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Biodegradation of Cypermethrin by *Micrococcus* sp. strain CPN 1

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Abstract A bacterium capable of utilizing pyrethroid pesticide cypermethrin as sole source of carbon was isolated from soil and identified as a *Micrococcus* sp. The organism also utilized fenvalerate, deltamethrin, perimethrin, 3-phenoxybenzoate, phenol, protocatechuate and catechol as growth substrates. The organism degraded cypermethrin by hydrolysis of ester linkage to yield 3-phenoxybenzoate, leading to loss of its insecticidal activity. 3-Phenoxybenzoate was further metabolized by diphenyl ether cleavage to yield protocatechuate and phenol as evidenced by isolation and identification of metabolites and enzyme activities in the cell-free extracts. Protocatechuate and phenol were oxidized by *ortho*-cleavage pathway. Thus, the organism was versatile in detoxification and complete mineralization of pyrethroid cypermethrin

Keywords Biodegradation · Cypermethrin · *Micrococcus* sp. strain CPN 1 · 3-Phenoxybenzoate · Protocatechuate

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Introduction

Cypermethrin[(+/−)-α-cyano-3-phenoxybenzyl(+/−)-*cis*,*trans*-3(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate] is a synthetic pyrethroid pesticide, widely used to control pests in cotton and vegetable crops. It is highly toxic to fish and aquatic invertebrates (Bradbury and Coats 1989). Cypermethrin has been classified as a possible human carcinogen by US Environmental protection agency (EPA). It adversely affects the central nervous system and cause allergic skin reactions and eye irritation. The oral LD_{50} in rats in 250 mg/kg. The microorganisms play a significant role in detoxifying pesticides in the environment. There are few reports on the degradation of pyrethriod insecticides in soils (Kaufman et al. 1981; Roberts and Standen 1977, 1981). However, much less is know about the pathway for the degradation of pyrethriod cypermethrin in different microorganisms. In this paper, we describe the isolation and characterization of a *Micrococcus* sp. that degrades cypermethrin.

Materials and methods

Chemicals

Cypermethrin, fenvalerate, deltamethrin and permethrin were obtained from Rallis India Ltd., Mumbai. 3-Phenoxybenzoic acid, 3-phenoxybenzaldehyde, protocatechuic acid, catechol, phenol, 4-

hydroxy-3-phenoxybenzoic acid and 2,3-benzoic acid were purchased from Sigma-Aldrich, USA. All other chemicals were of analytical grade.

Organism and growth conditions

The organism was isolated from pesticide-contaminated soil by an enrichment culture technique. It was grown on Seubert's mineral salts medium (Seubert 1960) containing 0.1% (wt/vol) cypermethrin as sole source of carbon, in 500 ml Erlenmeyer flask on a rotary shaker (150 rpm) at room temperature. Growth was measured turbidometrically at 660 nm. The cultures were maintained on Cypermethrin- mineral salts agar slants.

The identification of the Cypermethrin-degrading organism was done on the basis of its morphological, cultural and physiological characteristics. The biochemical tests were carried out according to Pelczar (1957) and Holding and Collee (1971). DNA isolation and determination of its G + C content from melting temperature was done as described by Marmur (1961) and Mandel and Marmur (1968).

Oxygen uptake studies

The oxygen uptake by whole cells of *Micrococcus* sp. grown on cypermethrin was performed in an Oxygraph fitted with Clark type Oxygen electrode (Hansatech, Germany). The cells were harvested in the early logarithmic phase by centrifugation at 12,000 × *g* for 20 min and washed twice with 0.05 M phosphate buffer pH 7.0. Oxygen uptake rates are expressed as nmol of O_2 consumed min^{-1} mg^{-1} of dry cells. The values were corrected for endogenous respiration.

Isolation and identification of metabolites

The metabolites were isolated from culture filtrate of the organism grown on cypermethrin (0.1% wt/vol) by extraction with ethylacetate before and after acidification to pH 2 with 2 N HCl and the residue obtained was dissolved in methanol. The residues were analyzed for metabolites by Thin layer chromatography on Silica gel G plates using the following solvent systems. (A) Chloroform-acetic acid (95:5 v/v), (B) Chloroform-acetic acid (98.5:1.5 v/v) and (C) Toluene-diethylether-acetic acid (75:25:1 v/v). The metabolites were visualized under UV light at 254 nm or by exposure to iodine vapours and also by spraying with 1% $FeCl_3$-$K_3Fe(CN)_6$ solution in water. Phenolic compounds gave blue colour on spraying with Folin–Ciocalteu's phenol reagent and bluish-green colour with 100 mM $FeCl_3$, which turned red on exposure to ammonia. Aldehydes were detected spraying with solution of 2,4-dinitrophenylhydrazine (0.1%) in 2 M HCl. UV-visible absorbance spectra were recorded with 'Hitachi' 150–20 spectrophotometer. The mass spectra were recorded with Jeol, MS-DS operated at 70 ev. Metabolites were analyzed by reversed phase HPLC, using a 5-μ spherisorb-ODS (C18) column (25 cm × 4.6 mm) acetronitrile and phosphate buffer (50 mM, pH7.0) as the mobile phase at a flow rate of 1 ml min^{-1}. Peaks were detected at 254 nm or scanned at 220–400 nm in a spectrophotometer.

Enzyme assay

Cell free extracts were prepared from the washed cells suspended in three volumes of 50 mM phosphate buffer, pH 7.0 by sonication (ultrasonic processor model XL 2010) for 5 min and centrifugation at 10,000 × *g* for 40 min at 4°C. The clear supernatant was used as crude extract for enzyme assays.

Esterase activity was assayed spectrophotometrically by measuring the decrease in absorbance at 233 nm due to disappearance of substrate, cypermethrin. 3-Phenoxybenzaldehyde dehydrogenase activity was assayed spectrophotometrically by increase in absorbance at 340 nm, owing to the formation of NADPH. 3-Phenoxybenzoate dioxygenase activity was assayed spectrophotometrically by measuring the increase in absorbance at 295 nm. Phenol hydroxylase activity was assayed according to Neujahar and Gaal (1973). Protocatechuate-3, 4-dioxygenase activity assayed according to MacDonold et al. (1954). Catechol-1, 2-dioxygenase activity was assayed according to Hayaishi et al. (1957). Catechol-2, 3-dioxygenase activity was assayed by the method of Kim et al. (1992). Protein was determined by the method of Lowry et al. (1951). One unit of enzyme activity was defined as the amount required to catalyze the formation or consumption of 1 μmol of product or substrate per min.

Results

Characterization of organism

Cypermethrin-degrading strain CPN 1 an aerobic, Gram-positive coccus that was catalase and oxidase positive. The organism reduced nitrate, hydrolyzed gelatin and casein, assimilated acetate, citrate, succinate, benzoate and oxalate, did not produce acid from glucose, lactose and sucrose. There was no hydrolysis of starch and no DNase activity. The strain was able to grow in medium containing 5% NaCl. The G + C content of DNA from the bacterial strain was found to be 68.8 moles%. Thus according to Bergey's Manual of Determinative Bacteriology, 9th edition (Holt 1993), the strain CPN 1 was tentatively identified as *Micrococcus* sp.

Growth on various aromatic compounds

The *Micrococcus* sp. strain CPN 1 utilized pyrethriod pesticides cypermethrin, fenvalerate, deltamethrin and perimethrin, phenol, 3-phenoxybenzoate, protocatechuate and catechol as growth substrates. The growth of *Micrococcus* sp. strain CPN 1 on cypermethrin (0.1% wt/vol) as the sole carbon source is shown in Fig. 1. The organism utilized cypermethrin at 1 g/l as the sole energy source.

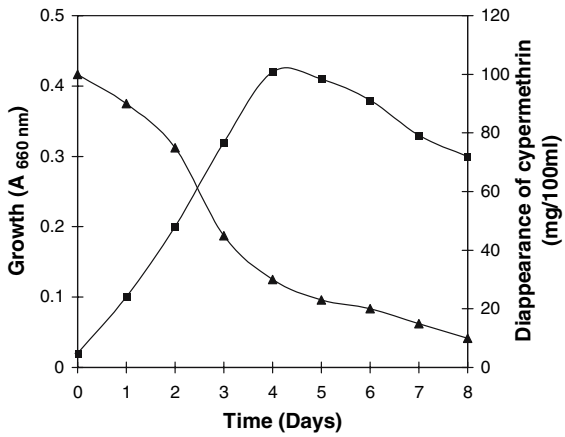

Fig. 1 Utilization of Cypermethrin (▲-▲) during growth (■-■) of *Micrococcus* sp.strain CPN 1

Identification of metabolites

The analysis of the culture extracts of *Micrococcus* sp. strain CPN 1 grown on cypermethrin by Thin layer chromatography (TLC) revealed presence of three compounds I, II and III. The Rf values of compounds I, II and III corresponded with those of authentic 3-phenoxybenzoate, protocatechuate and catechol respectively (Table 1.). These compounds were purified by preparative TLC and analyzed by HPLC. Mass spectra of isolated compound I corresponded well with that of authentic 3-phenoxybenzoate.

Oxidation of metabolites by whole cells

The whole cells of *Micrococcus* sp. strain CPN 1 grown on cypermethrin readily oxidized cypermethrin, fenvalerate, 3-phenoxybenzaldehyde, 3-phenoxybenzoate, protocatechuate, phenol and catechol, but glucose-grown cells failed to oxidize any of these compounds. On the other hand, the compounds such as 4-Hydroxy-3-phenoxybenzoate and 2,3-dihydroxybenzoic acid were not oxidized by the organism (Table 2.)

Enzyme activities in cell-free extracts

The cell-free extracts of *Micrococcus* sp. grown on cypermethrin contained the activities of esterase, 3-phenoxybenzaldehyde dehydrogenase, 3-phenoxybenzoate dioxygenase, phenol hydroxylase, protocatechuate-3,4-dioxygenase and catechol-1,2-dioxygenase but not catechol-2,3-dioxygenase (Table 3).The cell-free extracts of glucose-grown cells did not contain any of these enzyme activities. These results have indicated that the enzymes of cypermethrin degradation were induced by growth of the organism on cypermethrin.

Discussion

It is evident from the results that the organism degraded cypermethrin by initial hydrolysis of its ester linkage to yield 3-phenoxybenzoate. The hydrolysis of ester linkage destroys the insecticidal activity of cypermethrin leading to its detoxification. 3-Phenoxybenzoate was further metabolized by diphenylether cleavage, to yield protocatechuate and

Table 1 Chromatographic and spectral properties of metabolites of cypermethrin by *Micrococcus* sp. CPN 1

Property		Isolated compound I	Authentic 3-Phenoxy benzoic acid	Isolated compound II	Authentic protocatechuic acid	Isolated compound III	Authentic catechol
1.	TLC: R_f values different in solvents						
	A	0.61	0.61	0.09	0.09	0.26	0.26
	B	0.48	0.48	0.28	0.28	0.38	0.38
	C	0.35	0.35	0.12	0.12	0.20	0.20
2.	Melting point (°C)	147°C	147°C	202°C	202°C	104°C	104°C
3.	HPLC: retention time (min)	1.6	1.6	2.17	2.17	1.97	1.97
4.	UV absorption λ_{max} in methanol (nm)	215, 292	215,292	260,295	260,295	221,278	221,278

[a] Solvent systems A, B and C are as described in materials and methods

Table 2 Oxidation of various compounds by cells of *Micrococcus* sp. strain CPN 1 grown on cypermethrin

Substrate (1 μmol)	Oxygen uptake[a] (nmoles $min^{-1}mg^{-1}$ of dry cells)
Cypermethrin	60
3-Phenoxybenzaldehyde	34
3-Phenoxybenzoic acid	50
Protocatechuic acid	45
Phenol	70
Catechol	80
4-Hydroxy-3-phenoxybenzoic acid	–
2,3-Dihydroxybenzoic acid	–

[a] The values are corrected for endogenous respiration rates

Table 3 Specific activities of enzymes in the cell-free extract of *Micrococcus* sp. strain CPN 1 on Cypermethrin

Enzymes	Specific activity (Units/mg of protein)
Esterase	0.100
3-Phenoxybenzaldehyde dehydrogenase	0.273
3-Phenoxybenzoate dioxygenase	0.341
Phenol hydroxylase	0.514
Protocatechuate-3, 4-dioxygenase	0.649
Catechol-1, 2-dioxygenase	0.678
Catechol- 2, 3-dioxygenase	0.00

phenol. The oxygen uptake and enzymatic studies have confirmed that 3-phenoxybenzoate, protocatechuate,phenol and catechol are the intermediates in the degradation of cypermethrin by *Micrococcus* sp. strain CPN 1. The presence of high activities of protocatechuate-3,4-dioxygenase and catechol-1,2-dioxygenase in the cypermethrin-grown cells has indicated that protocatechuate and catechol were further oxidized through *ortho*-cleavage pathway. The proposed pathway for the degradation of cypermethrin by *Micrococcus* sp. strain CPN 1 is shown in Fig. 2.

The initial reactions involved in the degradation of cypermethrin to 3-phenoxy benzoate in *Micrococcus* sp. strain CPN 1 appears to be similar to that described in other bacteria (Kaufman et al. 1981; Roberts and Standen 1977, 1981). However, the further metabolism of 3-phenoxybenzoate in *Micrococcus* sp. strain CPN 1 differs from that in other organisms. 3-Phenoxybenzoate was metabolized to 4 -hydroxy-3-phenoxybenzoic acid in *Bacillus cercus*, *Pseudomonas fluorescen* and *Achromobacter* sp. (Maloney et al. 1988) and in soil bacteria (Roberts and Standen 1981). On the other hand, *Micrococcus* sp. strain CPN 1 metabolized 3-phenoxy benzoate to protocatechuate and phenol, similar to that reported in *Pseudomonas pseudoalcaligenes* POB310 (Halden et al. 1999, 2000).

Thus, the *Micrococcus* sp. strain CPN 1 was versatile in complete biodegradation of the toxic pyrethriod pesticide cypermethrin in the environment.

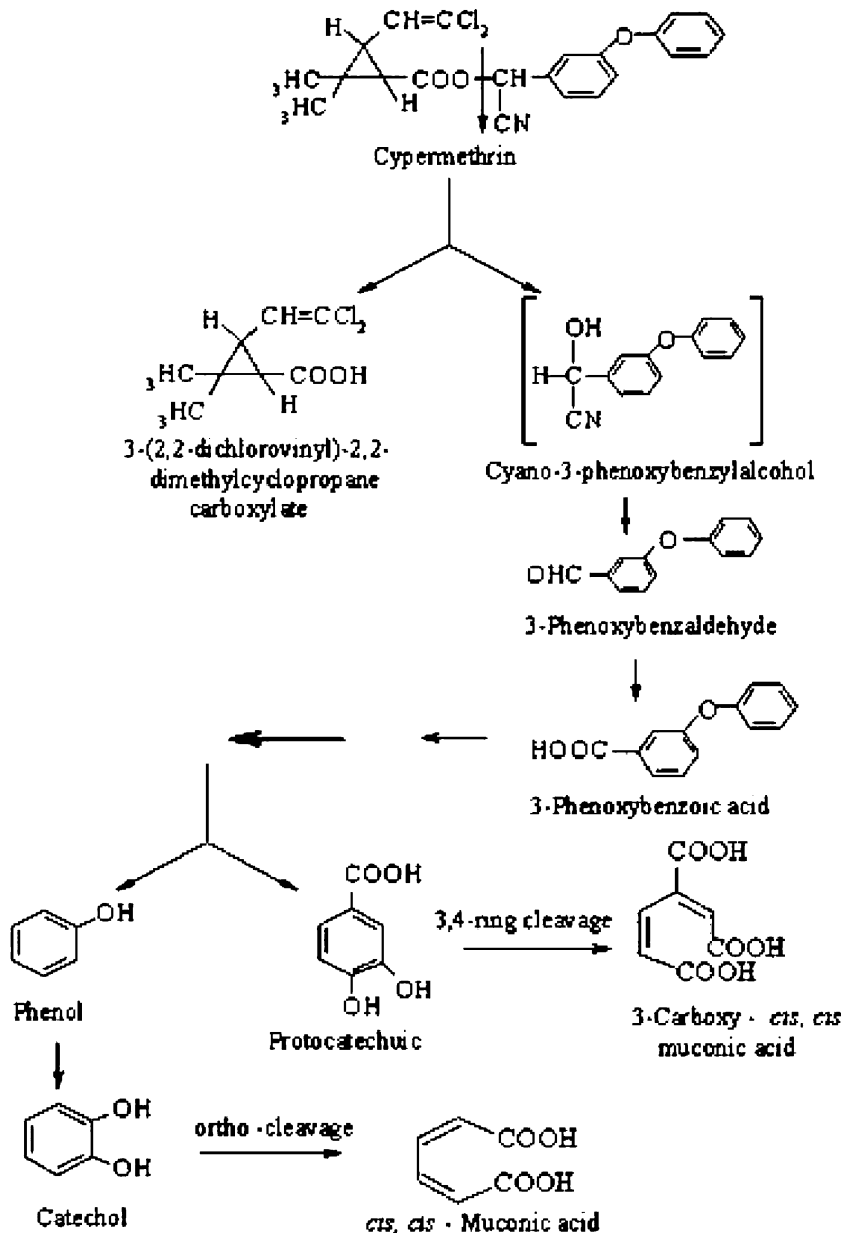

Fig. 2 Proposed pathway for the degradation of Cypermethrin by *Micrococcus* sp.strain CPN 1

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