

PRODUCTION OF 6-AMINOPENICILLANIC ACID IN IMMOBILIZED ENZYME REACTOR WITH ELECTRODIALYSIS

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Phenylacetic acid, an inhibitory product, was formed through the hydrolysis of penicillin G by immobilized penicillin acylase. In this study, electrodialysis was applied to remove phenylacetic acid continuously from the reaction mixture and to enhance the efficiency of the reaction.

The permeation rate of phenylacetic acid, in this electrodialysis system was found to be much faster than those of two other compounds, penicillin G and 6-aminopenicillanic acid. Therefore, the membranes selected here might be suitable for continuous removal of phenylacetic acid from the reaction mixture for penicillin G hydrolysis. However, the permeation rate of phenylacetic acid was significantly influenced by the penicillin G concentration; it decreased with increasing penicillin G concentration. A theoretical model was developed to describe this phenomenon and was applied to the hydrolysis of penicillin G by means of a combination of immobilized penicillin acylase and electro dialysis. The experimental data were compared to those predicted by a model using independently determined parameters such as the Michaelis constant, inhibition constants and permeation rate constants. Fairly good agreement was found between the theoretical and experimental data. When a 100 mol·m⁻³ penicillin G solution was used as the substrate, the time required to reach 98 % conversion could be reduced to 24.7 % by using the combination with electrodialysis.

Introduction

6-Aminopenicillanic acid (6APA) is a basic raw material for the industrial production of semisynthetic penicillins containing the β -lactum structure. It has been produced through the deacylation of penicillin G (Pen-G) using immobilized penicillin acylase (IPA).

In this enzymatic reaction, the pH of the reaction mixture decreases with increase in phenylacetic acid (PAA), which is produced as a by-product. Also, the deacylation of Pen-G is severely inhibited by PAA. Accordingly, it is important to maintain a low concentration of PAA by removing it from the reaction mixture continuously. However, there have been no reports concerning a bioreactor for the hydrolysis of Pen-G equipped with a device for continuous removal of PAA, though there have been many on immobilization^{2, 4, 7, 8)} and reaction kinetics^{6, 9)}.

Electrodialysis involves a membrane system in which low-molecular weight organic and inorganic ions are transported through an ion-selective membrane by means of a direct electric current. The most common application of electrodialysis has been in the food and dairy industry^{1, 3)}.

In this study, the application of electrodialysis to the hydrolysis of penicillin G is investigated in order to

remove PAA continuously from the reaction mixture, and the efficiency of this system is discussed.

1. Materials and Methods

1.1 Immobilized enzyme

Immobilization of PA on hydrogenated porous polyacrylonitrile fibers was carried out as described previously⁴⁾. The enzyme from *Bacillus megaterium* B-400 was purified through adsorption on Celite No. 560 and elution with 24 % ammonium sulfite. Hydrogenated porous polyacrylonitrile fibers (diameter, 0.2 to 0.5 × 10⁻² cm; length, 1 m) were activated with glutaraldehyde and then used for immobilization of PA. The immobilization yield and specific activity of the immobilized PA were 82 % and 1910 units·g⁻¹ of support respectively. Enzyme activity of IPA was assayed by estimating the 6-APA liberated from Pen-G by the method of Bomstein and Evans¹⁾. One unit of enzyme was defined as the amount producing 1 μ mol of 6APA min⁻¹ at 34°C. IPA fibers were cut into approximately 1.0 cm lengths prior to use.

1.2 Apparatus for the combination of enzyme hydrolysis and electrodialysis

Figure 1 shows a schematic diagram of the combination system of enzyme hydrolysis and electrodialysis. The inside of the electrodialysis module is shown in Fig. 2. The electrodialysis module was internally separated

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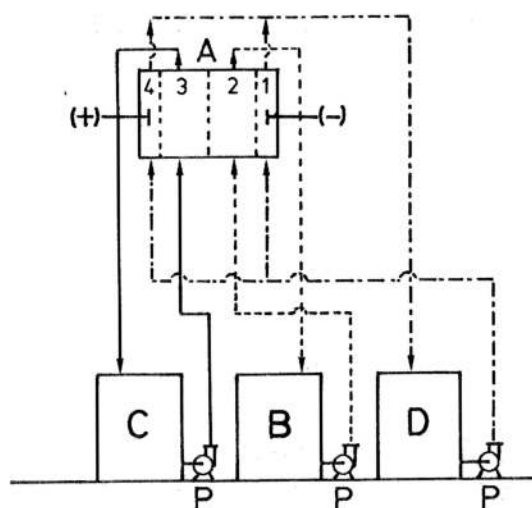


Fig. 1 Schematic diagram of the electrolysiser
 A: Electrolysis and reactor unit
 1. Cathode compartment 2. Reaction compartment with IPA 3. Compartment for concentrated solution 4. Anode compartment
 B: Vessel for reaction mixture
 C: Vessel for concentrated solution
 D: Vessel for electrode solution

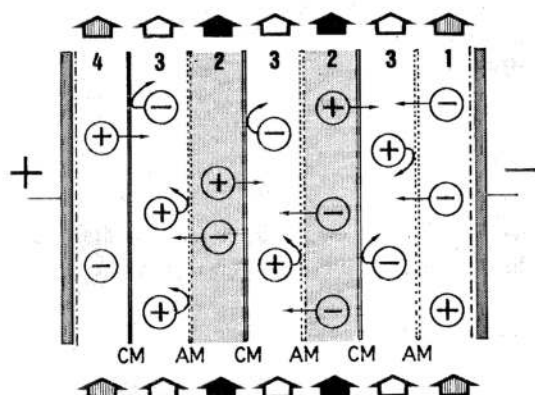


Fig. 2 Reactor combined with electrodialysis
 1. Cathode compartment 2. Reaction compartment with IPA 3. Compartment for concentrated solution 4. Anode compartment
 AM: Anion-exchange membrane, CM: Cation-exchange membrane, ■: Immobilized enzyme (IPA),
 ◀: Reaction solution, ◀◀: Concentrated solution,
 ◀◀◀: Electrolyte solution

into four compartments—a cathode compartment, a reaction compartment containing IPA, a compartment for the concentrated solution, and an anode compartment—by means of anion-exchange and cation-exchange membranes. Cation-exchange membranes, CMV, and anion-exchange membranes, such as AMV, ASV, ASR and DSV, were purchased from Asahi Glass Co. The size of the membranes was $0.15 \times 0.24 \text{ m}^2$ (effective area, 0.021 m^2). The characteristics of the ion-exchange membranes are shown in Table 1. Immobilized enzyme was placed in the reaction compartment. The hydrolysis of Pen-G was carried out by circulating the reaction mixture between the reaction compartment containing IPA in the electrolysiser and vessel B in Fig. 1, at a flow rate of

Table 1. Characteristics of ion-exchange membranes

Membranes	Membrane thickness $\times 10^3$ [m]	Bursting strength $\times 10^{-4}$ [kg·m ⁻²]	Resistance of area $\times 10^4$ [Ω·cm ²]	Transportation value		
				T-Na ⁺	T-Cl ⁻	T-SO ₄ ²⁻
CMV	0.13-0.15	3-5	2.0-3.5	0.92 <		
AMV	0.11-0.15	3-5	2.0-3.5		0.94 <	
ASV	0.11-0.15	3-5	3.0-4.5		0.95 <	0.008 >
ASR	0.11-0.15	3-5	2.0 >		0.94 <	
DSV	0.13-0.15	3-5		$U_{\text{H}_2\text{SO}_4} = 2.4-4.0 \text{ R} = 1.001$		
CMV	Cation-exchange membrane (Na)			$U_{\text{H}_2\text{SO}_4}$	[mol·m ⁻² ·h ⁻¹]	
AMV	Anion-exchange membrane (Cl)			R	$U_{\text{H}_2\text{SO}_4}/U_{\text{H}_2\text{SO}_4}$	
ASV	Anion-exchange membrane (Cl)					
ASR	Anion-exchange membrane (Cl)					
DSV	Anion-exchange membrane (Cl)					

(diffusion dialysis)

$2.5 \times 10^{-3} \text{ m}^3 \cdot \text{min}^{-1}$, with a Masterflex pump (Cole-Parmer Instrument Co., Chicago, IL). PAA, a byproduct of Pen-G hydrolysis by IPA, was removed from the reaction mixture continuously and accumulated as a concentrated solution through the membranes by electrodialysis. The concentrated solution containing PAA was circulated between the compartment for the concentrated solution and vessel C by the Masterflex pump. The electrolyte solution was circulated among the cathode and anode compartments and vessel D. During the reaction, the pH of the solution in the vessel for the reaction mixture, B, was maintained at 8.4 by adding $4 \times 10^3 \text{ mol} \cdot \text{m}^{-3}$ of NaOH. The reaction temperature was 34°C. The enzymatic reaction was carried out using $100 \text{ mol} \cdot \text{m}^{-3}$ of benzyl-penicillin potassium in $40 \text{ mol} \cdot \text{m}^{-3}$ of borate buffer as the substrate. Electrodialysis was carried out at a constant voltage of 10 V between the electrodes.

1.3 Analytical methods

The concentrations of Pen-G, PAA and 6APA in the reaction mixture and the concentrated solution were determined by HPLC on a Lichrosorb RP-18 column ($0.4 \text{ mm} \phi \times 15.0 \text{ cm}$) (Merck), using a solvent system of $300 \text{ mol} \cdot \text{m}^{-3} \text{ KH}_2\text{PO}_4$ -acetonitrile (8: 2 v/v) at a flow rate of $8.0 \times 10^{-7} \text{ m}^3 \cdot \text{min}^{-1}$ for analysis of Pen-G and PAA, and $10 \text{ mol} \cdot \text{m}^{-3}$ phosphate buffer (pH 7.0) - acetonitrile (100: 1 v/v) at the flow rate of $6.0 \times 10^{-7} \text{ m}^3 \cdot \text{min}^{-1}$ for analysis of 6APA and PAA. Detection the absorption at 230 nm was performed of with a UV detector.

1.4 Chemicals

Benzylpenicillin potassium ($1580 \text{ unit} \cdot \text{mg}^{-1}$) was purchased from Meiji Seika Co. (Tokyo). Other chemicals used were of analytical grade and were obtained commercially.

2. Results and Discussion

2.1 Selection of anion-exchange membranes

Before the reaction, we investigated suitable ion-exchange membranes for removing PAA from the reaction mixture. Figure 3 shows the results of electrodialysis of phenylacetic acid using various anion-exchange membranes. As can be seen in this figure, the permeation

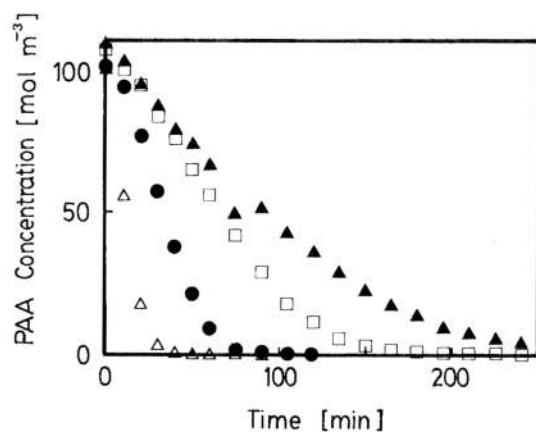


Fig. 3 Electrodialysis of PAA using various membranes.
 \triangle : DSV, \bullet : AMV, \square : ASR, \blacktriangle : ASV
 Voltage difference between membranes: 10 V
 Temperature: 37°C

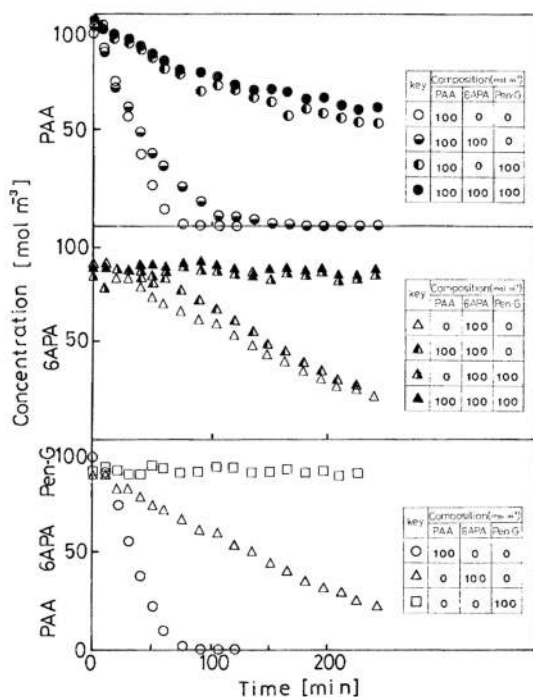


Fig. 4 Electrodialysis of Pen-G, 6-APA and PAA using AMV
 (a) Electrodialysis of PAA (b) Electrodialysis of 6APA
 (c) Electrodialysis of individual solutions of Pen-G, 6APA and PAA

rate of PAA through the DSV membrane was highest, but water also permeated through the membrane. Therefore, AMV was selected for the reaction system.

2.2 Permeation of PAA, 6APA and Pen-G on electrodialysis

Figure 4 shows the electrodialysis of each component in a mixed solution containing Pen-G, 6APA and PAA. In Fig. 4 (a), the effects of 6APA and/or Pen-G on the permeation of PAA are shown. It can be seen that the permeation of PAA was considerably influenced by Pen-G, compared with 6APA. The permeation of 6APA was scarcely observed in the presence of 100 mol·m⁻³ of Pen-G, as shown in Fig. 4 (b). However, the permeation of PAA or 6APA was little affected by the presence of

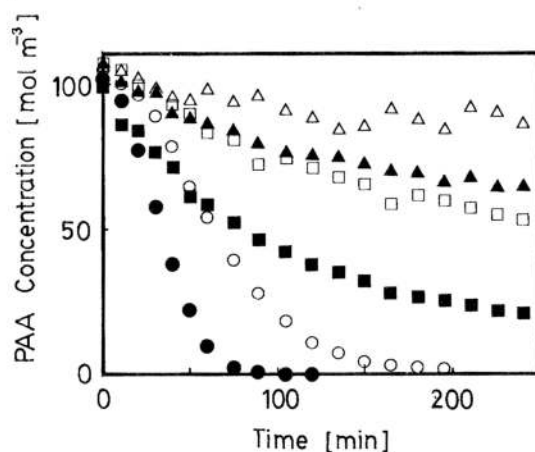


Fig. 5 Effect of the Pen-G concentration on electrodialysis of PAA using AMV. Concentration of Pen-G (mol·m⁻³)
 \bullet : 0, \circ : 10, \blacksquare : 50, \square : 100, \blacktriangle : 150, \triangle : 200

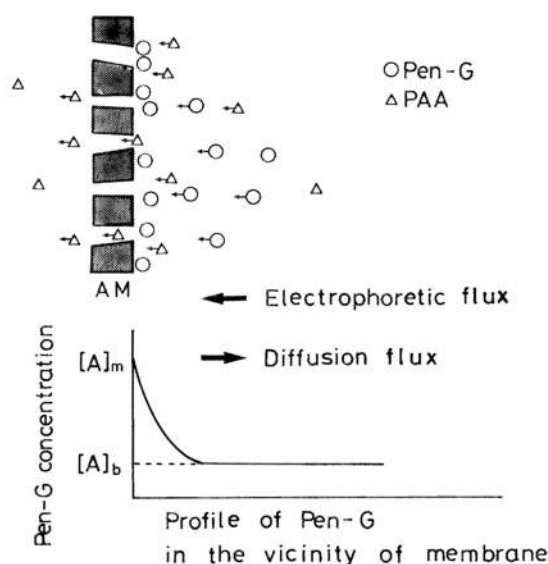


Fig. 6 Schematic model of ion transfer in the vicinity of the anion-exchange membrane

each other. Figure 4 (c) shows the results as to the permeation of PAA, 6APA and Pen-G measured separately and using each solution. As can be seen in this figure, the permeation rate of PAA in this membrane system was much higher than those of the other two compounds. The effect of Pen-G concentration on the electrodialysis of PAA is shown in Fig. 5. The permeation rate of PAA was found to be influenced significantly by the concentration of Pen-G.

2.3 Proposed model of the transport phenomenon on electrodialysis

The effect of the Pen-G concentration on the permeation rate of PAA was investigated with the following model.

A schematic model of ion transfer in the vicinity of an anion-exchange membrane is shown in Fig. 6. In this model, when ions in the solution move toward the membrane, the concentrated Pen-G ions at the surface of the membrane, which cannot permeate through the membrane, may prevent the permeation of PAA through the

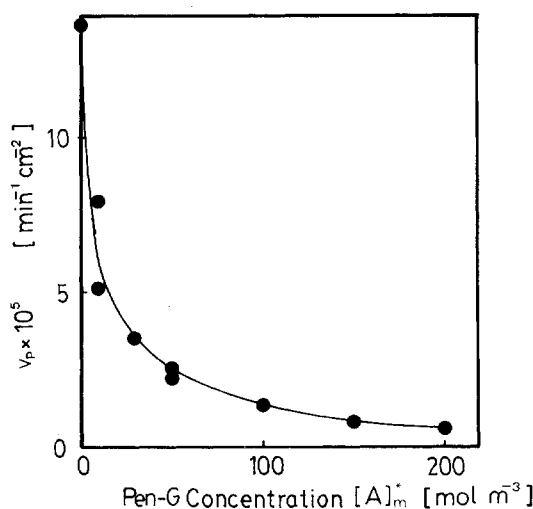


Fig. 7 Effect of Pen-G $[A]_m^*$ on v_p

membrane.

The flux of ions in electrodialysis is given by:

$$\begin{aligned} J &= -D \frac{dC}{dx} - zUC \frac{d\phi}{dx} \\ &= -D \frac{dC}{dx} + vC \end{aligned} \quad (1)$$

where

$$v = -zU \frac{d\phi}{dx}$$

The material balance of Pen-G in the vicinity of the membrane is described by the following equation, including diffusion and electrophoretic velocity components, as shown in Fig. 6.

$$\frac{d[A]_m}{dt} = K_L ([A]_b - [A]_m) - \frac{zUAS}{V} \frac{d\phi}{dx} [A]_b \quad (2)$$

If $[A]_m^*$ is defined by

$$[A]_m^* = \frac{K_L V}{\left(K_L V - zUS \frac{d\phi}{dx} \right)} [A]_b \quad (3)$$

substituting Eq. (3) into Eq. (2), the relationship between $[A]_m^*$ and $[A]_b$ can be represented by the following equation.

$$\frac{d[A]_m^*}{dt} = K_L ([A]_b - [A]_m^*) \quad (4)$$

If the Pen-G concentration in the liquid does not change, at the steady state

$$[A]_m^* = [A]_b \quad (5)$$

While, with regard to PAA, if the diffusion rate is assumed to be negligible compared with the permeation rate with electric force, the flux of PAA ions through the membrane can be given as

$$J = v_p [P] \quad (6)$$

v_p can be represented as a function of $[A]_m^*$ as

$$v_p = f([A]_m^*) \quad (7)$$

Figure 7 shows the effect of Pen-G concentration $[A]_m^*$ on the velocity of PAA permeation through the membrane, which was experimentally obtained by using various concentrations of Pen-G, $[A]_b = [A]_m^*$. As can be seen in this figure, the velocity of PAA permeation is considerably affected by the concentration of Pen-G.

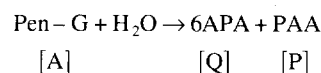
In the enzymatic reaction system, the concentration of Pen-G, $[A]_m$, at the surface of the membrane, which affects the permeation rate of PAA, changes with a decrease in the concentration of Pen-G, $[A]_b$, in the bulk solution during enzymatic reaction. The change in $[A]_m^*$ can be estimated from $[A]_b$ by using K_L in Eq. (4). K_L was experimentally estimated by the following method.

An electric current was introduced into the electrodialysis module containing a 50 mol·m⁻³ Pen-G solution. After 60 min, when the concentration profile of Pen-G in the vicinity of the membrane had reached the steady state, $[A]_m^*$ became 50 mol·m⁻³, by Eq. (5). At that time, the concentrations of Pen-G and PAA in vessel B in Fig. 1 were changed from 50 to 10 mol·m⁻³ and 0 to 100 mol·m⁻³, respectively, by replacing 4/5 of the total volume of the 50 mol·m⁻³ Pen-G solution with the same volume of a 125 mol·m⁻³ PAA solution. The ionic velocity of PAA, v_p , was calculated as a function of time using the flux of PAA, J , obtained in the above experiment. The time course of $[A]_m^*$ was estimated using the relationship between $[A]_m^*$ and v_p shown in Fig. 7. Furthermore, K_L was determined to be 0.02 min⁻¹ by Eq. (4) with the initial conditions $[A]_m^* = 50$ mol·m⁻³ and $[A]_b = 10$ mol·m⁻³, using the timecourse of $[A]_m^*$.

2.4 Hydrolysis of Pen-G by the immobilized enzyme system combined with electrodialysis

The hydrolysis of Pen-G to 6APA by means of the combination of immobilized enzyme and electrodialysis, as shown in Fig. 1, was carried out with 2.4×10^{-3} kg and 8.4×10^{-3} kg (dry weight of support) of immobilized PA, using 100 mol·m⁻³ of Pen-G as the substrate.

In the hydrolysis of Pen-G by PA, the following reaction takes place.



The material balances of Pen-G, 6APA and PAA in the immobilized-enzyme reactor are given by

$$V \frac{d[A]_b}{dt} = -rE \quad (8)$$

$$V \frac{d[Q]_b}{dt} = rE \quad (9)$$

$$V \frac{d[P]_b}{dt} = rE - JS \quad (10)$$

Here, only PAA is assumed to be removed from the reaction mixture during electrodialysis, where r is the enzymatic reaction rate and is given by⁶⁾

Table 2. Parameters used for the model simulation at 37°C and pH 8.5

$K_a = 3.57$	[mol·m ⁻³]
$K_{ip} = 1.15 \times 10^2$	[mol·m ⁻³]
$K_{iq} = 1.76 \times 10$	[mol·m ⁻³]
$K_{eq} = 7.83 \times 10^4$	[mol·m ⁻³]
$V_m = 1.25$	[mol·kg ⁻¹ ·min ⁻¹]

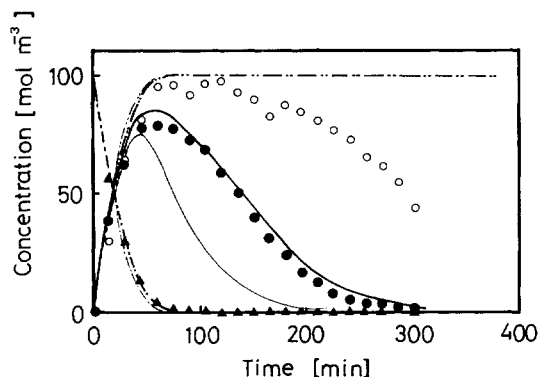


Fig. 8 Comparison between experimental data and calculated results with proposed model experimental data
Calculated curves with model with consideration of the profile for Pen-G
--- :Pen-G, — :PAA, ---- :6APA
Calculated curve with model without consideration of the profile for Pen-G
--- :Pen-G, — :PAA, ---- :6APA
Experimental data
▲ : Pen-G, ● : PAA, ○ : 6APA

$$r = \frac{V_m([A]_b - [P]_b[Q]_b / K_{eq})}{[A]_b \left(1 + \frac{[P]_b}{K_{ip}}\right) + K_a \left(1 + \frac{[P]_b}{K_{ip}} + \frac{[Q]_b}{K_{iq}} + \frac{[P]_b[Q]_b}{K_{ip}K_{iq}}\right)} \quad (11)$$

The above parameters⁶⁾ used for the simulation are shown in **Table 2**. From Eqs. (4) and (6)-(11), $[A]_b$, $[P]_b$ and $[Q]_b$ were numerically calculated using K_L and the parameters in Table 2. **Figure 8** shows a comparison of simulated and experimental results.

From this figure, it can be seen that the experimental data agree well with the calculated curves, considering the profile for Pen-G in the vicinity of the membrane (Pen-G shown by a dotted line and PAA by a solid line). In contrast, the calculated results for PAA concentration without consideration of the profile for Pen-G (broken line) exhibits a lower peak at an earlier reaction time compared with the experimental results. This is because the calculated permeation rate of PAA without consideration of the profile for Pen-G is higher than the experimental result.

Although no 6APA was assumed to permeate through the anion-exchange membrane in this model, it was found that a small amount did permeate through the membrane after Pen-G had been almost completely consumed.

Table 3 shows a comparison of the time required to reach 98 % conversion with and without combination with ED. As can be seen in this table, if the area of mem-

Table 3. Comparison of time required to reach 98 % conversion between with and without combination with ED

Amount of enzyme $\times 10^3$ [kg]	Area of membrane per enzyme [m ² ·kg ⁻¹]	Conversion [%]	Time required		
			With ED [min]	Without ED [min]	Reduction ratio [%]
8.4	2.05	90	52	53	1.9
		98	74	77	3.9
2.4	7.17	90	162	198	18.2
		98	216	287	24.7

Total area of membrane: 1720 cm²; temperature: 37°C; voltage: 10 V; initial concentration of Pen-G: 100 mol m⁻³; pH: 8.5; liquid volume: 3×10^{-3} m³

brane per amount of enzyme is large, the time required for completing the reaction is significantly reduced by using the combination with electro dialysis.

Therefore, the combination system comprising the immobilized enzyme and electro dialysis can be expected to be useful for the industrial production of 6APA.

Nomenclature

C	= concentration of ions	[mol·m ⁻³]
D	= diffusion coefficient of ions through membrane	[m ² ·min ⁻¹]
E	= amount of enzyme	[kg]
J	= flux	[mol·m ⁻² ·min ⁻¹]
K_a	= Michaelis constant	[mol·m ⁻³]
K_{eq}	= equilibrium constant	[mol·m ⁻³]
K_{ip}	= inhibition constant for 6-APA	[mol·m ⁻³]
K_{iq}	= inhibition constant for PAA	[mol·m ⁻³]
K_L	= mass transfer coefficient for molecular diffusion	[min ⁻¹]
r	= reaction rate per unit dry immobilized enzyme	[mol·min ⁻¹ ·kg ⁻¹]
S	= total surface area of membrane	[m ²]
U	= ionic mobility	[cm ² ·V ⁻¹ ·min ⁻¹]
U_A	= ionic mobility of Pen-G	[cm ² ·V ⁻¹ ·min ⁻¹]
V	= volume of reactor	[m ³]
V_m	= maximum reaction rate per unit dry immobilized enzyme	[mol·min ⁻¹ ·kg ⁻¹]
v_P	= ion velocity of PAA	[m·min ⁻¹]
v	= ion velocity	[m·min ⁻¹]
x	= distance	[m]
z	= charge number of ions	[-]
ϕ	= electric potential	[V]
$[A]$	= concentration of Pen-G	[mol·m ⁻³]
$[P]$	= concentration of PAA	[mol·m ⁻³]
$[Q]$	= concentration of 6APA	[mol·m ⁻³]

<Subscripts>

A	= Pen-G
b	= in bulk solution
m	= at membrane surface
P	= PAA

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