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연속 2단계 교반조 효소 반응장치의 최적화에 관한 연구

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Two-Stage Enzyme Reactor System for Production of 6-Aminopenicillanic Acid

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Abstract

For production of 6-aminopenicillanic acid by enzymatic hydrolysis of benzylpenicillin, two-stage continuous stirred tank enzyme reactor system was evaluated. Using proper mathematical model of the reaction kinetics and kinetic constants of soluble and immobilized forms of penicillin amidohydrolase, the reactor system was simulated with an aid of a computer and a set of optimum operating conditions was found. In comparison with the single stage system, the productivity of the two-stage enzyme reactor system was 50% higher than that of the single stage system under the same operating conditions. For the industrial applications, the reactor system using immobilized penicillin amidohydrolase was found to be far more advantageous in terms of productivity than that using soluble enzyme because of the reduced inhibitory effect of the product, 6-aminopenicillanic acid, on the immobilized enzyme as compared to the same effect on the soluble enzyme.

Introduction

Penicillin amidohydrolase(EC 3.5.1.11.) of *Bacillus megaterium* is an extracellular enzyme[1] which hydrolyses the amide linkage of the benzylpenicillin to yield the phenylacetic acid and 6-aminopenicillanic acid (6-APA)

which is used as the nucleus and the starting material for various semisynthetic penicillins. We have investigated the possibility of using immobilized penicillin amidase as a catalyst in a two-stage continuous enzyme reactor system for the production of 6-APA. In 6-APA production, it is desirable to raise the yield of the product to a level of theoretical yield since both the product and substrate have relatively high unit price and it is costly to separate and recover the unreacted substrate.

The choice of reactor for a particular enzyme-catalyzed conversion process depends not only on kinetics of the reaction but also on practical considerations. The performances of packed bed and continuous flow stirred tank reactor(CSTR) systems have been studied by many workers.[2-5] Theoretically, ideal plug flow reactor has much higher productivity than CSTR.[2] But the deviation from the ideality of such packed-bed type enzyme reactor system sometimes results in poor performance and low yield in practical applications.[6] Two-stage CSTR system with penicillin amidohydrolase is considered to be of practical importance since the productivity could be increased considerably as compared with the case where a single-stage CSTR system is used. Theoretical analysis of the enzyme reactor system showed this possibility. For this reason, we have evaluated the performance of two-stage CSTR type enzyme reactor system in which either soluble or immobilized enzymes is used. A mathematical model of the enzyme reaction kinetics was derived and the performance of the reactor system was simulated with an aid of a computer in order to optimize the productivity of the reactor system in terms of process variables.

Theory

1. Reaction kinetics. To derive a mathematical model for the penicillin amidohydrolase reactor system, it was necessary to find the rate equation which describes the enzyme reaction mechanism. In our previous report,[7] we discussed the rate equation model which takes the double inhibition effect of the products into consideration. The method of steady-state analysis of the enzyme reaction was used for derivation of the reaction rate expression.

The general uni-bi type of enzyme reaction kinetics described by Cleland[8,9] is applicable to derivation of the rate equation for the penicillin amidohydrolase reaction. King-Altman method[9] can also be used for the derivation of the rate equation. Our results are shown in Eq. (1).

$$V=V_{max}/\left\{1+\frac{K_m}{S}\left(1+\frac{A}{K_{iA}}\right)+\frac{P}{K_{iP}}\left(1+\frac{K_m}{S}\right)\right\} \quad (1)$$

By substituting the fractional conversion X into Eq. (1),

$$X=(S_0-S)/S_0,\ S=S_0(1-X),\ A=S_0\ X=P \quad (2)$$

we obtain the rate Eq. (3),

$$V=V_{max}S_0(1-X)K_{iA}\cdot K_{iP}/\{S_0(1-X)K_{iA}K_{iP}+K_mK_{iA}K_{iP}+K_mS_0XK_{iP}+S_0^2K_{iA}X(1-X)+S_0K_mK_{iA}X\} \quad (3)$$

The enzymatic hydrolysis of benzylpenicillin is represented by the Eq. (1) or by Eq. (3) in terms of fractional conversion.

2. Reactor kinetics. Based on the material balance of the reactor system shown in *Fig.* 1, and assuming that we have perfect mixing, isothermal operation, no side reaction of substrate and products during the reaction, and prolonged stability of the enzyme, a kinetic model of the reactor system can be derived and the result is shown in the following form:

first stage; $\frac{dX_1}{dt}=\left(-\frac{1}{\tau_1}\right)X_1+\frac{1}{S_0}V_1$ (4)

second stage; $\frac{dX_2}{dt}=(X_1-X_2)/\tau_2+\frac{1}{S_0}V_2$ (5)

Overall productivity; $\pi=\frac{S_0X_2}{\tau_1+\tau_2}$ (6)

where, τ_1 and τ_2 are the mean residence times of the substrate in the first and second stage respectively and S_0 is the initial substrate concentration. With the aid of a computer the differential equations (4) and (5) were integrated, and the results were used to predict the performance of the enzyme reactor system. As reported in the previous paper[7], the additional conversion achieved while the reaction mixture passes through the ultrafiltration system was found to be negligible under the actual operating conditions, where the mean space time of the reaction mixture in the thin channel ultrafilter unit was less than 0.001 hr. In deriving Eq. (4), it is assumed that the effect of substrate concentration of the recycle on the reactor performance is negligible since the concentration of substrate in the feed is far greater than that of the recycle when the conversion is greater than 95%. The separation efficiency of product by the ultrafilter system was found to be greater than 92% under the operating conditions employed, and the effect of product carried with the recycle on the reactor performance was not significant once a steady state condition with a conversion greater than 95% is achieved. Thus, we may still use the same kind of process arrangement as shown in *Fig.* 1, that is, the ultrafilter unit was used only as a separator of the product.

3. Two-stage and multistage CSTR enzyme reactor system. The penicillin amidohydrolase enzyme reaction shows that the reaction rate decreases as the fractional conversion or the reaction time increases. The order of reaction is equal to or less than unity at any time. The design equation for the CSTR system may be expressed as:

$$\tau/S_0=\left(-\frac{1}{V}\right)X \quad (7)$$

where, τ is the mean space time, S_0 the initial

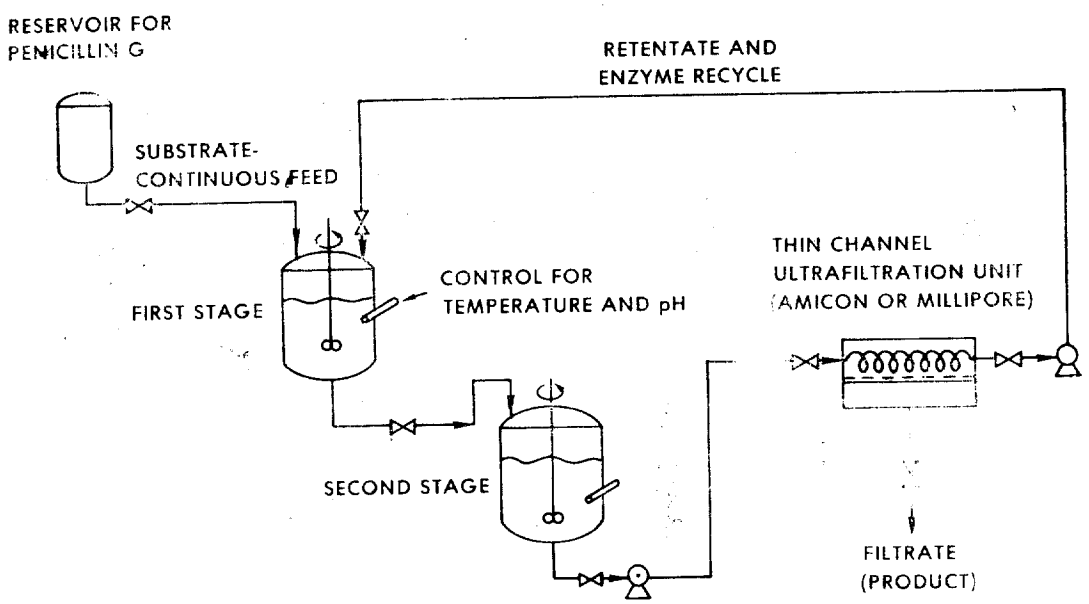


Fig. 1. A Model System for the Two-Stage Continuous Stirred Tank Enzyme Reactor System.

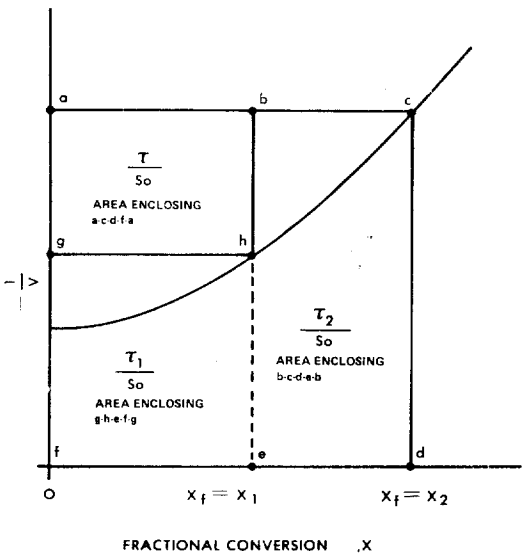


Fig. 2. Theoretical Comparison of Productivity of Two-stage CSTR System with that of Single Stage System.

or feed substrate concentration, V the reaction rate, and X the fractional conversion. The graphical representation of the design equation of CSTR system is shown in *Fig.* 2.

This figure shows that the area(acdfa) τ/S_0, represents the time required for the desired conversion. When we compare the times requred for a predetermined conversion(for example, 95%) with one and two-stage reactor system, we find that a longer time is required with one-stage reactor system from the relative sizes of the area. The area enclosed by a,c,d, and f represents the reaction time with single-stage reactor(τ/S_0) and the area enclosed by b,c,d,f,g, and h represents the reaction time with two-stage reactor system($\tau_1/S_0+\tau_2/S_0$).

Since the productivity of these enzyme reactor system may be expressed as.

$\frac{S_0X}{\tau}$ for a single stage reactor

$\frac{S_0X}{(\tau_1+\tau_2)}$ for a two-stage reactor

we can conclude that the productivity with two-stage reactor system is greater than that with a single-stage reactor system, since

$$\tau/S_0>(\tau_1+\tau_2)/S_0 \quad (8)$$

and $$S_0X/(\tau_1+\tau_2)>S_0X/\tau \quad (9)$$

We may extend this result of our analysis to a multistage system. However, we concluded that the reactor arrangement with more than three CSTR system becomes ineconomical due to significantly increased capital cost, since the gain from the improved productivity cannot outweigh the increase in the capital and operating costs with more than three-stage system.

Experimental

Basically the same experimental procedures as those employed in our earlier work[7] reported previously were used in this experimental work. Penicillin amidohydrolase was prepared from our mutant strain of *B. megaterium*(obtained from ATCC 14945). The medium consisted of casitone(Difco) 2.5%, yeast extract 0.5%, glucose 2%, benzoic acid 0.2% or phenylacetic acid 0.15%, and silicone antifoam 0.01%.

The optimal operating conditions of fermentation for cnzyme production were: initial pH of medium 7.0, temperature of cultivation 30°C, oxygen transfer rate greater than 55 m moles of oxygen per 1 per hr. The centrifuged supernatant from the fermentation broth was used as a crude form of the soluble enzyme. For preparation of the immobilized enzyme, the centrifuged supernatant was treated with 0.3% $CaCl_2 \cdot 2H_2O$(by weight). After solution of the $CaCl_2$, a mixture of 1%(by weight) each of bentonite and Hyflo-supercel were added. The pH was adjusted to 6.2 with HNO_3, and agitation was continued for one hour at

room temperature while maintaining the pH at 6.2. The mixture was then filtered on a Hyflo-precoated filter, and the penicillin amidohydrolase that was immobilized on bentonite was washed with 0.5% aqueous $CaCl_2 \cdot 2H_2O$ solution. The wet cake of immobilized enzyme was slurried in a mixture of water(40% of the original broth supernatant) and 0.1% toluene, the pH was adjusted to 8.0 with 10% NH_4OH, the mixture was warmed to 40°C and the hydrolysis reaction of benzylpenicillin was carried out.

The kinetic constants of soluble and immobilized penicillin amidohydrolase are determined using the enzyme so prepared, and these kinetic constants are used for this study. The specific activity of the enzyme is defined as the units of enzyme activity per mg of protein. One unit of enzyme is defined as the activity of enzyme that is equivalent to one micromole of product formed per minute under the specified reaction conditions.

The model reactor system is arranged as is depicted in *Fig.* 1. A combination of two enzyme reactors and an ultrafilter(Amicon) was considered as a model system for the continuous enzyme reactor. Benzylpenicillin is fed continuously into the first enzyme reactor that is connected to the second enzyme reactor and the ultrafilter in series. The reactor system is initially charged with the enzyme solution prior to feeding of the substrate. The retentate containing the enzyme and unreacted substrate is recycled back to the first stage

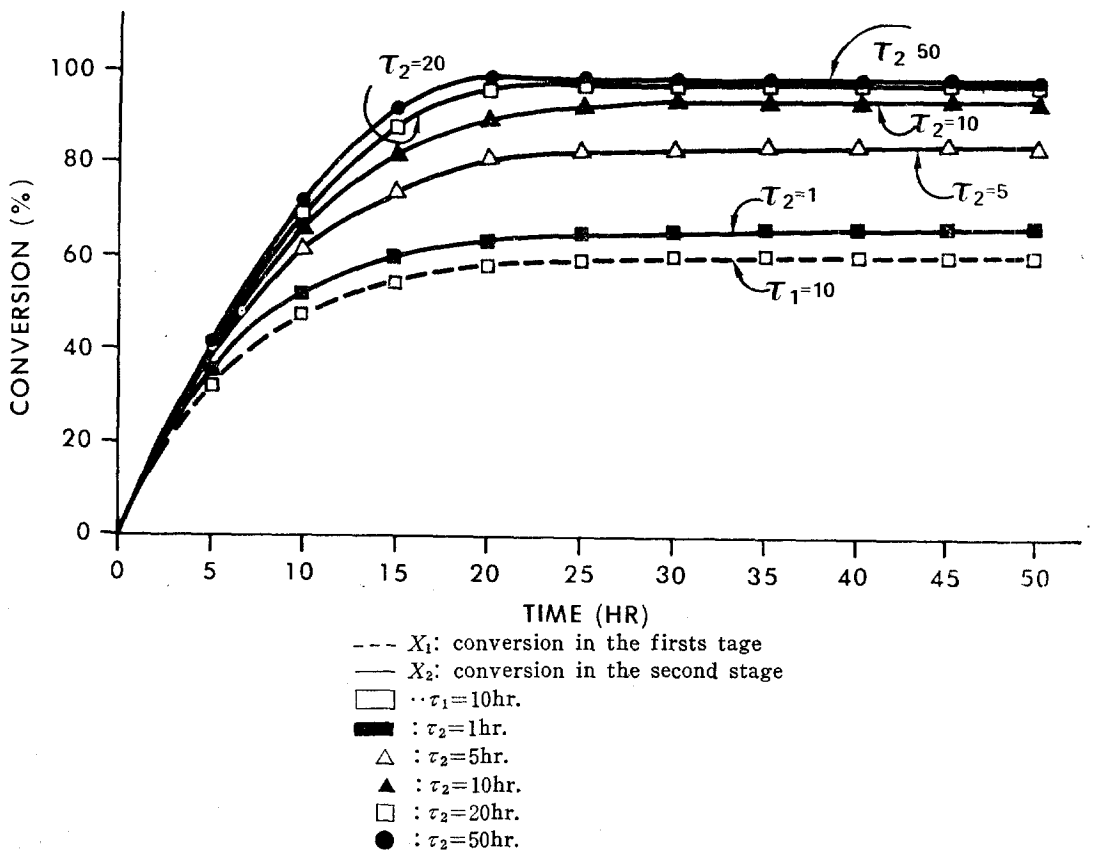


Fig. 3. Profile of Conversion in Two-Stage Reactor System. (τ_1=10hrs., fixed)

and the product and part of unreacted substrate is taken out as a filtrate from the ultrafilter unit. The mean residence time of the reaction mixture in the enzyme reactor system was adjusted or controlled by the flow rate of the substrate feed.

For further details of experimental procedures our earlier report[7] should be referred to.

Results and Discussion

Through this kind of simulation study based on a mathematical model which describes the physical system reasonably well, we can reduce the required experimenlta work considerably. We could find a great deal of information concerning the performance of the reactoı system from the results of this simulation study. *Fig.* 3 shows the progress of reaction of the immobilized enzyme system for a given initial substrate concentration, 0.2 M, for a fixed space time of the first stage, 10 hr., and for varying space times of the second stage. We can find easily that the steady-state is achieved within 30 hr. of reactor operation.

To compare the soluble enzyme with the immobilized enzyme, *Figs.* 4 and 5 are shown. The effects of initial substrate concentration on conversion and productivity are presented in these figures. *Fig.* 4 shows that, for both types of enzyme preparation, the percent conversion falls rapidly as the initial substrate concentration, S_0, increases. The conversion by the soluble enzyme seems to fall more rapidly than that by the immobilized enzyme. As shown in *Fig.* 5, when the feed concentration is less than 0.1 M, productivity of immobilized enzyme reactor system shows little difference from that of the soluble enzyme reactor system. But when S_0 is higher than 0.1 M, the productivity of 6-APA by the immobilized enzyme is greater than that by the soluble enzyme. The value of K_{iP} of the immobilized enzyme is greater than that of soluble enzyme by one order of magnitude as can be seen in *Table* 1. This means that the use of immobilized enzyme is far more advantageous than soluble enzyme for higher productivity for this enzyme reactor system. This conclusion is the same as for the single-stage CSTR system.[7]

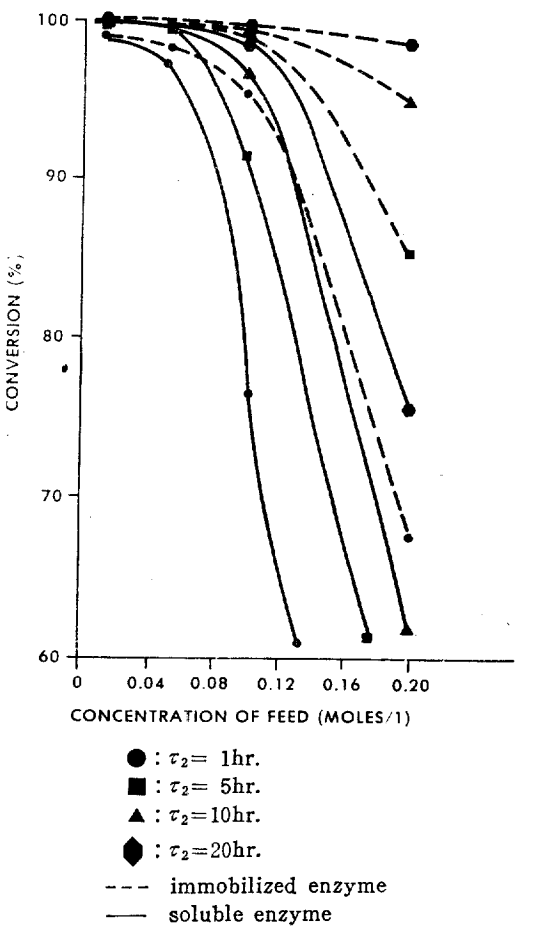


Fig. 4. Effect of the Substrate Concentration of the Feed on the Overall Conversion for Soluble and Immobilized Enzyme when First Stage Space Time is Fixed at 10 hrs.

Table 1. Kinetic Constants of Penicillin Amidase Poduced from B. Megaterium. Initial Enzyme Loading was 6×10^3 Units/l for both Soluble and Immobilized Enzyme Reactors.

Kinetic constants	K_m(M)	K_{iP}(M)	K_{iA}(M)
Soluble Enzyme	4.5×10^{-3}M	2.6×10^{-2}M	0.45M
Immobilized Enzyme	6.0×10^{-3}M	2.5×10^{-1}M	0.62M

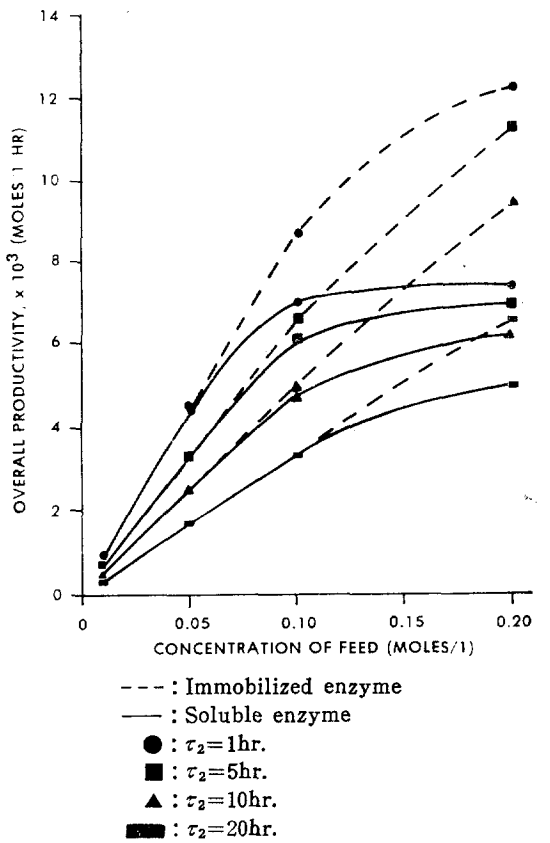


Fig. 5. Effect of the Substrate Concentration of the Feed on the Overall Productivity for Soluble and Immobilized Enzyme when First Stage Space Time is Fixed at 10 hrs.

Figs. 6, 7 and 8 show the productivity as a function of space times, τ_1 and τ_2, and initial substrate concentrations S_0. The overall isoconversion lines are also superimposed on these plots. From *Fig.* 6, we can find the values of τ_1 and τ_2 which corresponds to the maximum productivity of the reactor system for a given initial substrate concentration and the conversion requirement. For example, if we desire 95% or higher conversion, the optimal values of τ_1 and τ_2 can be determined from *Fig.* 6. These optimal operating conditions that correspond to the maximum productivity are found to be 10 to 12hr. for τ_1 and 6 to 10 hr. for τ_2 when initial substrate concentration is 0.2 M. To find the initial substrate concentration which corresponds to the maximum productivity, *Fig.* 7 was prepared. From this plot, the initial substrate concentration which

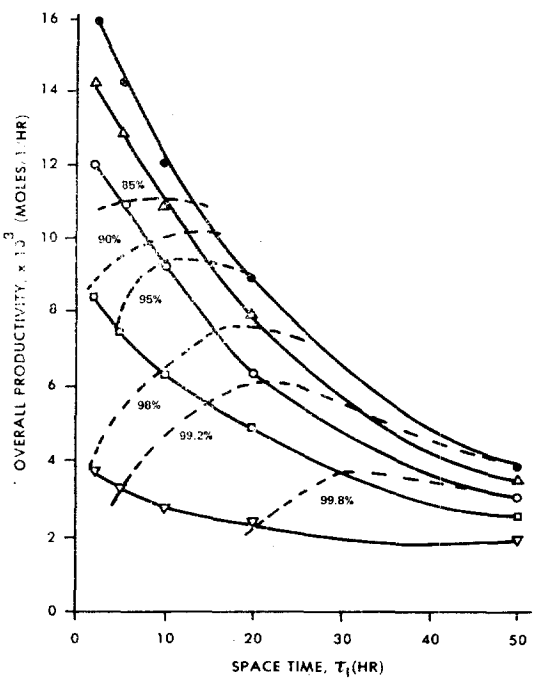


Fig. 6. Effect of the First Stage Space Time on the Overall Productivity for Immobilized Enzyme when Substrate Feed Concentration is Fixed at 0.2 M

gives the maximum point of productivity for 95% conversion is about 0.2M and τ_2 in the range of 6 to 10 hr. when the first stage space time, τ_1, is fixed at 10 hr. From *Fig.* 8, we can find the values of initial substrate concentration in the same region, and find also the value of τ_1 when τ_2 is fixed at 10 hr. All the results on productivity as a function of S_0, τ_1 and τ_2 are summarized for both

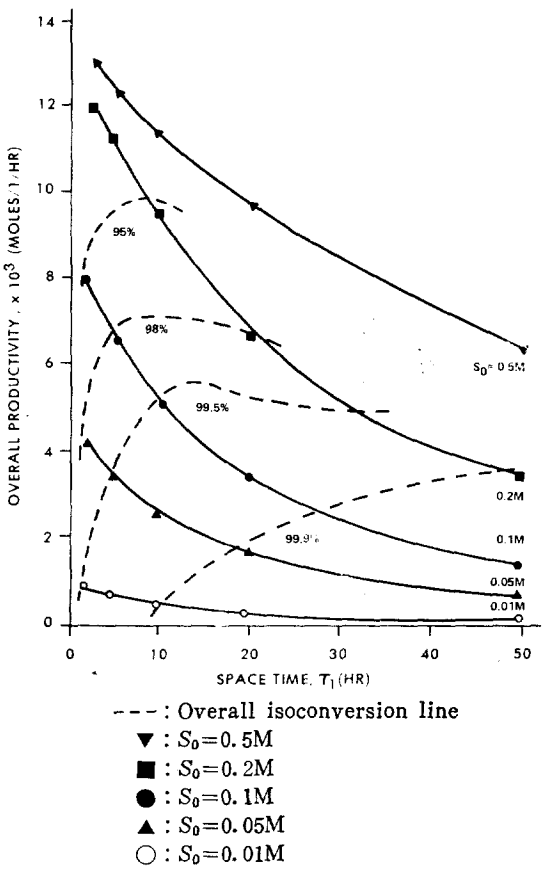


Fig. 7. Effect of the Second Stage Space Time on the Overall Productivity for Immobilized Enzyme when First Stage Space Time is Fixed at 10 hrs.

the immobilized and soluble forms of enzyme in *Fig.* 9.

One may find other optimal operating conditions for a higher conversion than 95% from these results. For example, the optimal conditions for 97, 98 and 99% conversions may be found, however, the values of productivity

Table 2. Optimal Operating Conditions for the Two-Stage CSTR Immobilized Penicillin Amidase System.

S_0(M)	τ_1(hr.)	τ_2(hr.)	Steady state conversion (%)	Overall productivity
0.18	10	8	95.1	9.5×10^{-3}

achievable are far less than the productivity achieved at 95% conversion. The process economy for the productivity with 95% conve-

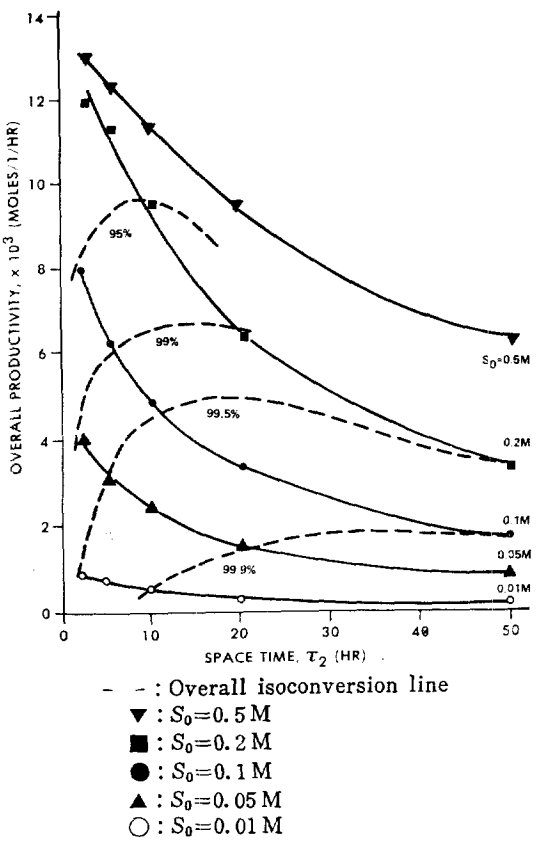
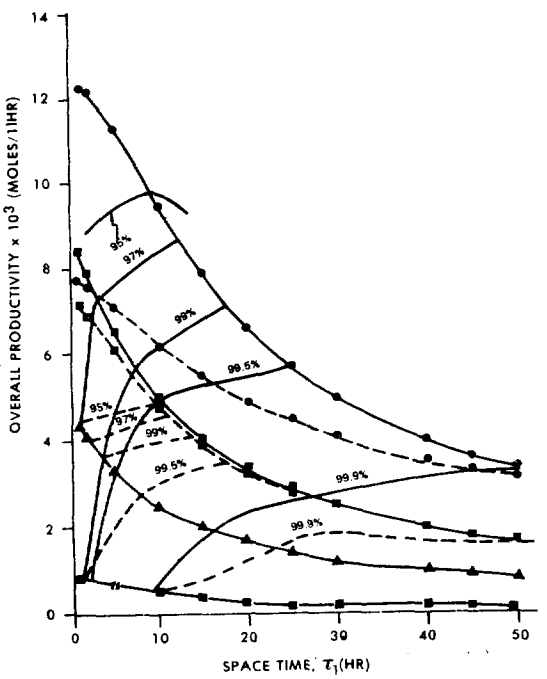


Fig. 8. Effect of the First Stage Space Time on the Overall Productivity for Immobilized Enzyme when Second Stage Space Time is Fixed at 10 hrs.

Table 3. Comparison of Productivity of 2-Stage CSTR System with that of Single Stage System.

	S_0(M)	X (fractional conversion)	Space time (hr.) required for the desired conversion	productivity*
Two-stage	0.18	0.95	$18(\tau_1+\tau_2)$	9.5×10^{-3}
Single-stage	0.18	0.95	$27(\tau)$	6.3×10^{-3}

*Productivity is defined as the number of moles of product formed per liter of reactor working volume per hour.



(95%) : Overall isoconversion line for immobilized enzyme

(95%) : Overall isoconversion line for soluble enzyme

● : S_0=0.2 M

■ : S_0=0.1 M

▲ : S_0=0.05 M

▬ : S_0=0.01 M

—— : Productivity for immobilized enzyme

- - - : Productivity for soluble enzyme

Fig. 9. Effects of the Substrate Concentration and the First Stage Space Time on the Overall Productivity for both the Soluble and Immobilized Enzyme when the Second Stage Space Time is Fixed at 10hrs.

rsion is far more favorable to that for the productivity with a conversion higher than 95%.

By the simulation method and optimization technique described in this paper, we determined the optimal operating conditions for the two-stage penicillin amidase enzyme reactor system. This result is shown in *Table* 2. *Table* 3 shows that two-stage CSTR system gives as much as 50% higher productivity than that of single-stage for a given initial substrate concentration and for a required conversion.

Conclusion

In production of 6-aminopenicillanic acid(6-APA) by enzymatic hydrolysis of penicillins the productivity obtained with the two-stage CSTR system using immobilized penicillin amidase was 50% higher than that obtained with a single-stage system. The optimum operating policy found from the computer simulation was that the feed concentration of substrate was 0.2M, the mean residence times of substrate in the first and second stage were 10 hr. and 8 hr., respectively, when conversion requirement was higher than 95%. Thus, the two-stage continuous enzyme reactor system using immobilized penicillin amidase for the production of 6-APA was found to be of practical interests.

Nomenclature

V Reaction rate(M/hr)

K_m Michaelis-menten constant(M)

K_{iP} Product inhibition constant of 6-APA (M)

K_{iA} Product inhibition constant of phenylacetic acid(M)

S_0 Initial substrate concentration(M)

τ Mean residence time of single stage CSTR system(hr)

τ_1 and τ_2 Mean residence times of substrate in the first and second stage reactor of two-stage CSTR system(hr)

π Overall productivity(moles/l/hr)

X Fractional conversion

A Concentration of phenylacetic acid in the reaction mixture(M)

P Concentration of 6-APA in the reaction mixture(M)

S Concentration of substrate, benzylpenicillin (M)

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