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Utilization of Phenylalanine and Phenylacetic Acid by *Pseudomonas solanacearum*

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ABSTRACT

Pseudomonas solanacearum utilized phenylalanine as sole source of carbon and nitrogen. Phenylalanine-grown cells rapidly oxidized phenylacetic acid and homogentisic acid. Gentisic and protocatechuic acids were also oxidized, after an initial lag. Phenylacetate-grown cells rapidly oxidized gentisic acid, slowly oxidized *o*-hydroxyphenylacetic acid and catechol, and did not oxidize *m*-hydroxymandelic and *m*-hydroxyphenylacetic acids. Phenylalanine was degraded via phenylacetic acid-r-lactone and β ketoadipate; Rothera's test indicated *ortho* cleavage of the aromatic ring.

Index Entries: Degradation; aromatic substances; phenylalanine; phenylacetic acid; *Pseudomonas solanacearum*.

INTRODUCTION

In plants and microbes, the routes of catabolism of phenylalanine (1) differ. Several fungi belonging to basidio-mycetes, ascomycetes, and deuteromycetes degrade phenylalanine via cinnamic acid (2) by producing phenylalanine ammonia lyase (PAL) (*1*). Phytopathogenic and symbiotic bacteria do not produce PAL, so do saprophytic bacteria (2). But bacteria have developed other pathways to utilize phenylalanine. Kunita

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(3) reported that *Pseudomonas fluorescens* utilized phenylalanine via phenylacetic acid (3). Catabolism of 3- and 4-hydroxyphenylacetate by the 3, 4-dihydroxyphenylacetate pathway was reported by Cooper and Skinner (4) in *Escherichia coli*.

The first step in the bacterial degradation of phenylacetic acid is its conversion to *p*-hydroxyphenylacetic acid (*p*-HPAA) (4). Further degradation of *p*-HPAA proceeds through hydroxylation at 3-carbon, yielding 3, 4-dihydroxyphenylacetic acid (5). According to Sparnins et al. (5), an unidentified soil bacterium hydroxylated *p*-HPAA at C-1 to give homogentisic acid (6), with simultaneous migration of the side chain substituent according to the equation:

$$p\text{-HPAA} + O_2 + \text{NADH} \rightarrow \text{HGA} + H_2O + \text{NAD}$$

Similarly, *Pseudomonas acidovorans* degraded *p*-HPAA to homogentisic acid (6). These cleavages resulted in the mitigation of their toxic effects. Therefore, we were interested in studying the ability of *Pseudomonas solanacearum*, a bacterium pathogenic on a variety of plants, to degrade aromatic substances as part of the mechanism of pathogenesis.

MATERIALS AND METHODS

The isolate obtained from the Centre's collection was grown on Dye's (7) medium. The cells were collected by centrifugation at 10,000*g* for 10 min at 4°C. Again the pellet was washed twice with double distilled water and stored in sterile distilled water at 4°C.

Phenylalanine and Phenylacetic Acid as Sole Carbon Source

The bacterium was grown on 2 m*M* of phenylalanine/phenyl acetic acid amended with 100 mL liquid Dye's medium without glycerol. Phenylalanine/phenylacetic acid-grown cells (A_{540} = 1.0, 24 h old) were inoculated and the flasks were incubated at 30°C in an orbital shaker at 100 strokes/min. Five milliliters aliquots were removed at 6-h intervals and the absorbance was measured at 540 nm in a Spectronic 20 model colorimeter.

Phenylalanine as Nitrogen Source

P. solanacearum was grown on Dye's agar medium, replacing ammonium phosphate with 2 m*M* phenylalanine. Growth was measured between 24 and 48 h of incubation at 30°C.

Manometry

Oxidation of aromatic substances was studied by direct manometric method (8). Cells were grown for 48 h on Dye's agar medium without

glycerol, but amended with 2 m*M* phenylalanine/phenylacetic acid. Cells equivalent to 2.85 mg dry wt, suspended in 2 mL 0.025*M* buffer, pH 7.4, were added to the main chamber of a Warburg flask. The side arm contained 0.5 mL of the substrate (2 μmol). The center well received 20% KOH solution and a small strip of folded filter paper. The flasks were arranged on the Warburg apparatus, operating at 75 strokes/min at 30 ± 0.5°C. The endogenous respiration and oxygen consumption caused by autooxidation of the substrate in the absence of cells were subtracted from the appropriate values of O_2 uptake in presence of the substrate.

Isolation of Intermediates of Phenylalanine Degradation

Intermediates were isolated according to Abitha Devi et al. (*9*). Cells were grown at 30°C in Dye's medium containing 2 m*M* phenylacetic acid for 48 h with continuous agitation. The cells were harvested by centrifugation, washed in 0.05*M* phosphate buffer (pH 7.2), and resuspended in the buffer containing 4 m*M* of either phenylalanine or phenylacetic acid in a total volume of 200 mL. The mixture was incubated for 18 h with phenylalanine and 12 h with phenylacetic acid at 30°C in an orbital shaker. The reaction was terminated by the addition of 5 mL of 1*N* HC1 and centrifuged at 20,000*g* for 10 min to sediment the cells. The supernatant was extracted twice with equal volumes of peroxide free ether. The combined ether extract was extracted twice with 50 mL (each) of 5% sodium bicarbonate. The bicarbonate fraction was adjusted to pH 2.0 with 10*N* HC1 and extracted twice with equal volumes of ether. Both neutral and acidic fractions were dried, evaporated separately to dryness, redissolved in 1 mL ethyl acetate, and analyzed by TLC and paper chromatography. Solvent systems used were: 15% acetic acid (vv) and benzene:acetic acid:water (10:7:3, upper phase). Spots on chromatogram were located under UV light and developed by spraying diazotized sulphanilic acid or *p*-nitroaniline, followed by 10% Na_2CO_3.

Degradation of ^{14}C-Phenylalanine

Degradation of ^{14}C-phenylalanine (ring labeled; supplied by Bhabha Atomic Research Centre, Bombay) was studied to confirm the intermediates of degradation pathway. The replacement culture contained 1 μCi/mL of ^{14}C-phenylalanine (ring labeled). Culture fluids were extracted. Spots were eluted from chromatogram, dissolved in 5 mL of ethyl acetate, concentrated, and rechromatographed to remove traces of contaminants. After rechromatography, spots were eluted in ethyl acetate, passed through a celite column, and eluted with methanol. The methanol extract was evaporated under reduced pressure and the residue was analyzed by infrared, nuclear magnetic resonance, and mass spectroscopy. Intermediates isolated from ^{14}C-phenylalanine were checked for the presence of radioactivity in a Beckman-50 liquid scintillation counter (Fullerton, CA).

Rothera's test (*8*) was performed to detect keto groups.

Table 1
Growth of *P. solanacearum* on Phenylalanine and Phenylacetic Acid Amended Dye's Medium

Carbon and/or nitrogen source	Growth after 48 h
Glycerol + ammonium phosphate	+ + + + +
Phenylalanine[a] + glycerol + ammonium phosphate	+ + + + +
Phenylalanine[b] + glycerol	+ + + + +
Phenylalanine[c] + ammonium phosphate	+ +
Phenylalanine[a]	+ + + + +
Phenylacetic acid + glycerol	+ + + + +
Phenylacetic acid	+ + + + +

+ +, very slow growth rate; + + + + +, unretarded growth.
[a]Served as both Carbon and Nitrogen source either in presence of readily utilizable Carbon and Nitrogen source or in their absence.
[b]Served as Nitrogen source in presence of readily utilizable Carbon source.
[c]Served as Carbon source in presence of readily utilizable Nitrogen source.

RESULTS

P. solanacearum utilized phenylalanine as sole carbon (C) and nitrogen (N) source. It utilized phenylacetic acid as the C source. Growth almost equaled the growth on glycerol, when phenylalanine was used as the N source (Table 1). However, growth was less when phenylalanine was used as the C source in presence of a readily utilizable N source, ammonium phosphate. There was an initial lag of 6 h in phenylacetic acid-amended medium and the lag was 18 h in phenylalanine-amended medium (Fig. 1).

Oxidation of Aromatic Substances by *P. solanacearum*

Cells grown on phenylalanine oxidized phenylacetic acid and homogentisic acid (Fig. 2). It oxidized protocatechuic acid and gentisic acid after an initial lag of 15 min, but slowly. A distinct lag period of oxidation was evident with catechol and mandelic acid. Phenylacetic acid-grown cells oxidized gentisic acid without any lag (Fig. 3). It oxidized mandelic and homoprotocatechuic acids immediately, but at a reduced rate. *p*-hydroxyphenylacetic acid and protocatechuic acid were oxidized after an initial lag. The rate of oxygen uptake was initially low and increased after 75 min. *o*-hydroxyphenylacetic acid and catechol were oxidized slowly. *m*-hydroxyphenylacetic acid and *m*-hydroxymandelic acid did not support growth.

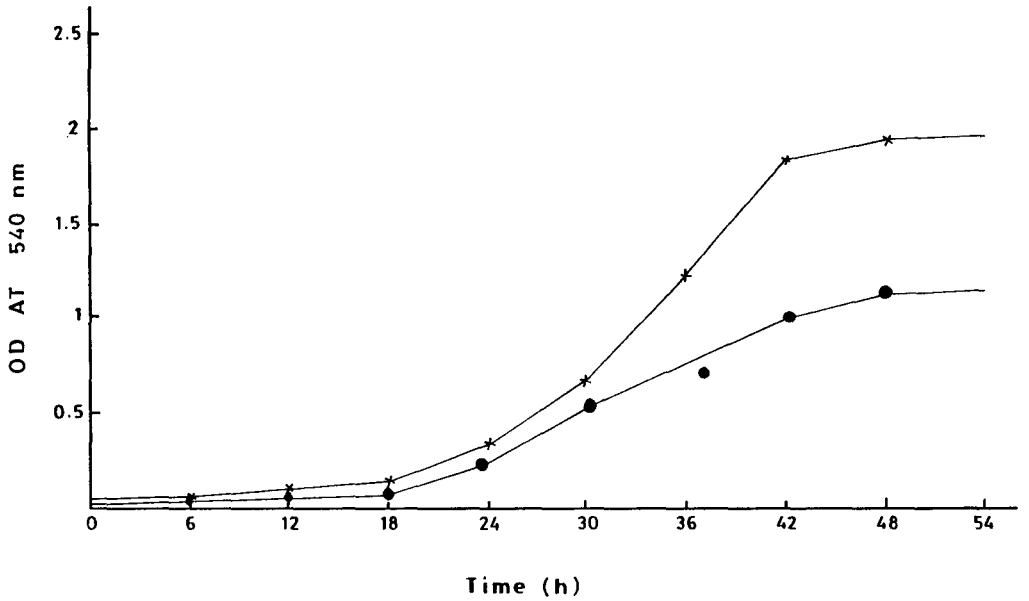


Fig. 1. Growth pattern of *P. solanacearum* on phenylalanine (●—●) and phenylacetic acid (x—x).

Intermediates of Phenylalanine Degradation Pathway

Four spots were located on TLC plates and on paper chromatograms with Rf values of 0.98, 0.81, 0.30, and 0.13 in benzene:acetic acid:water solvent. The first spot, with Rf value of 0.98, matched with phenylacetic acid. Mass spectral analyses confirmed its identity as phenylacetic acid (Fig. 4A,B). The second spot, with Rf value of 0.81, indicated the presence of a keto group. UV spectrum analysis revealed that it had no aromatic stretch and was identified as B-ketoadipic acid. The third spot, with Rf value of 0.30, was identified as *p*-hydroxyphenylacetic acid (Fig. 4C,D). The fourth spot, with Rf value of 0.13, was not identified. Similar results were obtained during phenylacetic acid utilization by *P. solanacearum*.

Since none of the hydroxylated aromatic substances were located in chromatogram, the disappearance of five of the most common dihydric phenols, catechol, protocatechuic acid, gentisic acid, homogentisic acid, and homoprotocatechuic acid, was investigated.

Phenylalanine-grown cells readily utilized homogentisic acid and the rate of degradation was faster than other substrates. Gentisic acid utilization was least (Fig. 5). Phenylacetic acid-grown cells utilized protocatechuic acid rapidly, but utilized only traces of gentisic acid (Fig. 6).

Rothera's test confirmed that the ring of all the dihydroxyphenols was cleaved via the *ortho*-pathway. Presence of B-ketoadipic acid confirmed the *ortho* cleavage.

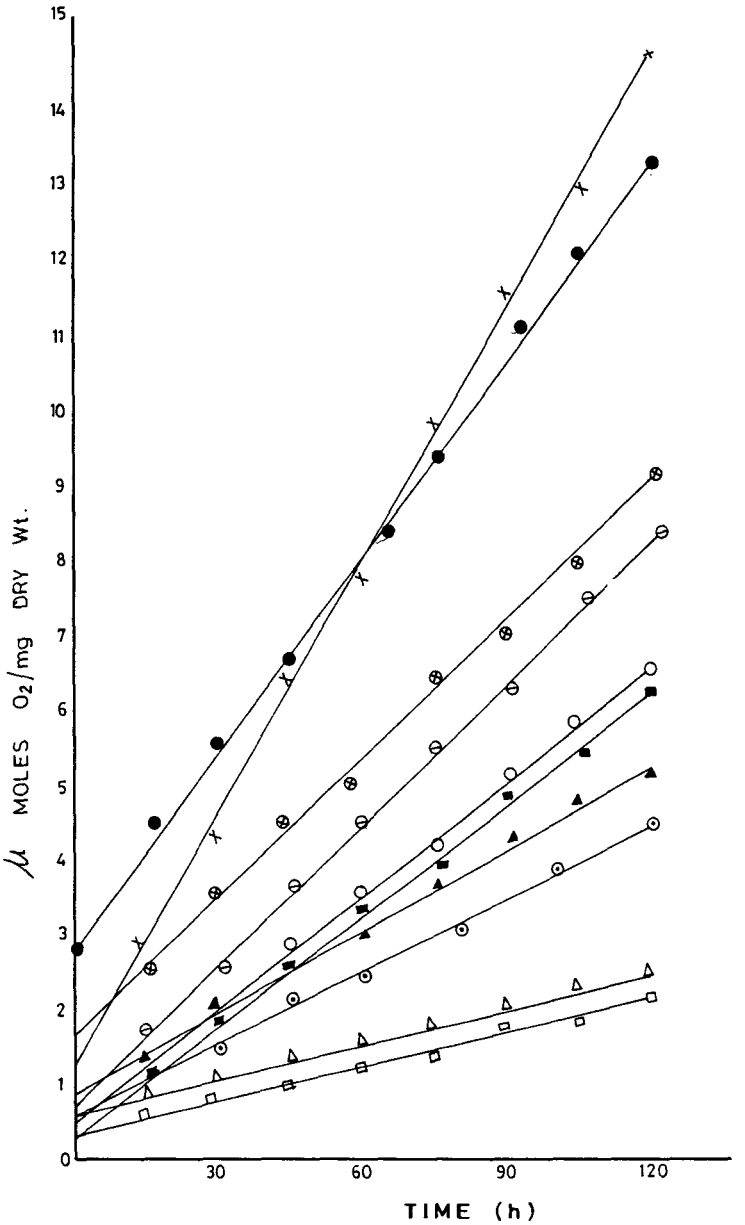


Fig. 2. Oxidation of aromatic substances by phenylalanine grown cells of *P. solanacearum*. ⊙—⊙, Control; △—△, catechol; ⊗—⊗, gentisic acid; ■—■, m-hydroxymandelic acid; ●—●, homogentisic acid; ⊘—⊘, homoprotocatechuic acid; □—□, mandelic acid; x—x; phenylacetic acid; ○—○, protocatechuic acid; ▲—▲, trans-cinnamic acid.

DISCUSSION

P. solanacearum utilized phenylalanine and phenylacetic acid as sole C source; however, growth was low compared with glycerol-amended medium. Phenylalanine supported the growth of *P. solanacearum* as N source.

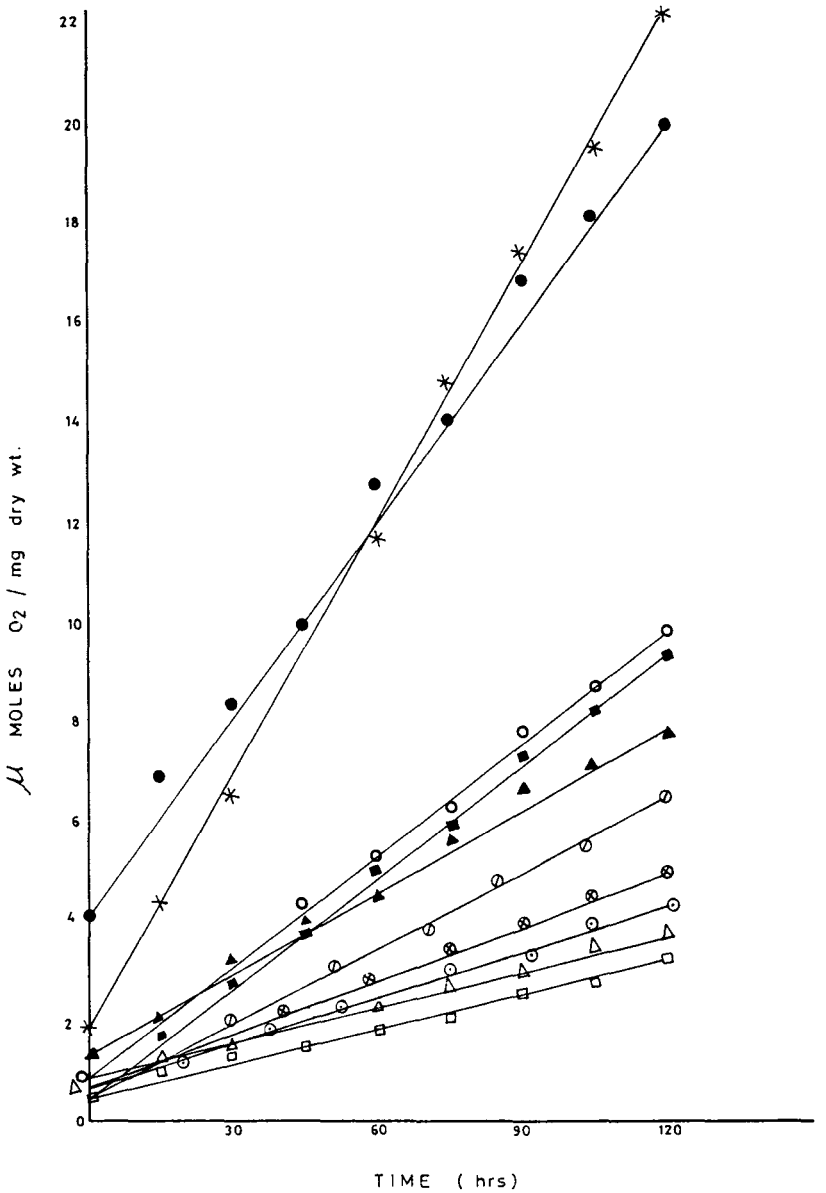
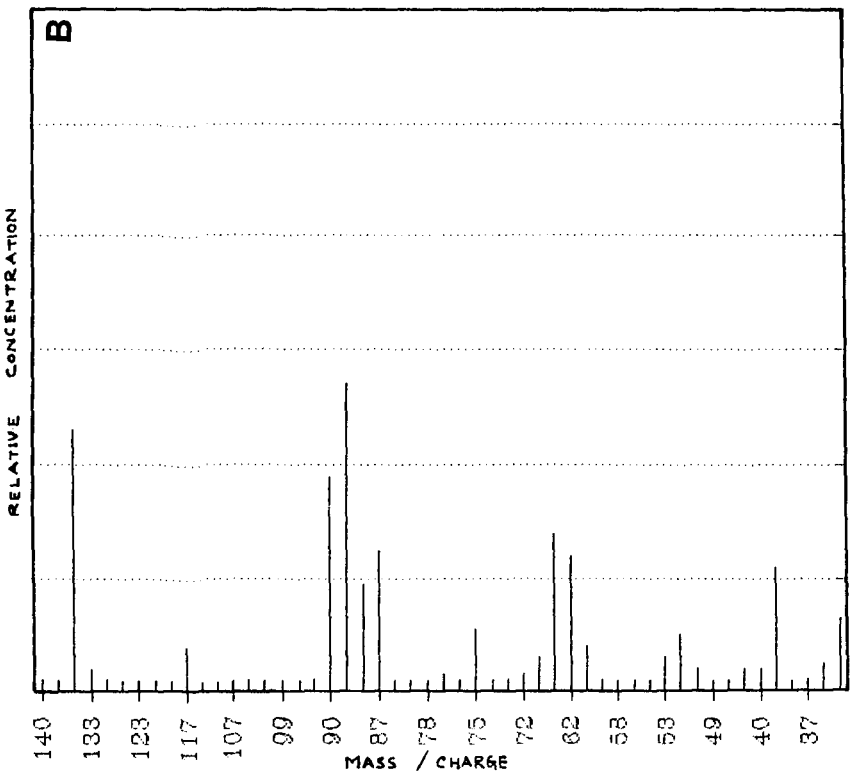


Fig. 3. Oxidation of aromatic substances by phenylacetic acid grown cells of *P. solanacearum*. ⊙—⊙, Control; ⊗—⊗, catechol; ●—●, gentisic acid; □—□, m-hydroxymandelic acid; △*△, m-hydroxyphenylacetic acid; ⊘—⊘, o-hydroxyphenylacetic acid; ■—■, p-hydroxyphenylacetic acid; ○—○, homoprotocatechuic acid; *—*, mandelic acid; ▲—▲, protocatechuic acid.

Phenylalanine-grown cells oxidized phenylacetic acid and homogentisic acid rapidly. The rate of disappearance of homogentisic acid indicated that its degradation was maximum and rapid by phenylalanine-grown cells compared with other dihydric phenols. But gentisic acid was utilized after an initial lag. Arunakumari and Mahadevan (*10*) found that when-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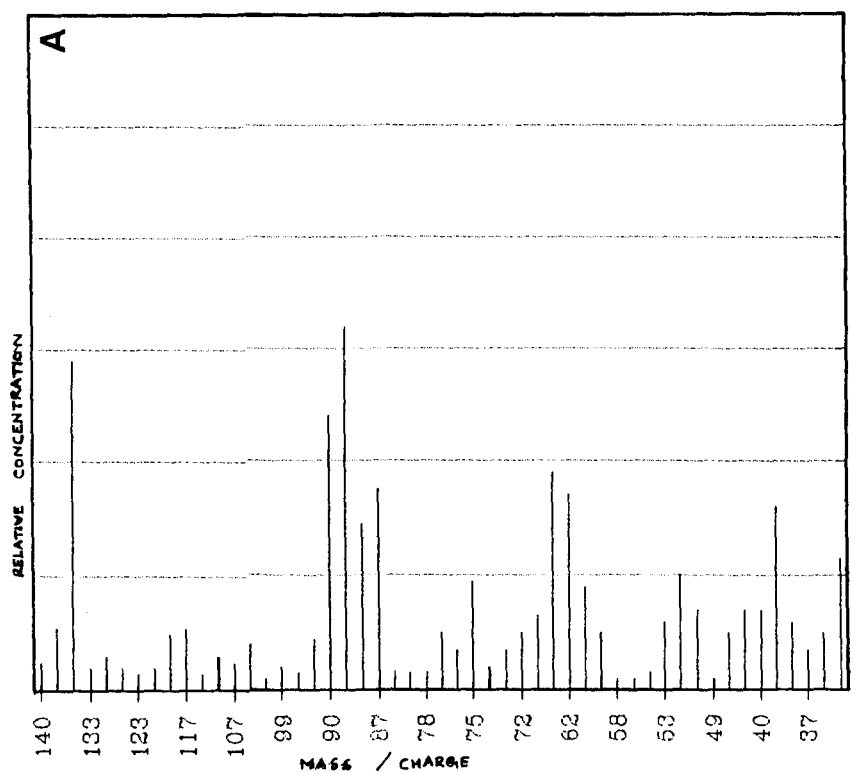
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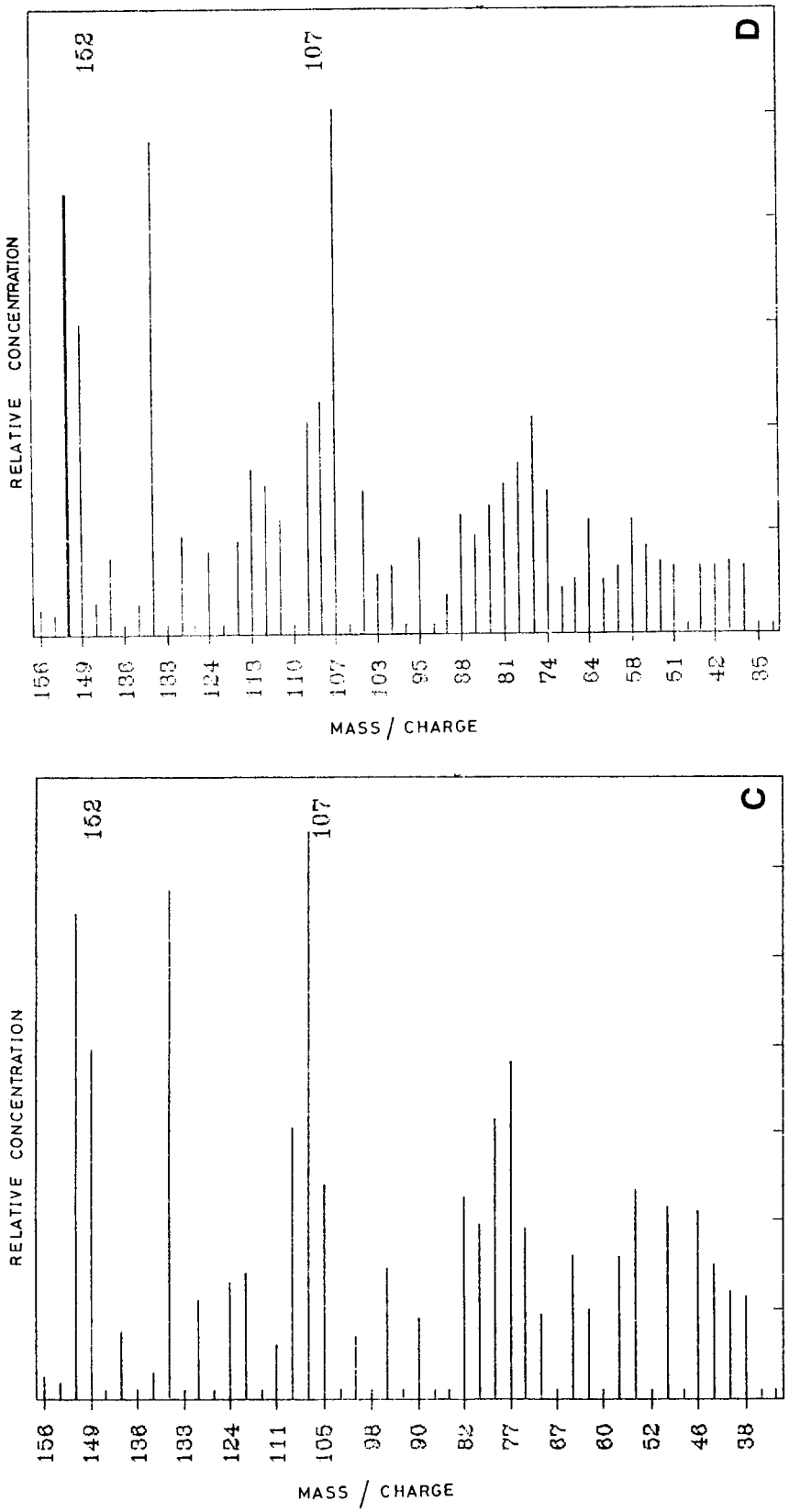

Fig. 4. MS of phenylacetic acid: (**A**) authentic; (**B**) sample; and *p*-hydroxyphenylacetic acid (**C**) authentic, (**D**) sample.

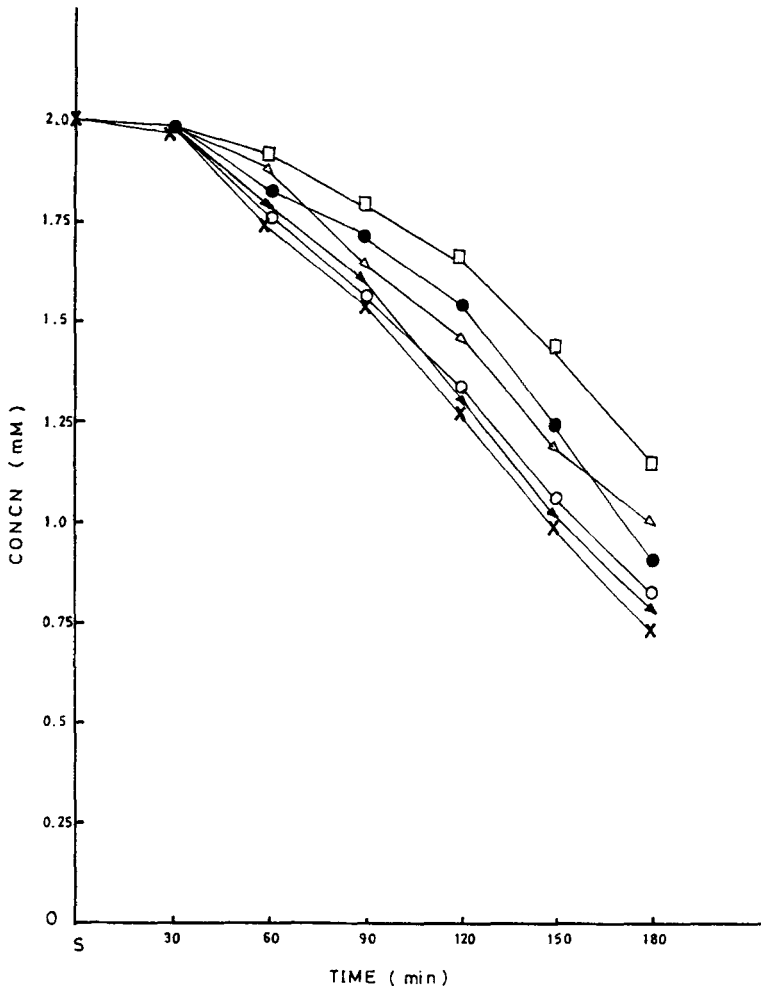

Fig. 5. Disappearance of aromatic substances from the medium by phenylalanine grown *P. solanacearum*. ●—●, Catechol; □—□, gentisate; △—△, homoprotocatechuate; x—x, homogentisate; ○—○, p-hydroxybenzoate; ▲—▲, protocatechuate.

ever homogentisic acid was oxidized by *P. solanacearum*, gentisic acid was also oxidized, or *vice versa*. This might be caused by the simultaneous induction of homogentisate or gentisate oxygenase by either of the substrates. Phenylacetic acid-grown cells rapidly utilized both gentisic and homogentisic acids.

None of the hydroxyphenylacetic acids was oxidized rapidly by either phenylalanine or phenylacetic acid-grown cells. The toxicity of *m*-hydroxyphenols to the bacterium was evident.

The rate of decrease in O.D. of dihydric phenols indicated that homogentisic acid could be an intermediate in phenylalanine degradation. Further, homogentisic acid was oxidized rapidly by phenylalanine-grown

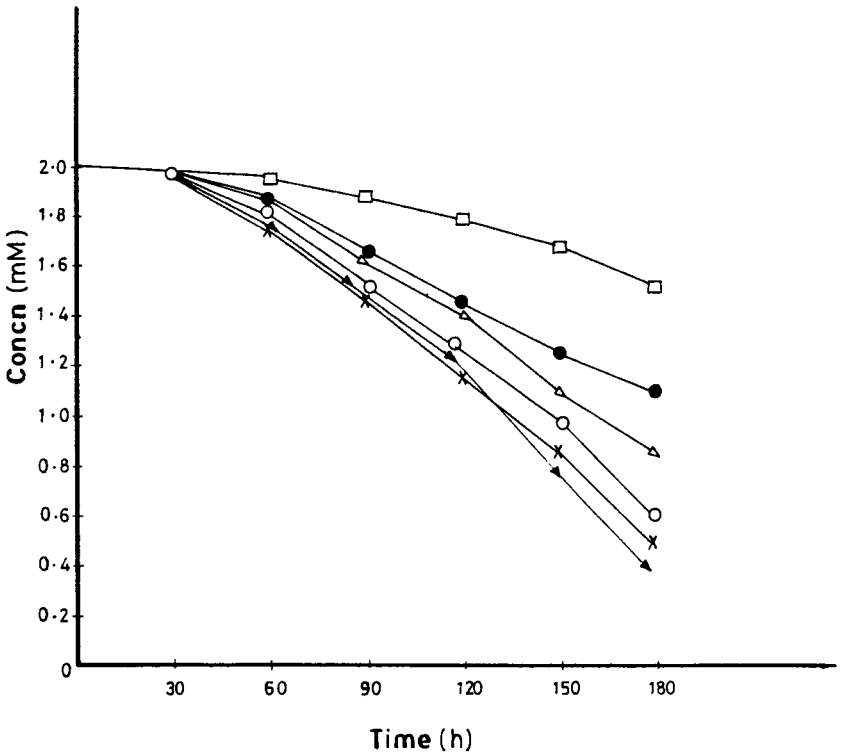

Fig. 6. Disappearance of aromatic substances from the medium by phenylacetic acid grown *P. solanacearum*. x—x, Catechol; □—□, gentisate; ○—○, homoprotocatechuate; ●—●, homogentisate; △—△, p-hydroxybenzoate; ▲—▲, protocatechuate.

cells. The conversion of phenylacetic acid to homogentisic acid by *P. fluorescens* strain K_2 is known (*11,3*). *P. solanacearum* grew rapidly on protocatechuic acid compared to gentisic acid, and the rate of oxidation of gentisic acid by the cells was very high when compared with protocatechuic acid. We could not therefore find a correlation between substrate oxidation and growth rate.

Bacterial degradation of phenylacetic acid has been shown to proceed through two separate pathways. The first involves the intermediate homogentisic acid (*11–13*), and the second pathway involves 3,4-dihydroxyphenylacetic acid. Dagley et al. (*14*) showed that phenylacetic acid-grown *Pseudomonas* catalyzed the conversion of 3,4-dihydroxyphenylacetic acid to pyruvic acid, fumaric acid, and β-Ketoadipic acid.

Adachi et al. (1964) reported the hydroxylation of *p*-hydroxyphenylacetic acid to form 3,4-dihydroxyphenylacetic acid, and the product of this ring fission was identified as δ-carboxymethyl-α-hydroxymuconic semialdehyde in *Pseudomonas ovalis*. A similar ring fission product was formed by the oxidation of 3,4-dihydroxyphenylacetic acid in *Pseudomonas* sp. (*13,14*).

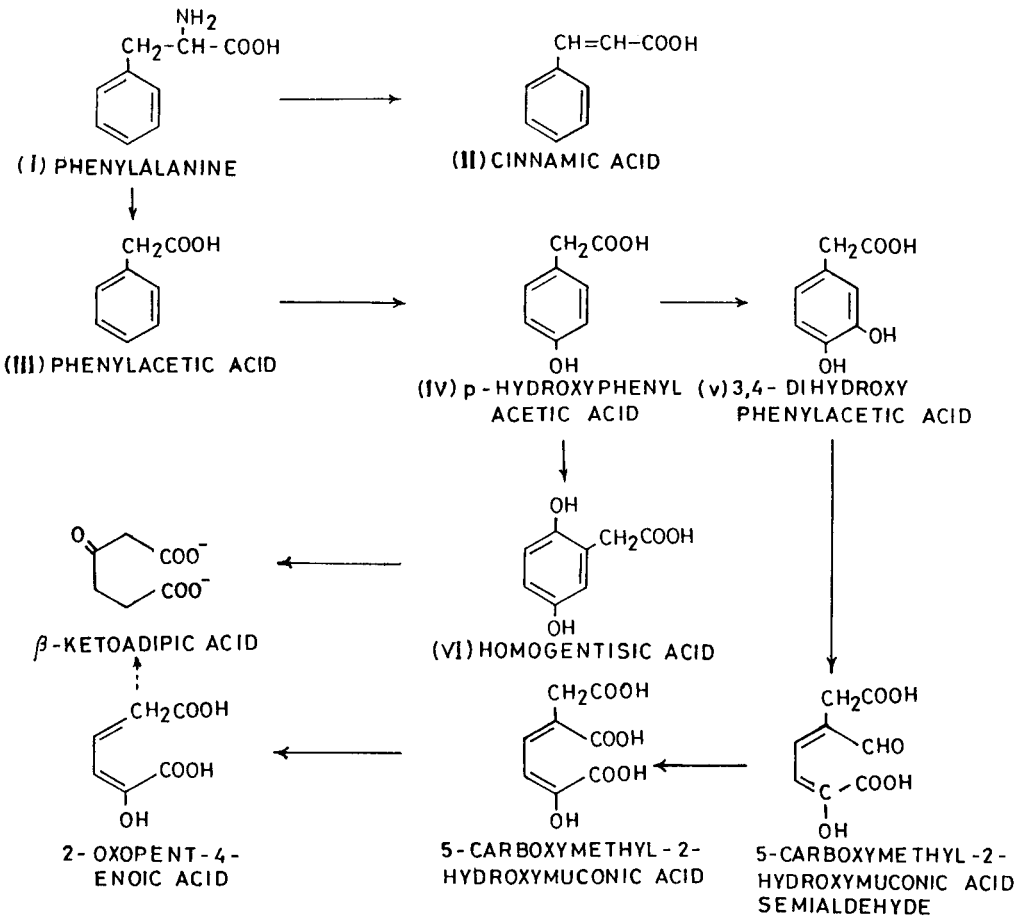

Fig. 7. Degradation pathway of phenylalanine and phenylacetic acid by *P. solanacearum*.

Cooper and Skinner (4) reported that *p*-hydroxyphenylacetate is catabolized by *E. coli* via *meta* cleavage route, with 3,4-dihydroxyphenylacetate as the ring fission product, identical to that used by various pseudomonads and *Acinetobacter* sp. (*5,11,15*). 3-Hydroxyphenylacetate is also catabolized by the same route in *E. coli*; 3-hydroxyphenylacetic acid is hydroxylated by other organisms to give 2,5-dihydroxyphenyl acetate, which is catabolized to fumarate and acetoacetate (*4*). Phenylacetic acid catabolism by *Pseudomonas* sp. follows a pathway that utilizes *p*-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid as intermediates (*16*). The tentative pathway of phenylalanine degradation by *P. solanacearum* is presented (Fig. 7).

Degradation of phenylalanine and related compounds is an important metabolic character of *P. solanacearum* in terms of phytopathogenicity. The ability of *P. solanacearum* to degrade phenylacetic acid and related aromatic compounds promotes its ability to infect and sustain its survival in the host plants, in the presence of a wide range of aromatic substances found both inside and outside the cells of host plants.

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