미생물 페니실린 아미다제에 관한 연구

(Ⅰ) *E. coli* 로부터 효소생산 조건의 최적화

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Studies on Microbial Penicillin Amidase

(Ⅰ) Optimization of the Enzyme Production from *Escherichia coli*

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Abstract

To maximize the production of penicillin amidase from *Escherichia coli* (ATCC 9637), the media composition and several factors affecting the enzyme production during fermentation were studied. The optimal media composition was found to be; 3.5% tryptone, 1.5% monosodium glutamate and 0.5% yeast extract. The addition of 0.15% phenylacetic acid as an enzyme inducer at the initial stage of cultivation increased the enzyme productivity about 5 fold. It was found that the enzyme activity reached maximum within 16hr of cultivation. The maximum production of the enzyme obtained was about 102.5 units/l broth under the optimized condition. The enzyme production was markedly increased by the optimization as compared with those previously reported.

Introduction

Penicillin amidase (Benzylpenicillin amidohydrolase, E. C. 3, 5, 1, 11) catalyzes the hydrolytic reaction of the side chain of benzylpenicillin to give 6-aminopenicillanic acid (6-APA) and phenylacetic acid (PAA). The reaction of enzymatic hydrolysis is shown in Fig. 1. This enzyme is of considerable commercial importance, since many semisynthetic penicillins are prepared from 6-APA.

Many species of microorganisms, such as bacteria, fungi, and actinomycetes are good sources of penicillin amidase. Particularly, the enzyme from bacteria such as *Escherichia coli*[2,3,4] and *Bacillus megaterium*[5] are known to be useful for the practical purpose. Besides them, many highly produ-
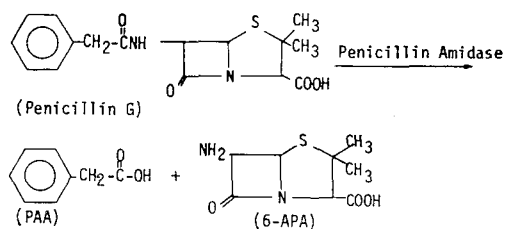

Fig. 1. **Enzymatic hydrolysis of benzylpenicillin.**

ctive microbial strains for penicillin amidase have been obtained by the extensive mutation and screening, and they have been used to produce 6-APA in an industrial scale.

In the present study, *E. coli* ATCC 9637 was chosen as an enzyme source. The effect of media composition and other factors on the enzyme

production through fermentation were studied in an attempt to optimize the fermentation condition for the maximization of the enzyme productivity.

Materials and Methods

1. Fermentation process.

A strain of *E. coli* (ATCC 9637) was used as a source of the enzyme in this experiment. The seed culture was cultivated in 250m*l* Erlenmeyer flask containing 50m*l* of medium composed of 3.5 % tryptone, 1.5% monosodium glutamate, 0.5% yeast extract at 30°C for one day in a reciprocal shaker.

50m*l* of the fermentation medium with various compositions was dispensed in 250m*l* Erlenmeyer flask, and was autoclaved at 121°C for 15 minutes. Except otherwise mentioned, pH was adjusted to 7.0 before autoclaving. Each flask was inoculated with 1% (v/v) of seed culture and cultivated for a day in a reciprocal shaker at 30°C. The bacterial cells were harvested after centrifugation and used for the enzyme source. The bench scale study on the enzyme production was carried out by using 500m*l* capacity fermentor (Bioflo Model C30, New Brunswick Scientific Co., Inc. U.S.A.) or a 5*l* jar fermentor (Marubishi, Japan).

2. Determination of the enzyme activity.

The activity of whole cell enzyme was determined by measuring the amount of 6-APA according to p-DAB method by Balasingham et al[6].

The reagent solution was prepared by mixing 1m*l* of 0.5(w/v) p-DAB in methanol to 6m*l* of acetate buffer made with 20% (v/v) glacial acetic acid 4m*l* and 0.05N NaOH 2m*l*.

All reagents were freshly prepared just before enzyme assay. 400mg of wet cell was suspended in 3.5m*l* of 0.2M borate buffer (pH8.2), and 1m*l* is added to 3m*l* of 20mM penicillin solution in 0.2M borate buffer. After 1hr of incubation at 40°C in an agitated vessel, 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 absorbancy at 415nm was measured by Spectronic 20 (Baush & Lomb) after incubation for 5 minutes. 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. 6-APA in the reaction mixture was identified by paper chromatography using butanol-acetic acid-water (4: 1: 1) mixture as a developing solvent, and the color was developed by the starch-iodine method of Thomas[7].

3. Determination of cell weight.

The cell paste was obtained from the culture broth by centrifugation, and dried in dry oven at 105°C until constant cell weight was obtained.

4. Materials

Potassium benzylpenicillin (1595 i.u./mg), 6-aminopenicillanic acid and phenylacetic acid were purchased from Sigma Chemical Co. (U.S.A.), and p-dimethylaminobenzaldegydehyde (p-DAB) from Aldrich Chemical Co. (U.S.A.). Components of culture media such as tryptone, peptone, yeast extract and casamino acid were purchased from Difco (U.S.A.) and mono sodium glutamate (MSG) from Miwon Co. Other chemicals used in this research were of the extra pure grade from Wako Chemical (Japan).

Results

Effect of nitrogen source

Three kinds of nitrogen sources such as yeast extract, peptone, and tryptone were evaluated. The effect of yeast extract on the production of penicillin amidase is shown in Fig. 2. The optimal concentration of yeast extract was between 0.3 to 0.6% in the presence of 2% peptone, 1% MSG and 0.2% PAA as basal media. With 0.5% yeast extract, 1% MSG and 0.2% PAA as basal midia, maximum enzyme activity was 32 units/50m*l* broth when 2% pepton ewas used (Fig. 3), and as high as 75 units of enzyme activity was attained when 3.5% tryptone was employed (Fig. 4). The difference in the effect on microbial growth was not significant between two nitrogen sources. Except mentioned otherwise, 3.5% tryptone and 0.5% yeast extract were employed as nitrogen sources for the following experiments.

Effect of carbon source
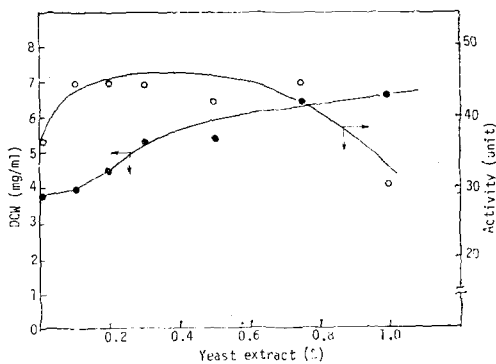
Glucose, MSG and glycerol were tested as

Fig. 2. Effect of yeast extract on the production of penicillin amidase. Media composition; peptone 2%, MSG 1%, PAA 0.2%.
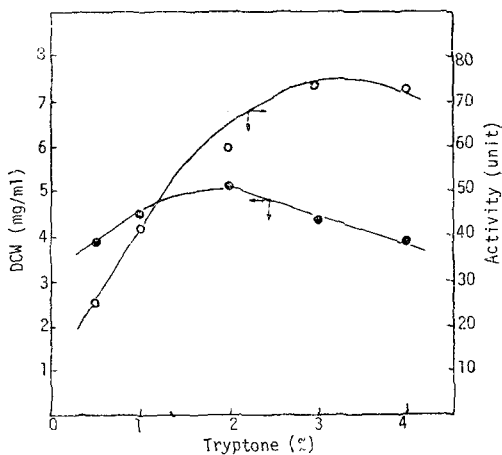

Fig. 3. Effect of peptone on the production of penicillin amidase. Media composition; yeast extract 0.5%, MSG 1%, PAA 0.2%.
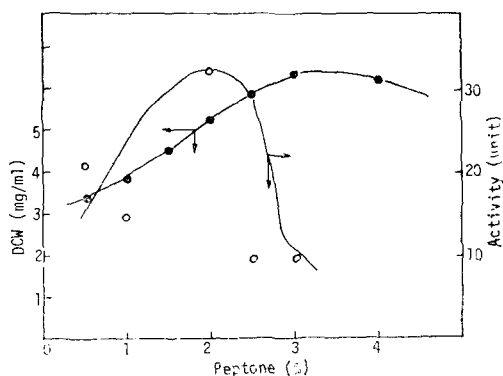

Fig. 4. Effect of tryptone on the production of penicillin amidase. Media composition; yeast extract 0.5%, MSG 1%, PAA 0.2%.

Table I. Effect of Carbon Source on the Production of Penicillin Amidase.

Carbon source	6-APA Production (μmole/mgDCW)	
	0.5%	1%
MSG	0.392	0.493
Glucose	0.058	0
Glycerol	0.206	—

Fermentation media was composed of 3.5% tryptone, 0.5% yeast extract and 0.2% PAA besides the carbon source used in this experiment.
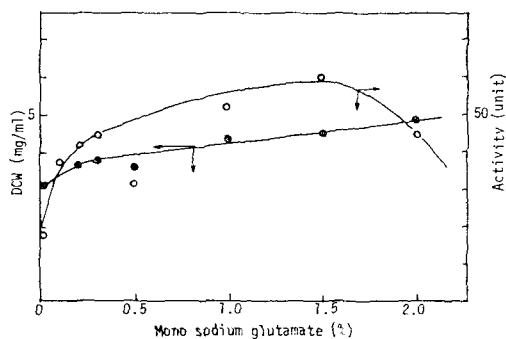

Fig. 5. Effect of MSG on the production of penicillin amidase. Media composition; tryptone 3.5%, yeast extract 0.5%, PAA 0.2%.

carbon sources. As shown in Table I, glucose and glycerol exerted inhibitory effect on the production of penicillin amidase. It was found that MSG was the best among the tested for the enzyme production. The optimal concentration of MSG was 1.5 % as shown in Fig. 5, and a slight inhibitory effect was noted above this concentration.

Effect of enzyme inducer

Although phenylacetic acid is known as the inducer of penicillin amidase, it was also reported that it exerted inhibitory effect at high concentration[8]. The effect of phenylacetic acid concentration and its addition time were investigated since these are considered important factors on the production of penicillin amidase.
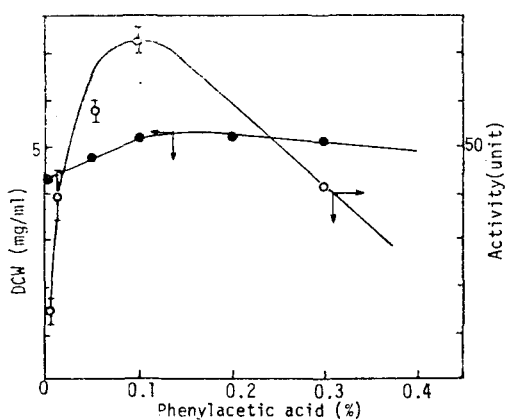
As shown in Fig. 6, the enzyme activity incre-

Fig. 6. Effect of phenylacetic acid on the production of penicillin amidase. Media composition; tryptone 3.5%, yeast extract 0.5%, MSG 1.5%.

Table Ⅱ. Effect of Adding Time of Inducer on the Production of Penicillin Amidase.

PAA adding time after inoculation (hr)	Dry cell wight (mg/ml)	Enzyme Activity (units/mg DCW)
0	7.5	0.596
6	7.4	0.441
12	7.3	0.323
18	7.6	0.052
24	7.4	0.043
No PAA	7.4	0.045

Fermentation condition: initial pH 7.0, the fermentation media composed of 3.5% tryptone, 0.5% yeast extract, 1.5% MSG. 0.15% of PAA was added at the various time indicated.

ased about 5 fold by adding 0.1% phenylacetic acid. At lower concentration, the degree of stimulation was proportional to the amount of phenylacetic acid. However, the stimulatory effect was significantly decreased at higher concentration. The strongest stimulatory effect was achieved when phenylacetic acid was added at the initial stage of fermentation (Table Ⅱ). The addition time of the inducer had no great effect on the growth of E. coli.

Effect of initial pH

Fig. 7 shows the effect of initial pH on the microbial growth and the production of penicillin amidase. The initial pH of media containing 3.5% tryptone, 0.5% yeast extract, 1.5% MSG and 0.1% phenylacetic acid were varied between 5 to 9 before autoclave. The cell growth and the enzyme production were greatly inhibited at pH 5. Although there is no marked effect on the cell growth above pH 6.0, the enzyme activity was significantly affected by alkaline pH.
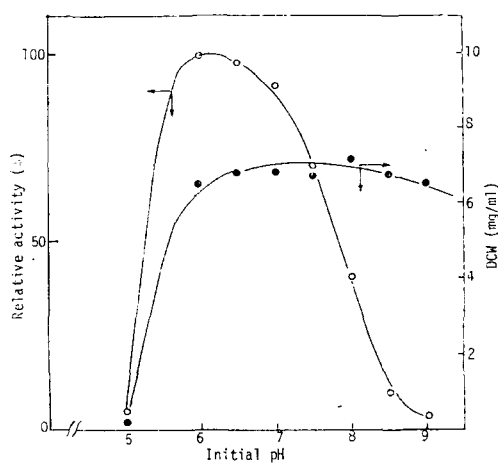

Fig. 7. Effect of initial pH of medium on the production of penicillin amidase. The pH was adjusted before sterilization and the media composition used was as described in Table III.

Time course of the production of penicillin amidase.

Employing the media composition optimized as shown in Table III, time course of enzyme prod-

Table Ⅲ. Comparison of Media Composition and Productivity of Penicillin Amidase

	Marancenbaum[8] et al.	Sato[9] et al.	Szentirmai[7]	Dogaru[10] et al.	Present experiment
Composition of media	glucose 1% peptone 1% Y. E. 1% KH_2PO_4 1.5% $(NH_4)_2SO_4$ 0.2% $MgSO_4 \cdot 7H_2O$ 0.025%	MSG 0.5% peptone 2% Y. E. 0.5% KH_2PO_4 0.3% $MgSO_4 \cdot 7H_2O$ 0.02% $FeCl_3 \cdot 6H_2O$ 0.02% PAA 0.2%	PAA 0.2% MSG 1.5% Y. E. 0.4%	urea 0.5% lecithin 0.05% NaCl 0.3% C. S. L. 3% PAA 0.15%	tryptone 3.5% MSG 1.5% Y. E. 0.5% PAA 0.15%
Dry cell weight (mg/ml)	7.12	7.0	3.0	8.1	6.76
Specific Act. (umol/hr/mg DCW)	0.023	0.35	0.64	0.135	0.61
Total Act. * (unit/l)	3.9	60.4	52.2	27.4	102.5

uction, pH and cell growth were investigated using 500ml jar fermentor (BioFlo model C 30). It is well known that penicillinase, which inactivates penicillins hydrolyzing the β-lactam ring of penicillin, may be produced alongside with penicillin amidase. It was confirmed that the penicill inase was not produced in the optimized media and fermentation condition, since no penicilloic acid was detected by paper chromatography[6].

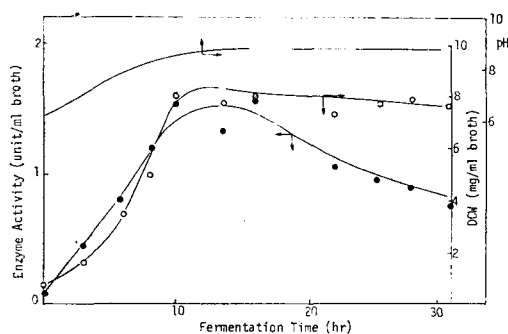As shown in Fig. 8, enzyme activity reached maximum within 16hr of fermentation. The enzyme

Fig. 8. Time course of the enzyme production. Fermentation condition; temperature 30°C, agitation speed 400rpm, aeration 1VVM, pH not adjusted (initial pH after inoculation was 6.3).

production increased in parallel with the cell growth. The pH increased at a constant rate within 10hrs of incubation and reached 9.7 at the stationary phase of the cell growth.

Discussion

In the present study, the effect of media composition and other factors on the penicillin amidase production from *E. coli* were investigated to maximize the enzyme productivity.

Among the carbon sources tested, glucose and glycerol exerted a strong inhibitory effect on the production of penicillin amidase (Table I), and it may be ascribed to the effect of catabolite repression as reported by Szentirmai[8].

Phenylacetic acid, the enzyme inducer, had a great stimulatory effect on enzyme production within the concentration range of 0.15% (Fig. 6). Although the effect on the cell growth was not great the enzyme production was significantly reduced at high phenylacetic acid concentration. It suggested that the concentration of enzyme inducer is a major influencing factor on enzyme productivity.

As shown in the initial pH effect on enzyme production the decrease in activity at acidic pH may be ascribed to the inhibition of cell growth. The decrease in enzyme activity at high pH appears

to be due to the enzyme inactivation, since no inhibitory effect on cell growth was found.

The media composition optimized in this experiment is shown in Table III. Compared with the other published results, enzyme productivity was significantly increased by media optimization. Dry cell weight per unit broth volume was about the same, but the specific activity was greatly enhanced. The total activity was 102.5 units/l about 2 fold increase compared with Szentirmai[8], and Sato et al[10], or 3 fold increase compared with Marancenbaum et al[9], and Dogaru et al[11].

In experiment on the time course of enzyme production, it was obvious that the enzyme activity decreased after 16hrs of incubation. The enzyme inactivation may be ascribed to high pH of fermentation broth in analogy with the experimental results of the initial pH effect (Fig. 7). It is reported that the phenylacetic acid became metabolized to other inactive forms during fermentation. The concentration of phenylacetic acid is expected to decrease with the time course of fermentation. It is expected that the enzyme productivity will be greatly enhanced by controlling the pH, and by maintaining the constant level of phenylacetic acid in fermentation broth.

요 약

대장균으로부터 페니실린 아미다제의 생산을 최적화하기 위하여 배지조성 및 발효 중 효소생산에 미치는 제반영향들에 대하여 연구하였다. 최적배지조성은 3.5% 트립톤, 1.5% 글루타민산 소듐, 0.5% 이스트추출물 그리고 0.15%의 페닐초산이었다. 효소생산 유도물질로서 0.15%의 페닐초산을 발효초기에 가해줄 때 효소생산성이 약 5배 증가하였다. 최적화된 배지 및 발효조건에서 발효 16시간만에 최대의 효소생산을 나타내었고 이때의 생산성은 약 102.5 units/l 발효액이었다. 이러한 최적조건에서 발효할 때 현재까지 보고된 효소생산성과 비교하여 상당한 생산능력의 향상을 보였다.

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