APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1981, p. 773-778
0099-2240/81/110773-06$02.00/0

Vol. 42, No. 5

Production of L-Phenylalanine from *trans*-Cinnamic Acid with *Rhodotorula glutinis* Containing L-Phenylalanine Ammonia-Lyase Activity

SHIGEKI YAMADA,* KOICHI NABE, NOBUHIKO IZUO, KATSUHIKO NAKAMICHI, AND ICHIRO CHIBATA

Research Laboratory of Applied Biochemistry, Tanabe Seiyaku Co., Ltd., 3-16-89, Kashima, Yodogawa-ku, Osaka 532 Japan

Received 5 May 1981/Accepted 4 August 1981

An enzymatic method using L-phenylalanine ammonia-lyase (EC 4.3.1.5) for the rapid conversion of *trans*-cinnamic acid to L-phenylalanine has been investigated. With *Rhodotorula glutinis*, enzyme activity as high as 0.3 U/ml of culture broth was obtained. The enzyme activity was kept stable for a relatively long time during cultivation by the addition of L-isoleucine. Optimization of the parameters of the conversion reaction resulted in accumulation of 18 mg of L-phenylalanine per ml of reaction mixture. The conversion yield from *trans*-cinnamic acid was about 70%. The method may provide a rapid and practical way to produce L-phenylalanine useful as an essential amino acid.

L-Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5), an enzyme found in a variety of plants, catalyzes the deamination of L-phenylalanine to give *trans*-cinnamic acid, which is further transformed in the plants into lignins and the related polyphenols. The presence of PAL in yeast was demonstrated for the first time by Ogata et al. (10, 11); since then, other microorganisms have been found to show PAL activity (1, 7, 9, 12). Also, the enzymatic characteristics of PAL from microorganisms have been extensively studied. However, practical applications such as the biosynthesis of L-phenylalanine from *trans*-cinnamic acid have rarely appeared in literature, even though reversibility of the enzyme reaction has been suggested by some workers (6). The reason for this lack of published effort is probably that the activity of PAL in microorganisms is relatively low and decays rapidly after an initial increase during fermentation (1, 5, 8).

In this report, the induction and stabilization of PAL activity during the cultivation of *Rhodotorula glutinis* were investigated, and the optimum conditions for the conversion of *trans*-cinnamic acid into L-phenylalanine were determined to develop a workable enzymatic method for the commercial production of L-phenylalanine.

MATERIALS AND METHODS

Microorganism and cultivation methods. *R. glutinis* IFO 0559, which was reported to show high PAL activity (11), was used throughout this work. Stock cultures were maintained on an agar slant containing 1.0% yeast extract, 1.0% peptone, 0.5% NaCl, 0.05% L-phenylalanine, and 1.5% agar (pH 6.0). For seed culture, a 100-ml portion of the same liquid medium in a 500-ml flask was sterilized and then inoculated with one loopful of freshly prepared stock culture. The cultures were incubated at 30°C for 20 h on a reciprocating shaker. For PAL formation, unless otherwise noted, two kinds of basal medium were used. Sorbitol medium was composed of 2% sorbitol, 1% $(NH_4)_2SO_4$, 0.5% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 6H_2O$, 0.001% NaCl, and 2% L-phenylalanine (pH 6.0). The optimum culture medium for the production of PAL was composed of 1% yeast extract, 1% peptone, 0.5% NaCl, 0.05% L-phenylalanine, and 0.5% L-isoleucine (pH 6.0). A 100-ml portion of each test medium in a 500-ml flask was autoclaved at 120°C for 10 min, inoculated with 1.0% of the seed culture, and incubated at 30°C for 18 h on a reciprocating shaker operated at 140 strokes per min with a 7-cm stroke.

Preparation of cells. The cells harvested by centrifugation from 100 ml of the culture broth were washed once by suspending in 10 ml of 0.9% saline and resuspended in water in a final volume of 5.0 ml. One milliliter of this cell suspension usually contained about 150 mg of dry cells and showed about 5.6 U of PAL activity. The cell suspension was immediately used as a source of enzyme activity in further experiments. The cell suspension showed the same activity in the presence of 0.005% cetylpyridinium chloride as that of cell-free extracts prepared by sonic oscillation (5°C, 15 min, 10 kilocycles, 200 W).

Assay of PAL activity. Activity of PAL was assayed by a modification of the method of Kalghatgi and Subba Rao (7). The standard assay mixture contained 25 mM L-phenylalanine, 25 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.8), 0.005% cetylpyridinum chloride, and 0.25 ml of the cell suspension in a final volume of 5.0 ml. The

reaction was carried out at 30°C for 10 min, and *trans*-cinnamic acid formation was determined by following the increase in absorbance at 278 nm. One unit of PAL activity was defined as that activity which converts 1 μmol of L-phenylalanine to *trans*-cinnamic acid per min. The specific activity was expressed in terms of units per milligram of dry cells, and total activity was expressed in terms of units per milliliter of culture broth.

Formation of L-phenylalanine from *trans*-cinnamic acid. Unless otherwise noted, *trans*-cinnamic acid was converted to L-phenylalanine as follows. A reaction mixture of 1.0 ml of the cell suspension and 4.0 ml of substrate solution was incubated at 30°C for 1 h. For the preparation of the substrate solution, 740 mg of *trans*-cinnamic acid was dissolved in 45 ml of 28% ammonium hydroxide. The resulting solution was adjusted to pH 10.0 with HCl and diluted with distilled water in a final volume of 80 ml. The concentration of *trans*-cinnamic acid and ammonia was varied according to each experimental design. After the reaction, the cells were removed by centrifugation. L-Phenylalanine and *trans*-cinnamic acid in the supernatant were determined as described below.

Analytical methods. Cell growth was determined turbidimetrically at 660 nm in a Hitachi electric photometer EPO-B and was expressed as dry cell weight (milligrams per milliliter) using a previously determined relationship between turbidimetric reading and dry cell weight.

Identification and determination of L-phenylalanine were performed both by the method of microbioassay using *Leuconostoc mesenteroides* P-60 (13) and by thin-layer chromatography using the ascending technique on a Merk 60 F_{254} plate (solvent, 1-butanol–acetic acid–water, 4:1:1, vol/vol). The plates were developed for 4 h and air dried for 30 min to remove the solvent. The chromatograms were sprayed with ninhydrin and heated at 105°C for 5 min. The colored spots were measured with a Shimadzu dual-wavelength chromatogram scanner CS-910 with a wavelength of 620 nm for the sample and 500 nm for the reference and compared with known quantities of authentic L-phenylalanine. A more accurate quantitative determination of L-phenylalanine was made by using a Hitachi KLA-3B amino acid analyzer (resin, Hitachi Custam Resin; eluting buffer, citrate buffer [pH 3.25]; temperature, 55°C). Quantitation of *trans*-cinnamic acid was achieved by thin-layer chromatography, with 20:1:2 isopropanol-ammonium hydroxide-water (vol/vol) as a solvent. The plates after development were air dried for 30 min and heated in an oven for 10 min at 110°C to remove the solvent. The chromatograms were directly assayed with the chromatogram scanner with a wavelength of 278 nm for the sample and 360 nm for the reference and compared with known quantities of authentic *trans*-cinnamic acid.

Chemicals. Yeast extract was purchased from Oriental Kobo Industries Co. Ltd. (Tokyo, Japan). The other reagents were purchased from Katayama Chemical Industries Co. Ltd. (Osaka, Japan).

RESULTS

Culture conditions for the formation of PAL.

(i) Induction of PAL by L-phenylalanine. *R. glutinis* IFO 0559 could grow abundantly in a simple chemically defined medium consisting of a source of carbon such as sugar or sugar alcohol, a source of inorganic nitrogen, and mineral salts; for example, the medium containing 2% glucose or sorbitol also contained 1% $(NH_4)_2SO_4$, 0.5% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 6H_2O$, and 0.001% NaCl. However, PAL activity in *R. glutinis* was not induced in such a medium without L-phenylalanine. Clear induction of PAL activity was observed by addition of L-phenylalanine in sorbitol medium as shown in Fig. 1. On the other hand, no PAL activity was found in a medium containing glucose even in the presence of L-phenylalanine.

(ii) Effect of natural organic nutrients on PAL formation. The maximum enzyme activity in the cells grown in sorbitol medium varied with amount of natural organic nutrients added to the medium. The effects of yeast extract and peptone on the formation of PAL are shown in Table 1. From the above experiments, the nature of the carbon source, and the concentration of L-phenylalanine, yeast extract and peptone seemed to be very important factors for the formation of PAL in *R. glutinis*. Among various combinations, the following composition was suitable for practical production of PAL by *R. glutinis*: 1.0% yeast extract, 1.0% peptone, 0.05% L-phenylalanine, and 0.5% NaCl (pH 6.0). The rate of growth and the formation of PAL in this medium are shown in Fig. 2. PAL activity was detectable after 10 h of growth, reached a maximum around 16 to 18 h, and then rapidly reduced when growth reached stationary phase. When a little amount of L-isoleucine (for instance, 0.5%) was added to this medium, however, PAL activity increased somewhat and was sustained for a relatively long time as shown in Fig. 3. DL-Isoleucine was nearly equally effective.

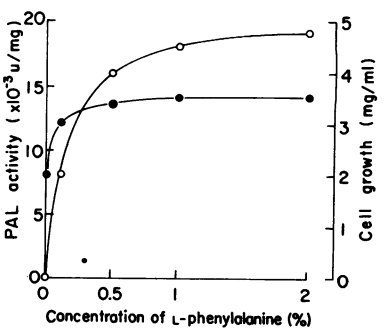

FIG. 1. *Induction of PAL by L-phenylalanine. L-Phenylalanine was added to the sorbitol medium, and cultivation was carried out at 30°C for 18 h. Symbols: ○, PAL activity; ●, cell growth.*

TABLE 1. *Effect of natural organic nutrients on PAL formation*

Addition (%)[a]		Total activity (10^{-3} U/ml)	Sp act (10^{-3} U/mg)
Yeast extract	Peptone		
0	0	14	12.8
0	0.1	33	18.6
0	0.5	79	27.4
0	1.0	92	28.7
0	1.5	97	25.4
0.1	0	43	19.4
0.1	0.1	71	23.0
0.1	0.5	123	33.2
0.1	1.0	133	28.9
0.1	1.5	107	27.5
0.5	0	114	30.0
0.5	0.1	128	32.0
0.5	0.5	139	30.8
0.5	1.0	159	30.5
0.5	1.5	148	28.4
1.0	0	147	30.0
1.0	0.1	157	30.1
1.0	0.5	164	28.8
1.0	1.0	190	31.0
1.0	1.5	136	25.1
1.5	0	154	26.1
1.5	0.1	164	28.7
1.5	0.5	150	25.5
1.5	1.0	154	26.2
1.5	1.5	137	22.9

[a] Yeast extract and peptone were added to the sorbitol medium containing 0.5% L-phenylalanine. Cultivation was carried out at 30°C for 18 h.

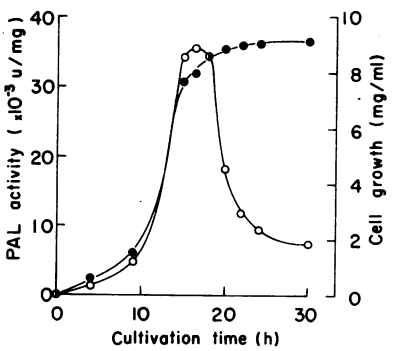

FIG. 2. *Typical time course of the formation of PAL. Cultivation was carried out in the medium containing 1% yeast extract, 1% peptone, 0.05% L-phenylalanine, and 0.5% NaCl (pH 6.0) at 30°C. Symbols: ○, PAL activity; ●, cell growth.*

Thus, the optimum composition of culture medium for the production of PAL by *R. glutinis* was determined as described in Materials and Methods. Under the optimum condition, maximum activity (0.3 U/ml of culture broth and 0.035 U/mg of dry cells) was attained at 18 h, before the culture entered the stationary phase.

Conditions for the conversion of *trans*-cinnamic acid to L-phenylalanine by intact cells. To establish the optimum conditions for the enzymatic conversion of *trans*-cinnamic acid to L-phenylalanine by PAL activity of intact cells, the following parameters were investigated.

(i) Effect of *trans*-cinnamic acid concentration on initial velocity of conversion. Initial velocity of the conversion was varied with the concentration of *trans*-cinnamic acid (Fig. 4). The apparent K_m value was 4.2 mM for *trans*-cinnamic acid. Substrate inhibition was observed at a level of *trans*-cinnamic acid higher than 60 mM. The substrate concentration appropriate for the conversion was 30 to 60 mM.

(ii) Effect of ammonia concentration on initial velocity of conversion. As shown in Fig. 5, the apparent K_m value of the reaction by intact cells was very high, 3.17 M, for ammonia. Therefore, it is advantageous to carry out the

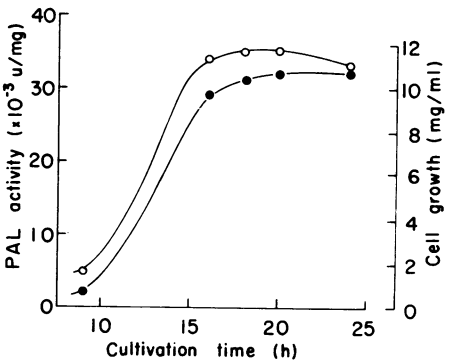

FIG. 3. *Effect of addition of L-isoleucine on stability of PAL during cultivation. Cultivation was carried out in the optimum medium described in the text at 30°C. Symbols: ○, PAL activity; ●, cell growth.*

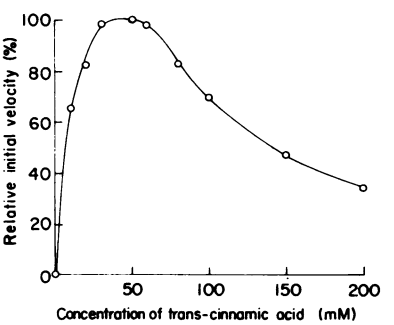

FIG. 4. *Effect of trans-cinnamic acid concentration on initial velocity of conversion. Reaction mixture contained 1 ml of cell suspension and 4 ml of the substrate solution prepared according to the method described in the text. Reaction was carried out at 30°C for 1 h.*

reaction at concentrations higher than 7 M.

(iii) Effect of pH on initial velocity of conversion. The effect of pH on the conversion rate of *trans*-cinnamic acid to L-phenylalanine by intact cells was investigated in the presence of 50 mM *trans*-cinnamic acid and 7 M ammonia. As shown in Fig. 6, maximum activity was observed at pH 10.0. On the other hand, the optimum pH for the deamination of L-phenylalanine to *trans*-cinnamic acid was 8.8.

(iv) Effect of ammonia concentration on conversion yield. The conversion of *trans*-cinnamic acid was carried out with various concentrations of ammonia. The conversion yield was found to increase with increasing ammonia concentration. At a level of ammonia concentration higher than 7.5 M, the conversion yield reached 72% of quantitative conversion (Fig. 7). Further conversion, however, was hardly attained even when a large excess of cells was used and the incubation time was sufficiently prolonged.

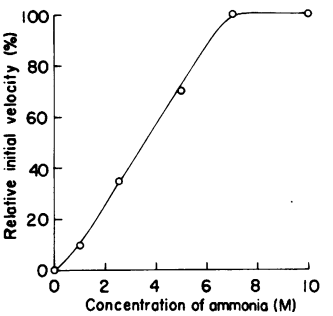

FIG. 5. *Effect of ammonia concentration on initial velocity of conversion. Reaction mixture contained 1 ml of cell suspension and 4 ml of the substrate solution prepared according to the method described in the text. Reaction was carried out at 30°C for 1 h.*

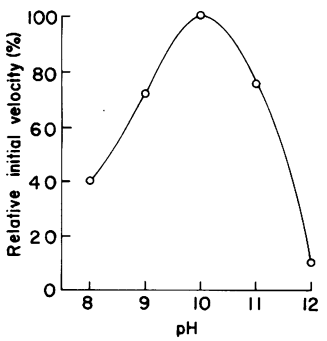

FIG. 6. *Effect of pH on initial velocity of conversion. Reaction mixture contained 1 ml of cell suspension and 4 ml of the substrate solution prepared according to the method described in the text. Reaction was carried out at 30°C for 1 h.*

From the above experiments on the enzymatic properties of *R. glutinis* IFO 0559, it seems preferable that the conversion of *trans*-cinnamic acid at a concentration of 30 to 60 mM be carried out at pH 10.0 in the presence of 7.5 M ammonium ion. From the standpoint of economical L-phenylalanine production, however, it is desirable to increase the concentration of substrate so as to increase the amount of the product. Therefore, the practical production of L-phenylalanine was carried out at a concentration of substrate so as to increase the amount of the product. Therefore, the practical production of L-phenylalanine was carried out at a concentration of 150 mM *trans*-cinnamic acid, where the conversion velocity was reduced to one-half the maximum velocity.

Time course of the conversion of *trans*-cinnamic acid to L-phenylalanine. A typical production of L-phenylalanine from *trans*-cinnamic acid is illustrated in Fig. 8. A 40-ml volume of cell suspension containing 20 g of dry cells was prepared from 2,000 ml of the broth cultured under the optimum conditions. A substrate solution was prepared as follows: 4.4 g of *trans*-cinnamic acid was dissolved in 90 ml of 28% ammonium hydroxide, and then the solution was adjusted to pH 10.0 with about 23 ml of 36% hydrochloric acid and diluted with distilled water to a final volume of 160 ml. The incubation mixture, containing 40 ml of the cell suspension and 160 ml of the substrate solution, was incubated at 30°C with shaking. L-Phenylalanine increased in proportion to the consumption of *trans*-cinnamic acid. During the reaction, the pH was maintained at 10.0, which is optimum for the enzyme reaction. After 24 h, about 70%

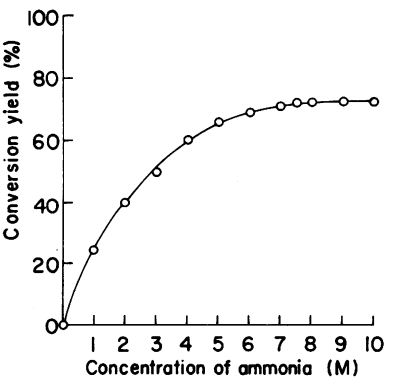

FIG. 7. *Relationship between ammonia concentration and conversion yield. Reaction mixture contained 1 ml of cell suspension and 4 ml of substrate solution prepared according to the method described in the text. Reaction was carried out at 30°C for 20 h.*

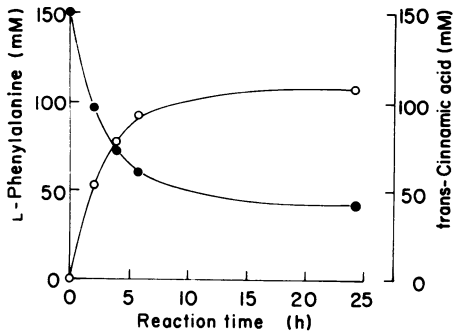

FIG. 8. *Conversion of trans-cinnamic acid to L-phenylalanine by PAL in R. glutinis IFO 0559. The reaction was carried out at 30°C. Symbols: ○, L-Phenylalanine; ●, trans-cinnamic acid.*

of *trans*-cinnamic acid was converted to L-phenylalanine. The reaction mixture contained 3.5 g of L-phenylalanine, and 1.3 g of *trans*-cinnamic acid remained. The accumulated L-phenylalanine did not decompose or racemize during the incubation. After the reaction, however, PAL activity of the recovered cells fell to 20% of initial activity.

Isolation and identification of L-phenylalanine. A 200-ml sample of the incubation mixture, containing 3.5 g of L-phenylalanine and 1.3 g of *trans*-cinnamic acid, was centrifuged to remove the cells. The excess ammonia was removed in vacuo from the supernatant. The solution was adjusted to pH 1.8 with HCl and filtered to remove deposited *trans*-cinnamic acid. The filtrate was passed through a column packed with 500 ml of Amberlite IR-120 (H^+ form) to adsorb L-phenylalanine. After a water wash, L-phenylalanine was eluted from the column with 0.3 N NH_4OH. The eluant was evaporated to dryness in vacuo to give crude crystals of L-phenylalanine (3.5 g, 100%). The crude crystals were dissolved in a small volume of 1.5 N NaOH and recrystallized by addition of 50% acetic acid to give pure L-phenylalanine (2.4 g, 69%), showing a single spot by thin-layer chromatography. The melting point was 259°C decomposition. The optical rotation was $[\alpha]_D^{20} = -34.3°$ (c = 1, water). This value was equivalent to 100% optical purity. Elemental analysis corresponds to $C_9H_{11}N_1O_2$; calculated: C, 65.43%, H, 6.71%; N, 8.48%; found: C, 65.43%; H, 6.71%, N, 8.48%.

DISCUSSION

Ever since yeast was found to contain PAL, the formation of this enzyme by a variety of microorganisms has been reported (1, 7, 9, 12). The PAL activity in most of these microorganisms, including *R. glutinis*, was found to be unstable during cultivation (5). Thus the observation, shown in Fig. 3, that L-isoleucine addition stabilizes PAL activity in culture is noteworthy. Since DL-isoleucine was nearly equally effective, D-isoleucine seemed to be effective to some extent. Detailed experiments on the peculiar behavior of isoleucine isomers are in progress, but we postulate that isoleucine isomers inhibit an inactivation system of PAL, such as that suggested in plant tissues (2–4, 14, 15). Additionally, while PAL induction in the resting cells by L-phenylalanine and other compounds is under study, PAL induction by isoleucine isomers is also observed. It may be that PAL induction by L-isoleucine is maintained for a longer time during fermentation than that by L-phenylalanine.

With respect to the action of PAL, most previous reports have studied in detail the catalytic role of PAL in the conversion of L-phenylalanine to *trans*-cinnamic acid. The authors of British patent no. 1,489,468 (Pfizer Inc., published 19 October 1977) and U.S. patent no. 3,957,580 (Pfizer Inc., published 18 May 1976) have reported the utilization of PAL for the production of L-phenylalanine by the enzymatic conversion of *trans*-cinnamic acid.

The authors of the British patent claim that the normal reaction between *trans*-cinnamic acid and L-phenylalanine is an 80:20 equilibrium in favor of *trans*-cinnamic acid and that a yield of L-phenylalanine approaching the theoretical conversion, 20% L-phenylalanine, may be obtained by employing a large mass of cells and excess ammonium ions. In the actual examples of this patent, however, both the conversion yield and the final concentration of L-phenylalanine were less than 5% and 2.5 mg/ml, respectively. On the other hand, the method of this report was able to increase the conversion yield to more than 70% and to accumulate L-phenylalanine at a concentration of about 18 mg/ml. This apparent discrepancy may be attributable to differences in optimization of the conditions for the conversion reaction. The most important reason for high productivity seems to be that the strong substrate inhibition at higher concentrations of *trans*-cinnamic acid (>150 mM) was avoided and extremely high ammonia concentrations were used at high pH. The conversion yield was dependent on the concentration of ammonia but not on the molar ratio of ammonia to *trans*-cinnamic acid. After the reaction, however, PAL activity of the recovered cells was markedly reduced by high concentration of ammonia and high pH of the reaction mixture. Accordingly, reuse of the cells seemed to be impractical.

On the other hand, the authors of the U.S. patent list the immobilization methods for *R. glutinis* having PAL activity. However, details

of the reaction, the conversion yield, and the final concentration of L-phenylalanine are obscure. Although the experimental data were not presented, we also prepared *k*-carrageenan-immobilized cells of *R. glutinis* by the method described previously (16). The activity yields of the immobilized cells were about 64% of the intact cells. The immobilized cells could be reused, but their activity was reduced to about 30% of the initial activity, as was the case with the intact cells. Accordingly, up to the present, it seems that the use of immobilized cells offers no advantage over the batch method with intact cells.

Strictly speaking, it requires more data to discuss the economics of the method. Furthermore, reuse of the intact cells or the stabilization of the immobilized cells is an important problem for the perfection of an ideal process. However, the reported data show that one can readily and practically prepare L-phenylalanine as an essential amino acid by enzymatic conversion of *trans*-cinnamic acid with PAL in *R. glutinis*. As a commercial process, this is of potentially great value to the amino acid industry.

LITERATURE CITED

1. **Bezanson, G. S., D. Desaty, A. V. Emes, and L. C. Vining.** 1970. Biosynthesis of cinnamic acid and detection of phenylalanine ammonia-lyase in *Streptomyces verticillatus*. Can. J. Microbiol. **16**:147–151.
2. **Creasy, L. L.** 1976. Phenylalanine ammonia-lyase inactivating system in sunflower leaves. Phytochemistry **15**: 673–675.
3. **Duke, S. O., R. E. Hoagland, and C. D. Elmore.** 1980. Effects of glyphosate on metabolism of phenolic compounds. V. L-α-Aminooxy-β-phenylpropionic acid and glyphosate effects on phenylalanine ammonia-lyase in soybean seedlings. Plant Physiol. **65**:17–21.
4. **French, C. J., and H. Smith.** 1975. An inactivator of PAL from gherkin hypocotyls. Phytochemistry **14**:963–966.
5. **Fritz, R. R., D. S. Hodgins, and C. W. Abell.** 1976. Phenylalanine ammonia-lyase. Induction and purification from yeast and clearance in mammals. J. Biol. Chem. **251**:4646–4650.
6. **Havir, E. A., and K. R. Hanson.** 1968. L-Phenylalanine ammonia-lyase. II. Mechanism and kinetic properties of the enzyme from potato tubers. Biochemistry **7**:1904–1914.
7. **Kalghatgi, K. K., and P. V. Subba Rao.** 1975. Microbial L-phenylalanine ammonia-lyase. Purification, subunit structure and kinetic properties of the enzyme from *Rhizoctonia solani*. Biochem. J. **149**:65–72.
8. **Kalghatgi, K. K., and P. V. Subba Rao.** 1976. Regulation of L-phenylalanine ammonia-lyase from *Rhizoctonia solani*. J. Bacteriol. **126**:568–578.
9. **Moore, K., P. V. Subba Rao, and G. H. N. Towers.** 1968. Degradation of phenylalanine and tyrosine by *Sporobolomyces roseus*. Biochem. J. **106**:507–514.
10. **Ogata, K., K. Uchiyama, and H. Yamada.** 1966. Microbial formation of cinnamic acid from phenylalanine. Agric. Biol. Chem. **30**:311–312.
11. **Ogata, K., K. Uchiyama, and H. Yamada.** 1967. Metabolism of aromatic amino acid in microorganisms. Part I. Formation of cinnamic acid from phenylalanine. Agric. Biol. Chem. **31**:200–206.
12. **Parkhurst, J. R., and D. S. Hodgins.** 1971. Phenylalanine and tyrosine ammonia-lyase activity in *Sporobolomyces pararoseus*. Phytochemistry **10**:2997–3000.
13. **Steele, B. F., H. E. Sauberlich, M. S. Reynolds, and C. A. Baumann.** 1949. Media for *Leuconostoc mesenteroides* P-60 and *Leuconostoc citrovorum* 8081. J. Biol. Chem. **177**:533–544.
14. **Tan, S. C.** 1980. Phenylalanine ammonia-lyase and the phenylalanine ammonia-lyase inactivating system. Effect of light, temperature and mineral deficiencies. Aust. J. Plant Physiol. **7**:159–167.
15. **Tanaka, Y., K. Matsushita, and I. Uritani.** 1977. Some investigations on inactivation of phenylalanine ammonia-lyase in cut injured sweet potato root tissue. Plant Cell Physiol. **18**:1209–1216.
16. **Tosa, T., T. Sato, T. Mori, K. Yamamoto, I. Takata, Y. Nishida, and I. Chibata.** 1979. Immobilization of enzymes and microbial cells using carrageenan as matrix. Biotechnol. Bioeng. **21**:133–145.