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미생물 페니실린 아미다제에 관한 연구

(II) *E. coli*의 균체 고정화 페니실린 아미다제의 특성 및 반응조에 관한 연구

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Studies on Microbial Penicillin Amidase

(II) Characteristics and the Reactor Performance of Whole Cell Immobilized Penicillin Amidase of *Escherichia coli*

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Abstract

Whole cell penicillin amidase of *Escherichia coli* was immobilized by entrapment in gelatin followed by extrusion and crosslinking with glutaraldehyde. The immobilized enzyme preparation demonstrated the recovery yield of activity up to 70% and good stability during storage and operation. The half life of activity decay during the operation was estimated to be about 50 days. The optimum pH and temperature for both of immobilized and soluble enzyme are 8.5 and 50°C, respectively. No significant change was demonstrated in the effect of pH and temperature, but the increase in heat stability at high temperature was observed in the case of the immobilized enzyme. It was found that the plug flow reactor could be operated favorably since the pH drop along the column path due to the reaction product was minimized by employing substrate solution with moderate buffer strength. The optimal condition of reactor operation was discussed with regard to the effect of substrate concentration and the residence time on the conversion efficiency and productivity.

Introduction

Penicillin amidase (Benzylpenicillin amidohydrolase, E.C. 3,5,1,11) is an enzyme to which much attention has been paid by many workers for its use in the industrial production of 6-aminopenicillanic acid, the nucleus of many semisynthetic penicillins of therapeutic use. For practical use of this enzyme in industrial scale, various kinds of immobilization methods with different enzyme sources have been reported (Table I). These include covalent bonding to ion exchanger[1,2,3,7], adsorption to celite[4], entrapment in cellulose acetate fiber[5], entrapment in acrylamide gel[6], copolymerization with acrylamide[8], covalent bonding to nylon fiber[9]. As an enzyme source, several microorganisms such as *Escherichia coli* and *Bacillus megaterium* are known to be useful for the practical purpose. Other microbial sources of penicillin amidase were also well documented[12].

Although the kinetics of soluble and immobilized enzyme and their reactor performances in a stirred tank reactor have been intensively studied[3,4,9], the quantitative study on the reactor performance in a plug flow reactor has not been extensively carried out. It is well known that pH adjustment is necessary for the reaction of penicillin amidase due to the acidic reaction product, phenylacetic acid.

One of the main problems involved in a plug flow reactor is that maintenance of pH of the substrate feed within a narrow range by the addition of alkali solution is rater difficalt.

In the course of studies on penicillin amidase, the fermentation condition of *E. coli* (ATCC 9637) for the production of penicillin amidase was carried out[10]. In the present study, whole cell containing the enzyme was immobilized by entrapment in gelatin crosslinked with glutaraldehyde previously developed in our laboratory[11]. The enzymatic characteristics and the performance of the immobilized enzyme in a batch as well as in a plug flow reactor is hereby reported. The optimal condition of reactor operation was also discussed with emphasis on the effect of substrate concentration and residence time alongside with the effect of pH drop.

Table 1. Immobilization Methods of Penicillin Amidase from Various Microbial Sources.

Immobilization Method	Enzyme Source	References
Covalent bonding to DEAE-cellulose derivative	*E. coli* (ATCC 9637)	1
Covalent bonding to DEAE-cellulose derivative	*E. coli* (NCIB 8743A)	2, 3
Adsorption to celite	*B. megaterium* (ATCC 14945)	4
Entrapment in cellulosetriacetate fiber	*E. coli* (ATCC 9637)	5
Entrapment in acrylamide gel	*E. coli* (ATCC 9637)	6
Covalent bonding to XAD-7 ion exchange resin	*E. coli*	7
Copolymerization with acrylamide	*E. coli*	8
Covalent bonding to nylon fiber	*B. megaterium* (ATCC 14945)	9

Materials and Methods

Materials

Potassium benzylpenicillin (1595 i.u./mg), 6-aminopenicillanic acid, were purchased from Sigma Chemical Co. (U.S.A.). p-Dimethylaminobenzaldehyde (p-DAB), DEAE-cellulose, and gelatin were purchased from Aldrich Chemical Co. (U.S.A.). Other chemicals used in this research were of the extra pure grade from Wako Chemical (Japan).

Enzyme source

Whole cell penicillin amidase was obtained by culturing *E. coli* (ATCC 9637) cells as described in the previous paper[10]. 28*l* jar fermentor (New Bruswick, Model CMF-128S) was used for large scale enzyme production. The media was composed of MSG 1.5%, tryptone 3.5%, yeast extract 0.5 %, phenylacetic acid 0.15%. The agitation speed was 400 rpm with the aeration rate 0.5VVM.

After cultivation for 1 day at 30°C, the cells were harvested by continuous centrifuge, and stored at 4 °C until use for immobilization. No enzyme activity decay was noticed during storage over 2 months.

Determination of enzyme activity

The activity of whole cell or immobilized enzyme was determined by measuring the amount of 6-APA according to p-DAB method by Balashingham et al[2]. All reagents were freshly prepared just before assay. After 1hr of reaction at 40°C in an agitated vessel containing substrate (20mM penicillin G in 0.2M borate buffer, pH 8.2) and whole cell or immobilized enzyme, the reaction was stopped by boiling for 5 minutes. One m*l* of supernatant after centrifugation was added to 6m*l* of p-DAB reagent, and the optical density at 415nm was measured after 5 minutes.

In case of column reactor, 1 m*l* of effluent was pooled and 6 m*l* of p-DAB reagent was added

without boiling.

One unit of enzyme is defined as the activity of enzyme that is equivalent to one μmole of 6-APA per an hour under the specified condition.

Enzyme immobilization

The immobilization procedure is described in Fig. 1. The cell paste, the water content of which is about 85%, is mixed with aqueous gelatin solution and cellulose material such as DEAE-cellulose. The amount of gelatin added was determined by the desired rigidity of the immobilized enzyme product. The mixed enzyme paste was casted into a pellet form with the aid of an extruder, and treated with 5% glutaraldehyde solution for 1 min. to crosslink the gelatin matrix. The pellet was washed thoroughly with distilled water and dried overnight. The dried product was ground in a mortar and sieved in a desired size. The average total recovery of the enzyme activity was about 70%.

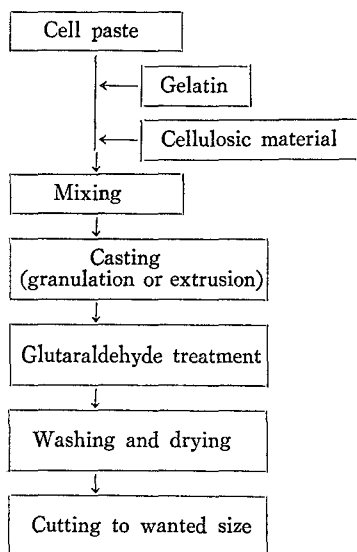

Fig. 1. Immobilization procedure of whole cell penicillin amidase.

Reactor operation

For batch reaction, immobilized enzyme pellet was swollen for 30 min in 0.2M borate buffer (pH 8.2) before reaction. After adding substrate solution, the reaction mixture was incubated at 40°C, except where otherwise stated, in a reactor agitated with a magnetic stirrer. The enzyme activity was determined with a given time interval.

A packed bed column reactor (1.3×12cm) was used for the continuous operation. A sintered glass filter was fitted at the bottom of the column, and a preheating unit was connected to the inlet end of the column to maintain the isothermal condition. The substrate solution was continuously fed at constant speed by a peristaltic pump. In an attempt to attain efficient performance in a plug flow reactor system, pH drop effect along the column path was minimized by employing buffer solution (0.2M phosphate buffer, pH 8.3).

Results

Characteristics of the immobilized enzyme

General properties of the immobilized enzyme were compared with those of cell-free enzyme preparation. As shown in Fig. 2, the optimum pH was 8.5 and a marked decrease in the enzyme activity was observed in the alkaline pH region.

The overall pH-activity profile was almost identical for both enzyme preparations. The effect of temperature on the activity of the immobilized enzyme was not different from that of cell-free enzy-

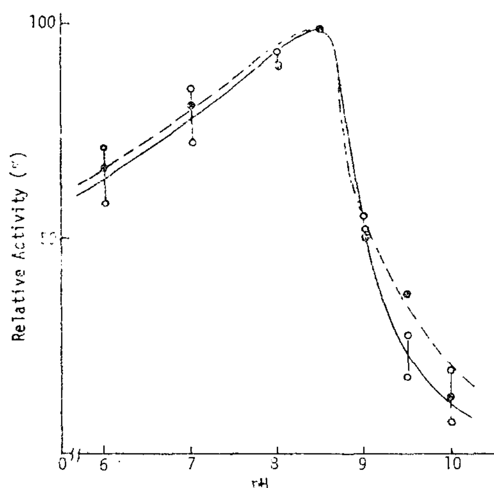

Fig. 2. Comparison of the effect of pH on the reaction rate between the cell free and immobilized enzyme.

(-○-) cell free enzyme, duplicated, (-●-) immobilized enzyme. 0.2M phosphate buffer for pH 6 to 8.5; and 0.2M borate buffer for pH 9.0 to 10.0.

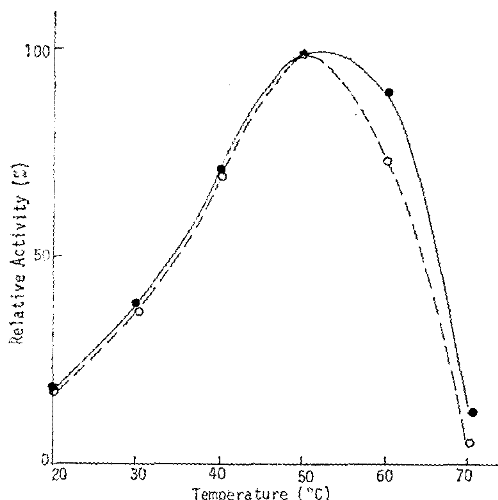

Fig. 3. Comparison of the temperature effect on the reaction rate between the whole cell and immobilized enzyme.
(-○-) whole cell enzyme;
(-●-) immobilized enzyme.

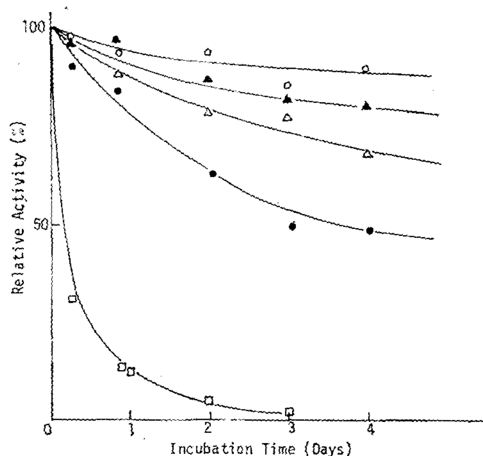

Fig. 4. The effect of pH on the storage stability of the immobilized enzyme.
The incubation temperature was 40°C.
(-●-) pH 5 (0.1M acetate buffer);
(-△-) pH 6 (0.1M phosphate buffer);
(-○-) pH 7 (0.1M phosphate buffer);
(-▲-) pH 8 (0.1M phosphate buffer);
(-□-) pH 10 (0.1M bicarbonate buffer).

me below 50°C. However, it demonstrated a slight increase in the enzyme activity in the region of high temperature (Fig. 3).

The thermal stability of the immobilized enzyme in a wide range of pH at 40 °C is shown in Fig. 4. The thermal stability gradually increased up to pH 7.0 and sharply decreased at alkaline region. It revealed the maximum stability at pH 7, which is lower than the optimal pH of the enzyme reaction. The operational stability of the immobilized enzyme in a plug flow reactor is show in Fig. 5. It is readily seen that the operational stability follows the pattern of a typical exponential decay, and the half life was found to be about 50 days. Disintegration in physical form of the immobilized enzyme pellet was not found during the operation.

Batch reactor operation.

The time course of conversion of benzylpenicillin in a batch reactor for the whole cell and the immobilized enzyme preparation is shown in Fig. 6 and 7, respectively.

When 74mg/*ml* reaction mixture of whole cell enzyme was used at 40 °C and pH 8.3, it was observed that 5mM substrate solution was converted completely within 0.5 hr of reaction and it took about 2hr for the complete conversion of 30mM substrate solution.

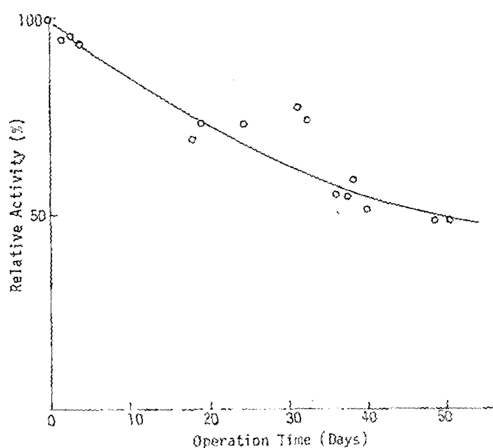

Fig. 5. Operational stability of the immobilized enzyme.
30mM substrate solution was continuously fed to the plug flow reactor containing 1g (wet wt.) of the immobilized enzyme. Feed rate, 10*ml*/hr; temperature, 40°C.

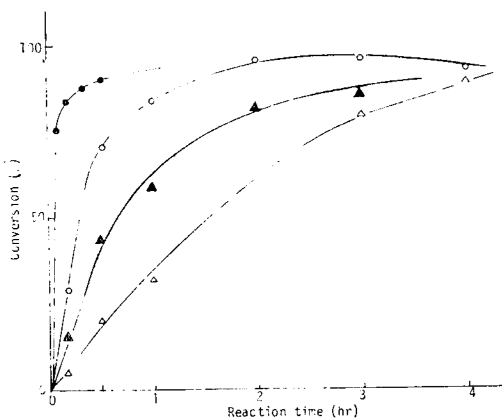

Fig. 6. Time course of conversion using the whole cell enzyme.
The enzyme reaction was carried out at 40°C, pH 8.3(0.2M borate buffer), with cell concentration 74mg DCW/m*l* reaction mixture. The total reaction volume was 20m*l*. The substrate concentration; (●) 5mM, (○) 30mM, (▲) 50mM, (△) 100mM.

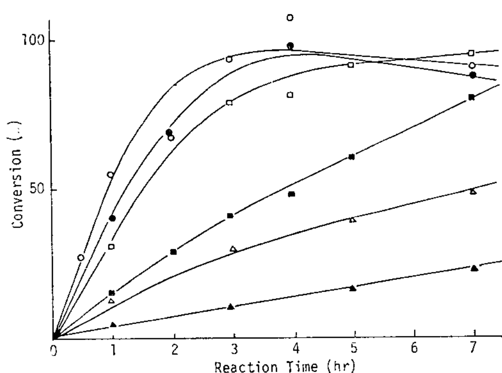

Fig. 7. Time course of conversion using the immobilized enzyme.
The enzyme reaction was carried out at 40°C, pH 8.3(0.2M borate buffer) with continuous stirring. 400mg of immobilized enzyme was added to 20m*l* of substrate solution; (○) 5mM, (●) 10mM, (□) 15mM, (■) 30mM, (△) 50mM, (▲) 100mM.

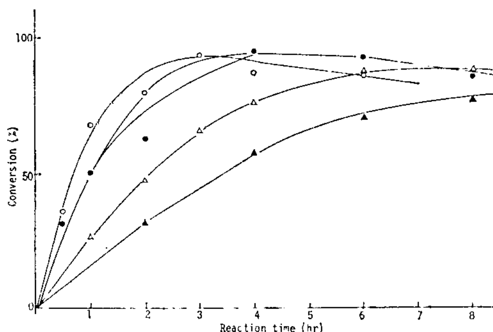

Fig. 8. Time course of conversion using the immobilized enzyme with different amount of enzyme loading.
Substrate concentration; 15mM, reaction volume; 20m*l*, enzyme loading; (○) 800 mg, (●) 400mg, (△) 200mg, (▲) 100mg.

The complete conversion of 15mM substrate required about 5 hrs when 20 mg/m*l* of immobilized enzyme was used. It was noted in both cases that the degree of conversion decreased gradually after reaching a certain maximum level.

The Michaelis-Menten constant (Km) of the immobilized enzyme in a batch reactor was found 3.8 mM, whereas that of the cell-free enzyme was 2.9mM. The maximum conversion rates of the two enzyme preparations were 340 μmole benzylpenicillin/hr/g of dry cell and 225 μmole benzylpenicillin/hr/g of immobilized enzyme, respectively. The effect of enzyme loading on the conversion efficiency in the immobilized enzyme reaction is shown in Fig. 8. The increase in the conversion efficiency was propartional with the enzyme concentration, and the decrease in conversion at a larger reaction time was evident as the case shown in Fig. 6 and 7.

Plug-flow reactor operation

Experiments on the reactor performance in a plug flow reactor was carried out in a column packed with 1g (dry weight) of the immobilized enzyme pellet after swelling. The bed height of the immobilized enzyme column was 3.5cm. The time required for

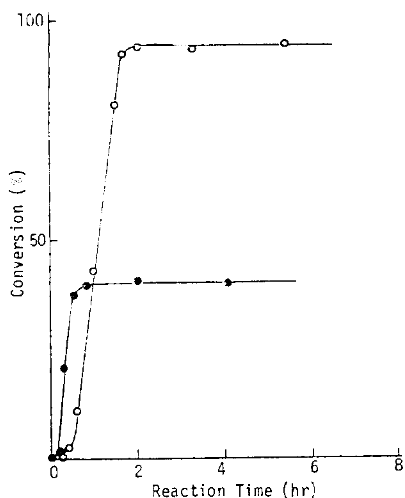

Fig. 9. Attainment of steady state in a plug flow reactor.
(○) 15mM substrate concentration, flow rate 8.2ml/hr
(●) 30mM substrate concentration, flow rate 17.0ml/hr

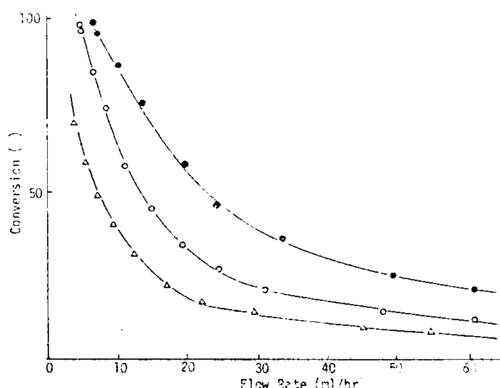

Fig. 10. Steady state conversion in a plug flow reactor as a function of space time and substrate concentration.
Substrate concentration; (●) 15mM, (○) 30mM, (△) 50mM

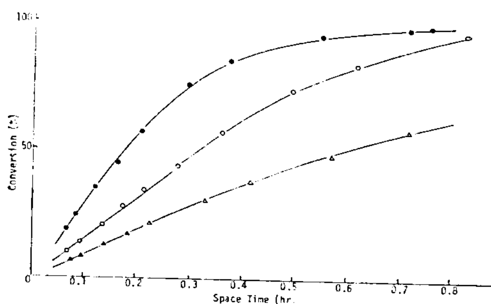

Fig. 11. The conversion efficiency in a plug flow reactor as a function of space time and substrate concentration.
Substrate concentration; (●) 15mM, (○) 30mM, (△) 50mM

attaining steady state was different with different flow rate (Fig. 9).

As shown in Fig. 9, 94% conversion was attained in 2hrs as 15 mM substrate solution was fed at a flow rate of 8.2 ml/hr. It was, however, found that the steady state could be obtained when the effluent volume was 3 to 4 times of the reactor volume regardless of the different feed rate and the substrate concentration.

The steady state conversion at different flow rates for three different substrate concentrations is shown in Fig. 10. As shown in Fig. 10, the degree of hydrolysis increased as flow rate became slower, and reached above 90% at flow rate 10ml/hr for 15mM substrate solution and at 6ml/hr flow rate for 30mM substrate solution. When the conversion is replotted against the space time, τ, it is demonstrated that the higher conversion can be obtained at the lower substrate concentration in a given space time, or at the longer space time in a given substrate concentration (Fig. 11).

Optimization of the performance of the plug flow reactor.

As shown in Fig. 12, pH of the effluent solution decreased gradually with the increased space time or with the increased substrate concentration even when a moderate buffer was used. The pH of the effluent solution dropped to 7.1 from the initial pH 8.3 when 50mM substrate in 0.2M phosphate buffer was applied. At this point, the fractional conversion of the substrate was about 70%. The relative activity at this pH was about 88% of that

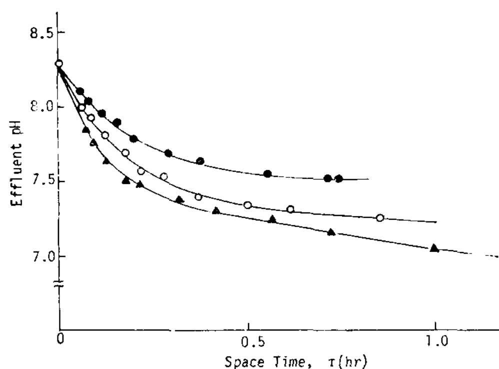

Fig. 12. pH Drop in a plug flow reactor as a function of space time and substrate concentration.
Substrate concentration; (●) 15mM, (○) 30mM, (△) 50mM

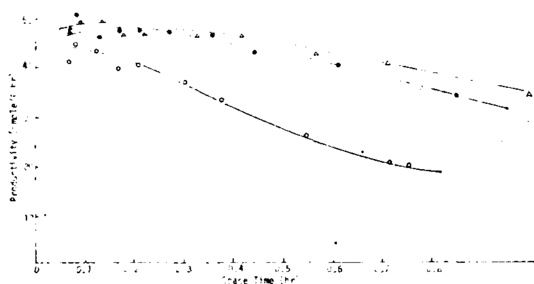

Fig. 13. Productivity of immobilized penicillin amidase plug flow reactor system.
The productivity was calculated as $= \frac{So\,X}{\tau}$ where So; initial substrate concentration, X; conversion, τ; residence time. Substrate concentration; (○) 15 mM, (●) 30mM, (△) 50mM

observed at the optimal pH. It is also evident that the enzyme activity decreases more when the higher fractional conversion is attained since the effect of pH drop becomes more severe. However, the use of moderate buffer solution has a great advantage over the substrate solution without buffer, where the pH drop amounts to as low as pH 4. Fig. 13

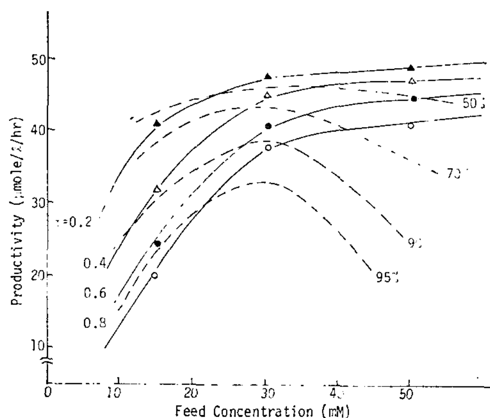

Fig. 14. Productivity of immobilized penicillin amidse in a column reactor system as a function of feed concentration and residence time.
Dotted line shows isoconversion line. Residence time; (▲) 0.2hr, (△) 0.4hr, (●) 0.6hr, (○) 0.8hr.

shows the productivity as a function of space time and substrate concentration. For any desired level of conversion of any substrate concentration of the feed, one can calculate the productivity as a function of space time. The productivity, SoX/τ, is defined as μmoles of 6-APA produced per liter per hour. It was demonstrated that the productivity decreased gradually as the space time increased. It was, however, of interest to note that the decrease in the productivity was more drastic in case of 50mM substrate feed than those of the lower concentration of substrate feed (15mM and 30mM). The result can be explained by the fact that the pH drop becomes more severe when the higher concentration of substrate feed is applied. The result shown in Fig. 14 represents, to a limited extent, an optimization of the process in terms of the productivity and the keyoperating parameters. The dotted line represents the isoconversion line. When the space time is 0.2hr, the conversion is too low to be practical although the productivity appears higher than any other cases. When the space time is 0.4hr, the conversion reaches 90%

only in case when the feed concentration is below 10mM. When the space time is set at 0.8hr, the conversion reached over 90% with the feed concentration up to 33mM.

Discussion

The method using glutaraldehyde crosslinking has been widely applied to the immobilization of enzymes from various microbial sources. There have been several reports on the immobilization of whole cell enzymes by direct crosslinking of cell mass[14], or entrapment with gelatin followed by crosslinking with glutaraldehyde[15]. According to the present immobilization process, the size of the immobilized enzyme pellet can be controlled as desired by using an adequate pore size in an extruder. The hardness of pellet can also be controlled by the treating time with glutaraldehyde. The use of cellulosic material, such as DEAE-cellulose in preparing enzyme pellet is considered to be useful due to the increased porosity of the immobilized enzyme pellet, which can minimize the internal diffusion effect.

No great change in enzyme characteristics by immobilization was found except for the slight enhancement in stability at high temperature, and the increase in the numerical value of Michaelis-Menten constant. As shown in batch experiments, the conversion gradually decreased after attaining a certain maximum value as the space time becomes longer. It is ascribed to the degradation of 6-APA in aqueous media by the cleavage at lactam ring due to the alkaline pH of the reaction solution. The degradation of 6-APA and benzylpenicillin at alkaline pH has been well noted[16]. It suggests that the space time should not exceed a certain point where the degree of 6-APA degradation becomes dominant over the 6-APA production by the enzymic hydrolysis of penicillin. It strongly suggests that the degree of product degradation should be considered as one of the major factor influencing the productivity.

It is also concluded the use of moderate buffer solution even in case of stirred tank is recommended to increase the operational stability of immobilized enzyme[17]. The pH of the reaction mixture in a stirred tank reactor can be readily controlled by way of alkali titration by pH stat method, while it is difficult to control the pH drop in a plug flow reactor. In this experiment, however, it was demonstrated that the pH drop can be minimized within a certain pH range by using buffer solution with moderate strength. Since one cannot completely eliminate the pH drop even though a buffer solution is employed in a plug flow reactor, the optimization of the reactor performance requires careful consideration of various factors such as substrate concentration, residence time, desired conversion yield and buffer strength used.

In view of the effect of pH drop in a column reactor and the pH dependent stability of the immobilized enzyme, the use of somewhat lower initial pH than normally used in the experiment deserves further investigation in terms of enzyme stability sacrificing the conversion efficiency as little as possible. From the optimization process of a plug flow reactor, some valuable information of the operational mode of a reactor may be drawn. The conversion efficiency where the space time is below 0.2hr is thought impractical although the productivity is shown higher than any other cases. The critical feed concentration where the conversion efficiency reaches 90% is 27mM when the space time is 0.6hr, and 33mM when the space time is 0.8hr. The conversion efficiency seems practical only in cases where the key operating parameters such as the feed concentration and the residence time are met.

In an industrial reactor, higher catalyst contents could be used to reduce the residence time of the labile benzylpenicillin and 6-APA in the reactor.

Although the immobilized enzyme demonstrated the maximum stability at pH 7.0, it is necessary to operate the reaction at a higher pH to achieve maximum conversion because of the higher optimum pH and the effect of pH drop during the operation. Immobilization of penicillin amidase increased its stability at the temperature range above 50°C. In practice, however, the optimum temperature for the deacylation process is determined not by the

stability of the enzyme, but by the stability of benzylpenicillin and 6-APA.

A batch reactor can be operated reusing the immobilized enzyme several times without a great loss of enzyme activity usually accompanied by the degradation or abrasion of physical form of the immobilized enzyme pellet due to the mechanical agitation.

In early study[17] on the 6-APA production by the immobilized penicillin amidase in a continuous four state tank reactor, it was suggested that the addition of alkali solution to maintain the constant pH level was a major factor in the operational stability of the immobilized penicillin amidase. The loss of the enzyme activity was increased by both decreased stirring speed and buffer concentration. It may be ascribed to the increase in local pH, in which the decrease in enzyme activity becomes significant as is the case of our own experimental result (see Fig. 4). In a stirred tank, the results at high stirring speed is satisfactory, but the agitation speed is limited due to the attrition of the immobilized enzyme particle caused by the mechanical agitation. In the present work, we have operated satisfactorily two types of reactor, a stirred batch and a continuous plug flow reactor.

It is reported that the design of a suitable plug flow reactor is rather difficult because the pH adjustment in a narrow range by alkali addition is essential[3]. It is shown in the present study, that the difficulties involved in the pH drop can be overcome by employing buffer solution with moderate strength. The decrease in the enzyme activity due to the pH drop was not significant in the experimental range.

요 약

대장균이 생산하는 페니실린 아미다제를 젤라틴에 포괄시켜 사출한 후 글루트알데히드로 가교하여 고정화하였다. 이렇게 하여 만들어진 고정화효소는 약 70%의 높은 효소역가를 나타내었고 보관 및 반응조에서 좋은 안정성을 보여주었다. 반응조내에서의 효소역가 반감기는 약 50일이었으며 최적 pH 및 온도는 각각 8.5와 50°C로 나타났다. 효소역가에 미치는 pH 및 온도의 영향은 고정화하기 전과 큰 차이가 없었으나 고온에서의 안정성이 증가되었다. 기질용액으로 완충액을 사용하여 column을 사용하는 관형식 반응조에서의 반응생성률에 기인하는 pH 감소효과를 최소한으로 줄이므로써 효소반응조를 최적화하였다. 반응조 조작상의 중요한 인자 즉 기질농도, 체류시간, 반응생성물로의 전환율 및 이에 따르는 생산성을 pH 감소효과와 연관시켜 최적반응조건을 논의하였다.

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