Production of 6-Aminopenicillanic Acid by \textit{Kluyvera citrophila} KY 3641

By Ryo Okachi, Masanaru Misawa and Takashi Nara

Tokyo Research Laboratory, Kyowa Hakko Kogyo Co.,
Asahimachi, Machidashi, Tokyo

Received October 13, 1971

\textit{Kluyvera citrophila} KY 3641 cultivated aerobically more than 48 hr produced penicillin acylase. Sucrose, monosodium glutamate and aspartic acid stimulated the growth of the cells, whereas glucose, fructose, maltose and lactose inhibited the growth and acylase production. Enzymic reaction was carried out with whole broth, too, instead of using separated intact cells. The cells maintained its acylase activity more than one month and could be used repeatedly. Acetone-dried or freeze-dried cell was also available for enzymic reaction. Identification of 6-APA was also described.

In the previous paper\textsuperscript{1,2} we reported the enzymic formation of 6-aminopenicillanic acid (6-APA) from penicillin G (Pc-G). Under an optimum condition \textit{Kluyvera citrophila} KY 3641 accumulated approximately 10 mg/ml of 6-APA from 21 mg/ml of Pc-G added as substrate.

Penicillin acylase of \textit{K. citrophila} KY 3641 is an intracellular and inducible enzyme and belongs to type II ('"Bacterial" Type) acylase by J. M. T. Hamilton-Miller (1966).\textsuperscript{3} This type of acylase has been found in various bacterial genera including \textit{Escherichia}, \textit{Pseudomonas}, etc.

However no report has so far been found on acylase formation by the genus of \textit{Kluyvera}. Acylase of this organism has many interesting characteristics which could not be found in other known acylase producing organisms.

We will report in this paper further investigations concerning various conditions of cell growth of \textit{K. citrophila} KY 3641 and enzymic cleavage of Pc-G to 6-APA.

\textbf{MATERIALS AND METHODS}

\textbf{Microorganism.} \textit{K. citrophila} KY 3641\textsuperscript{4-6} was used.

\textbf{Medium.} Bouillon medium\textsuperscript{1} was used throughout the work.

\textbf{Culture of the organism.} One drop of fresh cell suspension (ca. $1 \times 10^8$ cells/ml) from an active slant of \textit{K. citrophila} KY 3641 was seeded in 10 ml of bouillon medium in a 50 ml test tube. After incubation for 24 hr at 30°C on a shaker, each 2 ml was incubated in 20 ml of bouillon medium in a 250 ml Erlenmeyer flask, and incubated for 2~4 days.

\textbf{Cell growth.} Fermented broth was centrifuged and resuspended in deionized water, then optical density was measured at 660 nm by a photometer of Tokyo Koden Co., and the dry cell weight (DCW, mg/ml) was determined from a standard curve.

\textbf{Enzymic reaction.} The procedure reported previously\textsuperscript{1} was usually used with a little modification in each experiment.

\textbf{Assay of 6-APA.} For qualitative assay TLC\textsuperscript{1} was used, and the hydroxylamine method\textsuperscript{5} was used for quantitative assay.

\textbf{Freeze-dried and acetone-dried cell.} Cell grown in bouillon medium was washed twice with deionized water and made to wet paste.

\textsuperscript{1} Enzymatic Synthesis of \textit{D(-)-\alpha-Aminobenzylpenicillin} (Part II). See reference.\textsuperscript{1}
For freeze-drying, the cell paste was frozen at 
-20°C for 18 hr and dried up in vacuo by a freeze-
drying apparatus of Kyowa Vacuum Co.

The same cell paste was resuspended in a small 
amount of water to a heavy suspension, and 5 volumes 
of acetone was added. After stirring for 1 hr, cells 
were washed with acetone again and dried up in a 
desiccator.

RESULTS AND DISCUSSIONS

Effects of culture age

In bouillon medium K. citrophila KY 3641 
already reached to the maximum growth for 
24 hr (Table I), but the enzyme activity was 
low. Young cells cultivated less than 24 hr 
produced more penicilloic acid than 6-
APA in the reaction mixture.

When the organism was grown more than 
48 hr, the stable acylase activity was always 
observed and then pH of the broth was higher 
than 8.5.

Cells cultivated more than 24 hr produced 
no penicilloic acid in the reaction mixture.

<table>
<thead>
<tr>
<th>Age (hr)</th>
<th>pH</th>
<th>DCW (mg/ml)</th>
<th>6-APA (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>7.2</td>
<td>2.4</td>
<td>tr.</td>
</tr>
<tr>
<td>24</td>
<td>7.8</td>
<td>3.2</td>
<td>1.4</td>
</tr>
<tr>
<td>48</td>
<td>8.8</td>
<td>3.2</td>
<td>2.5</td>
</tr>
<tr>
<td>72</td>
<td>9.0</td>
<td>3.5</td>
<td>2.8</td>
</tr>
<tr>
<td>96</td>
<td>9.1</td>
<td>3.4</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Effects of aeration

Aeration did not give any significant effect 
on the growth and the enzyme activity of K. 
citrophila KY 3641 if it was grown aerobically 
(Table II), whereas, under anaerobic condi-
tions, cells completely lost acylase activity 
with the production of much penicilloic acid, 
though the cell growth was not decreased.

In aerobic growth, color of cells was light 
brown, but if it was cultured anaerobically it 
turned to dark green.

Effects of carbon source

Among various carbon sources added to 
bouillon medium, sucrose, monosodium glu-

<table>
<thead>
<tr>
<th>Basal medium</th>
<th>Carbon source</th>
<th>pH (mg/ml)</th>
<th>DCW (mg/ml)</th>
<th>6-APA (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bouillon</td>
<td>None</td>
<td>9.0</td>
<td>3.5</td>
<td>2.24</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>4.7</td>
<td>1.9</td>
<td>tr.</td>
</tr>
<tr>
<td></td>
<td>Glucose +0.5% CaCO₃</td>
<td>5.4</td>
<td>1.9</td>
<td>tr.</td>
</tr>
<tr>
<td></td>
<td>Fructose</td>
<td>4.7</td>
<td>1.1</td>
<td>tr.</td>
</tr>
<tr>
<td></td>
<td>Maltose</td>
<td>4.8</td>
<td>1.6</td>
<td>tr.</td>
</tr>
<tr>
<td></td>
<td>Sucrose</td>
<td>8.2</td>
<td>4.8</td>
<td>1.86</td>
</tr>
<tr>
<td></td>
<td>Lactose</td>
<td>5.4</td>
<td>2.2</td>
<td>tr.</td>
</tr>
<tr>
<td></td>
<td>Mannitol</td>
<td>4.6</td>
<td>3.5</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Sorbitol</td>
<td>8.8</td>
<td>4.0</td>
<td>2.20</td>
</tr>
<tr>
<td></td>
<td>MSG</td>
<td>8.6</td>
<td>4.6</td>
<td>1.90</td>
</tr>
<tr>
<td></td>
<td>Aspartic acid</td>
<td>9.0</td>
<td>4.2</td>
<td>1.94</td>
</tr>
<tr>
<td></td>
<td>K-Citrate</td>
<td>9.0</td>
<td>4.0</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>Na-Citrate</td>
<td>9.0</td>
<td>3.8</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>NH₄-Citrate</td>
<td>9.0</td>
<td>2.8</td>
<td>0.20</td>
</tr>
</tbody>
</table>

| a) Each 2% of carbon source was added after au-
| toclaving separately. After 3 days of incubation, cells 
| were washed and suspended to 3 mg/ml in the reaction 
| mixture. Pc-G, 10 mg/ml; 6-APA was assayed at 
| 4 hr period.

<table>
<thead>
<tr>
<th>Medium (ml)</th>
<th>Flasks</th>
<th>KGa Pm *</th>
<th>pH</th>
<th>DCW (mg/ml)</th>
<th>6-APA (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>250 ml Erlenmeyer flask</td>
<td>3.2 × 10⁻²</td>
<td>9.0</td>
<td>3.5</td>
<td>2.8</td>
</tr>
<tr>
<td>30</td>
<td>250 ml Erlenmeyer flask with buffer plate</td>
<td>1.8 × 10⁻¹</td>
<td>8.8</td>
<td>2.8</td>
<td>2.1</td>
</tr>
<tr>
<td>500</td>
<td>2 liter Erlenmeyer flask with buffer plate</td>
<td>1.0 × 10⁻²</td>
<td>8.8</td>
<td>2.0</td>
<td>2.5</td>
</tr>
<tr>
<td>1000</td>
<td>2 liter Erlenmeyer flask</td>
<td>5 × 10⁻³</td>
<td>7.6</td>
<td>2.7</td>
<td>tr.</td>
</tr>
</tbody>
</table>

a) KGa Pm; Oxygen absorption rate. Pc-G, 10 mg/ml; 6-APA was assayed at 4 hr period.
tamate (MSG) and aspartic acid remarkably stimulated the cell growth (Table III). Then, acylase activity was almost the same as the control. On the other hand, glucose, fructose, maltose and lactose inhibited the growth and acylase production. Although, mannitol or citrate salts did not inhibit the cell growth so much, the acylase activity was markedly suppressed. In this case pH was lower than 6.0, and when the pH was controlled to 8.0, the growth and acylase activity were not recovered.

When 6-APA formation was inhibited by those carbon sources, added Pc-G was cleaved to penicilloic acid in place of 6-APA.

6-APA accumulation in whole broth

*K. citrophila* KY 3641 was incubated in an Erlenmeyer flask for 48 hr, and the whole broth was then adjusted to pH 7.5 followed by the addition of Pc-G as a substrate. After incubation at 35°C on a shaker, accumulated 6-APA was assayed. As shown in Table IV, 6-APA was accumulated in various media.

When phenyl acetic acid (PAA) was added to medium the accumulation of 6-APA was the highest, though it took rather long time.

**TABLE IV. ACCUMULATION OF 6-APA IN WHOLE BROTH**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Cell growth*</th>
<th>Substrate</th>
<th>6-APA (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DCW (mg/ml)</td>
<td>Pc-G (mg/ml)</td>
<td>20 hr</td>
</tr>
<tr>
<td>Bouillon</td>
<td>3.3</td>
<td>30</td>
<td>5.0</td>
</tr>
<tr>
<td>Bouillon + MSG 2%</td>
<td>4.8</td>
<td>30</td>
<td>6.2</td>
</tr>
<tr>
<td>Bouillon + PAA 0.2%</td>
<td>3.3</td>
<td>30</td>
<td>5.5</td>
</tr>
<tr>
<td>Bouillon + PAA 0.2%</td>
<td>4.0</td>
<td>30</td>
<td>8.6</td>
</tr>
</tbody>
</table>

But when both MSG and PAA were added to medium it required shorter time for a maximum 6-APA formation.

No difference was found between 3% and 5% of Pc-G as substrate concentration. The conversion rate was hence better at 3% of Pc-G.

Based on these results, it became possible to accumulate a large amount of 6-APA in whole grown medium without separating cells from it.

**Preservation of intact cells**

After growing cells in the bouillon medium, the whole broth was centrifuged, and wet paste of cells was preserved at a low temperature for about one month. In this procedure, cells still maintained almost their enzymic ability to accumulate 6-APA from Pc-G (Table V).

**TABLE V. PRESERVATION OF INTACT CELLS**

<table>
<thead>
<tr>
<th>Preserved in</th>
<th>Preserved for (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Deep freezer at -15°C</td>
<td>2.7*1</td>
</tr>
<tr>
<td>Refrigerator at 4°C</td>
<td>2.7</td>
</tr>
</tbody>
</table>

\*1 Accumulated 6-APA (mg/ml) at 4 hr period of incubation. Each cell was suspended to 3 mg/ml before use. 10 mg/ml of Pc-G was added.

**Enzyme activity of cells used repeatedly**

The reaction mixture after usual enzymic reaction was centrifuged and cells were separated by washing with saline. These cells were used for the next batch as enzyme sources.

6-APA was accumulated almost the same amount as that in the first time (Table VI).

In the second or third reaction, weight of cell decreased about 10% than before. It was thus realized that cells could be used more than three times without loss of activity. On the other hand, heavily suspended cells were packed in a cellulose dialysable membrane.
### Table VI. Enzyme Activity of the Cell Used Repeatedly

<table>
<thead>
<tr>
<th>Run</th>
<th>Volume of reaction mixture (ml)</th>
<th>6-APA (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>50</td>
<td>5.65</td>
</tr>
<tr>
<td>2nd</td>
<td>45</td>
<td>5.20</td>
</tr>
<tr>
<td>3rd</td>
<td>40</td>
<td>5.03</td>
</tr>
</tbody>
</table>

Pc-G 15 mg/ml, cell concentration 8 mg/ml. 6-APA was assayed at 4 hr period.

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### Table VII. 6-APA Production by Freeze-Dried or Acetone-Dried Cell

<table>
<thead>
<tr>
<th>Cell</th>
<th>Cell concentration in enzymic reaction DCW (mg/ml)</th>
<th>6-APA (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze-dried</td>
<td></td>
<td>7.0</td>
</tr>
<tr>
<td>Acetone-dried</td>
<td></td>
<td>6.8</td>
</tr>
<tr>
<td>Intact</td>
<td></td>
<td>7.0</td>
</tr>
</tbody>
</table>

Pc-G, 10 mg/ml; 6-APA was assayed at 4 hr period.

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**Identification of 6-APA**

The supernatant of the reaction mixture was treated according to the procedure shown in Fig. 2. 2.4 g of white needle crystal was isolated from 750 ml of the reaction mixture. Over all purification yield was approximately 60%.

Final crystal was developed on thin layer chromatography to compare with an authentic 6-APA (Sigma Chem. Co., Ltd.). It showed the same Rf value as that of the reference 6-APA on two solvent systems.¹⁰

The high voltage paper electrophoresis was carried out at 3,000 V in formic acid-acetic acid buffer, pH 2.0, for 30 min. Both the isolated specimen and reference 6-APA moved 9.8 cm to cathode from origin.

According to the method described by J. Uri et al.,¹¹ paper disc was washed by 3% solution of sodium bicarbonate followed by dipping into 0.1% solution of isolated preparation or 6-APA. After drying in the air, each disc was dipped into 1% acetone solution of phenylacetyl chloride, and put on an agar plate seeded by *Staphylococcus aureus* 209 P. Each disc showed almost the same diameter of inhibition zone on the plate. Melting point was 209°C (decomposed). Elemental analysis was found: H; 5.63, C; 44.16, N; 12.98. Calculated for C₈H₁₂N₂SO₃: H; 5.60, C; 44.43, N; 12.95. IR spectrum is given in Fig. 3. From these analytical data, isolated crystal was identified to be 6-APA. The purity was determined to be 100.5% by the hydroxylamine method.⁸
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FIG. 2. Isolation of 6-APA.

Enzymic reaction mixture

Centrifuge

Cake

Supernatant

Adjust to pH 2.0 with 6 N H₂SO₄

Extract twice by the same volume of n-butyl acetate

Solvent layer

Water layer

Adjust to pH 7.0 with Ba(OH)₂

Centrifuge

Precipitate

Supernatant

Concentrate *in vacuo*

Add double volumes of methanol

Filter

Precipitate

Filtrate

Concentrate *in vacuo*

Adjust to pH 4.3 with 6 N HCl

Stand still in cold room for over night

Filter

Crystal

Filtrate

Wash with cold water

Dry up *in vacuo*

White needle crystal
REFERENCES