NEW BIOREACTOR FOR FIBROUS SUPPORT AND HYDROLYSIS OF PENICILLIN G

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Phenylacetic acid, a strong acid, was formed from the enzymatic hydrolysis of penicillin G to 6-amino-penicillanic acid. Because of the difficulty of maintaining the pH constant inside the reactor, the reaction mixture was circulated at a high flow rate between the packed bed reactor and the vessel for adjusting pH. The reactor and the vessel were connected via a pump. When an immobilized enzyme made of compressible fibers was packed in the packed bed reactor, the hydrolytic efficiency of the immobilized enzyme was approximately 0.5. The low efficiency of the immobilized enzyme was assumed to be caused by the aggregation of the immobilized enzyme due to the pressure generated by flow of the reaction mixture. Therefore, the aggregation in the packed bed reactor was analyzed experimentally using Kozeny-Carman's equation in order to estimate the distribution of the aggregation in the reactor. Furthermore, a new column devised specially to prevent aggregation of the compressible fibrous support was developed. A reaction time required for completing the hydrolysis (98 % of conversion) was adopted to estimate the efficiency of the entire immobilized enzyme packed in the reactor throughout the entire period of reaction. The efficiency of the immobilized enzyme packed in the new column of the reactor was concluded to be increased by approximately 1.8 times, compared with that of conventional packed bed columns.

Introduction

Immobilized enzymes are most commonly used in packed bed reactors. Most supports for immobilization tend to be deformed or compressed. Therefore, when an enzymatic reaction requires a high flow rate, not only the pressure drop across the compressible materials¹⁾, but also a low efficiency of immobilized enzyme caused by its aggregation emerge as severe problems. Using immobilized invertase entrapped by 15 wt% polyacrylamide gel, Furusaki and his colleagues²⁾ demonstrated that the apparent reaction rate of immobilized enzyme packed in a differential type reactor decreased markedly owing to aggregation of the immobilized enzyme, even when the superficial velocity of substrate solution was slow. However, the entire efficiency of the immobilized enzyme packed in the reactor was not analyzed.

Immobilized penicillin acylase (IPA) (EC 3.5.1.11) having a high specific activity was developed by glutaraldehyde crosslinking on hydrogenated porous polyacrylonitrile fibers (HPF), and has been used for the industrial production of 6-aminopenicillanic acid (6-APA) from penicillin G (Pen-G)⁴⁾. From this enzymatic hydrolysis, phenylacetic acid (PAA), a strong acid, is produced as a by-product. Because of the difficulty of maintaining the pH

constant inside the reactor, the reaction mixture was circulated at a high flow rate between the packed bed reactor and the vessel for adjusting pH, where the reactor and the vessel were connected via a pump. However, when IPA was packed in the packed bed reactor, the catalytic efficiency of the immobilized enzyme became low because of aggregation caused by the high flow rate. An immobilized enzyme prepared on a fibrous support can have high specific activity because fine fibers have a large surface area³⁾. Additionally, fine fibers could be easily recovered by means of filtration compared to fine particles. Therefore, a reactor suitable for fibrous immobilized enzymes is certainly important in the biotechnology industry.

In this work, an investigation on the aggregation of IPA fibers packed in a packed bed reactor was carried out experimentally considering the distribution of aggregation in the reactor. Furthermore, a column design devised specially to prevent the compressible fibrous support from aggregating and the results of the hydrolysis of Pen-G using the new column were described.

1. Materials and Methods

1.1 Chemicals

Benzylpenicillin potassium (1,580 unit/mg) (potassium Pen-G) was purchased from Meiji Seika Co. (Tokyo).

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Fig. 1 Schematic diagram of the equipment used for hydrolysis of Pen-G: (1) reservoir for 4*N* NaOH, (2) pump for pH regulation, (3) reservoir for temperature regulation, (4) pump for temperature regulation, (5) vessel for adjusting pH, (6) pump for circulation, (7) reactor of immobilized enzyme, (8) flow meter

Other chemicals used were of analytical grade, except for borate (technical grade), and were obtained commercially.

1.2 Immobilized enzyme

HPF was prepared by hydrogenation of porous polyacrylonitrile fibers (ratio of the copolymer of acrylonitrile (average molecular weight, 70,000- 80,000) and methyl acrylate, 9:1; diameter, 2.5×10^{-2} mm; length, 1 m; water regain, 1-2 ml/g) (Asahi Kasei Kogyo Co. (Osaka)). IPA was prepared by immobilizing penicillin acylase on HPF, as described in our previous paper⁴⁾.

1.3 Batch hydrolysis of Pen-G without film diffusion resistance and aggregation

The batch hydrolysis of Pen-G with IPA was assumed to be one without film diffusion resistance and aggregation. The hydrolysis reaction was initiated by adding 24,000 U IPA, which was cut into 1-cm-long pieces, into 2 l Pen-G solution (268 mM Pen-G dissolved in 40 mM of borate buffer), and continued until the residual Pen-G concentration was less than 5.4 mM under strong stirring. The hydrolysis was carried out at 34 °C and the pH maintained at 8.4 by adding 4*N* NaOH.

1.4 Hydrolysis of Pen-G

The equipment used was composed of the reactor packed with the immobilized enzyme and the vessel for adjusting the pH, as shown in Fig. 1. The batchwise hydrolysis of Pen-G was carried out by circulating the reaction mixture between the immobilized enzyme column and the vessel for adjusting pH. During the reaction, the pH was maintained at 8.4 by adding 4*N* NaOH and the temperature was kept at 34°C. Potassium Pen-G (268 mM) dissolved in 40 mM borate buffer was used as the substrate. The amount of IPA used was 12,000 U (approximately 60 ml in volume) per liter of Pen-G solution.

1.5 Packed bed column

Figure 2 shows the packed bed column used for the

Fig. 2 Illustration of the packed bed column: (1) outlet of circulation flow, (2) plate for separating the immobilized enzyme, (3) immobilized enzyme beds, (4) flow distribution bed in which 8-10-mm-of diameter of glass beads were packed, (5) plate, (6) empty layer, (7) inlet of circulation flow, (8) flow distributor

investigation on the aggregation of IPA. The column was composed of 6 IPA beds, a flow distribution bed at the bottom, in which 8 - 10-mm-diameter glass beads were packed, and an empty bed at the top of the column. Each bed of immobilized enzyme was 1,000 mm in diameter, 70 mm high, and 55 l in volume, and was separated by a latticed plate and a stainless steel net. Pressure gauges were equipped to measure the pressure drop at each bed.

1.6 Column with horizontal bars

Figure 3 shows the new column developed for fibrous support in order to prevent aggregation of the compressible fibrous support by circulating upward flow. The column, 110 mm deep, 240 mm wide, 590 mm high and 15 l in volume, had horizontal bars (108 mm long, 5 mm diameter) to hold the fibrous immobilized enzyme. The horizontal bars were set apart from one another 40 mm horizontally and 60 mm vertically, as shown in Fig.3-B. The assembly of columns is shown in Fig. 4. Each column was equipped with two valves at the top and bottom, and a flow meter at the top. Using the valve at the bottom, the circulation flow rate for each column could be regulated based on the activity of packed IPA.

1.7 Void fraction

The volume of IPA was calculated by measuring the diameter of IPA (the average diameter was 3.2×10^{-2} mm). The volumes of IPA in the packed state at various pressures were determined by measuring the thickness of IPA using a glass cylinder (110 mm diameter, 70 mm height) equipped with a latticed plate and a stainless steel net on both sides. In the glass cylinder, a polyethylene circular plate (108 mm diameter) was set under approximately 20 g of IPA. Aggregation of the IPA was accomplished with

Fig. 3 Illustration of the column with horizontal bars devised specially for fibrous support: (A) (1) outlet of circulation flow, (2) empty space, (3) horizontal bars to hold fibrous support for circulation flow (4) plate for supporting the horizontal bars, (5) stainless steel net, (6) flow distribution bed in which 8-10mm-of diameter of glass beads were packed, (B) position of horizontal bars, (1) horizontal bars

pushing up the circular plate with the upward circulation flow. Void fractions were calculated using the volumes of total IPA and of IPA in the packed state.

1.8 Determination of η_D

The ideal reaction rate of IPA at $Car = 134 \text{ mM}$ of Pen-G was measured using approximately 200 mg of IPA (1-cm-long pieces) and 4 l of 134 mM Pen-G solution (40 mM borate buffer) in the vessel under strong stirring for 30 min at 34 °C and pH 8.4. The actual reaction rate of IPA in the packed bed column under aggregation by circulating flow of various pressures at $Car = 134 \text{ mM}$ of Pen-G

Fig. 4 Schematic diagram of the equipment for hydrolysis of Pen-G using columns with horizontal bars specially devised for compressible fibrous support: (1) reservoir for 4N NaOH, (2) pump for pH regulation, (3) vessel for adjusting pH, (4) pump for circulation, (5) flow meter, (6) immobilized enzyme column with horizontal bars

Fig. 5 Time course of pH and conversion of Pen-G hydrolysis by using packed beds column (symbols): 20m³/h flow rate, ●: 50m³/h flow rate, ▲: 80m³/h flow rate, ○: solid line, hydrolysis without film diffusion and aggregation

was measured as follows: first approximately 10 g of IPA was packed on top of the layer of completely denatured IPA (approximately 50 g) in the glass cylinder used in measuring the void fraction. Next, 200 l of 134 mM Pen-G solution was circulated upward. Finally, after the pressure of circulation flow stabilized (about 10 min), the reaction rate was measured at 34 °C and pH 8.4 for 20-30 min. An effectiveness factor (at $Car = 134 \text{ mM}$ of Pen-G concentration), denoted by η_D , was represented as the ratio of the actual reaction rate to the ideal reaction rate.

1.9 Analytical methods

Concentrations of Pen-G, PAA and 6-APA in the reaction mixture were determined by HPLC on a Lichrosorb RP-18 (4 mm ϕ × 150 mm)(Merck) using the solvent mixture 0.3 M KH_2PO_4 - acetonitrile (8:2, v/v) at a flow rate of 0.8 ml/min for analysis of Pen-G and PAA, and 10 mM phosphate buffer (pH 7.0) -acetonitrile (100:1, v/v) at a flow rate of 0.6 ml/min for analysis of 6-APA and PAA. Detection was accomplished by absorption at 230 nm with a UV detector. One unit of enzyme was defined as that producing 1 μmole of 6-APA per min at 34 °C.

Fig. 6 Effect of circulation flow rate on pressure drop and pH drop across each bed

2. Results and Discussion

2.1 Hydrolysis of Pen-G using packed bed column

Ten million *U* of IPA (approximately 50 liters in volume) was packed in a bed of the packed bed column (Fig. 2). The hydrolysis of Pen-G to 6-APA was carried out with 268 mM Pen-G (250 kg) and 30 million *U* IPA (packed in 3 beds) by circulating the reaction mixture at 20, 50, and 80 m³/h flow rate (42, 106, and 170 cm/min superficial velocity, respectively). The time-course data of the pH at the outlet of column, and of the conversion (*Ca/Cao*) measured in the vessel are shown in Fig. 5. The time-course data of the conversion of the batch hydrolysis of Pen-G without film diffusion resistance and aggregation are also illustrated for comparison. The time-course of the pH was as follows: the pH decreased rapidly during the beginning of the reaction, reached a minimum value at 4–8 min reaction time, then increased gradually with the progress of the reaction and was asymptotic to 8.4. With the increase in flow rate from 20 to 50 m³/h, the minimum pH at the outlet increase from 7.12 to 7.80 (the pH at the inlet was 8.4), but the rate of hydrolysis also increased. However, the hydrolysis rates of the packed bed column at 50 and 80 m³/h flow rate were slower than that of the batch hydrolysis of Pen-G without film diffusion resistance and aggregation as shown in Fig. 5, although the pH at the outlet were 7.80 and 8.01, respectively (the hydrolysis rates of IPA at pH of 7.8, 8.01 and 8.40 are almost the same⁵⁾).

The pressure drop caused by the circulation flow across each bed packed with IPA is shown in Fig. 6. A compressed pressure of 1.0 to 1.5 kg/cm² was generated in

Fig. 7 Change of η_D at $Car = 134$ mM of Pen-G with pressure applied to IPA packed in bed

each bed by a flow rate of 50 to 80 m³/h (106 to 170 cm/min of superficial velocity). Furthermore, an almost equal pressure drop was generated in each bed in the column. Aggregation by applying the high circulation flow rate to the compressible fibers seemed to decrease the efficiency of IPA packed in the bed.

2.2 Analysis on aggregation

Since IPA was in the form of a very fine fibers, the circulation flow velocity used in this experiment was high enough to neglect the film diffusion resistance. Additionally, penicillin acylase was located in the outer region of IPA, from the surface to 6–8 μm deep, as described in our previous paper⁴⁾. Therefore, the reaction rate under strong stirring conditions could be assumed as the ideal reaction rate of IPA at $Car = 134$ mM of Pen-G. The change in η_D at $Car = 134$ mM of Pen-G with pressure applied to IPA packed in the packed bed column is shown in Fig. 7. When the pressure applied to IPA exceeds 0.5 kg/cm², η_D rapidly decreases to approximately 0.3, whereas η_D was 1 when the pressure applied to IPA was less than 0.5 kg/cm². An assumption made, illustrated in Fig. 8-A, was that when a bundle of IPA was compressed by more than *Pb* of pressure (turning pressure), one bundle of IPA was compressed into a fiber-like structure (*R*₂ was approximately 1 mm), because IPA was treated as a bundle of fibers. From Fig. 7, the *Pb* was 0.5 kg/cm².

Figure 8-B illustrates the distribution of pressure and void in compressible fibers compressed by upward circulation flow. At the plate surface, the compression pressure (*Px*) applied to fibers was maximum (*Pmax*) and the flow pressure (*Pi*) was 0 kg/cm² (*Pio*). On the other hand, at the surface of layered fibers, the compression pressure was 0 kg/cm² (*Pxo*) and the flow pressure was maximum (ΔP).

The mean effectiveness factor of the entire IPA packed in the bed, denoted by $\overline{\eta}_D$, is defined as

$$\overline{\eta}_D = (1/V_E) \{ \eta_D|_{Px < Pb} V_{Ex} + \eta_D|_{Px \geq Pb} (V_E - V_{Ex}) \} \quad (1)$$

where *V_E* and *V_Ex* are the volume of the entire IPA packed in the bed and of IPA compressed by a *Px* less than *Pb*, respectively. For estimating the ratio of (*V_Ex/V_E*), Kozeny-Carman's equation (2) was adopted.

(A)

(B)

Fig. 8 (A) illustration of compressed state of fibers, (B) illustration of compressed state of fibers packed in bed

$$u = (g_c / kS_v) \left(\xi x^3 / (1 - \xi x)^2 \right) (dP_x / dX) \quad (2)$$

where X is the length from the surface of layered fibers and ξx is the void fraction at P_x . The ratio of (V_{Ex}/V_E) is calculated from Eq. (3) assuming that Sv in Eq. (2) is constant.

$$V_{Ex} / V_E = \int_0^x (1 - \xi x) dx / \int_0^L (1 - \xi x) dx \quad (3)$$

where L is the thickness of the entire IPA. The relationship between the P_x and ξx was determined by the method described in section 2.7 as follows:

$$\begin{aligned} \xi x &= 0.37 P_x^{-0.32} & (P_x < 0.08) \\ \xi x &= 0.83 & (P_x \geq 0.08). \end{aligned} \quad (4)$$

The change in $\overline{\eta_D}$, calculated from Eq.(4) using 0.5 kg/cm^2 of P_b , with P_{max} is shown in **Fig. 9**. When P_{max} were 1.0 and 1.2 kg/cm^2 , η_D values were 0.5 and 0.42 , respectively, and were considered to agree well with the results of hydrolysis of Pen-G described above (Fig. 5).

2.3 Comparison of efficiency

The new column was characterized by horizontal bars which were equipped to render IPA immobile and to disperse the pressure generated by the circulation flow. The hydrolysis of Pen-G to 6-APA using 8 columns (30 million U IPA in total) with horizontal bars was carried out by packing uniformly 3.75 million U of IPA (18.75 l in volume under the non-compressed condition) into one column.

Fig. 9 Effect of maximum pressure applied to IPA on mean effectiveness factor of entire IPA packed in packed bed column. The mean effectiveness factor was calculated from Eq. (1) using 0.5 kg/cm^2 of P_b

Table 1 Hydrolysis of Pen-G using conventional packed beds column and new column with horizontal bars

Column	Superficial velocity (cm/min)	Pressure drop ^a (kg/cm ²)	pH ^b (-)	Reaction time required ^c (-)
Conventional packed beds column	42	0.23	1.28	> 3
	106	0.75	0.60	2.0
	170	1.35	0.39	2.2
New column	43	0.35	1.70	2.0
with horizontal bars	66	0.70	1.65	1.6
	87	1.20	1.30	1.3
	108	1.60	1.00	1.2
	130	2.50	0.55	1.1

^a Pressure drop across each bed of packed bed column and that of entire column with horizontal bars.

^b Maximum pH drop during the hydrolysis.

^c Reaction time required for completing hydrolysis (98 % of conversion) using packed bed column and column with horizontal bars. Reaction time required was compared with that of batch hydrolysis without pH drop, film diffusion resistance and aggregation as described in the text. as described in text.

The reaction time required for completing the hydrolysis (98 % of conversion) was adopted to estimate the efficiency of the entire IPA throughout the entire period of reaction. The results are shown in **Table 1**, where the reaction time required was compared with that of batch hydrolysis of Pen-G without film diffusion resistance and aggregation. In the case of hydrolysis using the column with horizontal bars, with the increase in circulation flow velocity from 43 to 130 cm/min , the reaction time required decreased from 2.0 to 1.1, while the pressure across the column increased from 0.35 to 2.50 kg/cm^2 . When the flow velocity of circulation was 130 cm/min , the ΔpH of the reaction mixture at the outlet of the column was a maximum of 0.55 (the pH decreased to 7.85 from 8.4). On the other hand, in the case of hydrolysis using the packed bed column, the minimum reaction time required was 2.0 and, at that time, the ΔpH of the reaction mixture at the outlet of the column was 0.60. From these results, it was clarified that the reaction time required was reduced from 2.0 to 1.1 when the column with horizontal bars was used instead of the packed bed column, that is, the former increased the efficiency by approximately 1.8 times.

A fibrous support is a typical support used in immobilization and can provide an immobilized enzyme with high specific activity. Additionally, fine fibers can easily be recovered from the reaction mixture compared with fine particles. However, fibers are highly compressible. The column with horizontal bars presented in this paper showed good properties of being able to hold fibrous supports immobilized and prevent compressible fibers from aggregation due to pressure generated by flow. The column with horizontal bars was concluded to increase the efficiency of IPA packed in it by approximately 1.8 times compared with that packed in the packed bed column.

Nomenclature

Ca	= Pen-G concentration	[mM]
Ca_0	= initial Pen-G concentration	[mM]
Car	= Pen-G concentration at fiber surface	[mM]
$F.V.$	= superficial velocity	[cm/min]
gc	= conversion factor	[kg m/Kg s ²]
k	= constant in Eq.(2)	[-]
L	= thickness of the entire IPA packed in the bed	[cm]
Pb	= turning pressure by which one bundle of IPA was compressed into a fiber-like structure	[kg/cm ²]
P_i	= flow pressure	[kg/cm ²]
P_{io}	= flow pressure on the support surface	[kg/cm ²]
P_{max}	= the maximum compressed pressure on the support	

	surface	[kg/cm ²]
P_x	= compressed pressure or/and compressed pressure at a distance x from the surface of layered fibers	[kg/cm ²]
R_1	= radius of a fiber	[cm]
R_2	= radius of a bundle of fibers under pressure exceeded Pb	[cm]
S_v	= specific surface area	[1/min]
u	= superficial liquid velocity	[cm/min]
V_E	= packed volume of IPA	[cm ³]
V_E^x	= packed volume of IPA compressed less than P_x	[cm ³]
X	= distance from the surface of layered fibers	[cm]
ΔP	= flow pressure at the surface of layered fibers	[kg/cm ²]
ξ_x	= void fraction at P_x of compressed pressure	[-]
η_D	= effectiveness factor of IPA determined by experiment	[-]
$\bar{\eta}_D$	= mean effectiveness factor of the entire IPA packed in the bed defined in Eq. (1)	[-]

Literature cited

- 1) Buchholz, K. and B. Godelman in *Enzyme Engineering*, Broun, G.G., G. Manecke and L.B. Wingard, eds. pp.89-92. Plenum Press, New York (1978)
- 2) Furusaki, S., Y. Okamura and T. Miyauchi: *J. Chem. Eng. Japan*, **15**, 148 (1982)
- 3) Ichijo, H., H. Uedaira, T. Suehiro, J. Nagasawa and A. Yamauchi: *Agric. Biol. Chem.*, **49**, 3591 (1985)
- 4) Ishimura, F. and H. Seijo: *J. Ferment. Bioeng.*, **71**, 140 (1991)
- 5) Suga, K., Y. Shiba, T. Sorai, S. Shioya and Ishimura, F.: *Ann. N.Y. Acad. Sci.*, **613**, 808 (1990)