Continuous Production of Urokinase by Alginate-Immobilized Cells of a Mutated MPGN Kidney Cell Line in an Airlift Bioreactor

Ravuri Bhavani Devi[1], Adinarayana Kunamneni[*1,2], Taadimalla Prabhakar[1], Francisco J. Plou[2], Poluri Ellaiah[1]

[1]Pharmaceutical Biotechnology Division, Department of Pharmaceutical Sciences, Andhra University, Visakhapatnam—530 003, India.
[2]Departamento de Biocatalisis, Instituto de Catalisis y Petroleoquimica (CSIC), Marie Curie 2, 28049—Madrid, Spain.

Urokinase (UK) production by free and calcium alginate immobilized cells was investigated in an airlift bioreactor (ALR). The average volumetric productivity with continuous fermentation (164.8 PU/ml/day) was found to be greater than the free cells (40.2 PU/ml/day). The total UK enzyme produced with continuous fermentation was found to be about 4.1-fold higher than with that of free cells. The immobilized mutated mesangio proliferative glomerulo nephritis (MPGN) kidney cell line showed a half-life of 30 days during continuous fermentation under airlift conditions.

Keywords Urokinase (UK); Mutated MPGN kidney cell line; Continuous fermentation; Cell immobilization; Airlift reactor (ALR)

Introduction

Urokinase (UK, EC 3.4.99.26) is a serine protease that activates plasminogen into plasmin, which in turn degrades fibrin clots. Hence UK finds its value as an important anti-thromboembolic drug. Plasmin has diverse physiological roles apart from its fibrinolytic role in the regulation of blood clotting [1,2], it has been implicated in complement activation, prohormone conversion, and the generation of localized extracellular proteolysis during tissue remodelling, cell migration, wound healing, carcinogenesis and neoplasia [3-8]. Cells from different origin are known to produce UK, but generally in low concentration. However, human kidney cells are reported as highest producers of UK [1,9].

In recent years, the need for urokinase (UK) production has increased significantly, and current production levels have not kept the same pace. To meet the increasing market need, research studies have been conducted to increase the bioreactor volumetric productivity by various approaches, such as media manipulation, bioreactor configuration and operating strategies. Hardly any information is available to correlate this basic insight with reactor configurations and operating strategies to maximize the UK productivity in the bioreactor.

An airlift reactor (ALR) can overcome the problems faced in the fluidized bed reactor (FBR) for the production of UK with immobilized cells. ALR differs from bubble column and FBR reactors by the presence of a draft tube. The main functions of the draft tube are increased mixing through the reactor and reduced bubble coalescence. The small bubbles in the reactor lead to an increase in surface area for oxygen transfer. Also, the draft tube equalizes shear forces throughout the reactor. This has been suggested to be the major reason why cells grown in airlift reactors gave high production than those grown in other types of reactors like stirred tank reactor (STR), spinner flask reactor (SPNR), packed bed reactor (PBR), FBR and perfusion reactor (PR). Different types of fermentation processes in these reactors were investigated mainly with respect to cell lines [10-12].

* Corresponding author: e-mail: adikunamneni@rediffmail.com, Phone: +34 915855479

The use of non-mechanically agitated bioreactors may contribute in reducing the process costs because they are considered to have a lower power requirement for comparable values of the oxygen mass transfer coefficient than the mechanically agitated vessels [13-15].

ALR was successfully operated in other fields for the production of protease using *Tetrahymena thermophila* [16], scleroglucan production by *Sclerotium glucanicum* [17], paclitaxel production by a cell suspension of *Taxus wallichiana* [18], enhanced anthocyanin production by grape callus [19], ginseng saponin and polysaccharide production by high density cultivation of *Panax nitoginseng* [20,21], production of recombinant interleukin-2 by BHK-1 cell line [22] and neomycin production using S*treptomyces marinensis* [23].

The authors laboratories have been engaged in developing an efficient technology for production and purification of urokinase. Medium formulation and strategic bioreactor operation have resulted in several folds increase in UK production. In the present investigation, it was planned to use a mutated mesangio proliferative glomerulo nephritis (MPGN) human kidney cell line G 109 for the preparation of biocatalyst by entrapment technique in alginate matrix and were used for continuous production of UK by ALR. Further, it is also decided to study the time course profile of viscosity, % torque and shear stress produced by the sparging based aeration system in ALR throughout the fermentation cycle. It is also decided to evaluate the duration of operational periods of ALR and half-life of immobilized beads.

Materials and Methods

Cell line

A mutated MPGN human kidney cell line G 109 (after 16^{th} passage), which produces UK was employed in the present study. It was isolated from MPGN patient's kidney biopsy samples [24] and a mutated cell line was developed in the University College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, India.

Preparation of inoculum

Ten milliliter of cell suspension from stock culture of mutated MPGN kidney cell line G 109 (after 16^{th} passage) was inoculated into 90 ml of Dulbecco`s modified eagle medium (DMEM) and incubated at 37°C in a shaker incubator (40 rpm) for 8 days. After incubation, the cell suspension (10×10^6 cells/ml) was used as inoculum for immobilization as well as for free cell fermentations.

Entrapment of cells in alginate

Cells were immobilized using sodium alginate by the ionotrophic method [25,26]. Two millilitres of cell suspension (10×10^6 cells/ml) was thoroughly mixed with 18 ml of sodium alginate slurry (3%, w/v) and the mixture was extruded into 40 ml of 0.2 M $CaCl_2$ solution to form spherical beads using peristaltic pump through small orifice. The beads then formed were cured for 2 h by incubating in 0.2 M $CaCl_2$ solution. The beads were thoroughly washed with sterile distilled water [27] and stored in saline solution (0.9% NaCl w/v solution) at 4°C for further use. The average diameter of the beads was 3.24 mm. All these operations were carried out under aseptic conditions.

Reactor system

A schematic diagram of the ALR, used in the present study, is shown in Fig.1. The reactor was made of glass with 37 cm height and 4.5 cm diameter, containing a concentric draft tube of 21 cm height and 1.5 cm of diameter. The working volume of the reactor was 450 ml. The air sparger was located at the bottom of the draft tube. The reactor was designed and fabricated indigenously at M/s, National Scientific Glass Works, Hyderabad, India. 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.

Fermentation in ALR

The alginate beads were loaded into the reactor with 450 ml of optimized production medium (unpublished results). The cells inside the beads were allowed to grow and activate for 16 days. The broth was drained from the reactor, filled with fresh production medium and continued the fermentation for UK production. The optimized medium was used for production strategies. The composition of modified optimised production medium is: DMEM supplemented with selenium, 20 ng/l; glucose,1 g/l; phenyl alanine, 5.9 g/l; ascorbic acid, 0.006 g/l; calcium chloride, 6.5 g/l; insulin, 5070 U/l and methyl jasmonate, 26.2 mM/l.

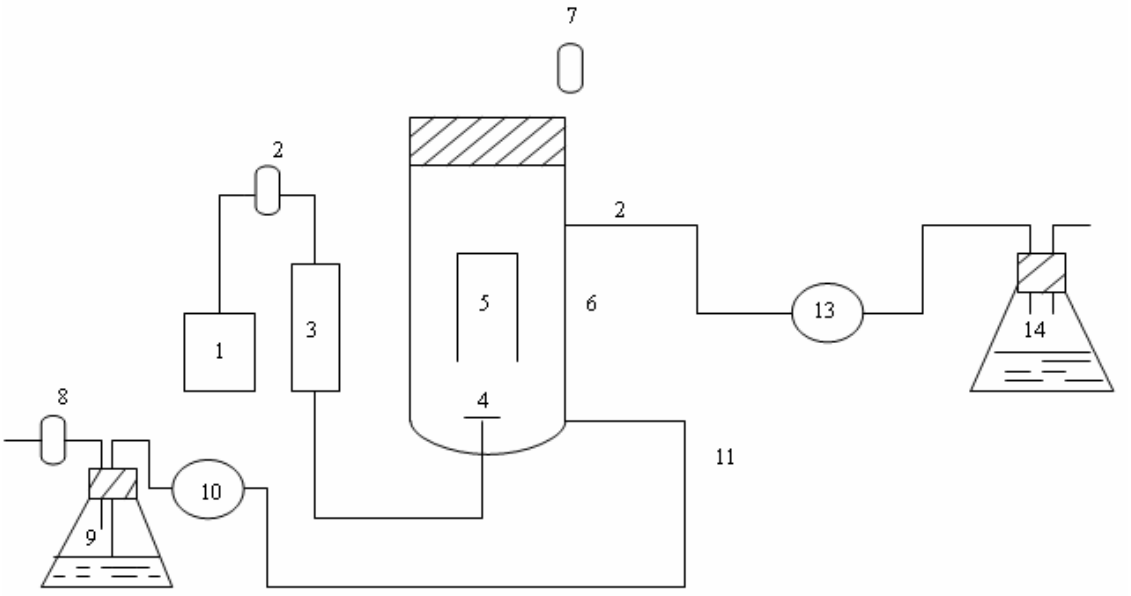


Fig. 1. A schematic diagram of the ALR in operation mode.

1	Air compressor	7	Air outlet filter
2 & 8	Air filters	9	Feeding flask
3	Humidifier	10 & 13	peristaltic pumps
4	Air sparger	11	Medium inlet
5	Draft tube	12	Effluent outlet
6	Reactor	14	Effluent collection flask

Continuous operation

The above batch operation was switched over to continuous operation mode by constantly adding the optimized production medium (Fig. 2). The over flow was collected constantly using peristaltic pump. The feed dilution rates were varied from 0.02 to 0.10 h^{-1}. The fermentation was continued with optimum dilution rate of 0.04 h^{-1}.

Analytical methods

UK activity was estimated by the modified method of Jesperson and Austrup [28]. A thin and stable fibrin clot was obtained by mixing 0.1 ml of thrombin solution (100 NIH U/ml), 0.1 ml of fibrinogen (5 mg/ml) and 0.6 ml of phosphate buffer (pH 7.5). For assay, discs of 5 mm diameter were prepared from Whatman no. 1 filter paper and sterilized at 160 °C for 1 h. The discs were dipped in test samples for 15 s, placed on the surface of the fibrin clot, incubated at 37 °C for 6 h and the area of lytic zones measured

with a planimeter. Activities were expressed in plough U/10×10^6 cells. All experiments were conducted in triplicate. The viable cell count was determined by a haematoxylin method [29].

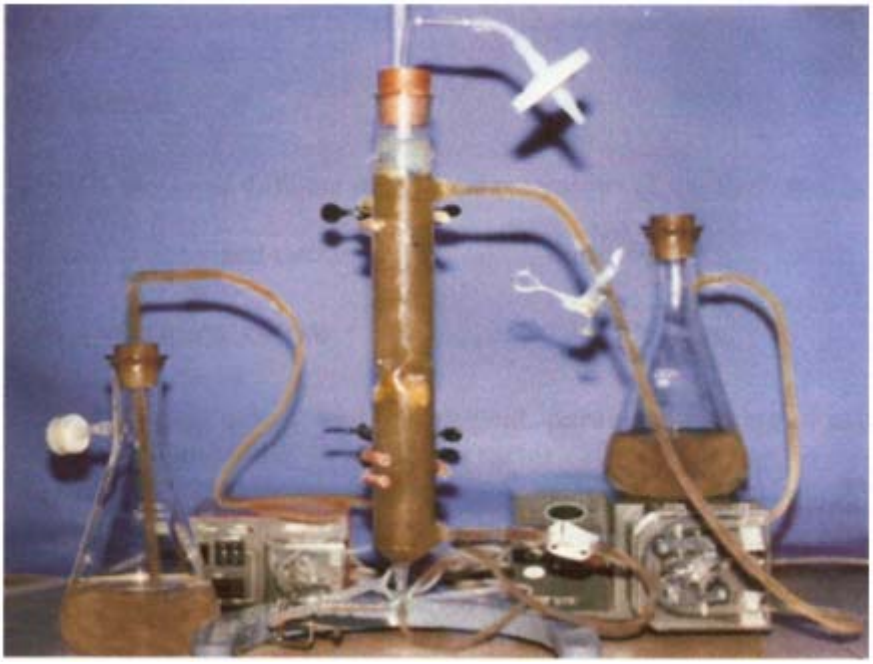


Fig. 2. Experimental set-up for ALR in continuous operation mode for UK production.

Results and Discussion

Studies on UK production and rheological parameters using mutated MPGN kidney cell line G 109 in ALR

The UK production aspects of free cells and immobilized cells of G 109 were evaluated for UK activity along with cell viability. The fermentation was carried out for 28 days as batch mode in ALR and samples were assayed during the fermentation cycle (Table 1). In the case of free cells, the maximum viable cell count reached to a level of 18.2×10^6 cells/ml. From the data, it was observed that the lag phase of G 109 cell line decreased in case of both free cells and immobilized cells (by 4 days) in bioreactors. In case of immobilized cells, the cell leakage increased gradually during the fermentation cycle.

The results showed that the UK production started on 4^{th} day incase of both immobilized cells and free cells and reached to a maximum titre (877 PU/ml) with immobilized cells in shorter time (16^{th} day) than the obtained (803 PU/ml) with free cells (20^{th} day) [Table 1]. On further incubation, negligible increment in the UK production was observed in both cases. The volumetric productivity of the immobilized cell fermentation is 54.8 PU/ml/day, while that of free cells is 40.2 PU/ml/day, which is 1.37 times higher than the free cell fermentation. This may be due to the stress imposed by the immobilized process, unlike in free cell fermentations.

Effect of airflow rate on UK production in ALR

Effect of aeration rates (0.5-3 vvm) on the production of UK with immobilized cells of G 109 in airlift bioreactor was studied. At 0.5 vvm, the volumetric productivity was 36.3 PU/ml/day while an increase in aeration up to 2 vvm, increased UK volumetric productivity was observed (Fig. 3). Further increase in

aeration (3 vvm) resulted in decreased volumetric productivity (37.4 PU/ml/day). From the results, it was concluded that the 2 vvm aeration was found to be optimum resulting in 55.3 PU/ml/day of volumetric productivity.

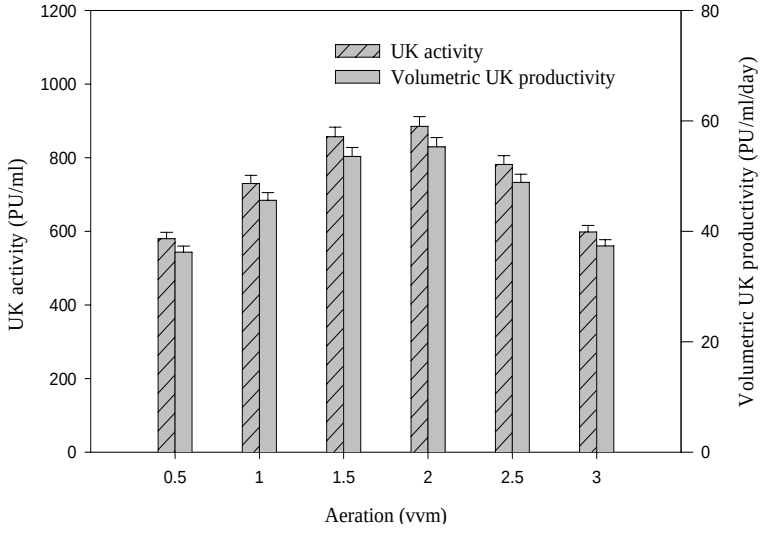
Table 1 Cell growth and UK production profiles with free cells and immobilized cells of G 109 in ALR.

Fermentation time (days)	Cell count/cell leakage ($\times 10^6$ cells/ml)		UK activity (PU/ml)		Volumetric productivity (PU/ml/day)	
	Free cells	Immobilized cells	Free cells	Immobilized cells	Free cells	Immobilized cells
0	-	-	-	-	-	-
4	8.9	2.7	96	115	24.0	28.75
8	14.6	3.9	215	382	26.9	47.5
12	16.8	5.9	376	605	31.3	50.4
16	17.4	6.7	587	877	36.7	54.8
20	18.2	7.0	803	854	40.2	42.7
24	16.2	8.3	776	835	32.3	34.8

Effect of dilution rate on UK production in ALR

An effective enzyme production is possible by optimizing the production rate in a given environment. Therefore, the impact of dilution rate/residence time on UK production with immobilized cells of G 109 was investigated in an ALR.

Experiments were conducted to evaluate the effect of dilution rate on UK production by immobilized cells of G 109 cell line, using the optimized production medium in an ALR (continuous mode). The results indicated that an increased UK titre was observed with an increase in dilution rates from 0.02 h^{-1} to 0.04 h^{-1} (Fig. 3). Further increase in dilution rate resulted in decreased volumetric productivity. The data suggested that the optimum UK production with alginate immobilized cells can be obtained at a dilution rate of 0.04 h^{-1} in an ALR.



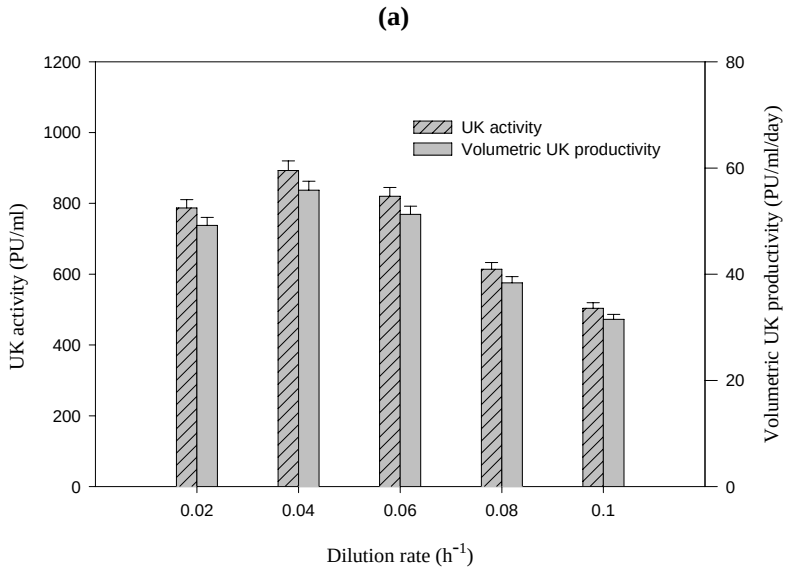


Fig. 3 Effect of aeration rate (a) and dilution rate (b) on UK production in ALR.

Continuous production of UK in ALR using alginate immobilized cells of G 109 cell line

Continuous production of UK with immobilized cells of mutated G 109 cell line was studied using optimized production medium in ALR. Initially the airlift reactor was operated for 16 days in batch mode and then shifted to continuous mode keeping 24 h of residence time (flow rate 18 ml/h; working volume 450 ml). The reactor was operated for 34 days without any attrition problems indicating that the alginate immobilized G 109 beads have half-life period of 30 days for the production of UK. The broth samples were collected every 2 days from the collection flask. The UK production increased gradually up to 16 days, there after the amount of UK was maintained constantly for 8 days and decreased later due to the sluggish mass in immobilized cells (Fig. 4).

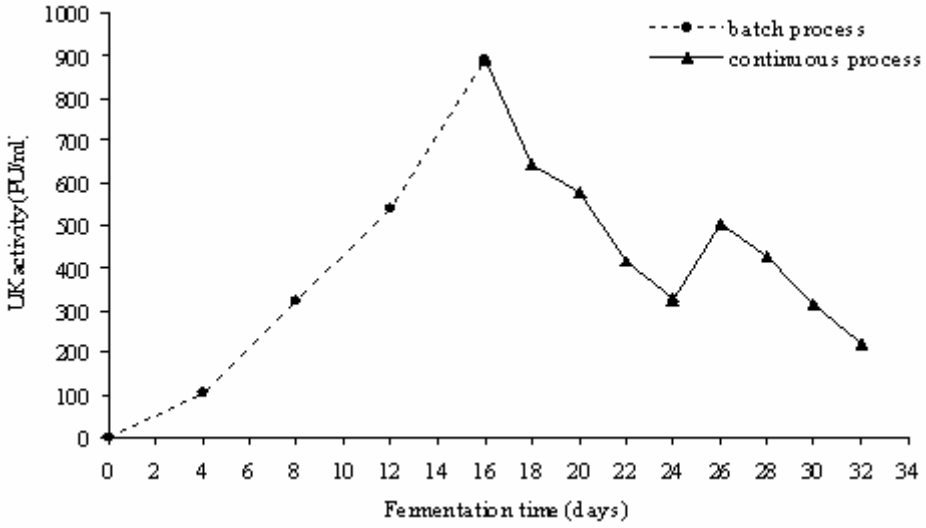
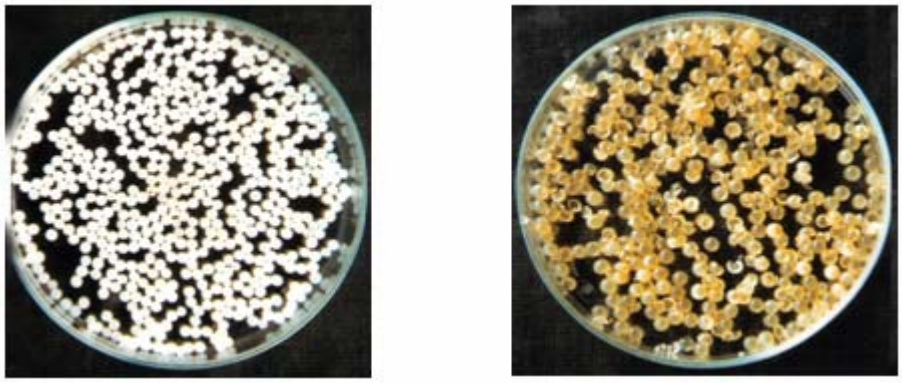


Fig. 4 Continuous production of UK by alginate immobilized cells of G 109 in ALR

On 24th day, feeding of production medium was stopped and the beads were washed continuously with sterile saline solution at a flow rate of 80 ml/h for 24 h. Later, the bioreactor was fed with fresh production medium and the immobilized cells were allowed to activate for 2 days. The rejuvenated cells were again subjected for continuous production of UK. As a result, the continuous cultivation for UK production was able to carried out for 34 days by using calcium alginate entrapped G 109 cells and in the optimized production medium by intermittent treatment with saline solution and reactivation of the cells. The immobilized cells were reactivated as shown by the increased production of UK. UK production gradually decreased with increase in operational time. This may be due to the decrease in cell viability caused by either aeration or agitation or both. However, conflicting observations were noticed in the literature. Continuous operation of the reactor with immobilized *Streptomyces* cells showed gradual decrease in actinomycin production [30]. Whereas, in the case of oxytetracycline production with polyurethane entrapped *Streptomyces* cells, the cells were intermittently treated with saline to improve the metabolite productivity [31]. The cells immobilized in alginate beads before fermentation and after fermentation are shown in Fig. 5.

A comparison of the UK production with free cells in batch fermentation and immobilized cells by continuous fermentation processes is shown in the Table 2. The average volumetric productivity with continuous fermentation (164.8 PU/ml/day) was found to be greater than that of the free cells (40.2 PU/ml/day). The total UK produced with continuous fermentation was found to be about 4.1-fold greater than with that of free cells. The overall results indicated that the continuous fermentation with immobilized cells was found to be the most economical than the batch process. The immobilized beads showed a half-life of 30 days during continuous fermentation under airlift conditions.



(a) (b)

Fig. 5 Appearance of G 109 cells immobilized in calcium alginate alginate before fermentation (a) and after fermentation (b), showing bead damage.

Table 2 Comparison of UK production with free cells (batch) and continuous fermentation processes in ALR.

Nature of cells	Total fermentation time (days)	Total UK titre (PU/ml)	Volumetric productivity (PU/ml/day)
Batch culture with free cells	20	803	40.2
Continuous culture with immobilized cells	34	5603	164.8

Acknowledgements One of the authors (R. Bhavani Devi) thanks to Indian Council of Medical Research (ICMR), India and Andhra University, India, for providing the financial support to carry out the research work.

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