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Production of L-Phenylalanine from Acetamidocinnamic Acid Using *Bacillus sphaericus* and *Paracoccus denitrificans*

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ABSTRACT

To establish an efficient production method for L-phenylalanine (L-Phe), the possibility of L-Phe production from acetamidocinnamic acid by a two-step enzyme reaction was investigated. *Bacillus sphaericus* N-7 (FERM-P no. 6746) was selected as an enzyme source for the acylase used to form phenylpyruvic acid from acetamidocinnamic acid. *Paracoccus denitrificans* IFO 12442 was selected as an aminotransferase source for the conversion of phenylpyruvic acid to L-Phe. By using both cell types, 0.46*M* L-Phe was produced in a molar yield of 92% from 0.5*M* acetamidocinnamic acid.

Index Entries: L-Phenylalanine, enzymatic production of and conversion of acetamidocinnamic acid to; acetamidocinnamic acid, L-phenylalanine production from; two-step enzyme reaction; L-phenylalanine production by; acylase activity in *Bacillus sphaericus;* aminotransferase activity in *Paracoccus denitrificans; Bacillus sphaericus,* useful acylase source for L-phenylalanine production; *Paracoccus denitrificans,* useful amino transferase source for L-phenylalanine production.

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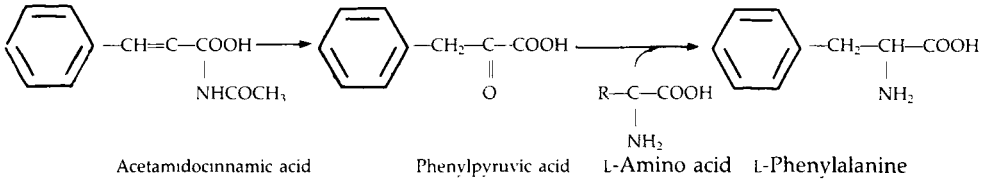
INTRODUCTION

L-Phenylalanine is now commercially produced on a large scale by a combination of chemical and enzymatic processes. Recently, many studies on the enzymatic production of L-Phe have been published (*1–6*). Our previous paper (*7*) described the isolation of *Alcaligenes faecalis* S-7 (FERM-P no. 6745) and *Bacillus sphaericus* N-7 (FERM-P no. 6746), which contain activity arising from the conversion of acetamidocinnamic acid to L-Phe. L-Phenylalanine formation system in both bacteria was found to be a two-step enzyme reaction as follows:



The first reaction is catalyzed by an enzyme "acylase" that splits the acetyl group of acetamidocinnamic acid and gives phenylpyruvic acid. The second reaction, catalyzed by aminotransferase, transfers the amino group of L-amino acid to phenylpyruvic acid and gives L-Phe. Succeeding these studies, we investigated the optimum conditions for the above two-step enzyme reaction, using *A. faecalis* S-7 and *B. sphaericus* N-7. By means of both strains, however, it was found difficult to elevate aminotransferase activity together with acylase activity. From these results we considered the possibility of using two microorganisms for L-Phe production: A first microorganism with high acylase activity to form phenylpyruvic acid from acetamidocinnamic acid, and a second microorganism with high aminotransferase activity to convert phenylpyruvic acid to L-Phe.

This paper reports the screening of microorganisms containing either higher acylase activity or higher aminotransferase activity, and the production of L-Phe from acetamidocinnamic acid using these two microorganisms.

MATERIALS AND METHODS

Microorganisms

This laboratory used a collection of 297 strains of bacteria.

Screening

The screening medium for acylase contained 0.2% acetamidocinnamic acid, 1% peptone, 1% meat extract, 1.25% yeast extract, and 0.5% NaCl. The screening medium for aminotransferase contained 0.5%

glucose, 1% peptone, 1% meat extract, 1.25% yeast extract, and 0.5% NaCl. Both media were adjusted to pH 7.0 with NaOH, distributed in 3-mL amounts to test tubes, and sterilized. After inoculation with test bacteria from the slant cultures, shaking incubation was carried out at 30°C for 24 h. The cells were collected by centrifugation, washed once with 3 mL of 0.9% saline, and resuspended in 3 mL of substrate solution to assay for acylase or aminotransferase activity.

Culture Methods

For stock culture, these strains were grown at 30°C for 1 d on an agar slant containing 0.5% glucose, 1% peptone, 1% meat extract, 1.25% yeast extract, 0.5% NaCl, and 1.5% agar, were adjusted to pH 7.0, and then stored at 5°C. Freshly prepared slant cultures were used for seed culture. *B. sphaericus* N-7, containing high acylase activity, was cultured in the following medium: 5% dextrin, 1% yeast extract, 0.5% peptone, 0.5% corn steep liquor, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.5% acetamidocinnamic acid, pH 7.0. *P. denitrificans* IFO 12442, containing high aminotransferase activity, was cultured in the following medium: 1% glucose, 0.2% $(NH_4)_2HPO_4$, 1.5% peptone, 1% yeast extract, 0.5% corn steep liquor, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2$, 0.00l% $FeSO_4 \cdot 7H_2O$, and 0.001% $MnSO_4 \cdot 6H_2O$, pH 7.0. A 50-mL portion of these media in a 500-mL flask was autoclaved at 120°C for 10 min, inoculated with one loopful of the freshly prepared stock cultures, and incubated at 30°C for 18–24 h on a reciprocal shaker operated at 140 strokes/min with a 7-cm amplitude.

Assay Methods

Acylase activity was measured by monitoring phenlpyruvic acid formation from a reaction mixture of 0.2*M* acetamidocinnamic acid, 5 m*M* aminooxyacetic acid, and 1.4 m*M* cetyltrimethylammonium bromide, pH 7.5, containing cells as the enzyme source, at 30°C for 30 min.

Aminotransferase activity was measured by monitoring L-Phe formation from a reaction mixture of 0.2*M* phenylpyruvic acid, 0.2*M* L-Asp, 0.1 m*M* pyridoxal-5′-phosphate, and 1.4 m*M* cetyltrimethylammonium bromide, pH 8.0, containing cells as the enzyme source, at 30° C for 1 h. One unit of enzyme activity was defined as that activity that produces 1 μmol of product/min.

Production of L-Phe from Acetamidocinnamic Acid

Unless otherwise noted, production of L-Phe from acetamidocinnamic acid was carried out as follows: A substrate solution containing 0.5*M* acetamidocinnamic acid, 0.5*M* L-Asp, 0.1 m*M* pyridoxal-5′-phosphate, and 1.4 m*M* cetyltrimethylammonium bromide, in a final vol of 100 mL, was adjusted to pH 8.0. One gram (dry cells) of *B. sphaericus*

N-7 cells and 250 mg (dry cells) of *P. denitrificans* IFO 12442 cells (acylase: aminotransferase, 1:1) were suspended in the substrate solution and the reaction mixture incubated at 30°C. The concentration of acetamidocinnamic acid was nearly in saturation at pH 8.0. After the reaction, the cells were removed by centrifugation. L-Phe and acetamidocinnamic acid in the supernatant were determined, as described below.

Analytical Methods

Cell concentrations were determined turbidimetrically at 660 nm in a Hitachi electric photometer EPO-B and expressed as dry cell weight (mg/mL) calculated from a standard curve.

Phenylpyruvic acid was determined by a method similar to that described by Katsuki et al. (*8*).

Acetamidocinnamic acid was determined using a Shimadzu LC-3A high-performance liquid chromatograph (HPLC) [column, Shimadzu zorbax SIL; carrier, 0.5% acetic acid in 40% methanol (vol/vol)].

L-Phenylalanine was determined using a Shimadzu LC-3A HPLC (column, Shimadzu zorbax SIL; carrier, 30% methanol), and also by microbioassay with *Leuconostoc mesenteroides* P-60 (*9*).

Chemicals

Acetamidocinnamic acid was prepared according to the method described previously (*7*). The other reagents were purchased from Katayame Chemical Industries Co. Ltd. (Osaka, Japan).

RESULTS AND DISCUSSION

Screening of Microorganisms Containing Acylase Activity and Microorganisms Containing Aminotransferase Activity

To obtain microorganisms containing high acylase or aminotransferase activities, 297 bacteria were tested. Among bacteria tested, comparatively high acylase activity was observed in two strains (Table 1). Characteristics of *A. faecalis* S-7 and *B. sphaericus* N-7 isolated from soil were described previously (*7*). On the other hand, aminotransferase activity was observed in a considerable number of bacteria belonging to the genera *Acinetobacter, Alcaligenes, Arthrobacter, Cornynebacterium,*

TABLE 1
Microorganisms Containing Acylase Activity

Microorganisms	Growth, mg/mL	Activity, 10^{-2} U/mg of cells
Alcaligenes faecalis S-7 (FERM-P no. 6245)	12.4	0.9
Bacillus sphaericus N-7 (FERM-P no. 6246)	11.2	1.0

Paracoccus, Proteus, and *Pseudomonas* (Table 2). As shown in Tables 1 and 2, among these bacteria, *B. sphaericus* N-7 showed highest acylase activity, and *P. denitrificans* IFO 12442 showed highest aminotransferase activity. Therefore, *B. sphaericus* N-7 was selected as a microorganism containing acylase activity, *P. denitrificans* IFO 12442 was selected as a microorganism containing aminotransferase activity, and these strains were used for further experiments.

Culture Conditions for B. sphaericus N-7 and P. denitrificans IFO 12442

To establish the most advantageous culture conditions for the formation of acylase activity in *B. sphaericus* N-7 and aminotransferase activity in *P. denitrificans* IFO 12442, effects of carbon source, nitrogen source, natural organic nutrients, and so on were investigated. *B. sphaericus* N-7 could grow abundantly in a medium consisting of carbon source, nitrogen source, and mineral salts; however, acylase activity in the cells was not induced in such a medium without acetamidocinnamic acid. Clear induction of acylase activity was observed by addition of acetamidocinnamic acid in a medium (Fig. 1). The optimum compositions of culture media for the formation of acylase activity in *B. sphaericus* N-7 and aminotransferase activity in *P. denitrificans* IFO 12442 were determined as described in the Materials and Methods section. Under these conditions, maximum acylase activity was 0.35 U/mL of broth and 0.04 U/mg of dried cell and maximum aminotransferase activity was 1.52 U/mL of broth and 0.16 U/mg of dried cell.

Conditions for Production of L-Phenylalanine from Acetamidocinnamic Acid, Using Two Microorganisms

To establish the most advantageous conditions for production of L-Phe from acetamidocinnamic acid by a two-step enzyme reaction, using two microorganisms, the following points were investigated.

TABLE 2
Microorganisms Containing Aminotransferase Activity

Microorganisms	Growth, mg/mL	Activity, 10^{-2} U/mg of cells
Acinetobacter calcoaceticus IFO 12552	11.7	12.1
Alcaligenes faecalis OUT 8025	9.6	2.7
Arthrobacter globiformis IFO 12956	3.6	11.0
Corynebacterium hydrocarboclastus ATCC 15592	4.8	5.1
Paracoccus denitrificans IFO 12442	9.8	13.9
Proteus vulgaris AHU 1472	9.2	5.1
Pseudomonas rubescens IAM 1510	11.2	2.9
Pseudomonas sckuylkilliensis IAM 1092	10.2	4.4

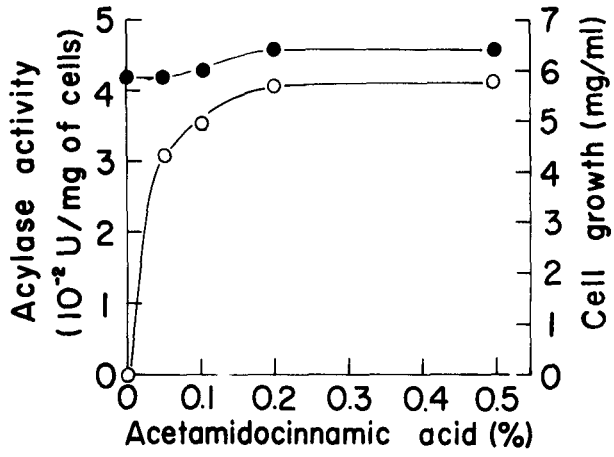


Fig. 1. Effect of acetamidocinnamic acid on acylase formation in *B. sphaericus* N-7. Acetamidocinnamic acid was added to the culture medium, and cultivation was carried out at 30°C for 24 h. Symbols: ○, acylase activity; ●, cell growth.

Effect of pH on Acylase Activity of B. sphaericus *N-7 and Aminotransferase Activity of* P. denitrificans *IFO 12442*

The effects of pH on the acylase activity of *B. sphaericus* N-7 and on the aminotransferase activity of *P. denitrificans* IFO 12442 were investigated (Fig. 2). The optimum pH of acylase activity was 7.5 and that of aminotransferase activity was 8.0–8.5. It was found that there were slight differences between the optimum pH of acylase and aminotranferase activities. The effect of pH on the conversion ratio of phenylpyruvic acid to L-Phe by aminotransferase activity was also investigated (Fig. 3). The

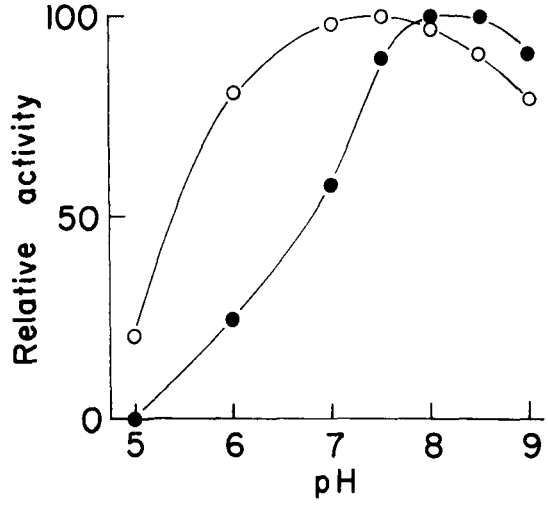


Fig. 2. Effect of pH on acylase activity and aminotranferase activity. The incubations were carried out under the standard conditions, except for the pH. Symbols: ○, acylase activity; ●, aminotransferase activity.

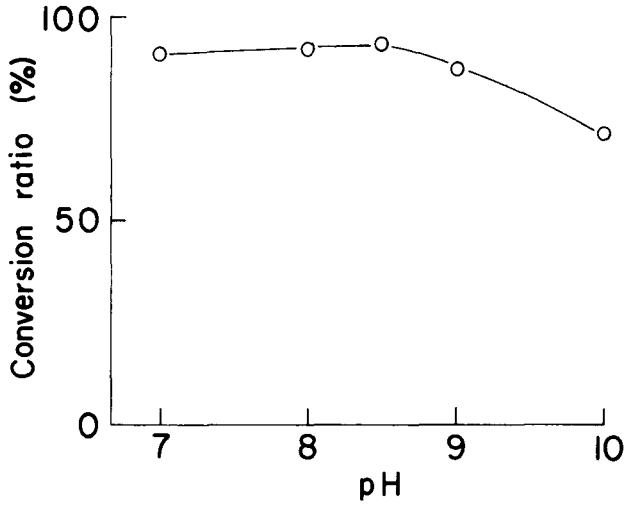


Fig. 3. Effect of pH on conversion ratio of phenylpyruvic acid to L-Phe. The reaction mixture containing 0.2*M* phenylpyurvic acid, 0.2*M* L-Asp, 0.1 m*M* pyridoxal-5'-phosphate, and 2.5 mg/mL *P. denitrificans* IFO 12442 cells, as aminotransferase preparation, was adjusted to the indicated pH with NH_4OH and incubated at 30°C for 3 d.

maximum conversion ratio was obtained at pH 8.0–8.5. Further conversion was hardly attained, even when enzyme preparation was freshly added. Generally speaking, from the standpoint of practical production, it is desirable to carry out a reaction in a single reactor at optimum pH. Therefore, it is most advantageous to carry out the two-step enzyme reaction at pH 8.0, where the initial velocity of acylase activity was reduced to 95% of the maximum velocity.

Effect of Amino Donor on Aminotransferase Activity of P. denitrificans *IFO 12442 and Conversion Ratio of Phenylpyruvic Acid to L-Phenylalanine*

The aminotransferase activity of *P. denitrificans* IFO 12442 with various amino acids and phenylpyruvic acid as the amino acceptor is presented in Table 3. Among amono acids tested, transamination of L-Glu and L-Asp were significantly higher than those observed for the other amino donors. To investigate whether the conversion ratio of phenylpyruvic acid to L-Phe was affected by the amino donor and its concentration, reactions using L-Glu and L-Asp were carried out at several concentrations (Fig. 4). When the reaction was carried out using L-Glu as an amino donor, the initial velocity was slightly higher than that using L-Asp. When 0.5*M* L-Glu was used, however, the reaction was stopped at about 60% of quantitative conversion, and further conversion was hardly attained, even when aminotransferase was added and the incubation time sufficiently prolonged. On the other hand, when L-Asp was used, more than 90% of phenylpyruvic acid was converted to L-Phe. This may be attributed to decomposition of the reaction product, oxaloacetic

TABLE 3
Effect of Amino Donor on
Aminotransferase Acitivity
of *P. denitrificans* IFO 12442[a]

Amino donor	Activity, 10^{-2} U/mg of cells
L-Aspartic acid	4.5
L-Glutamic acid	5.0
Others[b]	0–0.1

[a]The reaction mixture containing 0.2*M* phenylpyruvic acid, 0.2*M* each amino acid, 0.1 m*M* pyridoxal-5'-phosphate, 1.4 m*M* cetyltrimethyl ammonium bromide, and *P. denitrificans* IFO 12442 cells as enzyme preparation (pH 8.0) was incubated for 1 h at 30°C.
[b]Others; L-Ala, L-Arg, L-Cys, Gly, L-His, L-Ile, L-Lys, L-Met, L-Pro, L-Ser, L-Thr, L-Trp, L-Val

acid. The L-Asp showed higher productivity than L-Glu. On the basis of these data, subsequent experiments for L-Phe production from acetamidocinnamic acid were carried out using L-Asp as the amino donor.

Effect of Ratio of Two Enzyme Activities on Production of L-Phenylalanine from Acetamidocinnamic Acid

To determine the most advantageous ratio of acylase and aminotransferase activities for the production of L-Phe from acetamidocinnamic

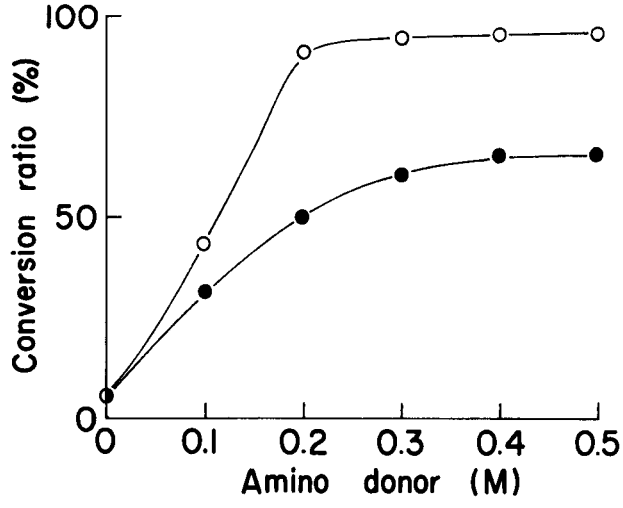


Fig. 4. Effect of amino donor on conversion ratio of phenylpyruvic acid to L-Phe. The reaction mixture containing 0.2*M* phenylpyruvic acid, amino donor (0–0.5*M*), 0.1 m*M* pyridoxal-5'-phosphate, and 2.5 mg/mL *P. denitrificans* cells, as aminotransferase preparation, pH 8.0, was incubated at 30°C for 4 d. Symbols: ○, L-Asp; ●, L-Glu.

acid, effects of increasing the aminotransferase activity on L-Phe formation was investigated (Table 4). A maximum initial velocity was observed when acylase and aminotransferase activities were mixed at a ratio of 1:3. On the other hand, practical conversion from acetamidocinnamic acid to L-Phe was best achieved when acylase and aminotransferase activities were equally mixed. From these results, a ratio of acylase: aminotransferase activities was designated as 1:1.

Production of L-Phenylalanine from Acetamidocinnamic Acid Using B. sphaericus *N-7 Cells and* P. denitrificans *IFO 12442 Cells*

Production of L-Phe from acetamidocinnamic acid, using two microorganisms, was carried out under the conditions described in the Materials and Methods section. A typical time course of L-Phe production from acetamidocinnamic acid is illustrated in Fig. 5. As shown in Fig. 5, acetamidocinnamic acid was consumed rapidly. On the other hand, L-Phe was accumulated gradually compared to acetamidocinnamic acid consumption. After 72 h, the reaction was finished, and 460 μmol/mL of L-Phe was accumulated in the reaction mixture (conversion ratio was 92% from acetamidocinnamic acid). The accumulated L-Phe did not decompose, even with prolonged incubation. The difference between the amount of consumed acetamidocinnamic acid and that of produced L-Phe in the process of this reaction was believed to be that phenylpyruvic acid (the intermediate of this reaction) was accumulated in the reaction mixture. The L-Phe accumulated in the reaction mixture was readily isolated and purified in the usual manner described in a previous paper (*1*). The yield of pure crystalline L-Phe from acetamidocinnamic acid was over 70%. This production method of L-Phe is very promising as a commercial process because acetamidocinnamic acid

TABLE 4
Effect of Ratio of Acylase Activity and Aminotransferase Activity on Production of L-Phenylalanine from Acetamidocinnamic Acid[a]

Activity ratio, aminotransferase/acylase	L-Phe formed, mol/L			
	1 h	6 h	16 h	24 h
0	0	0	0.008	0.010
1	0.012	0.078	0.160	0.181
2	0.016	0.090	0.165	0.179
3	0.020	0.098	0.163	0.182
4	0.020	0.101	0.167	0.181

[a]The reaction mixtures containing 0.2*M* acetamidocinnamic acid, 0.2*M* L-aspartic acid, 0.1 m*M* pyridoxal-5'-phosphate, 1.4 m*M* cetyltrimethyl ammonium bromide, *B. sphaericus* N-7 cells, as 0.35 U/L acylase activity, and *P. denitrificans* IFO 12442 cells, as aminotransferase activity (pH 8.0), were incubated at 30°C.

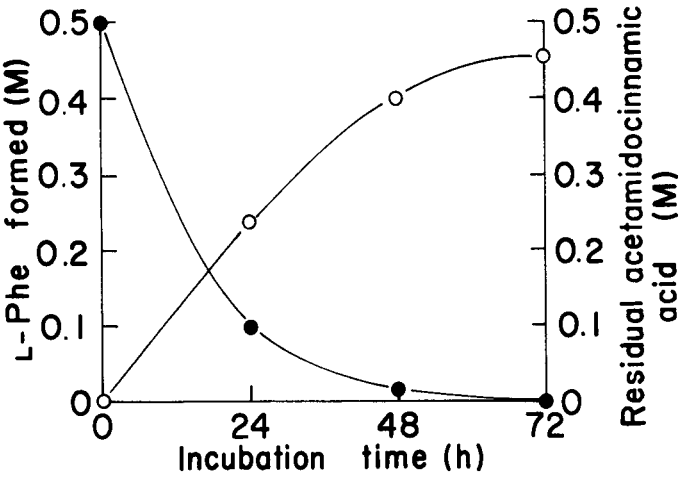


Fig. 5. Production of L-Phe from acetamidocinnamic acid by a two-step enzyme reaction using *B. sphaericus* N-7 cells and *P. denitrificans* cells. Symbols: ○, L-Phe; ●, acetamidocinnamic acid.

can be obtained at low cost, and the accumulated L-Phe can be easily isolated from the reaction mixture.

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