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Biodegradation of beta-cyfluthrin by *Pseudomonas stutzeri* strain S1

Nirmali Saikia[1], Subrata K. Das[2], Bharat K.C. Patel[3], Ram Niwas[1], Aqbal Singh[4] & Madhuban Gopal[1,*]
[1]*Division of Agricultural Chemicals, Indian Agricultural Research Institute, New Delhi 110 012, India;* [2]*Department of Biotechnology, Institute of Life Sciences, Nalco Square, Bhubaneswar 751 023, India;* [3]*School of Biomolecular and Biomedical Sciences, Faculty of Science and Technology, Griffith University (Nathan Campus), Brisbane, Australia 4111;* [4]*National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute, New Delhi 110 012, India*
*(*author for correspondence: e-mail: madhubangopal@hotmail.com)*

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Abstract

β-Cyfluthrin [α-cyano-4-fluoro-3-phenoxybenzyl-3(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate] pesticide has been in agricultural use in the recent years for controlling *Lepidopteran* pests affecting solanaceous crops. The extensive use of synthetic pyrethroids like β-cyfluthrin has resulted in wide spread environmental contamination. The purpose of this study was to isolate bacteria from soil and to determine their ability to degrade β-cyfluthrin and identify the intermediates in culture broth using spectroscopy. An aerobic bacterium capable of degrading β-cyfluthrin was isolated by enrichment culture. The 16S ribosomal DNA sequence of the isolate (strain S1) had 100% identity to the sequence from *Pseudomonas stutzeri*. Finally products formed during degradation of β-cyfluthrin have been identified as α-cyano-4-fluoro-3-phenoxybenzyl-3(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate (M.W. 341); 4-fluoro-3-phenoxy-α-cyanobenzyl alcohol (M.W. 243) and 3(2,2-dichlorovinyl)-2,2-dimethyl cyclopropanecarboxylic acid (M.W. 208).

Introduction

β-Cyfluthrin [α-cyano-4-fluoro-3-phenoxybenzyl-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-carboxylate] is a synthetic pyrethroid (Figure 2a) consisting of four active stereoisomers of the parent compound cyfluthrin (Leicht et al. 1996). This photostable synthetic pyrethroid has an ester and an ether linkage besides dichlorovinyl group attached to a cyclopropane moiety. Special feature of this compound is the presence of a carbon–fluorine bond that helps in overcoming resistance developed in insect by the use of xenobiotics (Naumann 1998). While the uses of organochlorine pesticides have declined, the use of synthetic pyrethroid has increased with time and they represent the most popular synthetic insecticides. The use of synthetic pyrethroid in India has increased by 42% during last 5 years.

Generally synthetic pyrethroids are recommended against *Lepidopteran* pests affecting solanaceous crops (Sinha & Gopal 2002). Various studies have demonstrated that synthetic pyrethroids are more efficient in controlling insect pest menace (Gopal et al. 1987) and have a favorable persistence profile than conventional insecticides (Mukherjee & Gopal 1992). β-Cyfluthrin has been tested against various pests of okra (Dikshit et al. 2002), cotton (Mukherjee et al. 2001), cabbage and mustard (Gopal et al. 2002b). Persistence of β-cyfluthrin on various crops has been studied and the pesticide though effective for controlling the insect pest leaves residues on vegetables that are harvested almost daily and some are even consumed raw. The presence of toxic residues over maximum residue limit (MRL: 0.1–0.5 mg/kg) of this insecticide in fruit, vegetables and green leaves is of concern to the human health (Codex Alimentarius Commission

2004). It therefore becomes imperative that each pesticide schedule on edible crops should be investigated for quantification of amount of residue.

Simple culinary process was tested for decontamination of β-cyfluthrin and was found quite efficient for day 0 and day 1 sample in reducing the pesticide residues from fruits. The limewater soaking was found better for decontamination of eggplant fruits than simple washing with tap water (Sinha & Gopal 2002). However, these treatments were not very effective at the later stage as the insecticide, though not a systemic insecticide, penetrated slightly in the skin of eggplant fruit. Therefore, an efficient method for detoxification of this compound is needed. There are only few reports on biodegradation of synthetic pyrethroids such as β-cyfluthrin by *Trichoderma viride* (Saikia & Gopal 2004; Sinha et al. 2002) and cypermethrin by *Pseudomonas fluorescens* (Grant & Betts 2003, 2004; Grant et al. 2002; Sakata et al. 1992). Preliminary studies with known microorganisms namely *Bacillus subtilis*, *Bacillus polymyxa, Klebsiella planticola* and *Proteus vulgaris* have shown maximum degradation of this compound up to 37.0% (Gopal et al. 2002a; Saikia 2003). Therefore, selection of a suitable microorganism was necessary for improving the level of degradation and detoxification of this pesticide.

To our knowledge, degradation of β-cyfluthrin by a soil bacterium has not been reported earlier. This paper describes the enrichment, isolation and characterization of a *Pseudomonas stutzeri* strain S1 able to degrade β-cyfluthrin. In addition, the products formed after degradation of β-cyfluthrin have been identified by spectroscopy.

Materials and methods

Chemicals and media

Analytical sample of β-cyfluthrin was procured gratis from Bayer India Ltd. All other chemicals used were analytical grade and purchased from E. Merck, Darmstadt, Germany and SRL Pvt. Ltd., Bombay, India.

Mineral salts medium that contained (in gram per liter) 2.0 g Na_2HPO_4, 0.75 g KH_2PO_4, 0.5 g $MgSO_4.7\ H_2O$, 1.0 g NH_4Cl was used for the isolation of bacterial strain. The pH was adjusted to 7.0 and β-cyfluthrin (50 μg/ml in acetone) was added to the medium. Liquid media without β-cyfluthrin were sterilized by autoclaving at 120 °C for 20 min. β-Cyfluthrin was sterilized by membrane filtration (Pore Size, 0.22 μm., Millex GP., Millipore, Bedford, Mass.) and added to the medium aseptically. Utilization of organic compounds including amino acids was determined in mineral salts medium containing substrates at concentrations of 1–5 g/l. For solid media 15.0 g of purified agar-agar powder (Difco, USA) per liter was added.

Enrichment and isolation

A variety of soil samples were collected from different area of an agrochemical industry manufacturing β-cyfluthrin at Chandigarh, India. The soil samples (25 g) were moistened with 5 ml of sterile distilled water and enriched by adding solid analytical sample of β-cyfluthrin (50 mg). After thorough mixing, the preparations were incubated in Petri dishes at 28 °C for 30 days. The growth of bacterium varied in different solvents, and it was not affected by acetone; β-cyfluthrin (5 mg/ml) stock solution was therefore made in acetone. Further enrichment was carried out in mineral salts broth (pH 7.0) supplemented with β-cyfluthrin (50 μg/ml). One gram of the enriched soil samples was transferred into 250 ml conical flasks containing 50 ml of enrichment medium. Cultures were incubated at 28 °C for 3 days in shaker (Adolf Kuhner AG, ISF-1-V, Schweiz) at 200 rpm. After three subsequent transfers into the same medium, serial dilutions of the samples of the flask were plated onto mineral salts agar plates supplemented with β-cyfluthrin (50 μg/ml) for isolation of individual colonies. Isolates exhibiting distinct colonial morphologies were isolated by repeated streaking on the same agar medium. Strain isolated from enrichment cultures were designated as S1 and ISD20.

Characterization and identification of isolates

Isolates exhibiting growth on β-cyfluthrin in pure cultures were characterized and identified using various biochemical tests and procedures as described (Cruickshank et al. 1975; Stolp & Gadkeri 1981). Further characterization of strains to genus or species level was performed by 16S rDNA gene analysis. DNA was prepared as described (Connolly & Patel 2002). The methods used for 16S rRNA gene amplification and sequencing has been

reported previously (Andrews & Patel 1996). Sequences generated during this work were assembled into one complete sequence using Bioedit (Hall 1999). The consensus sequence was manually corrected for errors and the most homologous sequences determined against the Gene Bank database using BLAST (Altschul et al. 1997) and using the 'Sequence Match' option against the Ribosomal Database Project (Maidak et al. 2001), were extracted and manually aligned. Sequence uncertainties were omitted from the analysis and phylogeny from 1322 unambiguous nucleotides determined as described previously (Andrews & Patel 1996).

Biodegradation test

Degradation studies were carried out at 28 °C in mineral salts medium containing β-cyfluthrin (50 μg/ml) taking three replicates. The effect of glucose on biodegradation of β-cyfluthrin was tested by supplementing (0.5%) glucose to the above medium. The bacterial growth was indicated by increase in turbidity in mineral salt media. At different time period's cultures were harvested for the determination of β-cyfluthrin consumption.

Isolation and characterization of β-cyfluthrin and its biodegraded products

TLC analysis
Cultures grown in mineral salts medium containing β-cyfluthrin was extracted with hexane. The extracts were dried over anhydrous sodium sulfate. A crude sample thus obtained was analyzed by thin layer chromatography on silica gel G plate using acetone: hexane (1:4 v/v) as the developing solvent. The plates were visualized under UV lamp and R_f value of each compound was recorded. Purification of individual metabolites was done by preparative TLC method using the glass plates of size 20 × 20 cm coated with silica gel G (1000 μm layer) and using the same developing solvent.

GLC analysis
Consumption of β-cyfluthrin with time period was carried out by gas liquid chromatograph using a Hewlet Packard instrument (Model 580, Series II) equipped with a Ni^{63} electron capture detector and an auto injector system connected to a HP computer (Model Vectra 05/165). The stationary phase consisted of HP-1 coated in a megabore column (10 m in length, 0.53 mm in diameter). The temperature of column, injector and detector were 250, 270 and 300 °C, respectively. The mobile phase was nitrogen gas maintained at a flow rate of 15 ml per minute. Two microliter samples were injected for the analysis.

Residue analysis and recovery

The media containing β-cyfluthrin was extracted, after addition of water saturated with sodium chloride, with hexane (3 × 15 ml). The organic solvent was passed through anhydrous sodium sulfate and evaporated to dryness. The residue was dissolved in hexane and subjected to GLC analysis, as given above. Efficiency of extraction and estimation of β-cyfluthrin was over 90%. It was found by fortifying mineral salt media with β-cyfluthrin at two concentrations 1 and 5 μg/ml.

^{1}HNMR
^{1}HNMR of β-cyfluthrin and its metabolites were obtained in a Varian Model EM-368L, 60 MHz instrument using a continuous wave mode. The samples were dissolved in deuterochloroform and tetramethyl silane was used as internal standard.

FD-MS
FD-MS of β-cyfluthrin and its metabolites were carried out in a Micromass Autospecs Ultima E Field-Desorption Mass Spectrometer under a calibration range of 50–500 da.

IR
An infrared spectrum was recorded on a Nicolett Fourier Transform Infra-red spectrophotometer (Model, Impact 400).

Nucleotide sequence accession number

The 16S rDNA sequence for strain S1 has been deposited in the Gene Bank under accession number AY485995.

Results and discussion

Identification of bacterial isolates

Bacterial strain obtained was a Gram negative, motile and rod shaped. Colonies on nutrient agar

plate of strain S1 were circular, smooth, round and creamy brown color. This is of the same morphology as described for *Pseudomonas stutzeri* (Palleroni et al. 1970; Stanier et al. 1966). Growth occurred in nutrient broth medium within a pH range of 6.5–9.0 with an optimum at pH 7.0. Strain S1 exhibited catalase, oxidase, urease, starch hydrolysis and nitrate reductase activities and could grow on Simmons citrate agar and MacConkey agar. Arginine dihydrolase, methyl red test, aesculin hydrolysis, Voges-Prosakauer, gelatin hydrolysis, indole production, H_2S production, phenylalanine deaminase and pigment production tests were negative. Strain S1 grew heterotrophically in mineral salts medium supplemented with glucose, sucrose, maltose, erythritol, mannitol, succinate, malate, fumarate, pyruvate, citrate, proline, leucine, tyrosine, glycine, alanine, glutamic acid, threonine and isoleucine. Fructose, arabinose, xylose, ribose, galactose, lactose, arabitol, dulcitol, adonitol, sorbitol, methanol, acetone, hexane, chloroform, serine, valine, histidine, lysine, methionine, arginine, phenylalanine, tryptophane, hydroxyproline, cysteine and aspartic acid did not support the growth of this organism. Analysis of the 16S rDNA gene sequences indicated that strain S1 was related to *Pseudomonas stutzeri* cluster within the radiation of genus *Pseudomonas* (*sensu stricto*) with the closest relative being *Pseudomonas stutzeri* genovar 7 (100% sequence identity).

Degradation of β-*cyfluthrin*

Time course of degradation of *β*-cyfluthrin by the strain S1 is presented in Figure 1. Result shows that about 94% degradation was observed with in 8 days. Plotting log of concentration of remaining *β*-cyfluthrin versus time drew straight lines. There is thus biphasic dissipation during the biodegradation of *β*-cyfluthrin by the strain S1. The graph shows rapid degradation of *β*-cyfluthrin during first 3 days, after a lag phase of one day, recording a half-life of 0.66 day without any additional organic source and later 16.95 day for the slower phase. The two-regression equations are $y = 2.4111 - 0.4529x$ (for initial fast dissipation; 1–2 days) and $y = 1.0533 - 0.0177x$ (for slow dissipation from third day onwards).

Elucidation of the structure of the unknown metabolites

Metabolite produced by the bacterial degradation of *β*-cyfluthrin was separated by thin layer chromatography. The R_f values of three metabolites were 0.79, 0.46 and 0.11 (Table 1) whereas the R_f value of the parent compound was 0.51. Two

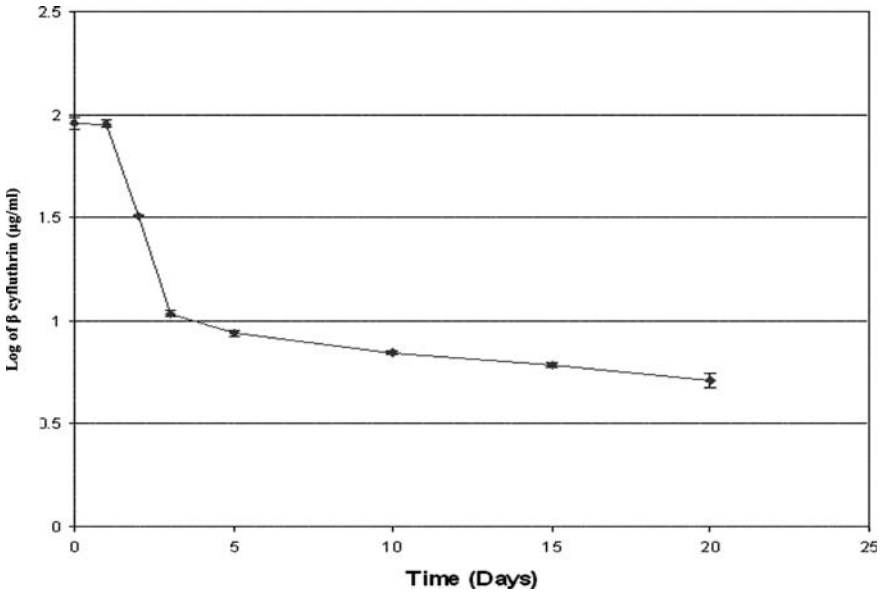
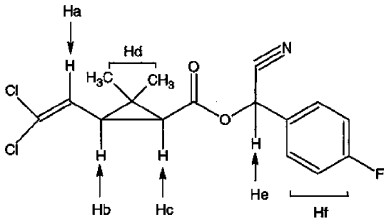
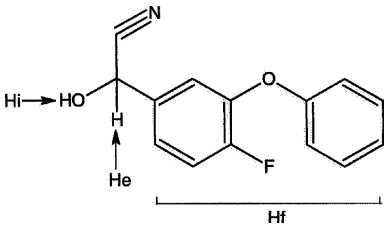
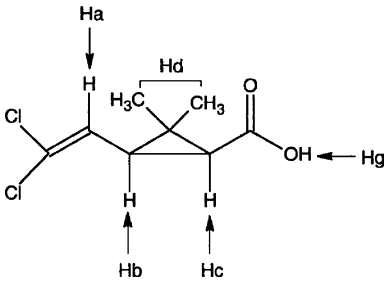


Figure 1. Time course of degradation of *β*-cyfluthrin by the strain S1 in mineral salts medium.

Table 1. Metabolites isolated during the degradation of β-cyfluthrin

R_f	Product no.	Chemical structure	Chemical name
0.79	I		α-cyano-4-fluorobenzyl-3(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate
0.46	II		α-cyano-4-fluoro-3-phenoxy benzyl alcohol
0.11	III		3(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid

additional spots below that of β-cyfluthrin (R_f 0.51) were of product II (R_f 0.46) and product III (R_f 0.11).

The formation of cyanohydrin (M.W. 243) and cyclopropane carboxylic acid derivative (M.W. 208) proves the breaking of ester bond linkage of the parent compound by the bacterium. It suggests that the bacterium contains enzymes having esterase activity. Cleavage of ether linkage resulted into the product I (M.W. 341), which was most non-polar and showed R_f of 0.79. NMR (Table 2) and FD-MS (Figure 2) confirmed the identity of these metabolites. In NMR spectra of this compound, four aromatic protons (δ 6.8–7.2) were present, suggesting a loss of the phenyl ring from the parent compound (M.W. 433). In the case of β-cyfluthrin

Table 2. ^{1}HNMR of β-cyfluthrin and its degradation products

Type of proton	δ- value			
	β-cyfluthrin	Product I	Product II	Product III
H_a	5.5 (1H)	5.5 (1H)	–	5.5 (1H)
H_b	1.6 (1H)	1.6 (1H)	–	1.6 (1H)
H_c	2.2 (1H)	2.1 (1H)	–	2.1 (1H)
H_d	1.2 (6H)	1.1 (6H)	–	1.1 (6H)
H_e	6.3 (1H)	6.3 (1H)	–	–
H_f	6.8–7.4 (8H)	6.8–7.2 (4H)	6.7–7.3 (8H)	–
H_g	–	–	–	10.0 (1H)
Hi	–	–	9.2–9.8 (1H)	–

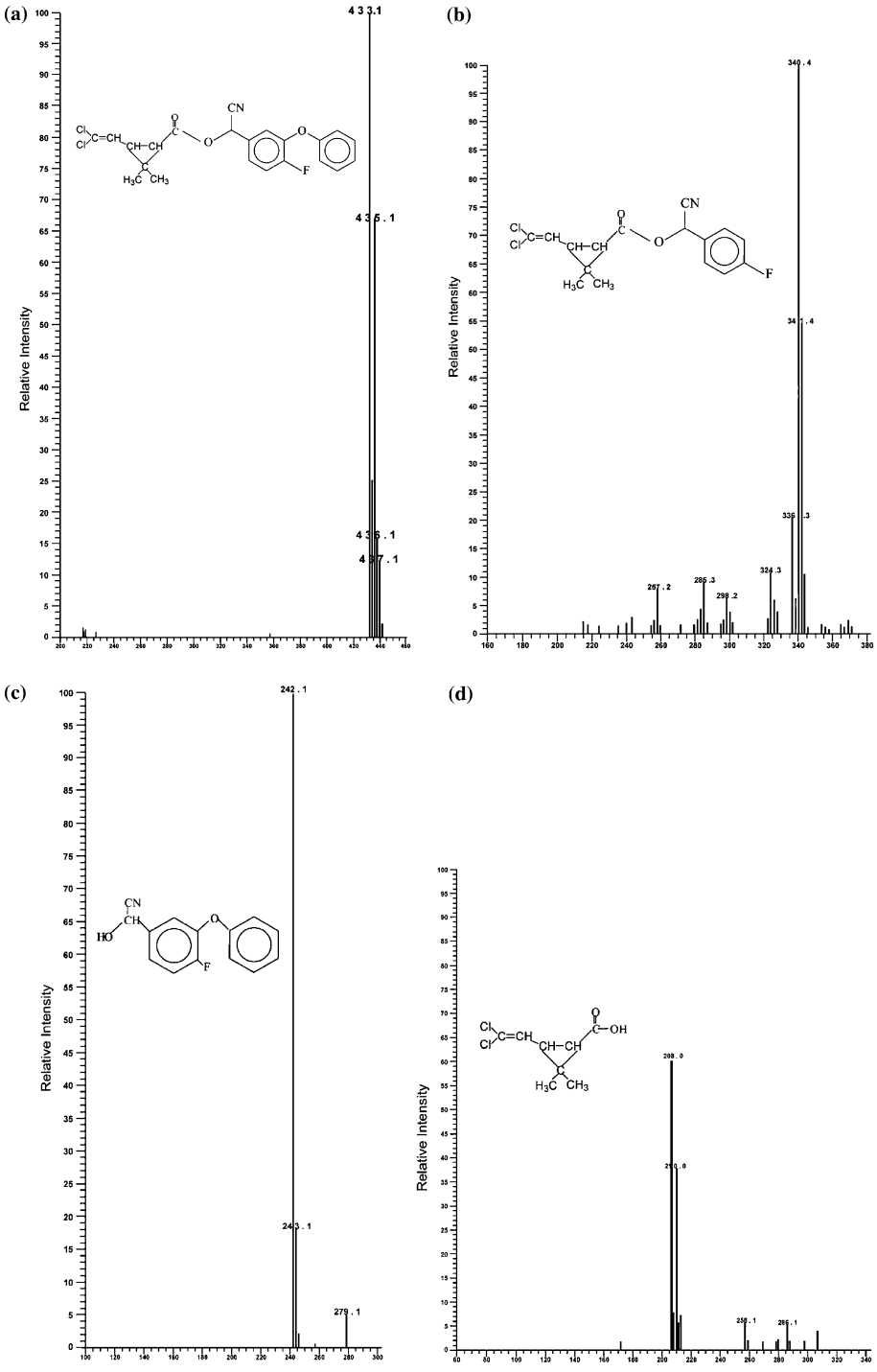


Figure 2. FD-MS spectra of the metabolites produced during β-cyfluthrin degradation using *Pseudomonas stutzeri* strain S1. (a) β-cyfluthrin (M.W. 433); (b) α-cyano-4-fluorobenzyl-3(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate (M.W. 341); (c) 4-fluoro-3-phenoxy-α-cyanobenzyl alcohol (M.W. 243) and (d) 3(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (M.W. 208).

(Figure 2a), the H_a proton was deshielded to δ 5.5 due to the magnetic anisotropy of vinylic group. Six protons from the two-methyl groups gave a singlet at 1.1 δ as H_d and protons due to H_b and H_c

present in cyclopropane ring gave two different singlet at δ 1.6 and 2.1 δ, respectively. H_e proton gave a singlet at δ 6.3 due to its direct bonding to electronegative oxygen and cyano group. Product I showed similar chemical shift as that of β-cyfluthrin (Table 2).

The NMR of metabolite (III) showed the presence of a proton around δ 10.0, which could be exchanged by D_2O during NMR, proving that the product is an acid. Presence of C=O was confirmed by IR, which showed a band at 1696.79 cm^{-1} and broad OH stretch at 2500–3500 cm^{-1}. It confirmed the breaking of ester bond present in β-cyfluthrin. The NMR of product II is relatively simple as it exhibited 8 protons in the aromatic region (δ 6.7–7.3) besides a proton deshielded due to its attachment to electro negative oxygen atom and inductive effect of adjacent cyano group. This product is not otherwise formed on carrying out the alkaline hydrolysis of the synthetic pyrethroid. The formation of cyanohydrin (M.W. 243) and cyclopropanecarboxylic acid derivative (M.W. 208) proved the cleavage of ester bond by the bacterium resulting in to detoxification of the insecticide. Although recovery of ^{14}C from the culture broth was over 90% during incubation of cypermethrin, degradation products retaining the ester linkage were not detected (Sakata et al. 1992). Major metabolite found in the culture broth was a hydrolysis product (1RS)-*cis*, *trans*-3-(2,2-dichlorovinyl)-2,2-dimethyl cyclopropane carboxylic acid. The R_f is 0.46 in n-hexane/toluene/acetic acid (3:15:2; v/v) and 0.33 in *n*-hexane/acetone (8:2; v/v) for the carboxylic acid. The R_f of the parent compound cypermethrin was 0.63 and 0.54 in respective solvent systems, implying that the major product formed is more polar than the parent compound. Khan et al. (1988) also reported that the main degradation product of another synthetic pyrethroid deltamethrin was 3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane carboxylic acid.

The product III (M.W. 208) formed is already documented as non-toxic Farka acid (Naumann 1998). Authentic data about toxicity of fluorinated analogue of *m*-phenoxybenzaldehyde is not available. Taking analogy from cypermethrin, it is inferred that fluorinated analogue of m-phenoxybenzaldehyde will also be less toxic than the parent compound β-cyfluthrin [LD_{50} (oral, rat) = 500 mg/kg (in polyethylene glycol) and it is 270 mg/kg (in xylene)]. Toxicity of m-phenoxybenzaldehyde is documented in www.parijattaagrochemicals.com/Metaphenoxy.htm as LD_{50} (oral, Rat) = 1500 mg/kg, whereas for cypermethrin it is 250 mg/kg. This clearly shows that the degradation product is less toxic than the parent synthetic pyrethroid.

Isolating the individual compound by preparative TLC and also subjecting the crude to FD-MS confirmed the identity of left over parent compound and the degradation products (Fig-

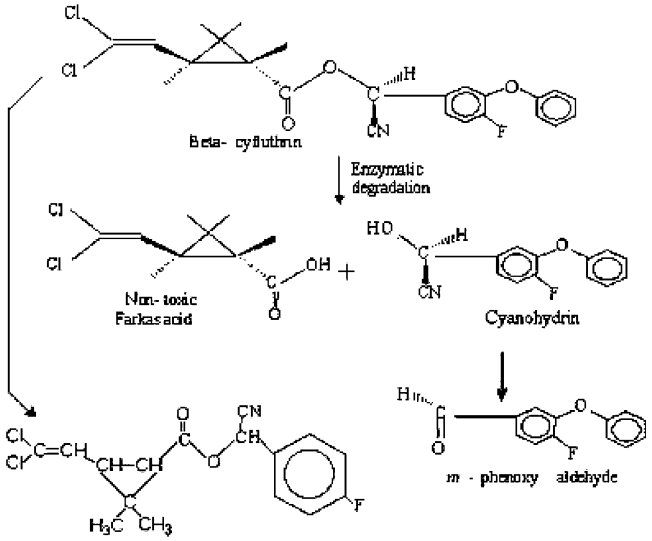


Figure 3. Proposed pathways for degradation of β-cyfluthrin by the strain S1.

ures 2a, b, c and d). The presence of a product having molecular weight (341) in FD-MS is more significant as ether bonds (C–O–C) are not easily cleaved even with chemical agents (White et al. 1996). It has also been documented that most soil bacteria have non-specific esterase so carbon ester compounds are known to undergo hydrolysis in soil (Kim et al. 2003; Shaw et al. 1980). It is interesting to note that both ester and ether bonds were cleaved by this bacteria. The product formed from ether cleavage is a new metabolite. The possible reason for cleavage of ether bond of β-cyfluthrin by the strain S1 may be due to the involvement of cytochrome P450 (Strauber et al. 2003). The breakdown pathway of β-cyfluthrin is summarized in Figure 3.

In conclusion, β-cyfluthrin besides being degraded to carboxylic acid and cyanohydrin also yield an ether cleavage product [having R_f higher (0.79) than the parent compound (0.51) in acetone: hexane 1:4 v/v solvent system]. In both the study hydroxylated products, formed by Fenton's oxidation were not present.

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