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Kinetic Parameter Studies of 6-Amino Penicillanic Acid (6-APA) Production by Agarose Immobilized Penicillin Acylase in a Packed Column Reactor

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Abstract

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 ß-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. Agarose is a natural polymer (carbohydrate) which is cheap, safe and easily available that makes it very suitable for enzyme immobilization. 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 parameters Vmax and Km values were calculated for both native and immobilized enzyme. The native enzyme showed Vmax=3.3μmol/min and Km value=18.18mM. The immobilized enzyme was packed in the column reactor to study the kinetic parameters by varying flow rate and different substrate concentration (according to the model of Lily et al 1966). For the immobilized enzyme the Km value=22.22mM. Cmax value was calculated using secondary plot of 1/C versus 1/Q to find the maximum capacity of the bioreactor. 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.

1.0 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 (Parmar et al 2000, Arroyo et al 2003). 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 ton/year (Van de Sandt et al 2000). There have been many reports, on *E.coli* for penicillin G acylase production (Shewale and Siva Raman, 1989). 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 (Vandamme E.J, 1998).

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 (Zarborsky O.R, 1973, Maria A.S et al 2004, Rajendhran J et al, 2004). In the

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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 (Alavaro et al 1990, Blanco et al 1989, Abian et al 2003). The accumulation of 6-APA in coarse of enzyme reaction has an inhibitory effect on penicillin acylase and so a continuous system of 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 was studied in the packed-column reactor according to the model developed by Lilly et al 1966. This model is important and applicable on the performance of immobilized enzyme and enabled us to determine the Cmax or maximum reaction capacity of the bioreactor.

2.0 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 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[19] 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 37^0 C and at pH 8.

Cell lysis was done with cetyl trimethyl ammonium bromide (0.05%). 1gm cells were suspended in 2mL of cetyl trimethly ammonium bromide in 50mM of phosphate buffer (pH 8) and incubated for 24 hours at 37^0C. Clear lysate from lysed cells was used as enzyme source after ammonium sulphate precipitation (50-70% saturation). 0.2mL 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[19].

The polymer material (agarose) at a concentration of 20g/L was prepared in 50mM phosphate buffer at pH 8. 5gm of wet cell mass was lysed in 10mL of 0.05% cetyl trimethyl ammonium bromide. After centrifugation the clear liquor was taken and immobilized in 2% of agarose. The loading capacity was 2.5mg penicillin acylase (partially purified) per gram of agarose gel. Chemical cross-linking was done with 5% glutaraldehyde. 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 10mL capacity column with diameter 1cm was used for packing the agarose immobilized enzyme.

3.0 Results and Discussion

Optimization of process parameters like temperature, pH and substrate concentration was carried out as initial part of work. The temperature 37ºC, pH 8 and 2% penicillin G substrate concentration were found to be optimum for 6-APA production. The 6-APA produced at different time intervals was assayed by hydroxylamine method (Batchelor et al, 1961). Substrate concentrations 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, Fig.2 and Fig.3).

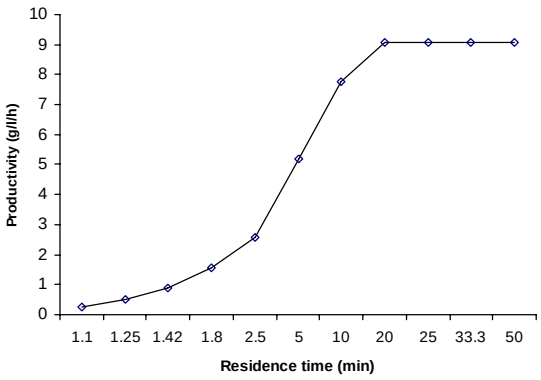

Fig. 1 Production of 6-APA from agarose immobilised pencillin acylase packed column by continuous feeding of 1% penicillin G substrate

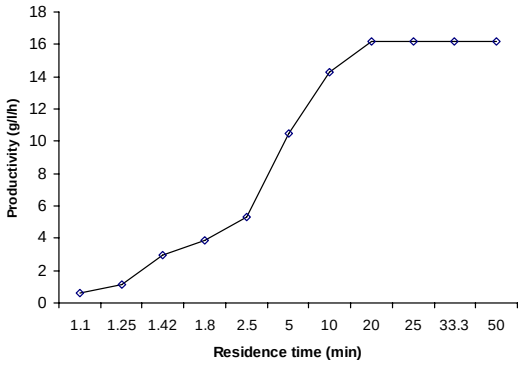

Fig. 2 Production of 6-APA from agarose immobilised pencillin acylase packed column by continuous feeding of 2% penicillin G substrate

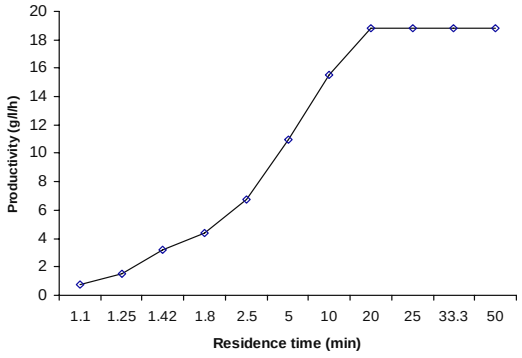

Fig. 3 Production of 6-APA from agarose immobilised pencillin acylase packed column by continuous feeding of 3% penicillin G substrate

Although 3% substrate concentration gave a higher yield of 18.79g/l/h of 6-APA at optimum residence time 20-33 min as compared to 16.2g/l/h of 6-APA yield by 2% substrate concentration but in terms of the yield factor (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} . Therefore, in terms of yield factor 2% substrate is preferable as optimum substrate concentration. The stability of the packed column in coarse of time varying from 3 days to 15 days was observed (Fig.4). The half life for immobilized enzyme is higher (5days) as compared to the native enzyme (543min), as previously reported. The decrease in yield of 6-APA in time coarse is due to the inactivation of the immobilized enzyme (Chan-su Rha et al 2005). For kinetic studies the Lineweaver-Burk plot was used to find out the Vmax (Maximum velocity) and Km (Michaelis-Menten

constant) values of both native penicillin acylase (partially purified) and agarose immobilized penicillin acylase (partially purified) as shown in Fig.5 and Fig.6 respectively.

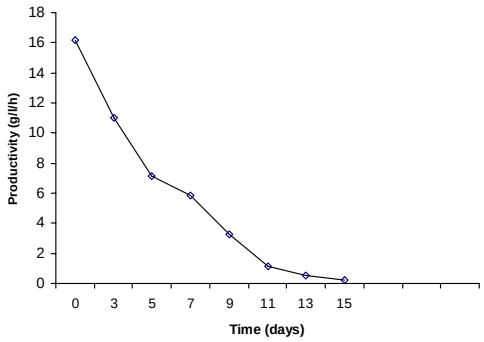

Fig. 4 Time course decrease of 6-APA production from agarose immobilized penicllin acylase packed column by feeding of 2% pencillin G substrate

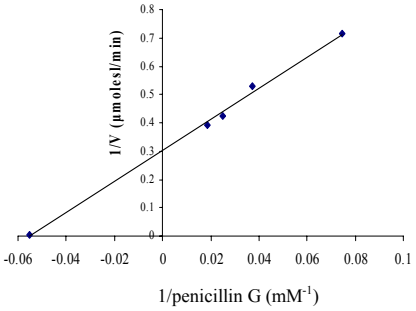

Fig. 5 Study of the Vmax and Km for native enzyme penicillin acylase enzyme (parially purified)

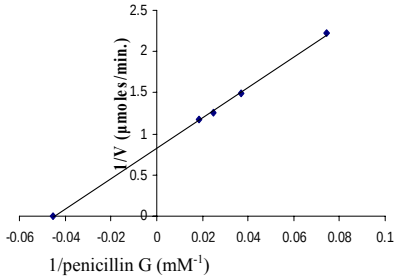

Fig. 6 Study of the Vmax and Km for immobilized penicillin acylase enzyme (partially purified)

The partially purified native enzyme showed Vmax = 3.3µmol/min and Km = 18.18mM. The partially purified immobilized enzyme showed Vmax = 1.25µmol/min and Km = 22.22mM. 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 1966. Based on this model for packed-bed bioreactors we have the following equation,

$$f[Ao] = C/Q + Km\,(app)\ln(1-f)$$

A plot of f[Ao] against ln (1-f) (where f is the fraction of substrate converted during passage over the reactor and [Ao] is the initial substrate concentration), resulted in a straight line with a slope. From the y-intercept of such plots the C (reaction capacity) values are found. Primary plots of f[Ao] against ln (1-f) were used to generate different C values. Secondary plot of 1/C against 1/Q gave a line with y-intercept yielding a Cmax (maximum reaction capacity) value of 0.1×10^{-3} mol min^{-1} as shown in Fig.7.

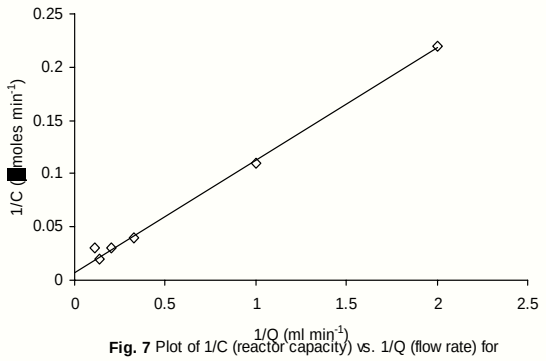

Fig. 7 Plot of 1/C (reactor capacity) vs. 1/Q (flow rate) for agarose immobilized penicillin acylase packed column by continuous feeding of penicillin G substrate in phosphate buffer (pH 8)

The Cmax values are considered to be free from diffusional effects. These diffusion free values are very useful to make comparison between the native enzyme and the performance of the immobilized enzyme (Sean Brahim et al 2002). The work carried out by us and presented in this manuscript is hence very useful and applicable to the industry.

4.0 Conclusion

The process of immobilization with a natural biopolymer like agarose was worked out by us and this is very useful because of the low cost and biodegradable nature of agarose unlike synthetic polymers. We have optimized process parameters and kinetic parameters. 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. The Cmax values give information on the performance of the immobilized enzyme to give diffusion free values. Therefore our work is particularly relevant to the pharmaceutical industry.

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