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Dissimilation of Ferulic Acid by *Bacillus subtilis*

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Abstract. *Bacillus subtilis* utilized ferulic acid and its intermediates vanillin, vanillic acid, and protocatechuic acid as sole carbon source. The enzymes of the ferulic acid degradative pathway such as deacetylase, vanillin oxidase, vanillate-*o*-demethylase, and protocatechuate 3,4-dioxygenase were inducible in nature. Concentration of the inducer profoundly influenced the induction of the enzymes involved in ferulic acid dissimilation.

Interest has recently expanded on the potential use of microorganisms to convert lignocellulose and lignin for industrial byproducts [11]. Ferulic acid is an important intermediate of lignin degradation [2]. Gram-negative bacteria degrade ferulic acid under both aerobic and anaerobic conditions [1, 9, 15]. The Gram-positive *Bacillus subtilis* dissimilates a variety of lignin model substances [6]. We present evidence on the degradation of ferulic acid by *B. subtilis*.

Materials and Methods

Chemicals. Ferulic acid, vanillin, vanillic acid, protocatechuic acid, and acetate kinase were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). All other chemicals were of the highest purity, available locally.

Organisms and culture conditions. *Bacillus subtilis* was isolated from virgin forest soil containing decaying wood, by enrichment culture with teak wood lignin as sole carbon source. It was maintained in Dye's medium [4] with glycerol as sole carbon source. Dye's medium contained: 1.0 g $(NH_4)_2HPO_4$; 2.0 g KH_2PO_4; 2.0 g KCl; 200 mg $MgSO_4 \cdot 7H_2O$; and 10 mg $Fe(NH_4)_2(SO_4)_2$ pH 7.2; distilled water, 1 liter. At 48 h of growth, the cells were subcultured.

Growth on aromatic substances. Growth and enzyme production experiments were performed in static cultures at 30°C. Ferulic acid, vanillin, vanillic acid, and protocatechuic acid were dissolved in 2 ml ethanol, and the final volume of the concentrated stock solutions was made up with distilled water. The solutions were filter-sterilized and aseptically added to Dye's medium so as to get the required final concentration. About 10^6 cells derived from a culture grown on glycerol medium were inoculated. Aliquots of 5 ml culture were withdrawn at regular intervals, and the turbidity was measured at 540 nm in a spectrophotometer.

Preparation of cell-free extract. Cells were grown on Dye's medium with aromatic substance (5, 10, and 20 m*M*) or glycerol (5 m*M*) as sole carbon source. After 36 h of incubation, the cells were collected by centrifugation, washed twice with 0.025 *M* phosphate buffer, pH 7.2, by centrifugation at 10,000 *g* for 10 min, and suspended in the buffer. They were broken in a MSE 150 Watt Ultrasonic disintegrator at 4 amplitude for 15 min; the suspension was centrifuged at 20,000 *g* for 30 min at 4°C, and the supernatant was used as crude enzyme. Protein in the extract was estimated with bovine serum albumin as standard [10].

Assays for enzyme activity. The side chain in ferulic acid is cleaved, and the two carbons of the side chain are released as acetate [14]. The cells were grown on ferulic acid (5 m*M*), and exponentially grown cells were used to prepare the enzyme. The assay system contained ferulic acid 0.5 ml (10 μmole); α,α'-dipyridyl (0.1 m*M*), 0.5 ml; 1 ml NAD (10 μmole); and 1 ml enzyme extract, incubated for 15 min at 30°C, and the reaction was stopped by the addition of 5.8 *N* (3 ml) perchloric acid. It was centrifuged at 10,000 *g* for 10 min; the supernatant was neutralized with 5 *N* KOH, kept on ice for 5 min, and centrifuged. Acetate in the supernatant was measured with acetate kinase [7].

Vanillin oxidase [3]. The reaction mixture contained 1 ml vanillin (10 μmole), 1 ml enzyme, and 1 ml phosphate buffer, 0.025 *M*, pH 7.2. Decrease in absorbance at 278 nm was followed in a recording spectrophotometer. Specific activity was expressed in terms of μmoles vanillin disappearing/min per mg protein.

Vanillate-*o*-demethylase [12]. The reaction mixture contained 1 ml vanillic acid (1 mg); α,α'-dipyridyl (10 μmole) 0.1 ml; 1 ml enzyme; and 3 ml phosphate buffer, 0.025 M, pH 7.2, and was incubated at room temperature for 2 h. The reaction was stopped by adding an equal amount of 10% TCA, extracted with peroxide-free diethyl ether, evaporated on a water bath at 60°C, and the residue was dissolved in ethanol. It was chromatographed, along with authentic protocatechuic acid on Whatman No. 1 filter paper, and developed in 15% acetic acid solvent. The protocatechuic acid spot was cut from an unsprayed chromatogram and eluted in water. To 2.5 ml of eluate, 1 ml 10% sodium tung-

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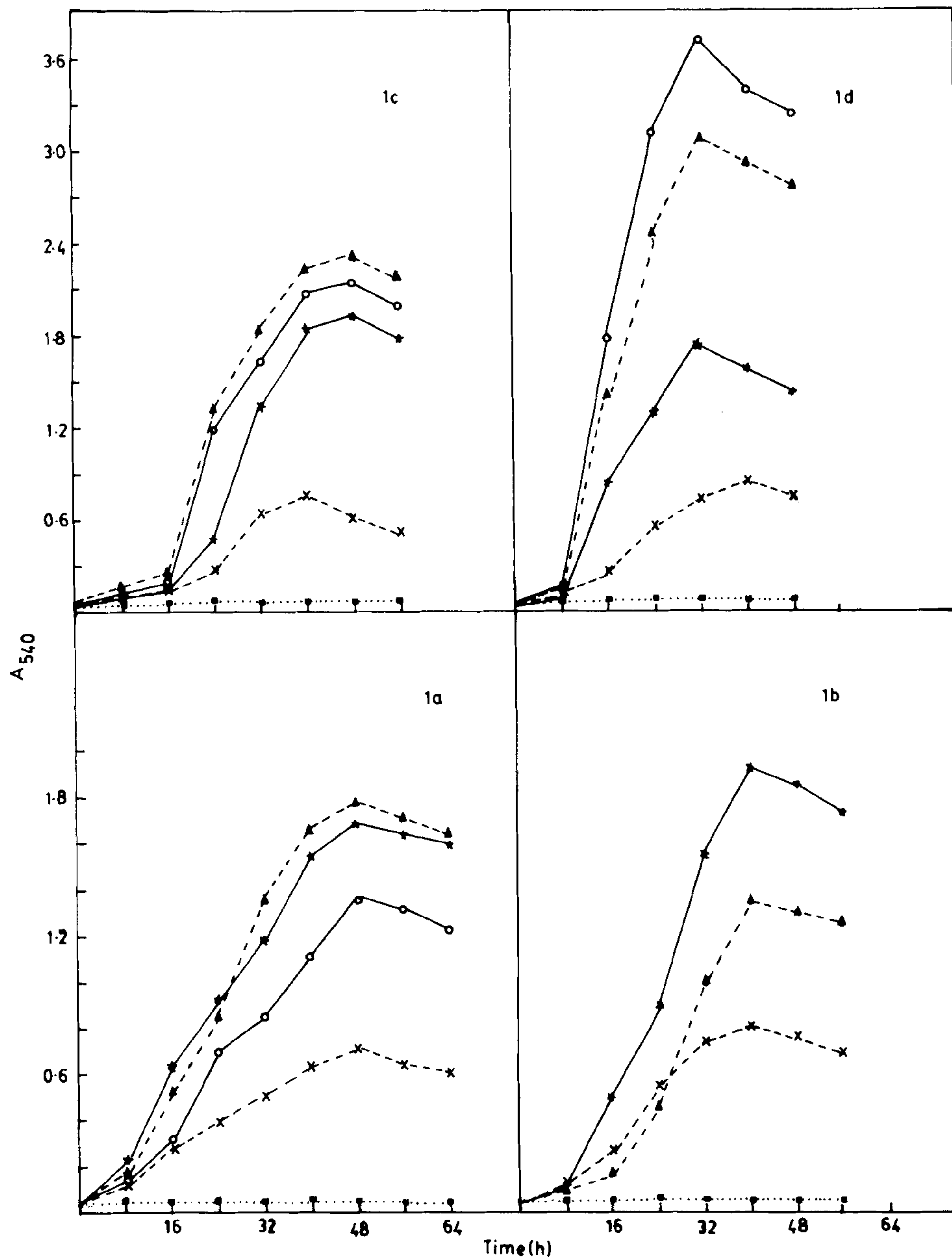

Fig. 1. Growth of *Bacillus subtilis* on ferulic acid and its intermediates: (a) ferulic acid; (b) vanillin; (c) vanillic acid; and (d) protocatechuic acid. ×---× 1 m*M*; ★—★ 5 m*M*; ▲---▲ 10 m*M*; ○—○ 20 m*M*; ■ · · · · · · ■ 40 m*M*.

state, 1 ml 0.5 *N* HCl, and 2 ml sodium nitrite (0.5%) were added and incubated for 5 min at room temperature. To it, 4 ml of 0.5 *N* NaOH was added and kept at room temperature for 30 min. The red-colored solution was read at 510 nm.

Protocatechuic acid standard was prepared by developing a known quantity of protocatechuic acid in paper chromatogram. The spot was cut and eluted in water, and the color was developed as mentioned before and estimated. The specific activity of vanillate-*o*-demethylase was expressed in terms of μmoles protocatechuic acid formed per mg protein.

Protocatechuate 3,4-dioxygenase [5]. The reaction mixture contained protocatechuic acid (10 μmole) 0.1 ml; 1.9 ml phosphate buffer, 0.025 *M*, pH 7.2; and 1 ml enzyme, and the decrease in OD at 290 nm (λ_{max} for protocatechuic acid) was followed in a recording spectrophotometer. The specific activity was ex-

Table 1. Induction of enzymes involved in ferulic acid degradation by *B. subtilis*

Inducer (5 m*M*)	Specific activity[a]			
	Deacetylase	Vanillin oxidase[c]	Vanillate-*o*-demethylase[d]	Protocatechuate 3,4-dioxygenase[e]
Ferulic acid	6.98	0.278	9.09	0.260
Vanillin	—	0.505	11.63	0.302
Vanillic acid	—	—	13.07	0.545
Protocatechuic acid	—	—	—	0.324
Glycerol	—	—	—	—

[a] Specific activity: μmoles substrate disappearing/min per mg protein.
[b] Deacetylase: μmoles acetate formed/10 μmole ferulic acid per mg protein. The reaction mixture contained 0.2 ml neutralized supernatant at pH 7.2; 0.3 ml water mixture (water; Tris HCl, 1.0 *M*, pH 7.4; $MgCl_2$, 1.0 *M*; 25 : 5 : 1 vol/vol); 0.35 ml hydroxylamine (4 *M*); 0.2 ml ATP (84 m*M*); 0.02 ml acetate kinase (20 U); and 0.13 ml water. Control received 0.15 ml water in place of the enzyme. The reaction was performed at 35°C in a water bath for 30 min; 1 ml of 10% TCA was added, followed by 4 ml of ferric chloride (60 m*M*), and the solution was read at 540 nm.
[c] Vanillin oxidase was assayed by decrease in OD at 278 nm. The reaction mixture contained 1 ml vanillin (10 μmoles), 1 ml enzyme, and 1 ml phosphate buffer 0.025 *M*, pH 7.2.
[d] Vanillate-*o*-demethylase: see *Materials and Methods*.
[e] Protocatechuate 3,4-dioxygenase was measured by decrease in OD at 290 nm.

pressed in terms of μmoles protocatechuic acid disappearing per min per mg protein.

Results

Bacillus subtilis utilized ferulic acid, vanillin, vanillic acid, and protocatechuic acid as sole carbon source, and growth was influenced by concentration. Ferulic acid supported the growth of *B. subtilis* up to 10 m*M*, and at 20 m*M* the growth rate declined (Fig. 1a).

Vanillin, 5 m*M*, favored maximum growth. After a lag of 8 h, the maximum growth was attained at 40 h (Fig. 1b). Increasing the concentration to 10 m*M* inhibited growth, and 20 m*M* was toxic. The lag period was prolonged at a concentration of 10 m*M*.

Increasing the concentration of vanillic acid from 1 m*M* to 10 m*M* increased the growth of *B. subtilis*, but 20 m*M* reduced it (Fig. 1c).

Protocatechuic acid was readily utilized by *B. subtilis*. After a lag of 8 h, maximum growth occurred at 32 h. Protocatechuic acid at 20 m*M* was the optimum concentration (Fig. 1d). Growth rate and yield of the cells were high in protocatechuic acid compared with other phenols. All the phenols were toxic at 40 m*M*.

Enzymes involved in ferulic acid dissimilation. Side-chain removal was the initial reaction employed by *B. subtilis* in the degradation of ferulic acid. Therefore, the enzyme deacetylase, involved in the removal of the side chain, was assayed. Only those cells from the ferulic acid culture released 6.98 μmoles of acetate from 10 μmoles of ferulic acid/mg protein (Table 1). These cells also contained vanillin oxidase (which converts vanillin to vanillic acid), vanillate-*o*-demethylase (which demethylase vanillic acid to form protocatechuic acid), and protocatechuate 3,4-dioxygenase (which cleaves protocatechuic acid).

Inducible nature of enzymes

Deacetylase. Ferulic acid (5 m*M*)-grown cells exhibited deacetylase activity, but cells grown on vanillin, vanillic acid, or protocatechuic acid lacked the enzyme activity (Table 1).

Vanillin oxidase. Both ferulic acid and vanillin induced vanillin oxidase, but vanillin was the best inducer (Table 1). Induction by ferulic acid resulted in about half the enzyme activity induced by vanillin.

*Vanillate-*o*-demethylase.* Vanillic acid was the best inducer of vanillate-*o*-demethylase (Table 1). Vanillin and ferulic acid also induced the enzyme, but to reduced levels.

Protocatechuate 3,4-dioxygenase. Protocatechuate 3,4-dioxygenase was induced by ferulic acid and its intermediates (Table 1). Vanillic acid was the best

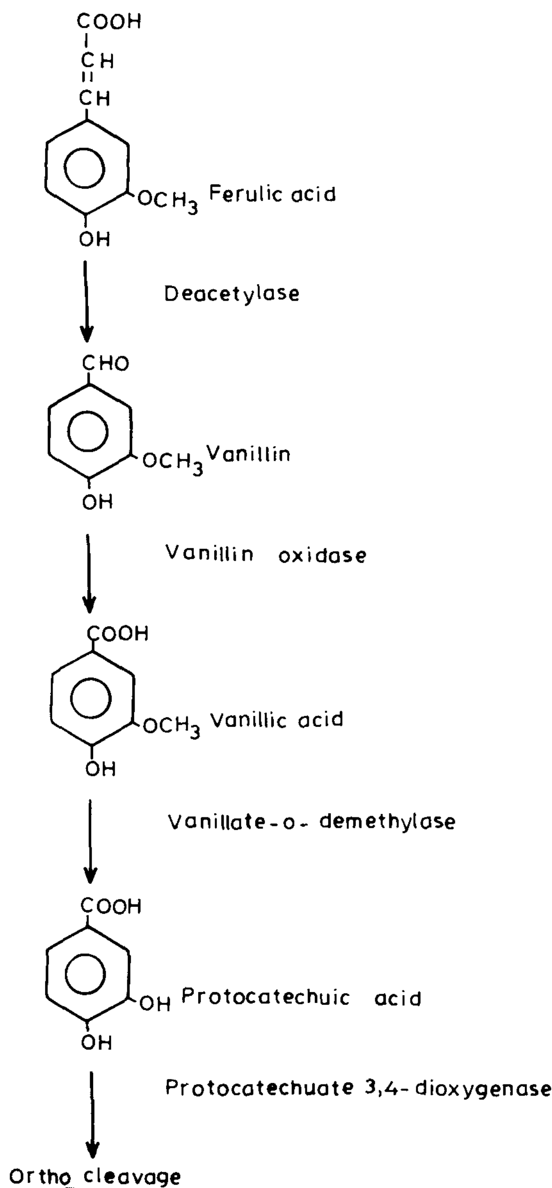

Fig. 2. Proposed pathway for the degradation of ferulic acid by *Bacillus subtilis*.

inducer, followed by protocatechuic acid. Vanillin and ferulic acid were poor inducers.

Glycerol-grown cells lacked enzyme activity.

Differential induction of enzymes involved in ferulic acid dissimilation. Bacillus subtilis showed significant differences in the utilization of ferulic acid and its intermediates. The optimum concentration of the substrate to support growth differed significantly; for ferulic and vanillic acids, it was 10 m*M*, 5 m*M* for vanillin, and 20 m*M* for protocatechuic acid (Fig. 1).

Ferulic acid. The enzyme extract prepared from the cells grown on 5 m*M* ferulic acid released 6.98 μmoles acetate/mg protein. Increasing the concentration of ferulic acid did not increase the release of acetate (Table 2). Cells grown on 10 m*M* ferulic acid exhibited high levels of vanillin oxidase and protocatechuate,3,4-dioxygenase. Moderate levels of vanillate-*o*-demethylase were estimated in 5 m*M* and 10 m*M* ferulic acid-induced cells, and high activity was recorded in cells grown in 20 m*M*.

Vanillin. Cells grown on vanillin (5 m*M*) did not contain any deacetylase, but exhibited maximum activity of vanillin oxidase, vanillate-*o*-demethylase, and protocatechuate 3,4-dioxygenase. However, enzyme levels declined in cells grown on 10 m*M* of vanillin (Table 2).

Vanillic acid. The maximum activity of vanillate-*o*-demethylase was recorded in cells grown on 5 m*M* vanillic acid, and higher concentrations did not enhance the induction. However, cells grown on 10 m*M* vanillic acid contained significant levels of protocatechuate 3,4-dioxygenase. These cells contained neither deacetylase nor vanillin oxidase (Table 2).

Protocatechuic acid. Significant increase in protocatechuate 3,4-dioxygenase activity was recorded with increase in the concentration of protocatechuic acid (Table 2). It did not induce deacetylase, vanillin oxidase, and vanillate-*o*-demethylase involved in ferulic acid dissimilation.

Discussion

Bacillus subtilis utilized ferulic acid, vanillin, vanillic acid, and protocatechuic acid as sole carbon source and employed the following reaction mechanisms in the dissimilation of ferulic acid: (a) shortening of side chain, (b) oxidation of aromatic aldehyde to acid, (c) demethylation, (d) hydroxylation, and (e) aromatic ring cleavage (Fig. 2). Side-chain removal is the first step in the cleavage of ferulic acid; the two carbons of the side chain were removed as acetate, resulting in the formation of the aromatic aldehyde, vanillin.

The conversion of ferulic acid proceeds through one of two pathways. By β-oxidation, cinnamic derivatives are converted to the corresponding benzoic acid derivative [16]. The side chain of these derivatives is shortened to the corresponding benzaldehydes [8, 15].

Acetic acid is released from the side chain of

Table 2. Differential induction of enzymes involved in ferulic acid dissimilation[a]

Inducer (mM)	Specific activity			
	Deacetylase	Vanillin oxidase	Vanillate-*o*-demethylase	Protocatechuate 3,4-dioxygenase
Ferulic acid				
5	6.98	0.278	9.09	0.260
10	7.50	0.329	9.15	0.310
20	6.05	0.228	10.84	0.198
Vanillin				
5	—	0.505	11.63	0.302
10	—	0.380	9.93	0.279
Vanillic acid				
5	—	—	13.07	0.545
10	—	—	12.70	0.645
20	—	—	11.64	0.493
Protocatechuic acid				
5	—	—	—	0.324
10	—	—	—	0.553
20	—	—	—	0.656

[a] Assay conditions were as mentioned in the footnotes of Table 1.

ferulic acid, measured with an exogenous supply of acetate kinase. The aldehyde, vanillin, was further oxidized to vanillic acid by vanillin oxidase. *Streptomyces viridosporus* oxidized aromatic aldehydes to substituted benzoic acids by aromatic aldehyde oxidase [3]. Vanillic acid was demethylated to protocatechuic acid by *Streptomyces* [13, 14]. Protocatechuic acid was cleaved by an *ortho* pathway enzyme, protocatechuate 3,4-dioxygenase.

The enzymes involved in ferulic acid dissimilation were inducible, and the maximum activity was usually influenced by the concentration of the inducer and its chemical nature.

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Literature Cited

1. Andreoni V, Galli E, Galliani G (1984) Metabolism of ferulic acid by a facultatively anaerobic strain of *Pseudomonas cepacia*. System Appl Microbiol 5:293–304
2. Crawford DL, Crawford RL (1980) Microbial degradation of lignin. Enzyme Microb Technol 2:11–22
3. Crawford DL, Sutherland JB, Pometto AL (1982) Production of an aromatic aldehyde oxidase by *Streptomyces viridosporus*. Arch Microbiol 131:351–355
4. Dye DW (1958) Host specificity in *Xanthomonas*. Nature 182:1813–1814
5. Fujisawa H, Hayaishi O (1968) Protocatechuate 3,4-dioxygenase I. Crystallization and characterization. J Biol Chem 243:2673–2681
6. Gurujeyalakshmi G (1986) Degradation of lignin substructure model substances by *Bacillus subtilis*. Doctoral Thesis, p 173
7. Holz G, Bergmeyer HU (1974) Determination (of acetate) with acetate kinase and hydroxylamine. In: Bergmeyer HU (ed) Methods of enzymatic analysis, vol 3, pp 1528–1532
8. Karanth NGK, Reber H (1979) Regulation of the utilization of 4-hydroxybenzoate and 4-hydroxycinnamate in batch and continuous cultures of *Pseudomonas testosteroni*. Arch Microbiol 120:97–103
9. Kawakami H (1980) Degradation of lignin-related aromatics and lignins by several pseudomonas. In: Kirk TK, Higuchi J, Chang HM (eds) Lignin biodegradation; microbiology, chemistry and potential applications. Boca Raton, Florida: CRC Press, vol 2, pp 103–125
10. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurements with Folin phenol reagent. J Biol Chem 193:265–275
11. Muthukumar G, Mahadevan A (1983) Microbial degradation of lignin. J Sci Ind Res 42:518–528
12. Nair PM, Vaidyanathan CS (1964) A colorimetric method for determination of pyrocatechol and related substances. Anal Biochem 7:315–321
13. Sutherland JB, Crawford DL, Pometto AL (1981) Catabolism of substituted benzoic acids by *Streptomyces* species. Appl Environ Microbiol 41:442–448
14. Sutherland JB, Crawford DL, Pometto AL (1983) Metabolism of cinnamic, *p*-coumaric and ferulic acids by *Streptomyces setonii*. Can J Microbiol 29:1253–1257
15. Toms A, Wood JM (1970) The degradation of *trans*-ferulic acid by *Pseudomonas acidovorans*. Biochemistry 9:337–343
16. Vollmer KO, Resener HJ, Grisebach H (1965) The formation of acetic acid from *p*-hydroxy cinnamic acid during its degradation to *p*-hydroxybenzoic acid in wheat shoots. Biochem Biophys Res Commun 21:221–225