CEPHALOSPORIN PRODUCTION WITH FREE & IMMOBILIZED CEPHALOSPORIUM *ACREMONIUM* CELLS & DETERMINATION OF OXYGEN TRANSFER RATE IN STIRRED TANK REACTOR

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ABSTRACT

Cephalosporin production by free and agar immobilized *Cephalosporium acremonium* cells was investigated in a stirred tank reactor. Cephalosporin production was increased two times with immobilized cells. In order to verify the oxygen transfer efficiency in STR, oxygen mass transfer coefficient was determined by using dynamic gassing out method at the end of steady state operation of the reactor after achieving cephalosporin production. The oxygen mass transfer coefficient ($K_L a$) obtained in STR with 20 % (V/V) agar immobilized beads, at 1 VVM (Unit volume of air per unit volume of the reactor per minute) of air flow rate and 250 RPM of agitator speed gave maximum amount of antibiotic production in the reactor. The oxygen mass transfer coefficient ($K_L a$) obtained in stirred tank reactor (STR) is $21 hr^{-1}$.

Keywords: Immobilization, Cephalosporin, *Cephalosporium acremonium*, Stirred tank reactor, dynamic gassing out method, Volumetric oxygen mass transfer coefficient ($K_L a$).

1. INTRODUCTION

Antibiotic production is a key area in applied microbiology and antibiotics are usually produced by batch / fed batch fermentation using free cell cultures. To enhance the productivity and to improve the economics, much attention has been paid on the improvement of the cultures employed in the antibiotic production. It is worthwhile to closely examine the fermentation strategies adopted to improve the productivity of the system and whole cell immobilization technology appears to be attractive for antibiotic fermentation. Immobilized microbial cells offer several advantages over free living cells in metabolic production. They can work as effective biocatalysts due to their characteristics of self generating and proliferation complex catalysts system. Several attempts have been made to immobilize various microbial species on different support matrices for antibiotic production. Among different methods employed, calcium alginate entrapment has shown considerable promise in antibiotic production. Reports are available on production of bacitracin [1], nikkomycin [2], neomycin [3], patulin [4, 5] erythromycin [6], actinomycin [7], candicidine [8] and cephalosporine [9] using immobilized cells [10].

Most available reports on antibiotic production using immobilized cells are limited to shake flask culture and only a few reports are available on bioreactor studies [11]. Earlier reports on immobilization of penicillin G - acylase in chitosan beads was studied by Braun, J [12] & Guisan [13]. But as of now, reports are only based on agar as gelling agent, [14] and use of agar beads for cephalosporin production is not yet reported.

The main objectives of the present investigation is to study about a) whole cell immobilization by entrapment in agar for cephalosporin production using *Cephalosporium acremonium* in Stirred tanks reactor and comparison of the same with free cells under similar conditions. b) Inadequate supply of oxygen is one of the major problem in industrial production of antibiotics. Efficient oxygen transfer is crucial especially for systems contaminating high biomass loading [15]. In antibiotic production oxygen demand is the key parameter hence oxygen supply plays a vital role in fermentation systems. It is difficult to meet oxygen demand especially in viscous fermentation broth. Special fermentor designs are required to enhance the rate of oxygen transfer in fermentation systems involving high oxygen demand. The design of a fermentor for optimum oxygen transfer requires a thorough understanding of the transfer and

utilization of oxygen in microbial systems [16]. The DO concentration of the fermentation broth has a profound effect on the performance of aerobic fermentation systems. The problem solving approach usually involves improvements in design of the bioreactor, agitator and sparger as well as the use of oxygen-enriched air [17].

Cephalosporium acremonium is sensitive to dissolved oxygen changes, and in the absence of dissolved oxygen, the cells starve. According to the earlier authors the cells are much more sensitive to oxygen starvation during the growth phase than during the production phase [18]. This indicates that the lack of the oxygen mainly influences the basic metabolism rather than biosynthesis of antibiotics. During the growth and production phases, dissolved oxygen is necessary. Dissolved oxygen depends on the specific interfacial area of the bubbles in submerged cultures, which is a function of the property of the medium (viscosity, coalescence promoting / repressing properties). Oxygen mass transfer is a function of process and cost. Hence estimation of oxygen mass transfer coefficient in stirred tank reactor for the production of antibiotics is essential for the design and development of a bioreactor for efficient and economical production of antibiotics [19].

The present study is aimed at determination of volumetric oxygen mass transfer co-efficient (K_La) using dynamic gassing out method in a stirred tank bioreactor with immobilized cells of *Cephalosporium acremonium* for the production of cephalosporin. This paper indicates the enhanced levels of cephalosporin production using immobilized agar beads, which is economically feasible and discusses the ways for improving reactor performances.

2. MATERIAL AND METHODS
2.1. Microorganism and Culture Conditions
In the present study the strain *Cephalosporium acremonium* ATCC 20339 was used and maintained on agar slopes, medium containing (gl^{-1}) of ; Soluble starch, 15; Yeast extract, 4.0; K_2HPO_4, 1.0; $MgSO_4$,1.0; pH: 6.5$\pm$0.2, temperature 25°C and subcultured at monthly intervals. Five ml of sterile distilled water was added to a well sporulated slant (7-day old) and spores removed by scrapping. The spore suspension prepared was then added to a 250 ml Erlenmeyer flask containing 45 ml of growth medium incubated on a rotary shaker at 180 rpm at 25°C. After 72 hrs of incubation, the culture broth was centrifuged; the cell pellet washed with sterile 0.5 % NaCl solution. This cell suspension was used as inoculum

for further experiments and also for the immobilization of whole cells.

2.1.1. Immobilization of whole cells
Cells were immobilized using agar by entrapment method [20]. 2 % agar was mixed with 0.2 % cells on dry weight basis (DCW) (W/V) and mixed well, to get uniform suspension, then this solution was dropped into paraffin oil in the bead form with the help of peristaltic pump (Watson and Marlow. U.K) through small orifice. The beads then formed were cured for 2 hrs by incubating in oil and washed twice with sterile saline (1 % NaCl W/V solution) and stored in saline solution at 4 $^\circ$C for further use. All these steps are carried out under aseptic conditions.

2.2. Fermentation in Stirred Tank Reactor
Experiments were conducted in batch STR (B. Braun Biostat, Germany) for the production of cephalosporin using agar immobilized cells of *Cephalosporium acremonium*. The composition of production media consists of (gl^{-1}): Sucrose, 80; Soya-bean meal, 60; $CaCO_3$, 1.5; DL-Methionine, 7.0; ammonia, 30. Sucrose was autoclaved separately. The salts were adjusted to pH 6.0 with NaOH/HCl prior to sterilization. The reactor and accessories were sterilized separately. The working volume of the reactor was 1 liter and filled with 20 % (V/V) of immobilized beads and the average size of the beads was 2.0mm. Continuous sterile air was supplied through air filters (Watman 50μ) at the rate of 1VVM (unit volume of air per unit volume of the reactor per minute) and impeller was provided for mixing with a speed of 250 RPM.

2.3. Analytical Methods
Samples were drawn in eppendrof tubes everyday under sterile conditions of antibiotic assay, carbohydrate estimation and pH. Cephalosporin concentration was determined by plate diffusion method using *E. coli* as test organism along with standard penicillin disc (10 mg, Hi-media) in every plate [21]. Carbohydrate was estimated by Anthrone method [22]. The volumetric mass transfer coefficient (K_La) was determined using the dynamic method by DO meter [23].

2.4. Determination of K_La by Dynamic Method of Gassing Out
Aeration and agitation are important variables to provide effective oxygen transfer rate during aerobic

bioprocesses. Hence, the knowledge of the volumetric mass transfer coefficient (k_La) is required. The determination of k_La in a bioreactor is essential in order to establish its aeration efficiency and to quantify the effects of operating variables on oxygen supply.

Oxygen transfer in aerobic bioprocesses is essential and any shortage of oxygen vastly affects the process performance. Therefore, oxygen mass transfer is one of the most important phenomena in the design and operation of mixing-sparging equipment for bioreactors. It can be described and analyzed by means of the volumetric mass transfer coefficient, k_La. The values of k_La are affected by many factors, such as geometrical and operating characteristics of the reactor (type of impeller, the geometry of the bioreactor, the agitation speed and the air flow rate), media composition and properties, concentration and microorganism's morphology and biocatalyst's properties as shown in Fig. 1.
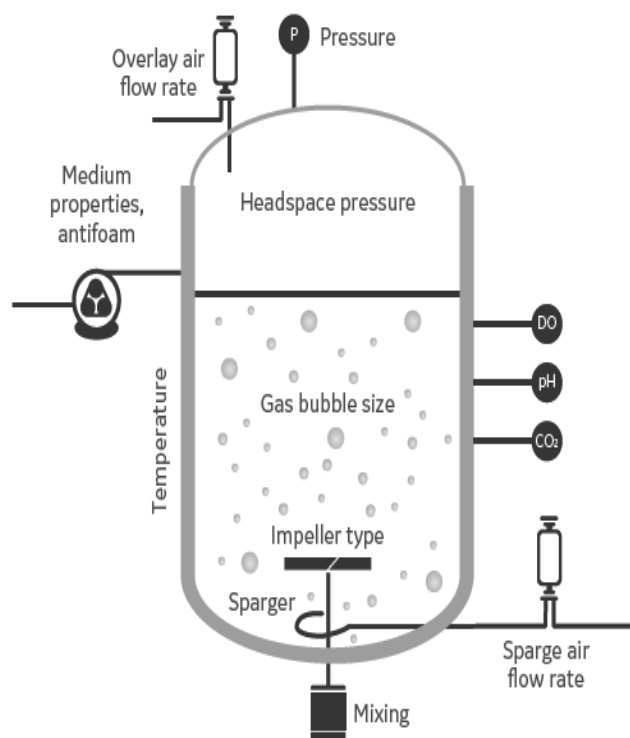
Fig. 1: Key factors that influence k_La values in a Bioreactor process.

This method is the simplest one since it requires only a dissolved oxygen probe [23]. It is mainly based upon the dynamic oxygen balance in a batch culture, which has the following form:

$$dC_L/dt = K_La\ (C^* - C_L) - QO_2X \qquad .....(1)$$

Where QO_2 is the rate of oxygen consumption per unit mass of cells (mm O_2 $g^{-1}h^{-1}$), rearranging equation (1) gives;

$$C_L = C^* - \frac{(QO_2X + dC_L/dt)}{K_La} \qquad .....(2)$$

The air supply is turned off at a certain time during fermentation and the variation of C_L with time is followed with the aid of a DO probe. Since, the term K_La ($C^* - C_L$) becomes zero when air is turned off, the C_L value decreases linearly with time according to equation (1). The slope of the C_L Vs time curve is a value of QO_2X. The air is then turned on and the increase in DO with time is followed. Having determined the QO_2X value, C_L is plotted against ($QO_2X + dC_L/dt$). The slope of this plot is equal to the reciprocal of K_La.

3. RESULTS AND DISCUSSION

3.1. Stirred tank reactor studies of cephalosporin production using *Cephalosporium acremonium*

Cephalosporin production using *Cephalosporium acremonium* under free and agar immobilized states was investigated in the stirred tank reactor. Immobilized cells have produced two-times more antibiotic than the free cells (fig.2).
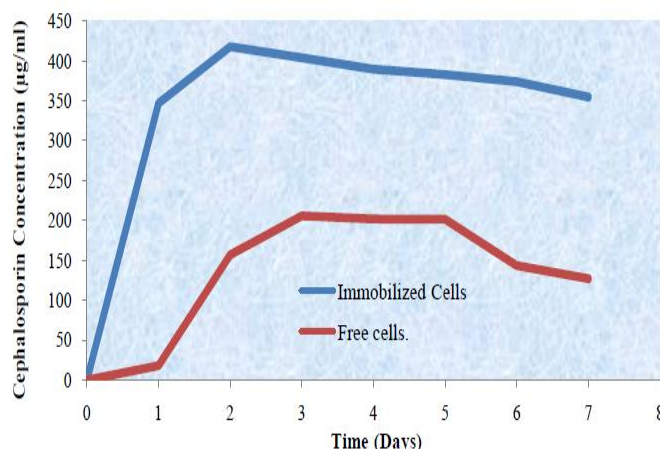
Fig. 2: Improved Cephalosporin Production by Agar Immobilized Cells Over Free Cells

The maximum amount of cephalosporin production (432.16 $\mu g\ ml^{-1}$) was produced at the end of 48 hrs by immobilized cells, whereas, the peak antibiotic production activity was observed after 72 hrs in the free cell fermentation. The volumetric productivity of the immobilized cell fermentation is 6.21 mg $l^{-1}\ h^{-1}$, which is 60 % higher than free cell fermentation (2.5 mg $l^{-1}\ h^{-1}$). Free cells showed a lag phase of 24 hrs, whereas immobilized cells commenced the production of antibiotic immediately. This is due to the induced stress

imposed by the immobilization process, which is not present in free cell fermentations.

The optimized production medium resulted in good yield of cephalosporin production the carbon and nitrogen (lactose and ammonia) concentrations were selected by conducting various experiments by varying carbon and nitrogen sources in the above production media. It was seen that enhanced supplementation of carbon and nitrogen sources are neither economic nor effective for cephalosporin production with immobilized cells of *Cephalosporium acremonium*. The observations suggest that antibiotic and biomass can be effectively decoupled by appropriately selecting the key nutrient concentration during fermentation. (10 g/l) of the sucrose in the media at zero hrs of fermentation indicates that under limited carbon sources supplementation, *Cephalosporium acremonium* cells can divert these sources to secondary metabolite production rather than cell mass production under immobilized conditions. Similarly observations were also noticed for patulin production with *P.urticae* by Berk et.al [4, 24] and antibiotic production with *Cephalosporium acremonium* by Kim et.al [25].

3.2. Determination of K_La values for STR.

Substrate and immobilized cells were transferred to STR. Air was bubbled continuously to the reactor and antibiotic concentration was monitored regularly and it was observed that, antibiotic activity started declining after 10th day onwards. Hence, the experiment was terminated at 10th day. On the same day experiment was carried out for the determination of K_La by dynamic method. In STR the dissolved oxygen was measured for every 30 seconds up 150 sec's. The dissolved oxygen (DO) concentration was stable at 6.21 mg.l^{-1}. Air supply was stopped at this condition (150 sec's) and decrease in DO was recorded until 600 sec's. The DO concentration decreased from 6.0 mg.l^{-1} to 5.6 mg.l^{-1} during this period. Again air supply was started to the reactor at this juncture (600 sec's) and DO was recorded. It was observed that DO recovered to 6.0 mg.l^{-1} at 950 sec's and remained stable further. The results were plotted and shown in fig. 3.

Specific rate of oxygen consumption over a volume of liquid (QO_2X) was determined by measuring slope of the curve during the absence of air supply from this graph. The rate of increase in DO (dC_L/dt) was determined at four points when the air supply was resumed on 600 sec's. Another plot was drawn (Fig. 4) between ($dC_L/dt + QO_2X$) Vs DO concentration. The slope of

this graph was the reciprocal of the volumetric mass transfer coefficient. The K_La value for the STR was found to be 21hr^{-1}. It is obvious that the values of k_La are not so sensible to the variation of the input air flow rate in comparison with variation of the agitation speed, especially in large reactors.
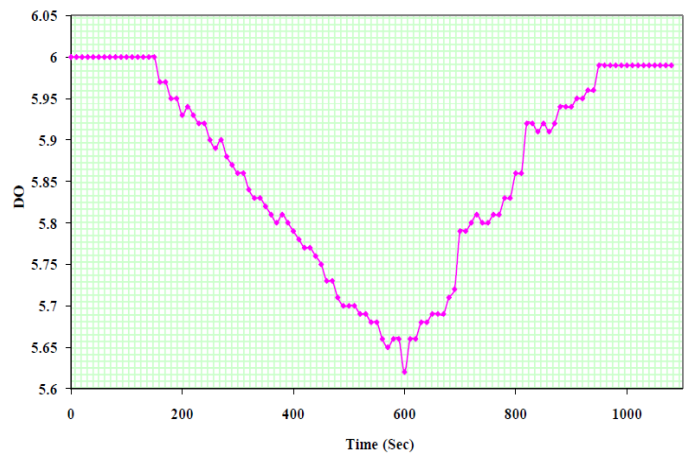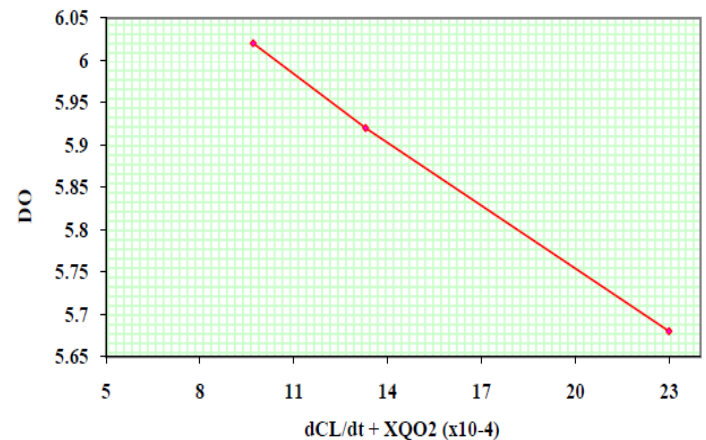

Fig. 3: Determination of K_La in STR

Fig. 4: Dynamic method for the determination of K_La values

Oxygen transfer Co efficient (K_La) studies reflect that confined cell fermentation maintains a much more favorable system, as the gas-liquid mass transfer properties of the culture become enhanced, possibly because of restructuring of the filamentous morphology of the cells.

The performance of the reactor in terms of oxygen mass transfer at a K_La value of 21hr^{-1}, has the advantages of the immobilized process demands a rigorous study of the limitation to mass transfer of the reagents, especially oxygen [26, 27] therefore, use of immobilized cells will increase the oxygen mass transfer rate [28, 29].

4. CONCLUSION

Current process for the production of cephalosporin is primarily dependent upon the use of very slow growing free cells and is not designed for the repeated use of the cells. However, this study clearly indicates that immobilized cells have potential to extend the production of cephalosporin yield for more number of days in STR and is more suitable for repeated use of fermentation cycles since; the entrapment of cells in agar beads provides mechanical protection to the cells by the matrix. Agar immobilized cells have shown two fold enhanced cephalosporin production compared to free cells in STR.

The most suitable method for k_La measurement requires taking into account the range of the variables as power input, volume and type of the reactor, characteristics of gas and liquid phase. The dynamic method is the most widely used, with a small degree of approximations for gas–liquid mixing in the reactor. This method is suitable for large scale bioreactors with errors less than 10 %, over the entire range of the operating conditions encountered in the industrial fermentor operations.

The high volumetric oxygen mass transfer coefficient performance in stirred tank reactor represented excellent performance in terms of good aeration and antibiotic production. Use of agar immobilized cells of *Cephalosporium acremonium* in stirred tank reactor is more economical for the production of cephalosporin.

Nomenclature

K_La = Volumetric oxygen transfer Coefficient, hr^{-1}.

C_L = Saturated dissolved oxygen concentration liquid, $mg.l^{-1}$.

C^* = Equilibrium dissolved oxygen concentration in liquid, $mg.l^{-1}$.

X = Biomass concentration in liquid, $mg.l^{-1}$.

QO_2 = Specific rate of oxygen Consumption $mMO_2 \ g^{-1} \ hr^{-1}$.

dc_L/dt = Change in DO concentration in liquid with time $m.gl^{-1} \ hr^{-1}$.

DO = Dissolved Oxygen

STR = Stirred tank reactor

5. ACKNOWLEDGEMENT

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