Penicillin Production in Continuous Stirred Tank Reactor by *Penicillium chrysogenum* Immobilized in Agar

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A previously developed immobilization technique involving agar as matrix is investigated further in penicillin production by *P. chrysogenum* ATCC 10238 at reactor level. Several modifications were found to decrease the germination lag time, including a higher spore concentration, an increased porosity of gel, and decreasing the mass transfer barrier. This approach enabled the production of penicillin from immobilized agar beads within 3–4 h from the onset of continuous system. A continuously immobilized cell stirred tank reactor produced penicillin in which production phase lasted up to 25 days in a steady state manner. The productivity of this system showed 2.5 times more stability than the productivity of the corresponding batch fermentation in stirred tank reactor.

Keywords:
Penicillin, Immobilized agar beads, *P.chrysogenum,* CSTR

Introduction

Continuous production of penicillin by immobilized *P.chrysogenum* ATCC 10238 was described by *El-Sayed* and *Rehm* using calcium alginate beads in a bubble column and conical bubble fermentor.[1a,b]

Immobilization of *P. chrysogenum* in K-carragenan beads in fluidized bed reactor was studied by *Deo* and *Gaucher.*[2] Jones et.al studied continuous production of penicillin by immobilized cells on celite and K-carragenan using fluidized bed reactor[3].

The continuous process with immobilized microorganism seems to have several advantages over a continuous fermentation with free cells. Strains of *P. chrysogenum* have been immobilized by entrapment in various hydrogels[4,5] and also by adsorption to inert supports such as celite. Different types of reactors and modes of fermentations have been used for this purpose. Despite the fact that a number of important antibiotics can be produced in continuous cultures (i.e. penicillin), essentially, all antibiotics are produced in batch culture processes. In this study efforts have been put to overcome some problems, associated i.e. by producing a product at minimum cost, which is achieved by maximizing the conversion of the raw material into the product and maximizing the volumetric productivity and also greater production. This paper describes the use of less expensive agar immobilization technique and a medium for the continuous production of penicillin over a period of 25 days in stirred tank reactor which has been developed.

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Materials & methods

Organism and Media

P. chrysogenum ATCC 10238 is used through out this study. Spore stocks were maintained on a sporulation medium containing (in g L^{-1}); Malt extract, 20; Glucose, 20; Peptone, 1.0. pH maintained at 6.5 $\pm$ 0.2 with NaOH / HCl. The growth phase medium contained (in g L^{-1}); Glycerol, 7.5; Peptone, 5.0 g; molasses, 7.5; Mg SO_4, 0.05; KH_2PO_4, 0.06; NaCl, 4.0.

The defined production phase medium for bioreactor contained (in g L^{-1}); Lactose monohydrate, 10; KH_2PO_4, 3.4; NH_4Cl, 1.21; phenyl acetic acid, 0.3; K_2SO_4, 3.95; $MgSO_4 \cdot 7H_2O$, 0.25; ammonia, 30. Lactose was autoclaved separately. The salts are adjusted to pH 6.0 with NaOH / HCl prior to sterilization.

Immobilization procedure

Aqueous spore suspension 8.4×10^8 spores ml^{-1} of 10.5 ml was added to 2 % agar solution swirled to obtain a homogeneous mixture; poured into paraffin oil in the bead form with the help of peristaltic pump. The suspension was cooled below the setting temperature of agar. After the beads have solidified, preparation was allowed to settle overnight at 30 °C. Oil layer was decanted, beads were filtered and washed with phosphate buffer solution and second time with water. All materials used were sterilized in an autoclave.

10 % beads were used for inoculation of 1 lit growth medium. Spore germination and mycelial pellet development were initiated by incubating the prepared biocatalyst in growth medium for 5 days at 27 °C.

Table 1 – *CSTR operating conditions in different runs*

Fermentation – run	Initial production medium (γ/g L^{-1}) in CSTR till 120 h	Daily replacement medium			
		Lactose γ/g^{-1} L^{-1}	Phenyl acetic acid γ/g^{-1} L^{-1}	Ammonia γ/g^{-1} L^{-1}	pH
Run-I	Lactose monohydrate, 10; KH_2PO_2, 3.4; NH_4Cl, 1.21; Phenyl acetic acid, 0.3; K_2SO_4, 3.95; $MgSO_4$, $7H_2O$ 0.25. pH 6.0	10.0	0.300	30.0	6.0
Run-II		0.20	0.006	0.60	6.0
Run-III		0.13	0.004	0.40	6.0

Continuous culture studies

For continuous cultures, the beads were first incubated as batch cultures for 120 h. Then fresh medium was transferred continuously to the reactor. The flow rate was 25.0 ml h^{-1}. The optimized air flow rate was 1 L L^{-1} min^{-1}. The feed medium had the following constituents (g l^{-1}); Lactose monohydrate, 0.13; Phenyl aceticacid, 0.004; and Ammonia 0.4 ml and pH 6. Conditions for CSTR are shown in Table 1.

Reactor dimensions

Operating volume: 1 dm^3, Liquid height: 16 cm, *L/D* (tank diameter): 4.338 cm

Aeration rate of 1 L L^{-1} min^{-1}; stirred speed 300 rpm.

Analysis procedures

Sugar's were estimated by Anthrone method[6], Penicillin was estimated by biological assay[7] Dry biomass was measured as the dry cell weight per liter of culture. Culture samples (5 mL) were centrifuged at 8000 rpm for 10 min. The cell pellet was resuspended in distilled water and filtered on pre weighed, dried by Whatman No.1 filter paper discs. The cells were dried at 75 °C for 24 h and the dry cell mass was determined. pH of the samples was recorded.

RESULTS

A number of operating parameters were varied in order to characterize the behavior of the reactor system under different conditions. The operating quantities tested included dilution rate (*D*), inlet feed concentration, fermentation time, and feed rates.

Kinetics of immobilized continuous stirred tank reactor with various operating parameters are conducted to compare performances with that of batch stirred tank reactor and to identity values for continuous system parameters that could provide higher performance. However as seen in fig 1, beyond D_c results in washout; cells are removed from the vessel at a rate faster than growth rate. This can limit the production of the CSTR, where there is no advantage for penicillin concentration when operated at these high dilution rates. At higher dilution rates, a large portion of carbon source goes into cell mass, and unless cell mass is recycled, the substrate is wasted. At the other extreme, as the dilution rate is lowered, one approaches batch culture. Thus, efficient method and optimized conditions were selected for substrate conversion and product accumulation, while applying for continuous culture to antibiotic production.

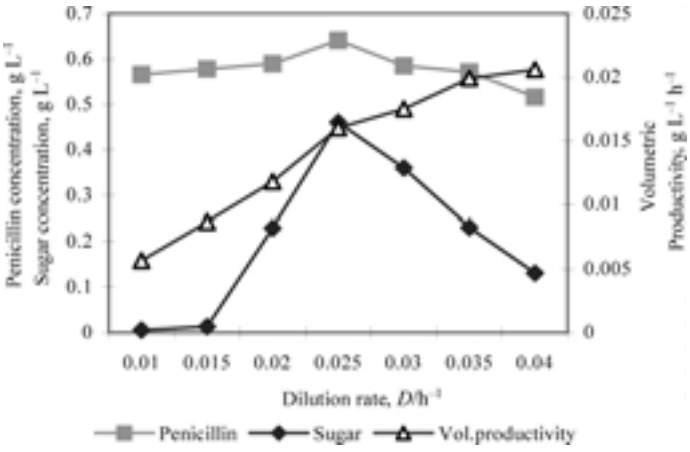
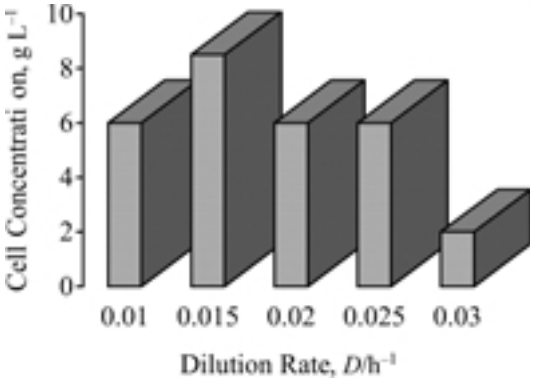


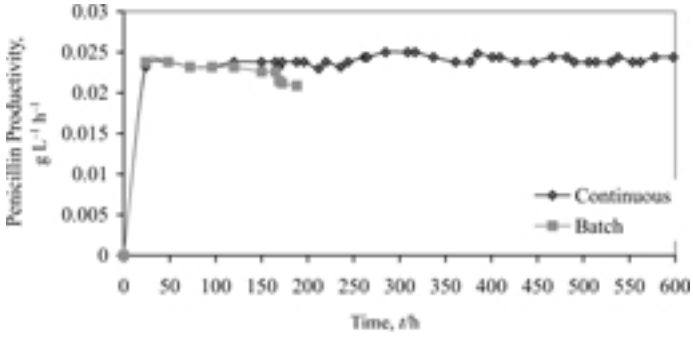
Fig. 1 – *Effect of dilution rate on fermentor penicillin concentration, sugar concentration & Volumetric productivity of CSTR*

Fig. 1 and 2 show that above D = 0.025 h^{-1}, consumption of more sugar limits the penicillin production, and over a certain significant dilution rate, the penicillin production is greater, this is primarily due to a required cell density in the reactor.

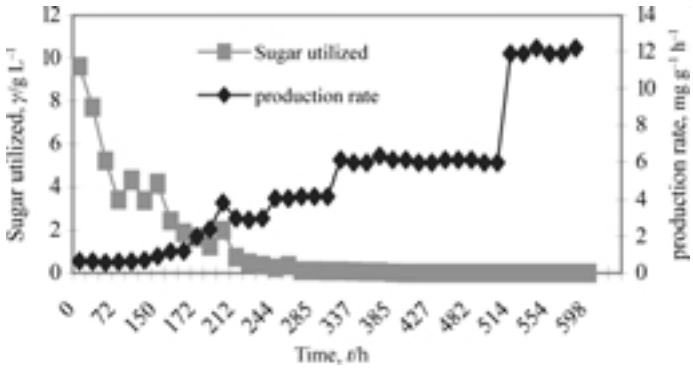
The secondary metabolites and cell concentrations are shown in fig. 1 and 2 as a function of dilution rate. The total cell concentration decreases monotonically as the dilution rate is increased, un-



F i g . 2 – *Effect of dilution rate on fermentor cell concentration g L^{-1}*



F i g . 3 – *Average volumetric productivity as a function of time. Inlet concentrations and dilution rates were selected to maximize productivity. Continuous system: lactose = 0.13 g L^{-1} d^{-1}, D = 0.025 h^{-1}*



F i g . 4 – *Sugar utilized by agar immobilized cells in continuous culture with a flow rate of culture medium of 22.7 ml h^{-1}*

like the constant cell mass found with microbial systems.

The maximum penicillin values of 0.641 g L^{-1} is reached at D = 0.025 h^{-1} for a flow rate of 25 mL h^{-1}. A maximum penicillin productivity of 0.026 g L^{-1} h^{-1} could be obtained with the substrate conversion which was found to be around 81.6 %. As the flow rate is increased further, higher substrate concentration is obtained, but penicillin yield is low, because most of the substrates are utilized for cell growth rather than for product formation and results in destabilization of beads.

In this system there are strong interactions between dilution rate, sugar utilization, and cell mass. We studied these variables in turn, where fig 2 compared with fig (1) shows the influence of sugar concentration and dilution rates on productivity.

It is clear that increase in the inlet feed, after attaining certain value, decreases the biomass productivity of the reactor. We, therefore, examined the influence of feed concentrations in replacement medium as shown in Table: 1, where it is found that 0.13 g L^{-1} of sugar in the feed at an optimal dilution rate gave the highest value of steady state volumetric productivity for a longer period of around 598 h. Table 1 indicates the different concentrations of nutrients used for replacement media. Penicillin production is the same and in steady state for all the 3 runs; but run-I could be operated only for 3 days, and run-II for 7 days, where as run-III can be successfully operated for nearly 25 days maintaining the stability of agar beads.

The less stability of run-I is due to rapid cell multiplication, which causes gradual swelling of beads by attaining higher cell growth after continuous feeding, and results in breakage of beads. The above studies indicate that run-III operating conditions are ideal for CSTR at D = 0.025 h^{-1}.

Fig. 3 indicates a typical continuous productivity curve with a medium flow rate of 25.0 mL h^{-1} and aeration rate of 1 L L^{-1} min^{-1}. In order to minimize the increase in biomass concentration, which is observed during the later stages of the batch runs, a continuous fermentation is carried out in order to utilize the nutrients for product yield and to minimize the cell biomass. Continuous feeding started after 120 h with a dilution rate of 0.025 h^{-1}. There is gradual increase in specific production rate and sugars are depleted as shown in fig. 4.

Discussion

The objective of immobilization of microorganisms is to allow their extended use as a catalyst. For biosynthesis of secondary metabolites, such as antibiotics, it is necessary to provide immobilized cells with a range of nutrients, which permits growth along with product formation.

The productivity and stability are comparable to those reported by earlier workers.[8] In batch systems there is fall in yield after a very short period, which can be overcome by CSTR. In CSTR the average specific growth rate (μ) is about 0.0235 h^{-1} and pH is maintained at 6.0 through out the reactor operation as described by.[9]

The effective concentration of active mycelia in agar beads results in higher volumetric penicillin productivity, assuming that the supply of C,N and phenyl acetic acid are not limiting, therefore, replacement media is modified to ensure that full potential of penicillin productivity is achieved by controlled growth near gel surface, which avoids the diffusion barrier for oxygen and nutrients.

Immobilization of cells in this case would provide a number of advantages; higher cell densities at lower broth viscosities would result in faster reaction rates of mass transfer. Higher yields by using immobilized agar beads achieved two-fold increase in penicillin to that of free cells. Similarly, in shake flask study maximum penicillin was produced at 72 h of fermentation and further fermentation results in decrease of product yield. CSTR, operated under optimized conditions, will result in penicillin production from 24 h of fermentation and remains stable till the end of fermentation i.e 600 h. Along with reduced power consumption and aeration requirements continuous operation would reduce the proportion of non productive growth phase, since cells are maintained in the production phase for an extended period; the use of immobilized cells would allow operation at higher dilution rates with no risk of wash out.[10] In order, to achieve immobilized cell technology to be successfully applied to fungal fermentations, new immobilization methods must be developed, which will overcome the limitations of loss of enzyme activity or the presence of unwanted free cells.[11]

ACKNOWLEDGEMENT

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References

1.a *El- Sayed A. H. M., Rehm H. J.* Appl. Microbiol Biotechnol. **26** (1987) 211.

b *El- Sayed A. H. M., Rehm H. J.* Appl. Microbiol Biotechnol. **26** (1987) 215.

2. *Deo Y.M., Gaucher G. M.,* Biotechnol. Bioeng. **26** (1984) 285.
3. *Jones A., Wood D. N. T., Razniewska, Gaucher G. M., Behie L. A.,* Can. J. Chem. Eng. **64** (1986) 547.
4. *Morikawa Y., Karube I., Suzuki S.,* Biotechnol. Bioeng. **21** (1979) 261.
5. *Deo Y. M., Gaucher G. M*., Biotechnol. Lett. **5** (1983) 125.
6. *Loewus F. A.,* Analytical. Chemistry **24** (1952) 21.
7. *Bandhopadhyay B., Humphrey A. E., Tayuchi, M*., Biotechnol. Bioeng. **9**(1967) 533.
8. *William P., Flanagan, et.al*. Biotechnol. Bioeng. **36** (1990) 608.
9. *Pirt S. J., KCM*. Ind. **34(1)** (1985) 13.
10. *Vandamme E. J*., J. Microb. Biotechnol. **1(2)** (1986) 102.
11. *Gbewonyo K., Wang D. I. C*., Biotechnol. Bioeng. **25** (1983) 2873.