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# Biodegradation of Endosulfan by a Soil Bacterium

H. M. Shivaramaiah[1,2] and I. R. Kennedy[2]

[1]Food Protectants & Infestation Control Department, Central Food Technological Research Institute, Mysore, India
[2]Faculty of Agriculture, Food, and Natural Resources University of Sydney, Sydney, Australia

A bacterium capable of metabolizing endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepine3-oxide) was isolated from cotton-growing soil and effectively shown to degrade endosulfan into endosulfan sulfate. The bacterium degraded 50% of the compound within 3 days of incubation. Endosulfan sulfate was the only terminal product and no other metabolites were formed during the incubation. Endosulfan and its metabolites were analyzed by gas chromatography. The metabolites formed indicated that the organism follows an oxidative pathway for metabolism of this pesticide. Therefore, the present study, microbial degradation of endosulfan by a soil bacterium, may provide a basis for the development of bioremediation strategies to remediate the pollutants in the environment.

*Key Words:* Endosulfan; Microbial degradation; Cotton; Soils.

## INTRODUCTION

Chlorinated organochlorine pesticides are one of the major groups of chemicals responsible for environmental contamination.[1] Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepine3-oxide), an organochlorine insecticide, is a mixture of the two isomers, $\alpha$ and $\beta$-endosulfan. It is a contact and stomach poison, used to control chewing and sucking insects, such as Colorado beetle, flea beetle, cabbage worm aphids and leaf hopper.[2,3] This insecticide is used to control insect pests on a wide range of crops, including cereals, cotton, coffee, fruits, oil seeds and vegetables. It is the most widely used insecticide in Australia to control *Heliocoverpa* species in cotton cropping.[4] Endosulfan residues have been detected in the atmosphere, soils, sediments, surface water and foods.[5]

Address correspondence to Dr. H.M. Shivaramaiah, Department of Food Protectants and Infestation Control, Central Food Technological Research Institute, Mysore 570020, India; E-mail: Shivramaiah@yahoo.com

The traditional approach to studies of microbial biodegradation often involves batch enrichment cultures using the compound of interest as a substrate, followed by isolation of pure culture, to grow on pesticide as a sole source of carbon and energy.[6] During the 1980s, a considerable number of bacterial strains were isolated that possessed the ability to degrade chloroaromatic compounds.[7]

Endosulfan degradation is affected by environmental conditions. Endosulfan transformed by bacterial and fungal cultures to endosulfan diol and endosulfan sulfate through oxidation with small amounts of endosulfan hydroxyether and endosulfan lactone.[8] Miles and Moy[9] have proposed a pathway wherein endosulfan is converted to endosulfan sulfate followed by endosulfan diol, endosulfan hydroxyether and endosulfan lactone (Fig.1). Formation of these metabolites has been confirmed by other investigators also.[10,11]. More recently, Sutherland *et al.*,[12] isolated and characterized an endosulfan and endosulfan sulfate-degrading mycobacterium.

As part of the development of an overall strategy to manage organochlorine pesticide residues, the present study was aimed to isolate and characterize soil bacteria capable of degrading endosulfan. The nature of degradation and metabolism of endosulfan by the isolated bacteria were examined using gas chromatography for identification of breakdown products.

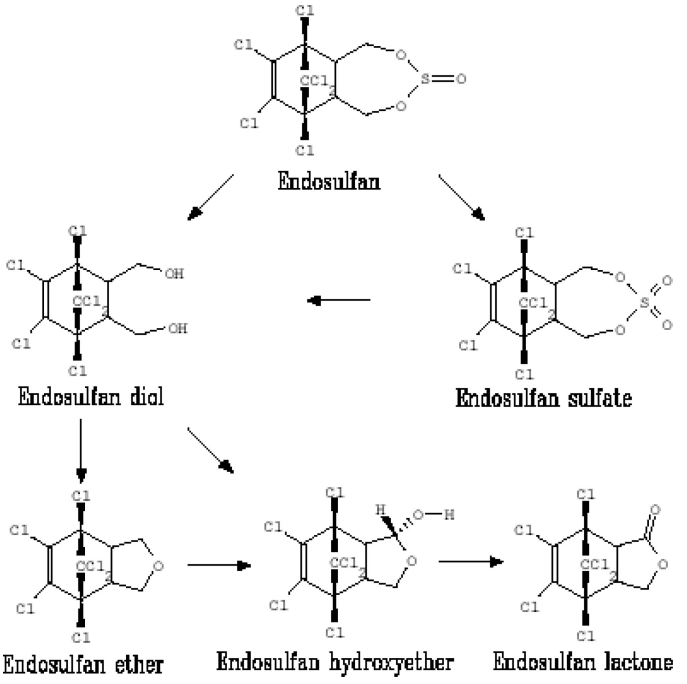


**Figure 1:** Schematic pathway of endosulfan degradation.

## MATERIALS AND METHODS

### Chemicals

All pesticide reference standards and metabolites were kindly provided by Hoechst, Germany. The pesticides and metabolites studied include endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepine3-oxide) isomers, endosulfan sulfate ($C_9H_8Cl_4S$), endosulfan diol ($C_9H_8Cl_6O$), endosulfan ether ($C_9H_6ClO_2$), endosulfan hydroxyether ($C_9H_4ClO_2$) and endosulfan lactone ($C_9H_4Cl_6O_2$). Anhydrous sodium sulfate and florisil (60–120 mesh) for column chromatography was purchased from E-Merck, Germany. Both sodium sulfate and florisil were dried in a muffle furnace at 550°C for 5 hours and stored in desiccators. The florisil was activated with distilled water (7%) prior to use. Solvents including hexane, acetone and methanol from Mallinckrodt were nanograde or HPLC grade. Agar nutrient broth, tryptone and peptone were purchased from Difco, Ltd, India..

### Soil Used for the Isolation of Degrading Bacteria

Soil used for the isolation of endosulfan-degrading bacteria was obtained from a cotton-cropping farm near Guntur, India. Surface soil (0–10cm) was removed using a spade and placed in plastic bags. The soil was transported to a laboratory, sealed in bags and stored at 4°C in a refrigerator until use.

### Soil Enrichment Technique for Isolation of Endosulfan-Degrading Bacteria

Soil samples taken by a core sampler from the top 10 cm of field plots were air dried to 20% (w/w) moisture content and passed through a sieve with 2 mm mesh. Fifty grams of each sample was placed in six glass dishes (5 cm deep × 9.5 cm diameter) and stacked one above the other in a closed container to maintain constant moisture conditions. The soil samples were treated with an aqueous suspension of endosulfan ($\alpha$ and $\beta$ isomers) to give a final concentration of 1000 ppm in soil and the contents were mixed gently and incubated at room temperature (26°C) for two weeks. The moisture content of the soil was kept constant by adding distilled water (60%) to dishes every two days to obtain their original weight. The insecticide treatment was repeated three times at two-week intervals.

### Isolation of Endosulfan-Degrading Organisms

An enrichment culture technique was used to isolate endosulfan-degrading organisms from soil obtained from the premises Lam Agricultural Research

Station, Guntur, India. Pretreated soil (5g) was added to sterile distilled water. Pour plate method was used for the isolation of bacteria using nutrient agar medium (beef extract 3 g, peptone 5 g, dextrose 5 g, NaCl 5 g, Agar 15 g, distilled water 1litre, pH 7) and incubated at room temperature (26°C). Four strains were isolated and sub-cultured. Pure cultures were obtained by repeated subculturing. The strains were maintained on nutrient agar slants. The colony characters were identified based on the colony morphology and staining characters. During the course of the investigation all the strains were tested for their ability to degrade endosulfan using pure culture isolates. The isolates S1, S2 and S4 were discarded when they apparently lost their degrading ability during repeated subculturing. One isolate (S3) had a stable endosulfan-degrading ability and was used in the experiment.

## Bacterial Identification

Biochemical tests were performed with Biolog identification (Biolog, Hayward, California) by manually-reading the plates. The pure culture was grown on nutrient agar medium. Colonies were characterized by staining and also based on morphological characters. The bacterial strain was first identified by testing gram staining and found to be gram negative. On microscopic observation it was found that the cells occurred in short rods in pairs typical of *Bacillus* species (Figs. 2 and 3).

## Growth of Isolated Organisms

Pure culture studies of different isolates obtained from endosulfan-spiked soil samples were carried out. Isolates were grown in Burkes mineral medium (dipotassium orthophosphate 0.2 g ($K_2HPO_4$), potassium dihydrogen orthophosphate 0.8 g ($KH_2PO_4$), magnesium sulfate 0.2 g (Mg $SO_4$)-, calcium chloride 0.1 g, ($CaCl_2$ 6 $H_2O$), ammonium sulfate-0.1 g ($NH_4SO_4$), yeast

**Figure 2:** Typical growth of soil bacterium strain (83) on nutrient agar.

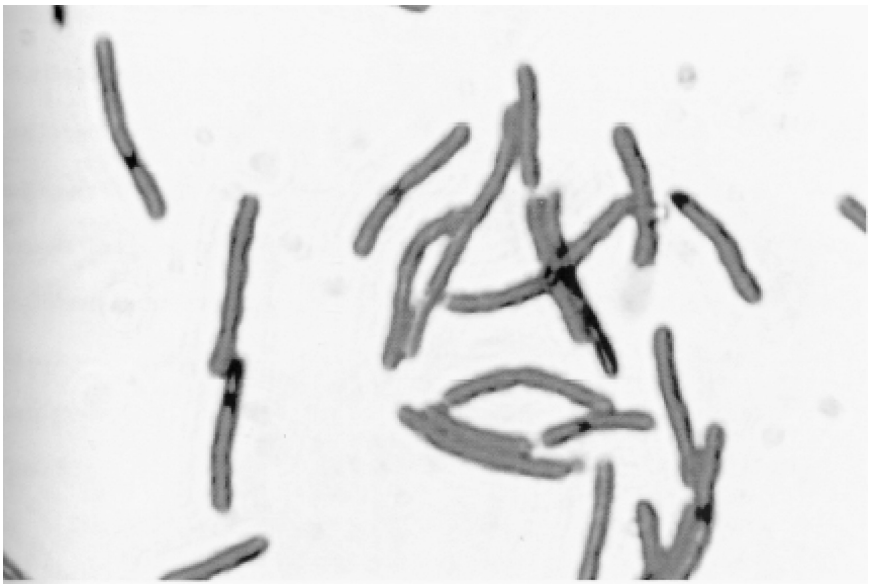

**Figure 3:** Bacteria (83) isolated from Indian soil.

extract-0.02% (0.2 g), and distilled water-1 litre, pH-7.6 glucose-1%). Each of the isolate was treated with different concentrations of carbon sources (i) endosulfan alone (20 ppm), (ii) endosulfan (50 ppm), (iii) endosulfan (20 ppm + glucose 0.1%), (iv) endosulfan (50 ppm + glucose 0.1%), (v) control. Growth studies were continued for 48 hrs.

## Measurement of Absorbance

The growth of bacteria in broths was measured by turbidity of the solution. A small volume of broth was placed in a 1.0 cm glass cuvette and its absorbance was read at 600 nm in a Uv-vis spectrophotometer.

## Pure Culture Studies on Metabolism of Endosulfan and Endosulfan Sulfate

Cultures to be assayed for their degradation of endosulfan were first sub-cultured in Luria broth nutrient medium and further transformed into Burkes Mineral Medium (BMM) prior to the commencement of assay. Erlenmeyer flasks (250 mL) in triplicate containing 100 mL BMM medium were stoppered with cotton wool plugs and autoclaved before use. Ten mL of the full grown bacteria in Luria broth was transferred into the flasks. Assays were performed in sealed flasks spiked with 5mg/g concentration of endosulfan ($\alpha$ and $\beta$-isomers) and endosulfan sulfate separately. Uninoculated flasks served as

controls. All flasks were covered with rubber stoppers held with Teflon tape. The flasks were incubated on a rotary shaker at 28°C and samples for pesticide residue analysis were removed aseptically.

### Extraction of Endosulfan from Samples

A 10 mL aqueous sample was homogenized with a Teflon homogenizer followed by 3 to 5 drops of concentrated hydrochloric acid. The homogenized sample was extracted thoroughly by vortexing with equal volume (10 mL) of hexane: acetone (80:20) and centrifuged at 2000 rpm for 25 min. The organic layer that separated was dried over anhydrous sodium sulfate prior to injection for gas chromatographic analysis using electron capture detection.

### Gas Chromatographic Analysis of Endosulfan and its Metabolites

A Hewlett Packard 5890 series gas chromatograph equipped with Ni (550 MBq) ECD electron capture detector was used to analyze the endosulfan and its metabolites. The auto injector injected one microlitre volume of each hexane extract. ADB 17 fused silica capillary column 30 m, 0.32 mm internal diameter 0.25 $\mu$m film thickness was fitted and a temperature program (55°C for one min- 180°C at 40 min 240°C at 20 min final time) used. Nitrogen was used as carrier gas at a column head pressure of 24 kpa giving a linear carrier flow of 4 cm with nitrogen as detector makeup gas (2 mL/ min). The injector temperature was 250°C and detector temperature was 280°C. The $\alpha$ endosulfan eluted at 23.05 min, $\beta$ endosulfan at 30.77 min and endosulfan sulfate at 38.50 min. Different metabolites were detected at different times of elution within a total chromatographic analysis of 50 min. The chromatograms were recorded and integrated using a Hewlett Packard computer and Chemstation software. External standards were used to quantify sample concentrations. The metabolites were detected and compared with that of standards.

## RESULTS AND DISCUSSION

### Degradation of Endosulfan by Bacteria

Results of the time course study of biodegradation of endosulfan by a soil bacterium (S3) are shown in Figure 4. Microbial degradation of endosulfan was observed for 6 days. A mixture of endosulfan isomers was extensively degraded in mineral medium containing rich cultures of bacteria. Degradation was determined by monitoring endosulfan disappearance by gas chromatography with electron capture detection. Substantial (nearly 50%) degradation of both the constituents (a and b) was observed in 3 days concomitant with an increase in bacterial biomass. Uninoculated controls retained >80% of the substrate

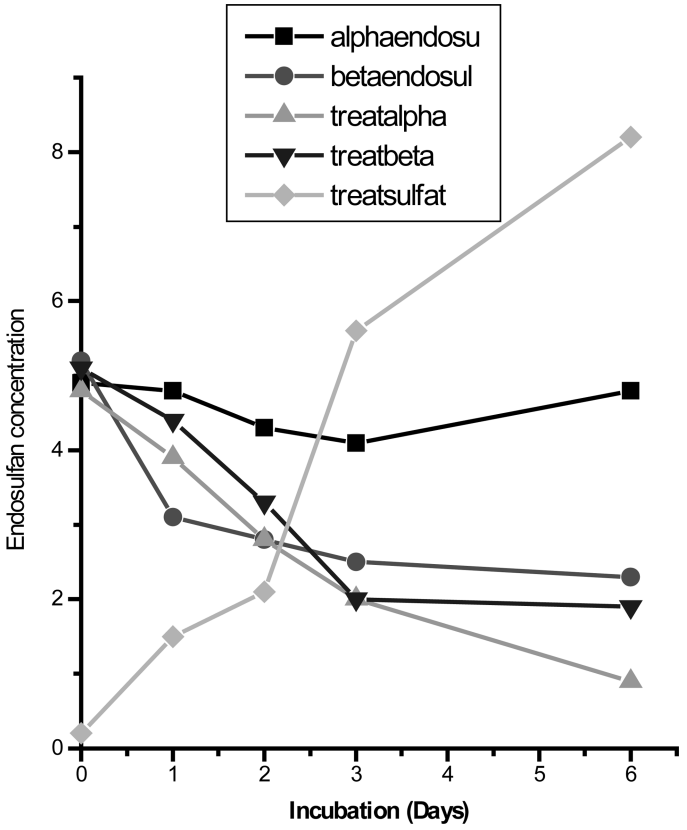


**Figure 4:** Degradation of endosulfan by soil Bacterium S3 in pure culture.

after 6 days of incubation, indicating that little chemical degradation or volatilization of endosulfan had occurred. However, GC analysis of acetone-hexane extracts of bacterial cultures indicated formation of endosulfan sulfate, which was the only major metabolite formed. The metabolite was identified on the basis of co-elution with an authentic standard. Endosulfan disappearance was concomitant with the formation of endosulfan sulfate metabolite (Table 1). The scheme of degradation is shown as in Figure 1. Oxidation product endosulfan sulfate is the only major metabolite, formed within one day of incubation and continues to accumulate throughout the incubation period. No other metabolite was observed during the incubation period.

In the case of bacterium S3, the proposed metabolic pathway for-endosulfan is that the parent endosulfan is oxidized to endosulfan sulfate, which is a terminal-end product. Endosulfan sulfate was detected as a major metabolite indicating that endosulfan degradation appears to have occurred through oxidation of endosulfan.

**Table 1:** Degradation of endosulfan by bacteria.

| Treatment | Endosulfan (a and b) remaining in broth (mg/mL) | | | | |
|---|---|---|---|---|---|
| | **Day-0** | **Day-1** | **Day-2** | **Day-3** | **Day-6** |
| | | | Control | | |
| Endosulfan Alpha | 4.9 ± 0.183 | 4.8 ± 0.179 | 4.3 ± 0.158 | 4.1 ± 0.167 | 4.8 ± 0.182 |
| Edosulfan beta | 5.2 ± 0.195 | 3.1 ± 0.184 | 2.8 ± 0.178 | 2.5 ± 0.160 | 2.30 ± 0.168 |
| Endosulfan sulfate | ND | ND | ND | ND | ND |
| | | | Inoculated with bacteria | | |
| Endosulfan alpha | 4.8 ± 0.159 | 3.9 ± 0.162 | 2.8 ± 0.148 | 2.0 ± 0.150 | 0.9 ± 0.162 |
| Edosulfan beta | 5.1 ± 0.139 | 4.4 ± 0.140 | 3.3 ± 0.155 | 2.0 ± 0.163 | 1.9 ± 0.170 |
| Endosulfan sulfate | 0.2 ± 0.045 | 1.5 ± 0.128 | 2.1 ± 0.110 | 5.6 ± 0.136 | 8.2 ± 0.135 |

ND = Not detectable.

## Incubation of Bacteria with Endosulfan Sulfate

Endosulfan sulfate, a metabolite of endosulfan, was incubated with bacteria in mineral media to determine whether the bacteria was capable of further degrading endosulfan sulfate. Table 2 indicates that there was no further degradation of endosulfan sulfate, supporting the hypothesis that it is in fact a terminal metabolite of endosulfan formed by these bacteria.

Only one of the isolated bacterial strain (S3) consistently degraded endosulfan. The results of endosulfan degradation indicate that the enzyme system responsible was probably a mono-oxygenase, converting endosulfan to endosulfan sulfate. Bacterial metabolism of endosulfan was demonstrated by both substrate disappearance and product formation.

According to previous studies, endosulfan degradation by soil microorganisms is of two types. Endosulfan transformed into endosulfan sulfate through oxidation or to endosulfan diol by hydrolysis. The bacteria isolated in the current study only converted endosulfan into endosulfan sulfate by oxidation.

In comparison with the previous reports on microbial metabolism of endosulfan,[12] our results indicate that the bacterium has a uniquely oxidized parent compound to sulfate. While several soil microbes have been shown to metabolize endosulfan into endosulfan sulfate by oxidation, endosulfan diol and several other metabolites by hydrolysis have also found to be formed. Miles and Moy[9] have proposed a pathway wherein endosulfan is converted into endosulfan sulfate as well as endosulfan diol, endosulfan ether, and endosulfan hydroxyether and endosulfan lactone. Formation of these metabolites has also been confirmed by Katayma and Matsumura[10] and Kullman and Matsumura[11] while working with fungi. However, Awasthi *et al.*[13] have reported aerobic degradation of endosulfan by bacterial co-culture without formation of any metabolites. Katayama and Matsumura[10] have shown that the cultures of *Trichoderma harzianum* were capable of producing endosulfan diol

**Table 2:** Incubation of endosulfan sulfate with bacteria.

| Treatment | Endosulfan sulfate remaining in broth (mg/mL) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Day-0 | Day-1 | Day-2 | Day-3 | Day-4 | Day-5 | Day-6 |
| Control (Media) | 4.9 ± 0.120 | 4.8 ± 0.137 | 4.8 ± 0.128 | 4.7 ± 0.136 | 4.8 ± 0.139 | 4.7 ± 0.140 | 4.8 ± 0.144 |
| Incubation with bacteria | 4.8 ± 0.128 | 4.7 ± 0.124 | 4.8 ± 0.130 | 4.8 ± 0.138 | 4.8 ± 0.145 | 4.8 ± 0.139 | 4.7 ± 0.136 |

as a principal metabolite. They suggested that a hydrolytic enzyme sulfatase is responsible for the indirect formation of endosulfan diol by hydrolysis of endosulfan sulfate.

The present study with the bacterial culture indicates that endosulfan sulfate is a terminal end product of oxidation that does not undergo further conversion under these conditions. These results suggest that endosulfan sulfate formation is due to direct oxidation of the parent compound itself and that it does not undergo hydrolysis. Endosulfan sulfate did not convert further under these conditions. Thus, these bacterial cultures fail to produce other metabolites like endosulfan ether, endosulfan hydroxyether and endosulfan lactone.

## CONCLUSION

Based on results of this study, it can be concluded that the degradation of endosulfan-to-endosulfan sulfate may occur by soil bacteria. Endosulfan sulfate is the terminal end product formed and no other metabolites were found during degradation. Endosulfan sulfate fails to degrade further when incubated with soil bacteria.

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