Enzymatic Production of L-Citrulline by
Pseudomonas putida

TOSHIO KAKIMOTO, TAKEJI SHIBATANI, NORIYUKI NISHIMURA, AND
ICHIRO CHIBATA

Department of Applied Biochemistry, Chemical Research Laboratory, Tanabe Seiyaku Co., Ltd., 962, Kashima-cho,
Higashiyodogawa-ku, Osaka, Japan

Received for publication 5 August 1971

To develop an efficient method for the production of L-citrulline, optimum conditions for the conversion of L-arginine to L-citrulline by microbial L-arginine deiminase and for production of the enzyme were studied. A number of microorganisms were screened to test their ability to form and accumulate L-citrulline from L-arginine. Pseudomonas putida was selected as the best organism. With this organism, enzyme activity as high as 9.20 units per ml could be produced by a shaking culture at 30°C in a medium containing glucose, ammonium phosphate, L-arginine hydrochloride, yeast extract, peptone, and inorganic salts. Appropriate addition of a surface active agent to the reaction mixture was found to shorten the time required for the conversion. A large amount of L-arginine hydrochloride was converted stoichiometrically to L-citrulline in 62 hr at 37°C. Accumulated L-citrulline was readily isolated in pure form by ordinary procedures with ion-exchange resins. Yields of isolated L-citrulline of over 90.5% from L-arginine hydrochloride were easily attainable.

Since Ackermann obtained citrulline from arginine by using putrid pancreas (1), many reports have appeared on the preparation of L-citrulline from L-arginine by arginine deiminase of microorganisms such as Pseudomonas aeruginosa (Bacillus pyocyaneus) (4, 15), Streptococcus faecalis (2, 5, 7, 8, 13, 14; V. A. Knivett, Proc. Int. Cong. Biochem., p. 86, 1952), Viridans streptococci (6), Clostridium perfringens (12), and bakers' yeast (10, 11). In most cases, arginine deiminase occurs together with ornithine transcarbamylase and carbamate kinase, composing the arginine dihydrolase system as shown below:

\[
\text{arginine + water} \xrightarrow{\text{arginine deiminase}} \text{citrulline + NH}_3
\]

\[
\text{citrulline + P}_\text{i}\xrightarrow{\text{orhithine transcarbamylase}} \text{ornithine} + \text{carbamylphosphate}
\]

\[
\text{carbamylphosphate} + \text{adenosine diphosphate} \xrightarrow{\text{carbamate kinase}} \text{adenosine triphosphate} + \text{CO}_2 + \text{NH}_3
\]

To accumulate citrulline, it is usually necessary to inhibit ornithine transcarbamylase activity by treatment of the cells with cetyltrimethyl ammonium bromide (CTAB) or acetone (5).

We have found that Pseudomonas putida produces high levels of arginine deiminase and has no ornithine transcarbamylase activity with intact cells or cells treated with CTAB. Accordingly, the present procedure, which produces an efficient conversion of L-arginine to L-citrulline, is an improvement over previous methods.

MATERIALS AND METHODS

Organisms. Screening tests were performed on 83 strains of bacteria, 31 strains of yeasts, 15 strains of molds, and 15 strains of actinomycetes, all from the collection in this laboratory. P. putida ATCC 4359 was selected for the fermentation experiments.

Screening experiments. Slant cultures of bacteria and actinomycetes were grown on a medium containing 0.25% peptone, 0.25% meat extract, 0.25% yeast extract, and 0.5% sodium chloride; cultures of molds and yeasts were incubated on malt extract medium. All organisms were grown for 24 hr at their respective optimal temperatures for growth.

The screening medium contained 1% glucose, 0.2% yeast extract, 0.2% peptone, 1.5% L-arginine hydrochloride, 0.15% NH$_4$Cl, 0.1% K$_2$HPO$_4$, 0.002% NaCl, 0.05% MgSO$_4$·7H$_2$O, 0.01% MnSO$_4$·4H$_2$O, and 0.0005% FeSO$_4$·7H$_2$O. The medium was adjusted to pH 6.0 with HCl, distributed in 3-ml amounts to test tubes, and sterilized. After inoculation with test organisms from the slant cultures, shaking incubation was carried out for 24 hr at 30°C. The cells were collected by centrifugation, washed with 0.9% saline, treated with CTAB (1 mg/ml), and mixed with 3 ml
of 30 mm L-arginine in 1 M acetate buffer (pH 6.0). The mixture was incubated for 16 hr at 37 C.

Routine identification and rough quantitative estimation of the formed amino acids were made by paper chromatography. The ascending method was used with Toyo filter paper no. 50 and 80% ethanol or t-butanol-methylmethylekton-formic acid-water (46:30:15:15). After development, the chromatograms were sprayed with 0.2% ninhydrin in 80% ethanol.

Fermentation experiments. Unless otherwise noted, fermentation experiments for enzyme formation were carried out as follows. The respective media were distributed in 200-ml amounts to shaking flasks (500-ml), sterilized, and inoculated with one loopful of the selected organism.

The cultures were incubated at 30 C for 24 hr with reciprocal shaking (140 rev/min, 8-cm stroke).

Methods of analysis. The assay of arginine deiminase was carried out by photometric measurements of citrulline liberated from L-arginine. The cells harvested by centrifugation from 3 ml of the broth cultured for 24 hr were washed with saline, treated with 3 ml of CTAB (1 mg/ml), and suspended in reaction mixture to a final volume of 3 ml. The reaction mixture contained 30 mm L-arginine in 1 M acetate buffer (pH 6.0) and was incubated for 1 hr at 37 C. L-Citrulline formed was assayed by the method of Archibald (3) using diacetylmonoxime.

One unit of L-arginine deiminase activity was defined as that activity which converts 1 µ mole of L-arginine to L-citrulline per min under the conditions of the assay.

Total enzyme activity, i.e., amount of formed enzyme, was expressed in terms of units per milliliter of broth, and specific activity was expressed in terms of units per milligram of dried cells.

No activity of L-arginine deiminase was found in the filtered broth of P. putida.

The assay of arginine was carried out by the modified method of Sakaguchi (9).

For the estimation of growth, the fermentation

| Genus              | No. of tested strains | No. of strains producing over 1 mg of citrulline per ml | Genus              | No. of tested strains | No. of strains producing over 1 mg of citrulline per ml |
|-------------------|-----------------------|-------------------------------------------------------|-------------------|-----------------------|-------------------------------------------------------|
| *Achromobacter*   | 10                    | 0                                                     | *Cryptococcus*     | 2                     | 0                                                     |
| *Aeromonas*       | 1                     | 0                                                     | *Debaryomyces*     | 2                     | 0                                                     |
| *Agrobacterium*   | 2                     | 0                                                     | *Endomyces*        | 2                     | 0                                                     |
| *Alcaligenes*     | 2                     | 0                                                     | *Endomycopsis*     | 1                     | 0                                                     |
| *Arthrobacter*    | 1                     | 0                                                     | *Eremothecium*     | 1                     | 0                                                     |
| *Bacillus*        | 3                     | 0                                                     | *Hanseniaspora*    | 1                     | 0                                                     |
| *Brevibacterium*  | 3                     | 0                                                     | *Hansenula*        | 1                     | 0                                                     |
| *Bacterium*       | 2                     | 0                                                     | *Kloeckera*        | 1                     | 0                                                     |
| *Corynebacterium* | 1                     | 0                                                     | *Lipomyces*        | 1                     | 0                                                     |
| *Erwinia*         | 1                     | 0                                                     | *Pichia*           | 1                     | 0                                                     |
| *Escherichia*     | 1                     | 0                                                     | *Rhodotorula*      | 1                     | 0                                                     |
| *Flavobacterium*  | 2                     | 0                                                     | *Saccharomycodes*  | 1                     | 0                                                     |
| *Kurthia*         | 1                     | 0                                                     | *Saccharomycopsis* | 1                     | 0                                                     |
| *Lactobacillus*   | 7                     | 2                                                     | *Schizosaccharomyces* | 1                     | 0                                                     |
| *Leuconostoc*     | 3                     | 2                                                     | *Torulopsis*       | 2                     | 0                                                     |
| *Microbacterium*  | 1                     | 0                                                     | *Trichosporon*     | 1                     | 0                                                     |
| *Micrococcus*     | 1                     | 1                                                     | *Trigonopsis*      | 1                     | 0                                                     |
| *Pseudomonas*     | 20                    | 5                                                     |                    |                       |                                                       |
| *Proteus*         | 3                     | 0                                                     |                    |                       |                                                       |
| *Sarcina*         | 8                     | 1                                                     | *Absidia*          | 1                     | 0                                                     |
| *Serratia*        | 2                     | 0                                                     | *Aspergillus*      | 2                     | 0                                                     |
| *Staphylococcus*  | 1                     | 1                                                     | *Chaetomium*       | 1                     | 0                                                     |
| *Streptococcus*   | 5                     | 2                                                     | *Cunninghamella*   | 1                     | 0                                                     |
| *Streptomonas*    | 2                     | 0                                                     | *Gibberella*       | 1                     | 0                                                     |
| *Mycobacterium*   | 2                     | 2                                                     | *Mortierella*      | 1                     | 0                                                     |
| *Nocardia*        | 3                     | 1                                                     | *Mucor*            | 2                     | 0                                                     |
| *Streptomycyes*   | 10                    | 5                                                     | *Neurospora*       | 1                     | 0                                                     |
| *Ashbya*          | 1                     | 0                                                     | *Oospora*          | 1                     | 0                                                     |
| *Bretanomyces*    | 1                     | 0                                                     | *Penicillium*      | 2                     | 0                                                     |
| *Candida*         | 2                     | 0                                                     | *Rhizopus*         | 1                     | 0                                                     |
|                   |                       |                                                       | *Trichoderma*      | 1                     | 0                                                     |
broth was diluted 1:20 with saline, and optical density was measured at 660 nm with a Hitachi photoelectric photometer (model 101). Dried cell weight was estimated from a standard curve which correlates optical density to weight of lyophilized cells.

RESULTS AND DISCUSSION

Screening experiments of organisms. Citrulline formation was observed with a considerable number of microorganisms when the reaction mixtures were incubated for 16 hr at 37 C (Table 1). Marked accumulation was found among strains of the genera Micrococcus, Pseudomonas, Sarcina, Staphylococcus, Lactobacillus, Leuconostoc, Streptococcus, Mycobacterium, Nocardia, and Strep- tomyces. No strain of yeasts and molds accumulated citrulline. Arginine deiminase activities were compared among the strains which produced high levels of citrulline (Table 2). Of the tested organisms, Pseudomonas putida showed the highest activity and was employed for the following experiments.

Cultural conditions for formation of L-arginine deiminase. To establish the most advantageous cultural conditions for the formation of L-arginine deiminase, various parameters were investigated with P. putida ATCC 4359.

Induction by L-arginine. When L-arginine, yeast extract, and peptone were omitted from the medium, enzyme activity of 0.03 unit per mg of dried cells was obtained, indicating that low levels of the enzyme were produced constitutively. Addition of yeast extract and peptone to the growth medium resulted in an enzyme activity of 0.4 units per mg of dried cells (Fig. 1). Furthermore, when 0.5% L-arginine hydrochloride was added to the above medium, highly induced enzyme activity (1.2 units per mg of dried cells) was obtained.

Effect of carbon sources. The effect of carbon sources at a concentration of 1% on the formation of the enzyme was investigated (Table 3).
Among the tested carbon sources, monosaccharides, such as glucose, mannose, and fructose, were found to be favorable for growth and for enzyme formation. Disaccharides and organic acids were less effective, and ethanol did not support production of the enzyme. Glucose was used as a main carbon source for subsequent experiments. The highest total activity was obtained at 2% glucose, whereas specific activity was highest at 1% concentration (Fig. 2).

Effect of nitrogen sources. To choose the most favorable nitrogen source, media containing various nitrogenous compounds were compared at a concentration of 0.05 M (Table 4). Secondary ammonium phosphate gave the most favorable results for the enzyme formation, and its optimum concentration was 0.03 M (Fig. 3).

Effect of ratio of glucose and ammonium ion. The above experiments revealed the advantage of glucose as carbon source and secondary ammonium phosphate as a supplementing nitrogen source. The ratio of glucose and secondary ammonium phosphate was examined. A carbon
atom-nitrogen atom ratio of 5.0, i.e., 2% glucose and 0.5% secondary ammonium phosphate, proved to be most favorable (Fig. 4).

**Effect of complex nutrients.** To investigate the most effective complex nutrient, experiments were carried out on media containing corn steep liquor, malt extract, peptone, tryptone, or yeast extract, each at a concentration of 0.2% (Table 5). Yeast extract and peptone gave the most favorable results, followed by tryptone.

The effect of the concentration of these nutrients added singly is described in Fig. 5 and 6. For the maximal production of the enzyme, high concentrations of these nutrients are required. When both yeast extract and peptone at low concentrations, i.e., 0.5%, were added to the medium, the highest enzyme formation, 9.2 units per ml of broth, was observed.

**Effect of shaking and temperature.** In preliminary experiments, shaking cultures gave much higher enzyme activity than stationary cultures.

| Complex nitrogen a | Growth (mg/ml) | Total activity b |
|--------------------|----------------|-----------------|
| None               | 4.60           | 2.44            |
| Corn steep liquor  | 0.80           | 0.02            |
| Malt extract       | 4.21           | 5.68            |
| Polypeptone        | 4.06           | 7.01            |
| Tryptone           | 4.30           | 6.76            |
| Yeast extract      | 5.00           | 8.65            |

a In addition to the above complex nutrients, all media contained 2% glucose, 0.5% L-arginine, HCl, 0.5% (NH₄)₂HPO₄, 0.1% KH₂PO₄, 0.002% NaCl, 0.005% MgSO₄·7H₂O, and 0.0005% FeSO₄·7H₂O.

b Units per milliliter of broth.

The effect of aeration was studied by varying the amount of media in the flasks. Maximal enzyme formation was attained with 220 ml of medium in a 500-ml flask (Fig. 7).

The effect of temperature was also studied at 25, 30, and 37°C. The best of these temperatures for enzyme formation was 30°C. Neither growth nor enzyme formation occurred at 37°C. Although growth occurred at 25°C, the enzyme activity was inferior to that observed at 30°C.

**Typical changes during fermentation.** The data for a typical fermentation under optimal conditions are given in Fig. 8. A 500-ml flask containing 220 ml of medium composed of 2% glucose, 0.5% yeast extract, 0.5% peptone, 0.5% L-arginine hydrochloride, 0.5% (NH₄)₂HPO₄, 0.1% KH₂PO₄, 0.002% NaCl, 0.05% MgSO₄·7H₂O, 0.01% MnSO₄·4H₂O, and 0.0005% FeSO₄·7H₂O, was used.
FeSO₄·7H₂O (pH 6.0) was inoculated with one loopful of the organism, and shaking incubation was carried out at 30°C. The enzyme was produced in parallel with growth, and maximum enzyme titer was attained at 24 hr, the point at which the culture entered the stationary phase. Addition of L-arginine hydrochloride at this point is considered to be the best for conversion to L-citrulline.

Throughout the culture period, the enzyme activity was found in the cells and not in the filtered broth. Maximum activity, 9.20 units per ml of medium and 2.05 units per mg of dried cells, observed in this culture of P. putida is much higher than that previously reported in S. faecalis (5), which showed a total activity of 2 units per ml of medium and a specific activity of 0.5 units per mg of dried cells.

**Conditions for the enzymatic formation of L-citrulline.** To establish the most advantageous reaction conditions for the enzymatic conversion of L-arginine to L-citrulline by L-arginine deiminase, various parameters were investigated.

**Effect of pH, temperature, and agitation on the enzymatic formation of L-citrulline.** The effects of pH and temperature on the enzymatic conversion of L-arginine to L-citrulline were investigated. As shown in Fig. 9, maximum activity was obtained at pH 6.0 and 50°C. At 50°C, however, the enzyme was gradually inactivated, whereas at temperatures lower than 37°C, the enzyme was stable for at least 10 days in the reaction mixtures. Therefore, it is most advantageous to carry out the reaction at pH 6.0 and 37°C. When the reaction mixture was agitated, the velocity of the enzymatic reaction appeared to be almost the same as that of the stationary incubation.

**Acceleration by surfactants.** In the above experiments, the cells had been treated with CTAB to increase the permeability of cell membranes and to inhibit ornithine transcarbamylase activity. P. putida, however, did not further decompose citrulline even in the absence of CTAB treatment, and the conversion rate of L-arginine to L-citrulline reached 100% at the end of the reaction under these conditions. Since CTAB did increase activity, however, the effects of other surfactants were examined (Table 6).

Of the tested surfactants, anionic and cationic types, especially CTAB and triethanolamine lauryl sulfate (TEALS), were the most effective. There were no surfactants which inhibited enzyme activity. By the addition of TEALS to the reaction mixture at concentrations of 0.02 to 0.03%, the duration of the enzymatic reaction could be reduced to one-third that without its addition.

**L-citrulline formation under optimal conditions.** A typical L-citrulline bioconversion is illustrated in Fig. 10. The experiment was conducted by incubating a mixture of 200 ml of the broth cultured under optimal conditions as enzyme source, 100 g of L-arginine hydrochloride, and 0.2 ml of 50% aqueous solution of TEALS at 37°C. L-Citrulline increased linearly with the consumption of L-arginine. During the reaction, the pH was maintained at around 6, which is optimal for the enzymatic reaction.

In this enzymatic procedure, neither formation of amino acids other than L-citrulline nor decomposition and racemization of accumulated L-citrulline occurred even with prolonged incubation. The L-citrulline formed was easily isolated by an ordinary procedure as follows. The reaction mixture was heated and centrifuged to remove the cells. The supernatant solution was passed through a column packed with Amberlite IR-120 (H⁺ form). The column was washed with water and eluted with 5% aqueous ammonia.
Table 6. Effects of surfactants on citrulline formation

| Surfactant | Main chemical component | Relative enzyme activity (%)\(^b\) of surfactant in concn of: |
|------------|-------------------------|---------------------------------------------------------------|
|            |                         | 2.5% | 0.25% | 0.025% | 0.0025% |
| NIKKOL SL-10 | None                  | 30   | 30   | 30     | 30     |
|             | Sorbitan alkylate     | 53   | 29   | 28     | 29     |
| SP-10      |                        | 54   | 39   | 28     | 30     |
| SS-10      |                        | 56   | 55   | 31     | 31     |
| BL-9 (EX)  | POE<sup>+</sup> lauril alcohol ether | 52   | 49   | 40     | 28     |
| BL-21      |                        | 57   | 42   | 40     | 32     |
| MGO        | Glyceryl monoalkylate | 79   | 72   | 73     | 30     |
| PMS-1C     | Propylene glycol monoalkylate | 64   | 61   | 33     | 31     |
| HCO-40     | POE castor oil derivative | 57   | 40   | 32     | 29     |
| TAMDS-40   | POE stearyl amide     | 60   | 39   | 31     | 33     |
| TAMDS-10   |                        | 52   | 38   | 32     | 30     |
| OTP        | Sodium di-2-ether hexyl-sulfosuccinate | 55   | 60   | 56     | 36     |
| SLP        | Lauryl phosphate      | 35   | 46   | 57     | 40     |
| SLS        | Lauryl sulfate        | 30   | 83   | 91     | 56     |
| KLS        |                        | 49   | 79   | 71     | 44     |
| SCS        | Cetyl sulfate         | 29   | 35   | 33     | 32     |
| SSS        | Stearyl sulfate       | 63   | 72   | 56     | 29     |
| TEALS      | Triethanolamine lauryl sulfate | 42   | 100  | 59     | 31     |
| CTAB       | Cetyl trimethylammonium bromide | 96   | 100  | 98     | 51     |
| Quatamine 86P | Stearyl trimethylammonium bromide | 32   | 77   | 97     | 47     |
| Lipomin SH | 2-Stearyl-1-oxyethylimidazoline sodium carboxymethyl hydroxide | 31   | 31   | 35     | 36     |
| Lipomin SA |                        | 32   | 77   | 97     | 47     |
| Anon BF    | Stearyl dimethyl-glycine | 25   | 50   | 76     | 71     |
| Anon BT    |                        | 30   | 49   | 42     | 24     |
| Levon 20   | Dioctyl diminoethylglycine | 31   | 39   | 81     | 63     |

<sup>a</sup> Washed cells (5 mg/ml) were treated with respective amounts of various surfactants for 10 min at 30°C, and excess surfactants were removed by centrifugation. Enzyme reaction was carried out by incubating a mixture containing 30 mM L-arginine, 1 M sodium acetate buffer (pH 6.0), and treated cells.

<sup>b</sup> Relative enzyme activity: 100% activity corresponds to 1.27 units per mg of dry cells.

<sup>c</sup> Polyoxethylene.

The eluted solution was concentrated in vacuo, and methanol was added. Crude crystals were collected by filtration and were recrystallized from aqueous methanol to yield 75.4 g of colorless crystals of L-citrulline: melting point, 222°C; \([\alpha]_D^{25} = +21.40^\circ\) (c = 10 in 1 N HCl). Analysis: C<sub>6</sub>H<sub>13</sub>N<sub>3</sub>O<sub>3</sub>; calculated: C, 41.13; H, 7.48; N, 23.99; found: C, 41.34; H, 7.42; N, 23.82. Yields of L-citrulline of over 90% from L-arginine were obtained. No amino acid other than L-citrulline was detected in the product by thin-layer chromatography.

**ACKNOWLEDGMENTS**

We are grateful to T. Takayanagi and K. Fujii of this company for their encouragement and to R. Yamaguchi and T. Kawahara for technical assistance.

![Fig. 10. Changes during the enzymatic reaction. Symbols: ●, L-arginine; ○, L-citrulline.](http://aem.asm.org/Downloaded from http://aem.asm.org/)

Downloaded from http://aem.asm.org/ on March 18, 2020 by guest
LITERATURE CITED

1. Ackermann, D. 1931. The biological breakdown of arginine to citrulline. Hoppe-Seyler's Z. Physiol. Chem. 203:66–69.
2. Akamatsu, S., and T. Sekine. 1951. Hydrolysis of arginine by *Streptococcus faecalis*. J. Biochem. (Japan) 38:349–354.
3. Archibald, R. M. 1944. Determination of citrulline and allantoin and demonstration of citrulline in blood plasma. J. Biol. Chem. 156:121–142.
4. Horn, F. 1933. The breakdown of arginine to citrulline by *Bacillus pyocyaneus*. Hoppe-Seyler's Z. Physiol. Chem. 216:244–247.
5. Knivett, V. A. 1953. L-Citrulline (from L-arginine). Biochem. Prep. 3:104–107.
6. Makisumi, S. 1964. Hydrolysis of arginine and γ-hydroxyarginine by arginine deaminase. Mem. Fac. Sci. Kyushu Univ. Ser. C. 5:107–115.
7. Oginsky, E. L., and R. F. Gehrig. 1952. The arginine dihydrolase system of *Streptococcus faecalis*. I. Identification of citrulline as an intermediate. J. Biol. Chem. 198:791–797.
8. Oginsky, E. L., and R. F. Gehrig. 1952. The arginine dihydrolase system of *Streptococcus faecalis*. II. Properties of arginine desimidase. J. Biol. Chem. 198:799–805.
9. Okuyama, N. 1969. Arginine, p. 284–287. In S. Akabori, T. Kaneko, and K. Narita (ed.), Tanpakushitsukagaku, vol. I Kyoritsu Shuppan, Tokyo.
10. Roche, J., and G. Lacombe. 1952. Arginine desimidase and the enzymatic formation of citrulline by yeasts. C. R. Soc. Seances Biol. Filiales 146:357–359.
11. Roche, J., and G. Lacombe. 1952. Arginine desiminase and the enzymatic formation of citrulline by yeast. Biochim. Biophys. Acta 9:687–692.
12. Schmidt, G. C., M. A. Logan, and A. A. Tytell. 1952. The degradation of L-arginine by *Clostridium perfringens*. J. Biol. Chem. 198:771–783.
13. Sekine, T. 1947. Hydrolysis of L-arginine by *Streptococcus faecalis*. J. Jap. Biochem. Soc. 19:79–85.
14. Slade, H. D. 1953. Hydrolysis of arginine by soluble enzymes of *Streptococcus faecalis*. Arch. Biochem. Biophys. 42:204–211.
15. Tomota, S. 1941. Arginine splitting by *Salmonella enteritidis* and *Bacillus pyocyaneus*. Tohoku J. Exp. Med. 41:317–321.