Continuous Production of 6-amino Penicillanic Acid (6-APA) by Agarose Immobilized Penicillin Acylase in a Packed Column Reactor

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Penicillin acylase, an industrially important biocatalyst catalyzes the conversion of penicillins to 6-amino penicillanic acid (6-APA) which is the main precursor for the production of semi-synthetic β-lactam antibiotics. The present work involves the continuous production of 6-APA in a packed column reactor by using agarose immobilized penicillin acylase as a block polymer. The strain *Escherichia coli* ATCC 11105 was used as enzyme source and penicillin G as substrate. The acidic nature of 6-APA has an inhibitory effect on the enzyme and so the continuous system of production is a better choice. To overcome this problem, penicillin acylase was physically entrapped on agarose gel. Kinetic quantities ζ_{max} and K_m values were calculated for both native and immobilized enzyme. The immobilized enzyme was packed in the column reactor to study the maximum capacity of the reactor in diffusion free condition by varying flow rate and different substrate mass fraction. F_{max} value was calculated using secondary plot of $1/F$ vs. $1/Q$ to find the maximum capacity of the bioreactor according to the model of Lilly et al. 1966. This study is very useful and applicable to the industry for the conversion of Penicillin G to 6-APA.

Key words:
Penicillin acylase, 6-APA, immobilization, agarose, packed column reactor.

Introduction

The hydrolysis of penicillin G to obtain 6-amino penicillanic acid (6-APA) is one of the most important examples of industrial applications of biocatalysts.[1,2] 6-APA is the main precursor for the production of semi-synthetic beta lactam antibiotics and is produced at a scale of ca. 10,000 ta^{-1}.[3] There have been many reports, on *E. coli* for penicillin G acylase production[4]. In the present study, we used *E. coli* strain ATCC 11105 for penicillin G acylase production. Immobilized penicillin G acylase accounts for 88 % of the world-wide 6-APA production, while the rest is produced by immobilized penicillin V acylase.[5]

Inorganic carriers like silica gel, alumina are known to be thermally and mechanically more stable[6] but the cost factor and non-biodegradable nature indicate the problems of their use. The application of an enzyme is often hampered by its reusability. This is because free enzyme as a biocatalyst, is lacking a long-term recycle from the reaction mixture, making the reuse of the enzyme impossible. Hence, the idea of immobilizing the enzyme on a suitable support has been of great industrial interest for many years.[7] Organic polymer carriers are the most widely studied materials because of the presence of rich functional groups, which provide essential interactions with the enzymes.[8] Polysaccharides such as dextran[9] and pectin have been used, but in terms of cost effectiveness, the use of carbohydrates like agar and agarose can be suggested.[10] Advantages of enzyme immobilization include a repeated use of an expensive enzyme, choice of batch or continuous processes, easy separation of the product and greater variety of reactor design.[6,11,12] In the present work, agarose was chosen as the support material for enzyme immobilization. The low cost and stability provided by agarose make it very suitable as an enzyme immobilization material and many researchers have described this.[13,14,15] The accumulation of 6-APA in the course of enzyme reaction has an inhibitory effect on penicillin acylase and so a continuous system for production is advantageous. In the present report, a packed-bed agarose immobilized reactor was analyzed and optimized for 6-APA production from penicillin G substrate. The immobilized enzyme kinetics were studied in the packed-column reactor according to the model developed by Lilly et al.[16] This model is important and applicable on the performance of immobilized enzyme and enabled us to determine the F_{max} or maximum reaction capacity of the bioreactor.

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Materials and methods

The chemicals used were all of analytical grade. 6-amino penicillanic acid was obtained from Fluka (Switzerland), Potassium benzyl penicillin was obtained from E Merk (Germany), Agarose and other chemicals were obtained from Qualigens fine chemicals, India.

Escherichia coli ATCC 11105 strain, used as the source of penicillin acylase, was obtained from the National Chemical Laboratory (NCL), Pune and maintained in nutrient agar medium.

For production of penicillin acylase enzyme, Luria broth media was used which contained yeast extract (0.5 %), tryptone (1.5 %), sodium chloride (0.5 %). Phenyl acetic acid (0.1 %) was used as precursor for enzyme production.

Hydroxylamine method[17] was used to determine enzyme activity. The activity of penicillin acylase can be determined by estimating 6-APA formed in the reaction mixture. One unit of enzyme activity is defined as the amount of enzyme required to produce 1μmol 6-APA per minute at 37 °C and at pH 8.

Cell lysis was done with cetyl trimethyl ammonium bromide (0.05 %). 1 mg cells were suspended in 2 mL of cetyl trimethly ammonium bromide in c = 50 mmol L^{-1} of phosphate buffer (pH 8) and incubated for 24 h at 37 °C. Clear lysate from lysed cells was used as enzyme source after ammonium sulphate precipitation (50–70 % saturation). 0.2 mL of aliquot from the partially purified enzyme was taken in test tubes and incubated with 2 % Penicillin G substrate for 20 min at optimum temperature. The amount of 6-APA formed was assayed by hydroxylamine method.

The polymer material (agarose) at a mass concentration of 20 mg L^{-1} was prepared in 50 m mol L^{-1} phosphate buffer at pH 8. 5 mg of wet cell mass was lysed in 10 mL of 0.05 % cetyl trimethyl ammonium bromide. After centrifugation the clear liquor was taken and immobilized in w = 2 % of agarose. The loading capacity was 2.5 mg penicillin acylase (partially purified) per gram of agarose gel. After the immobilization of the enzyme, chemical cross-linking was done with 5 % glutaraldehyde. Such a cross-linking results in the covalent binding of the enzyme to the agarose gel preparation providing stronger binding. The block polymer was forced through a net with defined mesh size. In this case, the mesh number was BSS 36, MM 425, ASTM 40, MIC 420. A 10 mL capacity column with diameter d = 1 cm was used for packing the agarose immobilized enzyme. The immobilized enzyme material occupied 8 mL of the column. H/T ratio is 8 which is greater than 3. Three different substrate mass fractions (w = 1%, 2%, 3%) were circulated through the column and the 6-APA formed in each case was estimated for different flow rates to optimize the residence time and also to determine the yield (y p/s) for 6-APA production. The yield factor gives the ratio of the product formed to the initial substrate concentration. The importance of determining yield factor is to find out the optimum substrate concentration that is needed for product formation. For determining the K_m and ζ_{max} values the substrate mass fraction was varied from 0.5 % to 3.0 % (Table 1). Enzyme kinetics followed the Michaelis-Menten equation. The Lineweaver-burk plot was used, slope and intercept of the I/ζ versus 1/[S] was used to determine the ζ_{max} (maximum reaction rate) and K_m (Michaelis-Menten constant) reflects the substrate affinity for the 6-APA production.

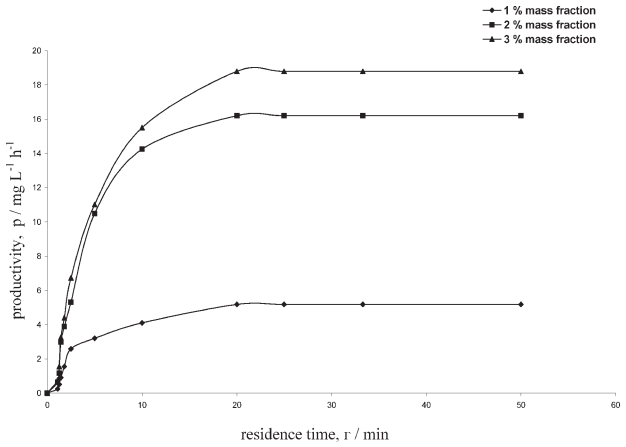
Table 1 – *Result for optimization of substrate mass fraction*

Substrate mass fraction, w/%	Enzyme activity, a/μmol min^{-1} mL^{-1}
0.5	1.4
1.0	1.8
1.5	2.7
2.0	3.2
2.5	3.1
3	3.1

Results and discussion

Optimization of process quantities like temperature, pH and substrate mass fraction was carried out as an initial part of the work. The temperature, T = 37 °C, pH 8 and w = 2 % penicillin G substrate mass fraction were found optimal for 6-APA production. The 6-APA produced at different time intervals was assayed by hydroxylamine method, Batchelor et al.[17] Substrate fractions varying from 1 % to 3 % were circulated through the column and 6-APA production was found in relation to the residence time in each case (Fig. 1).

Although w = 3 % substrate mass fraction gave a higher productivity of 18.79 mg L^{-1} h^{-1} of 6-APA at optimum residence time, Γ = 20–33 min as compared to 16.2 mg L^{-1} h^{-1} of 6-APA yield by 2 % substrate fraction but in terms of the yield (y p/s) the 2 % substrate showed y p/s = 0.81 h^{-1} and 3 % substrate showed y p/s = 0.62 h^{-1}. The 1 % substrate showed y p/s = 0.51 h^{-1}. The yield factor

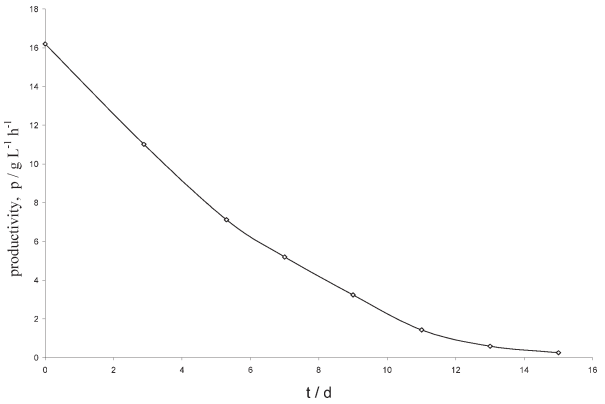


F i g . 1 – *Production of 6-APA from agarose immobilized penicillin acylase packed column (1. 8 cm bed volume) by continuous feeding of 1 %, 2 % and 3 % penicillin G substrate, pH 8, temperature 37 °C. Unit of substrate mass concentration is mg/100mL and Unit of enzyme activity is μmol min^{-1} mL^{-1}. The yield factors (y p/s) for 1 %, 2 % and 3% substrate concentrations are 0.51 h^{-1}, 0.81 h^{-1} and 0.62 h^{-1} respectively.*

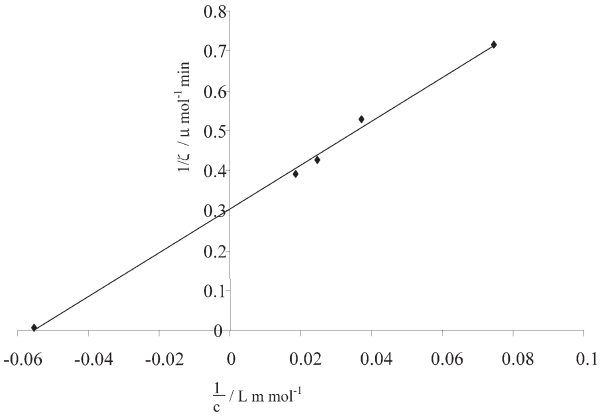
gives the ratio of the product formed to the initial substrate fraction. The importance of determining yield factor is to find out the optimum substrate concentration that is needed for product formation. Therefore, in terms of yield factor 2 % substrate is preferable as optimum substrate fraction. The stability of the packed column in course of time varying from 3 d to 15 d was observed (Fig. 2). The half life of immobilized enzyme is higher (5 d) as compared to the native enzyme (533 min), as previously reported.[18] The decrease in yield of 6-APA in time course is due to the inactivation of the immobilized enzyme[19] and diffusional barrier problem. In the case of batch mode of production, the accumulation of 6-APA has an inhibitory effect on the enzyme but in the case of continuous mode of production the 6-APA is continuously eluted out of the column and so in terms of stability the continuous system showed more stability of the agarose immobilized enzyme preparation (half life of 5 d) as compared to the agarose immobilized enzyme preparation in batch mode[18] (half life of 693 min). The activity and half life of immobilized enzyme can be improved by the use of organic solvents as suggested by certain researchers.[15,20]

For kinetic studies, the Lineweaver-Burk plot was used to find out the ζ_{max} and K_m (Michaelis-Menten constant) values of both native penicillin acylase (partially purified) and agarose immobilized penicillin acylase (partially purified) as shown in Fig. 3 and Fig. 4 respectively.

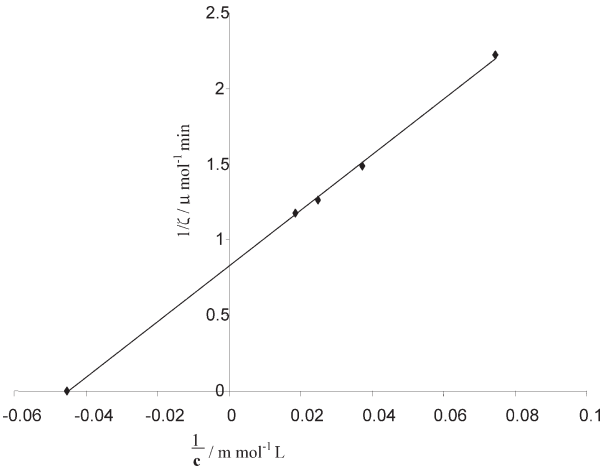
The partially purified native enzyme showed ζ_{max} = 3.3 μmol min^{-1} and K_m = 18.18 m mol L^{-1} . The partially purified immobilized enzyme showed ζ_{max} = 1.25 μmol min^{-1} and K_m = 22.22 m mol L^{-1}.



F i g . 2 – *Time course decrease of 6-APA production from agarose immobilized penicillin acylase packed column by feeding of 2 % penicillin G substrate, pH 8, temperature 37 °C and optimum residence time 20–33 min. The half-life of immobilized enzyme was found to be 5 d. Unit of substrate mass concentration is mg/100mL and Unit of enzyme activity is μmol min^{-1} mL^{-1}.*



F i g . 3 – *Study of the ζ_{max} and K_m for native penicillin acylase enzyme (partially purified). Results obtained for free enzyme by Lineweaver-Burk plot (1/ζ vs. 1/[S]) showed ζ_{max} = 3.3 μmol min^{-1} and K_m =18.18 m mol L^{-1} at pH 8, temperature 37 °C and optimum residence time, Γ = 20–33 min.*

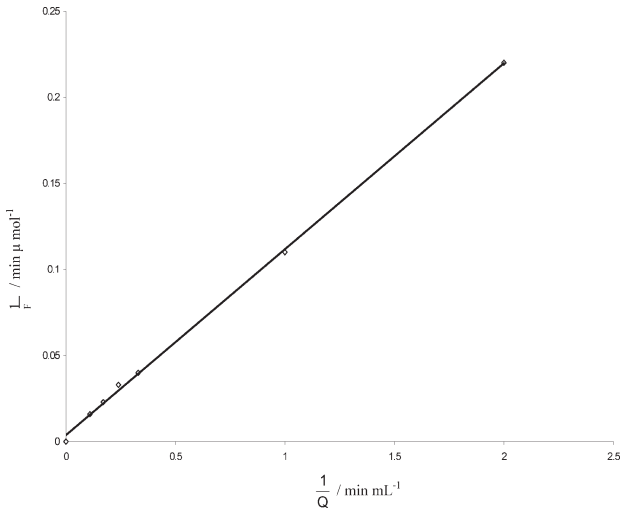


F i g . 4 – *Study of the ζ_{max} and K_m for agarose immobilized penicillin acylase enzyme (partially purified). Results obtained for agarose immobilized enzyme by Lineweaver-Burk plot (1/ζ vs. 1/[S]) showed ζ_{max} = 1.25 μmol min^{-1} and K_m = 22.22 m mol L^{-1} at pH 8, temperature 37 °C and optimum residence time, Γ = 20–33 min.*

In this manuscript, the results of 6-APA production at different flow rates were analyzed in terms of the kinetic equations developed by Lilly et al[16]. Based on this model for packed-bed bioreactors we have the following equation,

$$э[c_0] = F/Q + K_m\,(\text{app})\ \ln(1 - э)$$

A plot of $э[c_0]$ against ln $(1 - э)$ (where э is the part of substrate converted during passage over the reactor and $[c_0]$ is the initial substrate concentration), resulted in a straight line with a slope giving F value for a particular flow rate Q. For the same substrate concentration and different Q another F value is obtained. This way for different substrate mass fraction and different Q values we obtain different F values. Primary plots of $э(c_0)$ against $\ln(1 - э)$ were used to generate different F values. Secondary plot of $1/F$ against $1/Q$ gave a line with y-intercept yielding a F_{max} (maximum reaction capacity) value of $0.1.10^{-3}$ mol min^{-1} as shown in Fig 5.



F i g . 5 – *Plot of 1/F (reactor capacity) vs. 1/Q (flow rate) for agarose immobilized penicillin acylase packed column by continuous feeding of 2 % penicillin G substrate in phosphate buffer (pH 8), temperature 37 °C, and optimum residence time,* Γ *= 20–33 min. Different F values for different Q were obtained for different substrate concentrations using the equation* $э[c_0] = F/Q + K_m\,(app)\ ln\ (1- э)$. *Secondary plot of 1/ζ against 1/Q gave a straight line rate a* $\zeta_{max} = 0.1.10^{-3}$ *mol min*$^{-1}$.

Currently there is considerable interest in the preparation, properties and use of immobilized enzyme systems due to their potential industrial applications. However, the immobilized carrier acts a barrier for the substrate to come into contact with the enzyme. Using agarose as an immobilized material has a lesser problem of diffusion of substrate[15] and so agarose was chosen for enzyme immobilization. The F_{max} values are considered free from diffusional effects. These diffusion free values are very useful for comparison between the native enzyme and the performance of the immobilized enzyme.[21] The work carried out by us and presented in this manuscript is hence very useful and applicable to the industry.

Conclusion

The process of immobilization with a natural biopolymer like agarose was developed and is very useful because of the low cost and biodegradable nature of agarose unlike synthetic polymers. We have optimized process parameters and kinetic quantities. These provide important insight because a continuous system helps to overcome the problem of accumulation of 6-APA which is acidic in nature and has an inhibitory affect on penicillin acylase, so there is lesser stability in batch mode of production and the continuous mode of production is preferable. The F_{max} values give information on the performance of the immobilized enzyme to give us diffusion free values. In the present work, a partially purified enzyme was used so the results may vary from the purified enzyme. But from the cost-effectiveness point of view, since purified enzyme is expensive, our work is useful and can serve as a model for industrial application. A combination of immobilized enzyme and optimum use of some organic solvents can be suggested to improve the production of 6-APA. Therefore our work is particularly relevant to the pharmaceutical industry.

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List of symbols

a – enzyme activity, μmol mL^{-1} min^{-1}
c – concentration, mmol L^{-1}
d – diameter, cm
F – mole flow rate, mmol min^{-1}
H – height of reactor, cm
K_m – Michaelis constant, mmol L^{-1}
P – productivity constant. mmol L^{-1}
Q – volume flow rate, mL min^{-1}
t – time, h
w – mass fraction
ζ – rate of conversion, mmol min^{-1}
Γ – residence time, min
э – part

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