Biodegradation of aromatic carboxylic acids by *Pseudomonas mira*

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Abstract: Biodegradation of aromatic acids (ferulic, vanillic and sinapinic acids) by the soil bacterium *Pseudomonas mira* was studied by high-pressure liquid chromatography. The presence of glucose in the culture medium slowed down the degradation process but did not affect its mechanism. In addition to vanillic acid and hydroquinone, the products of degradation were found to include acetophenone derivatives. Probably, a mechanism capable of shortening the side chain by spontaneous decarboxylation of unstable 3-*keto*-3-phenylpropionic acid was present, in addition to the elimination of acetic acid via degradation of the cinnamic acid-type compounds.

Key words: Pseudomonas mira; Biodegradation; Ferulic acid; Sinapinic acid; Vanillic acid; Acetophenone derivative

Introduction

Biodegradation of aromatic compounds by soil bacteria (*Pseudomonas, Cellulomonas, Achromobacter*) was studied as early as 20 years ago by Kunc [1–3], who measured the consumption of oxygen by oxidation of the added aromatic compounds manometrically.

Vaughan and Butt [4] analysed bioconversion of *p*-coumaric acid, resulting in the formation of caffeic acid. The hydroxylase enzyme responsible for the conversion of cinnamic to *p*-coumaric acid was extensively studied by Hill and Rhodes [5] and Potts et al. [6]. In 1984, an enzyme catalyzing hydroxylation of ferulic acid to 5-hydroxyferulic acid was isolated by Grand [7].

The shortening of the side chain of cinnamic acids by elimination of the acetic acid molecule (so-called β-oxidation of cinnamic acids) was described by Kindl [8] and the corresponding enzyme responsible for this reaction had been characterized by Toms and Wood [9] when studying bioconversion of ferulic acid by *Pseudomonas acidovorans*. The shortening mechanism of the side chain of cinnamic acids was defined only recently by Hilton and Cain [10].

Materials and Methods

Microorganism

Strain V2 of *Pseudomonas mira* used for cultivation was obtained from the laboratory collec-

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tion of the Department of Ecology of the Institute of Microbiology, Czech Academy of Sciences, Prague, Czech Republic.

Medium

The microorganism was grown in a volume of 100 ml of either the medium I or II, having the following composition: Medium I (g/l) – $(NH_4)_2HPO_4$ 0.5, K_2HPO_4 0.4, $MgSO_4$ 0.1, NaCl 0.2, $FeCl_3$ 0.02, pH adjusted to 7.0. Medium II – 1.5 g of glucose per litre was added to medium I.

The microorganism was grown in the presence of one of the following aromatic acids: ferulic acid (0.1 or 0.5 g/l), vanillic acid (0.2 or 0.5 g/l) and sinapinic acid (0.5 g/l).

Cultivation

Inoculum. The bacterium was cultured on agar nutrient medium slants (Difco, State) enriched with vanillin (0.5 g/l) at 28°C for 24 h. After washing twice with a sterile Clark-Lubs buffer, pH 7 (29.5 ml 0.1 M NaOH and 50 ml 0.1 M KH_2PO_4, diluted with distilled water to a total volume of 100 ml) and centrifugation at 10 000 rpm, the bacteria were suspended in the same buffer to prepare an inoculum.

Cultivation and sampling. A volume of 100 ml of medium in 500-ml flasks was inoculated with 1 ml of bacterial culture to obtain the original absorbance value of about 0.1. The flask cultures were agitated on a reciprocal shaker (1.1 Hz) at 28°C.

Bacterial growth

5-ml samples were removed at time intervals (0–74 h) during cultivation. A 0.15-ml volume of 0.15 M sodium azide was added to the samples to prevent further growth. Half of each sample was centrifuged and the supernatant used as the blank value to measure the absorbance of the bacterial sample.

Absorbance measurement and glucose estimation

A UV-VIS Aerograph Variscan spectrophotometer (Varian AG, Zug, Switzerland) was used to measure the absorbance values. Samples removed from the cultures grown in the presence of ferulic and vanillic acids were measured at 540

nm; those from the cultures in which sinapinic acid was present, at 660 nm.

Glucose was estimated by using the glucose oxidase method (Oxochrom-glucose agent, Lachema, Brno, Czech Republic) [11].

Chemicals

Ferulic acid (Schuchardt, München, FRG) vanillic acid (Koch-Light, Colnbrook, UK) and sinapinic acid (Aldrich, Beerse, Belgium) that were used as the standards were analytical grade. The chromatographically pure 4-hydroxy-3-methohyacetophenone was obtained from the Institute of Biochemistry and Physiology of Microorganisms (Pushchino na Oke, CIS). The other chemicals, glucose, Oxochrom-glucose agent, ethanol for UV spectrometry, methanol for UV spectrometry, acetic acid and inorganic salts were analytical grade as supplied by Lachema, Brno, Czech Republic.

High-performance liquid chromatography

The aromatic carboxylic acids were determined by reverse HPLC method [12].

The identification of aromatic compounds in the mixture during the separation analysis was carried out both chromatographically by comparing their retention data with those of the pure standards and spectrophotometrically by Diode array detector Waters 990 (DAD). In case of some compounds, their structure either had to be verified or their presence identified by using another spectroscopic method like mass spectrometry.

Aromatic carboxylic acids present in the mixture were determined by a calibration method.

Preparative HPLC

Sample preparation. All 5-ml samples of the culture medium removed during cultivation that contained unknown compounds were combined and dried under vacuum. The dry sample was then dissolved in a volume of the mobile phase.

Compound isolation. 50-μl samples were repeatedly injected by using a 100-μl Hamilton syringe onto a metal semipreparative column (250 × 8 I.D.) filled with Separon SGX C18 (7 μm). Fractions containing unknown compounds were

collected during elution. Twenty fractions were combined and dried at 37°C under vacuum. The dry sample was then analysed by mass spectrometry to identify the unknown compound.

Mass spectrometry

Mass spectra were measured by using a Finnigan Mat 90 apparatus under the following conditions: energy, 70 eV; ionizing electrons, 1 mA; temperature of the ionizing source, 250°C; acceleration voltage, 5 kV; high-resolution measurement, error 5 ppm.

Results and Discussion

Vanillic acid was found to be a main product of bioconversion during cultivation of *Pseudomonas mira* V2 on both types of the medium containing ferulic acid. Other products of biodegradation in the medium containing a greater amount of ferulic acid (0.5 g/l) were represented by traces of hydroquinone and an unknown compound having a retention time (t_R) of 2.9 min.

Vanillic acid and hydroquinone were identified chromatographically on the basis of comparison of their retention data with those of the chemical standards and also by analysis of UV-spectra of

the compounds. An unknown compound $(t_R = 2.9)$ was isolated by chromatography on a semipreparative column and, after the excess mobile phase was evaporated at 37°C, the compound was identified by mass spectrometry to be an isomer of methoxyacetophenone.

Kinetics of the formation of the methoxyacetophenone isomer was expressed as a time function of the absorbance values measured at a wavelength of 274 nm. Table 1 summarizes the data concerning the kinetics of biodegradation of ferulic acid, formation of the corresponding degradation products and the bacterial growth in a medium containing 0.5 g/l of ferulic acid. The presence of glucose did not elicit any change in the metabolic pathway of ferulic acid.

The unknown compound of the acetophenone type was thought to be a degradation product formed either from vanillic acid or directly from ferulic acid by a different reaction mechanism. Therefore, other cultivations were carried out where vanillic acid was added to the medium.

Hydroquinone, an expected degradation product, was detected in the two media during the whole course of cultivation. A very low response of the UV-detector, as registered by the computer during the analyses, suggested the presence of this compound at extremely low concentrations. A subsequent DAD analysis confirmed the

Table 1

Growth of *Pseudomonas mira* and concentrations of aromatic compounds in the medium

Time (h)	Without glucose				With glucose				
	Ferulic acid (g/l)	Vanillic acid (g/l)	Isomer of methoxy-acetophenone (A_{274})	Growth (A_{540})	Ferulic acid (g/l)	Vanillic acid (g/l)	Isomer of methoxy-acetophenone (A_{274})	Glucose (g/l)	Growth (A_{540})
0	0.552	0.005	–	0.085	0.571	0.002	0.002	1.42	0.093
2	0.534	0.002	0.005	0.092	0.595	0.003	0.003	1.27	0.101
4	0.507	0.013	0.006	0.099	0.552	0.007	0.002	1.28	0.105
6	0.494	0.010	0.005	0.104	0.578	0.011	0.008	1.26	0.110
8.5	0.484	0.011	0.010	0.111	0.572	0.020	0.011	1.32	0.114
19	0.352	0.052	0.030	0.115	0.463	0.034	0.022	1.32	0.111
27	0.224	0.100	0.050	0.119	0.385	0.062	0.030	1.30	0.110
42.5	0.066	0.159	0.056	0.119	0.207	0.146	0.057	1.24	0.108
49.25	0.053	0.169	0.063	0.120	0.099	0.201	0.065	1.23	0.105
67	0.009	0.194	0.066	0.123	0.060	0.205	0.072	1.23	0.109

After 27 h of cultivation, traces of hydroquinone were detected but its concentrations were not quantified.

presence of hydroquinone on the basis of a similarity of the retention time compared to the standard and of the characteristics of the UV spectra. Hydroquinone can be a reaction product from biodegradation of vanillic acid. The formation of the product requires that the benzene ring become demethoxylated. The resulting p-hydroxy-derivative can be easily decarboxylated to produce hydroquinone [13]. The concentration of vanillic acid in the medium significantly decreased during cultivation. In addition to the minor amount of hydroquinone, which could not be considered the final product of biodegradation of vanillic acid, other compounds were evidently formed by splitting the aromatic ring. First, a compound containing conjugated double bonds [13] should have probably been formed, whose absorption maximum, calculated according to the Fieser-Woodward rules [14,15], was at a wavelength shorter than 250 nm. As no such compound having this absorption maximum in the UV-region was identified by DAD detection, the compound formed was assumed to immediately undergo further reactions in which the system of the conjugated double bonds was destroyed.

Thus, the assumption that the isomer of methoxyacetophenone was derived from ferulic acid was confirmed. This conclusion is in agreement with the latest data [10] whose analysis led

to a discovery and confirmation of a new metabolic pathway of cinnamic acids (Fig. 1).

The intermediate, a derivative of 3-keto-3-phenylpropionic acid, is a rather unstable compound capable of a spontaneous decarboxylation. After a short time, it can no longer be detected in the sample. A shift of the methoxy group on the benzene ring (methylation and demethoxylation) can take place simultaneously with the reactions on the side chain of ferulic acid. The envisaged demethoxylation of the benzene ring is in accordance with published results [16].

If the bacterium Pseudomonas mira was grown in the presence of sinapinic acid, we found that this compound could be used as the sole carbon source. An addition of glucose into the medium did not result in any change of the metabolic pathway of sinapinic acid. As compared to ferulic acid, the consumption of sinapinic acid was slower. With both acids, however, the decrease in the concentrations of the original aromatic acids was slowed down when glucose was added to the medium.

The only product of degradation of sinapinic acid that could be detected, 4-hydroxy-3-methoxyacetophenone, was identified on the basis of the corresponding retention time and the UV-spectrum characteristics compared to those of the chemical standard. The compound struc-
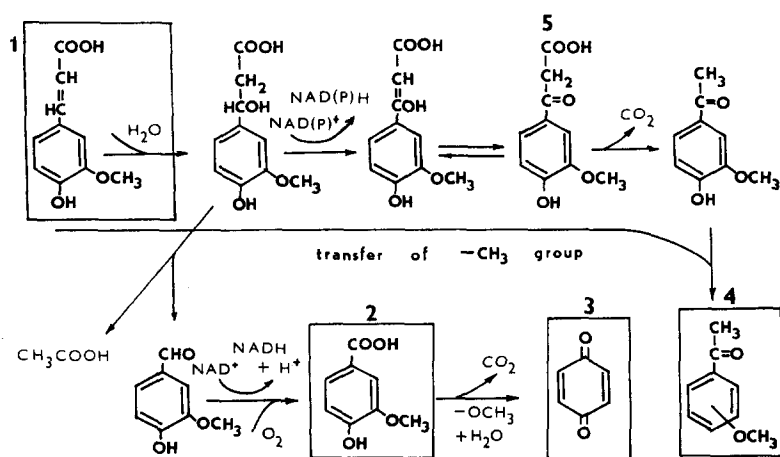

Fig. 1. Reaction scheme of decomposition of ferulic acid by Pseudomonas mira. 1, ferulic acid; 2, vanillic acid; 3, hydroquinone; 4, methoxy derivative of acetophenone; 5, derivative of 3-keto-3-phenylpropionic acid. Compounds in boxes were detected in culture medium.
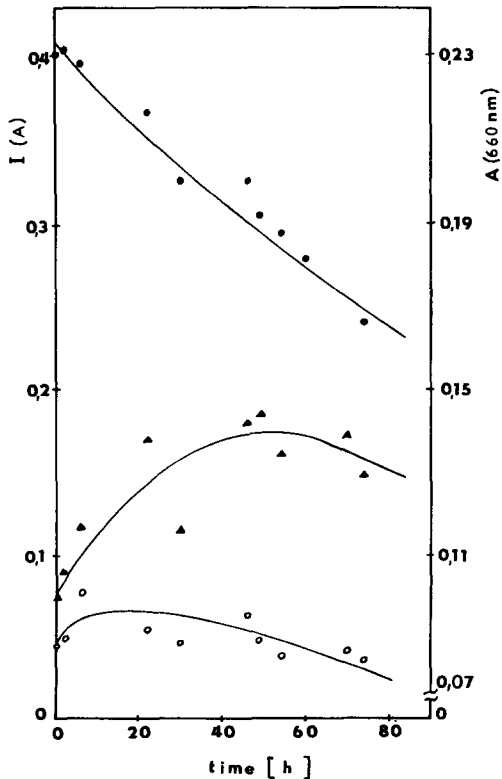
Fig. 2. Growth in the presence of sinapinic acid (0.5 g l^{-1}) at 28°C. (○) Growth curve (A_{660}). Concentrations of the components of the degradation mixture are expressed as the response of EC detector: (●) sinapinic acid, (▲) 4-hydroxy-3-methoxyacetophenone.

Decarboxylation is a process preferentially adopted by *Pseudomonas mira* V2 to decompose sinapinic acid. However, participation of another pathway including the formation of syringic acid cannot be excluded since even this compound can be immediately metabolized.

The soil bacterium *Pseudomonas mira* V2 was grown in a liquid medium with or without glucose in the presence of ferulic, vanillic or sinapinic acids. The bacterium was found to be capable of using the above-mentioned aromatic acids as the sole carbon source for growth. The addition of glucose into medium resulted in a slower consumption of the aromatic acids. The inhibitory effect of glucose on the enzymes responsible for catabolism of the aromatic compounds was thus established.

The effect of glucose on the decomposition of aromatic acids can, however, be different in various bacteria. In *Cellulomonas*, Kunc and Kotyk described a stimulation of the uptake of vanillic acid into the cell and the subsequent degradation of the compound in the presence of glucose [2,17].

Our results suggest an involvement of the glucose effect. The enzymes capable of splitting the aromatic compounds were synthesized by the bacterium as a result of the induction during the time the bacterial cultures were maintained on the media to which vanillin had been added. According to the principle of 'sequential induction', the bacteria can adapt themselves to vanillin and its metabolic products [13].

ture was confirmed by measuring the mass spectrum. The use of DAD connected with an electrochemical detector (EDLC) in series was found suitable to detect the compound. The EDLC detector exhibited a higher sensitivity as compared to the UV-detector and was also selective for this type of hydroxylated compounds. Though the response of the UV-detector to the degradation product was ten times weaker as compared to sinapinic acid, the responses of the electrochemical detector to sinapinic acid and the product tested were of the same order of magnitude. The time courses of the decrease in the concentration of sinapinic acid and of the formation of 4-hydroxy-3-methoxyacetophenone are shown in Fig. 2.

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