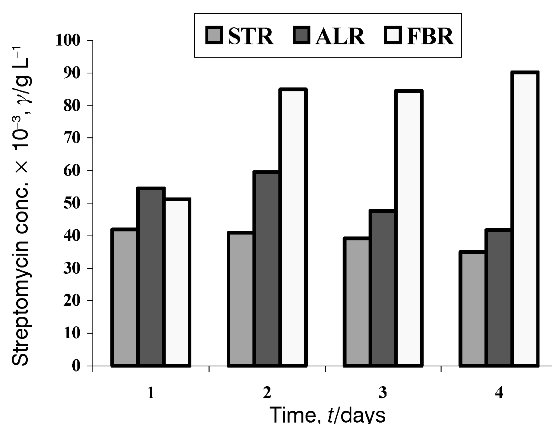


Fig. 5 – *Carbohydrate content (g l^{-1}) in ALR and FBR during the Repeated Batch fermentation for the production of streptomycin*

Figure 6 shows fraction of carbohydrate utilization. In STR 70-80 % of carbohydrate was utilized whereas 80-90 % utilisation was observed in, both, ALR and FBR. Maximum utilisation of carbohydrate was recorded in FBR up to 88-97 % during the 3rd -4th cycle. Figure 7 shows specific concentration of streptomycin per gm cells. This indicates that the FBR recorded more antibiotic when compared to ALR. The specific mass concentration of streptomycin had reached a maximum of 90 μg mL^{-1} during 4th day where maximum mass concentration of 800 μg mL^{-1} was estimated. Recently the application of these type of bioreactors have been significantly expanded parallel with the overall progress in the fields of bioengineering and genetic engineering. Therefore these types of reactors maintain high oxygen mass transfer rates without severely limiting the concentration of nutrients.[9] The superior performance of FBR has been also stressed by earlier workers in the area of wastewater treatment and in the production of antibiotics and phenol degradation etc. It was also stated by the earlier workers that tubular reactors represent a valuable contribution to the understanding of optimal conversions.[19] Apart from the aspects of commercial realisation, these reactors represent an effective method for determining bioprocess kinetics as well as evaluation of the physiological state of microbial cells.[6]



F i g . 6 – *Mass fraction of carbohydrate utilization in three systems of STR, ALR and FBR during the fermentation by repeated batch for 4 cycles*



F i g . 7 – *Specific mass concentration of Streptomycin (g l^{-1}) in STR, ALR and FBR during 4 cycles of Repeated batch fermentation*

Conclusion

Comparison of streptomycin production by immobilized cells in different bioreactors indicated that the average specific concentration of Streptomycin was higher in ALR and FBR than in STR. An enhanced antibiotic production was observed even with 50 % of diluted nutrient solution over four cycles, i.e. for 17 days using the same intact beads. The same was also evident from the percentage utilisation of carbohydrates, which was more in FBR followed by ALR. It may be concluded from the present study that the performance of FBR is superior to that of ALR interms of specific mass concentration of streptomycin with good utilisation of carbohydrates.

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