Bull. Environ. Contam. Toxicol. (2005) 75:1034–1040

DOI: 10.1007/s00128-005-0853-2

Bulletin of Environmental Contamination and Toxicology

Bioremediation of Endosulfan Using *Aspergillus terreus* and *Cladosporium oxysporum*

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Received: 9 February 2005/Accepted: 26 May 2005

Endosulfan (1,2,3,4,7,7-hexachlorobicyclo-2, 2,1-heptene- 2,3-bis-hydroxy methane-5, 6 sulfite is a broad-spectrum cyclodiene insecticide. It is used extensively throughout the world to control insect pests and nematodes of a wide range of crops including cereals, tea, coffee, cotton, fruits, oil seeds and vegetables (Lee et al., 1995). Technical endosulfan is a mixture of two stereoisomers i.e. alpha- and beta-endosulfan (Figure 1. I and II) in the ratio of 7: 3, is of great concern because of its persistence and extreme toxicity to aquatic invertebrates (Peterson et al., 1993, Verschuenren, 1983). Endosulfan has been classified as a moderately hazardous chemical (IPCS, Handbook on Endosulfan, 1984). Owing to its extensive usage, endosulfan residues are commonly found in the environment (Kullman et al 1996). Microbial degradation of endosulfan may play an important role in detoxifying the endosulfan-contaminated sites. There are a few reports on degradation of endosulfan by different groups of microorganisms (Martens, 1976; Miles et al, 1979; Katayama and Matsumura, 1993; Mukherjee and Gopal 1996).

In this study, we report the degradation of endosulfan by fungi, namely, Aspergillus *terreus* and *Cladosporium oxysporum*, isolated from field soil, under laboratory conditions.

MATERIALS AND METHODS

Cladosporium oxysporum was isolated from the egg masses of root knot nematodes, *Meloidogyne incognita.* The isolation of *Cladosporium oxysporum* was carried out by surface sterilizing the egg masses with 1000 μg/mL mercurous chloride and ethanol (95%) for 1 min. The egg masses were further rinsed with sterile water three times. The surface sterilized egg masses were placed on petri-plates containing potato- dextrose agar (PDA) medium under laminar flow. The petri-plates were incubated in the incubator at 25±2°C for 7 days. The fungi with distinct characteristics were observed. The fungal colony was purified for identification and characterized as *Cladosporium oxysporum* by ITCC, Division of Plant Pathology, IARI, and New Delhi. Cultures of *Aspergillus terreus* were isolated from the soil rhizosphere of root–knot nematodes infested brinjal plants.

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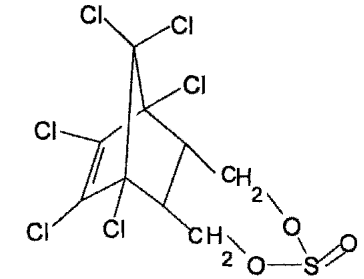

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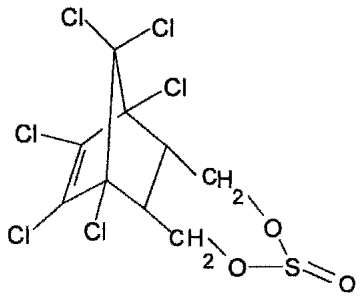

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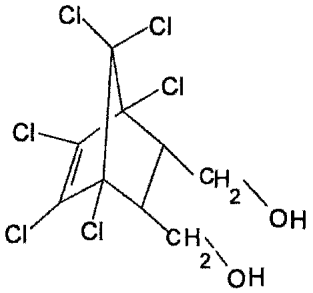

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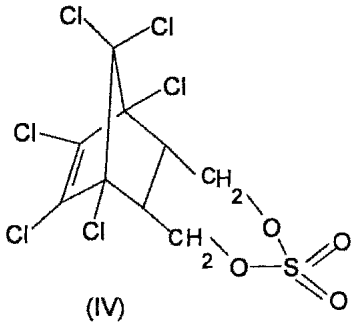

(IV)

Fig 1. Structure of α-Endosulfan (I) β-endosulfan (II), endosulfan diol (III) and endosulfan sulfate (IV)

The soil (1 g) was dispersed in sterile water and diluted serially to 1000 times. One aliquot of soil water suspension was pipetted into a petri dish containing PDA amended with a 0.001 mg of streptomycin sulphate. The petri-dishes were swirled gently to spread the suspension and the liquid medium allowed to cool and then incubated in an incubator at 25±2°C for 7-10 days. The fungi were characterised as Aspergillus *terrus* by ITCC, Division of Plant Pathology, IARI, New Delhi.

The fungi *Aspergillus terreus* and *Cladosporium oxysporum* (egg-parasitic) were grown and maintained in potato dextrose broth medium containing dextrose 20 g, potato, (peeled, sliced and cooked) 200 g and water 1 litre. Technical grade endosulfan was further purified by silica gel column chromatography (99% purity). All other chemicals used were of the highest analytical grade. Both the fungi were grown in 150 ml Erlenmeyer flasks containing 50 ml of potato

dextrose broth medium and incubated for three days in BOD at 25±2°C. All the conical flasks (150 mL capacity) were plugged with cotton and autoclaved at 15 atms pressure for 30 min. Endosulfan technical solution containing 100 µg in acetone added to eighty-four flasks under sterilized conditions of Laminar flow hood and allowed to evaporate under room temperature. 50 mL of the potato dextrose broth medium was added to each flask. The experiment was carried out in completely randomized design with five treatments and three replications. The treatments were: (i) Control 1 - medium + *Aspergillus terreus* inoculum, (ii) Control 2 - medium + *Cladosporium oxysporum* inoculum, (iii) Pesticide treatment 1 - medium + endosulfan, (iv) Pesticide treatment 2 – medium + endosulfan + *Aspergillus terreus* inoculum, (v) Pesticide treatment 3 – medium + endosulfan + *Cladosporium oxysporum* inoculum. The treatment (i) and (ii) served as blanks and treatment (iii) served as uninoculated control for studying the effect of fungus. The pH of the medium recorded after the addition of fungi and pesticide was 7.5.

Cultures were incubated for different growth periods of up to 15 days after addition of endosulfan. A set of heat killed control samples were prepared by autoclaving the cultures before adding endosulfan and were incubated along with the live culture for the same period of time. Appropriate control experiments were carried out with uninoculated medium. At intervals, samples in triplicate from control, heat-killed control and live cultures were removed and acidified to pH 2 with 1N HCl. The flasks were taken out periodically on 0, 1, 3, 5, 7, 10 and 15 for the estimation of endosulfan. The medium was centrifuged and then filtered. The filtrate was subjected to liquid-liquid partitioning thrice with hexane (30 mL). The mycelia residue left in the tube was transferred to a conical flask, to which hexane (20 mL) was added and shaken vigorously for 15 min in a shaker. The solvent was filtered through anhydrous sodium sulfate and stored. The process was repeated again with 20 mL hexane. The combined organic layer was dried and evaporated under reduced pressure to remove all the traces of hexane. The sample was made up in hexane (10 mL) and analyzed by GLC using a Hewlett Packard 5890 series II instrument. The column used was a megabore HP I (12 m long × 1 µ film thickness ×0.52 µm). The column temperature was maintained at 210 °C while the injector port and the detector were set at 250 °C and 300 °C, respectively. The carrier gas nitrogen flow was maintained at 20 mL min^{-1}. The retention time of alpha-endosulfan, beta endosulfan and endosulfan sulfate were 2.84, 4.03 and 5.26 min, respectively. The identity of the pesticide was confirmed by carrying out the GLC using a column of alternate polarity on specifically, glass column SE30 (2 m x 2 mm ID) coated on Chromosorb WHP. The column temperature was maintained 190 °C and the injector port and the detector were set at 210 °C and 250 °C, respectively. The nitrogen gas flow was 32 mL min^{-1}. The retention time of the alpha endosulfan, beta endosulfan and endosulfan sulfate was 3.01, 5.21 and 7.23 min, respectively.

The metabolite of endosulfan, endosulfan diol (Figure 1, III) was prepared in the laboratory to serve as authentic standard sample. Endosulfan (500 mg) was

dissolved in ethanol (30 mL) and subjected to alkaline hydrolysis with 10 per cent potassium hydroxide (15 mL). The reaction mixture was refluxed for 4 hour and worked up by neutralizing with dilute hydrochloric acid and further partitioning into diethyl ether thrice (3 x 30 mL). The organic solvent was removed and endosulfan diol was obtained as an oil (Shetty et al 2000) Rf 0.0726 (75:25 hexane-benzene), IR ν nujol cm^{-1} 3200 (OH), 1590 (C=C); NMR ($CDCl_3$: δ 3.9 (2H, d, CH_2-OH), 3.6 (2H, t, CH-CH2), 3.2 (2H, d, CH2-OH) and 2.35 (2H, bs, OH) (Canada National Research Council-CNRC, 1975). The endosulfan sulfate (Figure-I) (IV), the oxon metabolite of endosulfan was prepared by stirring endosulfan (100 mg) dissolved in ethanol with 10 % aqueous solution of potassium permanganate for 4-5 hr. The reaction mixture was worked up. The organic solvent evaporated and the compound was subjected to column chromatography over silica gel. The oxon metabolite (Figure1, IV) of endosulfan was obtained as pale yellow crystalline solid, Rf 0.54 (10% acetone -benzene), (IR ν KBr cm^{-1} 1700 (sulfate group) and 1600 (double bond). NMR ($CDCl_3$: δ 4.55 (4H, q, 2CH2-O) and 3.5 (2H, bs, 2C-H), 2.21 (t, 3H CH_3), 3.98 (d, 2H, CH_2), (8.20 (s, 1 H, Ar-H) (Shetty et al 2000, Canada National Research Council-CNRC, 1975). This was used as a reference standard for quantification of the presence of the endosulfan diol and the endosulfan sulfate (Figure 1, III and IV) formed during the dissipation experiment.

RESULTS AND DISCUSSION

The average percent recovery from three replicates spiked at 0.1 and 1 µg recorded was 92. The data in Table 1 revealed that total endosulfan (α+β) dissipated by day -1 to 1.72 mg kg^{-1} in the pesticide treatment -1 (uninoculated control) and 1.58 and 1.62 mg kg^{-1} in the pesticide treatment –2 and pesticide treatment –3, with *A. terreus* and *C. oxysporum,* respectively. The percent dissipation recorded in uninoculated control (pesticide treatment-1) was 8.02 by day-1, and 16.4 and 11.41 in pesticide treatments-2 and 3, with *A. terreus* and *C. oxysporum,* respectively.

The initial loss of the pesticide, though not significant, by day-3 the rate of dissipation increased to 29.6 percent in the *A. terreus* treated sample as compared only 17.2 percent in control-2 and 23.5 percent treatment with *C. oxysporum,* pesticide treatment -3. It was observed that both the fungi started metabolizing only after its incubation period of 5-10 days. Endosulfan sulfate, a toxic metabolite of endosulfan was detected on day-7 in the uninoculated control, at 0.08 mg kg^{-1} level while in the pesticide treatments- 2 and 3 endosulfan sulfate was detected on day-5 in levels of 0.08 mg kg^{-1} and 0.14 mg kg^{-1} in the *A. terreus* and *C. oxysporum* treatments, respectively. The endosulfan- sulfate dissipated with time and on day-7, it was recorded in amounts of 0.03 and 0.08 mg kg^{-1} (Table-1), respectively in treatments with *A. terreus* and *C.oxysporum.* However, no endosulfan diol was detected as was reported by Siddique et al., (2003). Degradation of endosulfan, was observed in treatments with fungi with *A. terreus* and *C. oxysporum*, recording a percent loss of 91 and 89, respectively by day-15,

Table 1. Effect of *Aspergillus terreus* and *Cladosporium oxysporum* on dissipation of endosulfan.

Isomer/ metabolite	Residues (mg kg^{-1})						
	0 d	1 d	3 d	5 d	7 d	10 d	15 d
Treatment 1 - Uninoculated control							
Endo-α	1.41	1.34	1.25	0.98	0.75	0.63	0.58
Endo-β	0.46	0.38	0.29	0.15	0.10	0.07	0.04
Endo-sulfate	-	-	-	-	0.08	0.05	-
Total	1.87	1.72 (8.02)	1.54 (17.2)	1.13 (39.5)	0.93 (50.2)	0.75 (59.8)	0.62 (66.8)
Treatment 2 – Inoculated with *Aspergillus terreus*							
Endo-α	1.36	1.23	0.99	0.54	0.26	0.11	0.09
Endo-β	0.53	0.35	0.34	0.25	0.18	0.12	0.07
Endo-sulfate	-	-	-	0.08	0.03	-	-
Total	1.89	1.58 (16.4)	1.33 (29.6)	0.87 (53.9)	0.47 (75.1)	0.23 (87.8)	0.16 (91.5)
Treatment 3 – Inoculated with *Cladosporium oxysporum*							
Endo-α	1.22	1.05	0.89	0.52	0.38	0.20	0.16
Endo-β	0.61	0.57	0.51	0.32	0.20	0.13	0.04
Endo-sulfate	-	-	-	0.14	0.08	-	-
Total	1.83	1.62 (11.4)	1.40 (23.5)	0.98 (46.4)	0.66 (63.9)	0.33 (81.9)	0.20 (89.0)

Endo: Endosulfan; Values are mean of three replications; Figures in parentheses are % dissipation

as compared to uninoculated control (pesticide treatment -1), where 66.8 per cent dissipation was observed (Figure 2). It is pertinent to mention that though the dissipation by both the fungi was in the range of 89-91 per cent by day –15, faster rate of dissipation was recorded with *A. terreus* as compared to *C. oxysporum* during day-1 to day-7, confirming the toxic behaviour of *A. terreus*. The treatments (i) and (ii) acted as a reference controls to eliminate any interfering peaks arising due to the co-extractives from fungi. The endosulfan diol (III) was not the major metabolite through the oxidative pathway. Endosulfan sulfate is toxic and persistent in nature (Stewart et al 1974) and has to be quantified in degradation studies. Kullman and Matsumura (1996) have been also reported similar results during transformation of endosulfan by a white rot fungus, *Phanerochaete chrysosporium* through producing more endosulfan sulfate than endosulfan diol. Martens (1976) found that sixteen fungi, 15 bacteria and 3 actinomycetes were found capable of metabolizing more than 30% of the applied endosulfan into endosulfan sulfate.

The majority of highly active fungi formed endosulfan sulfate as the major metabolite, whereas the majority of active bacteria formed endosulfan diol. Half-life recorded in Table 2 clearly establishes the ability of *both Aspergillus terreus and Cladosporium oxysporum* to degrade endosulfan.

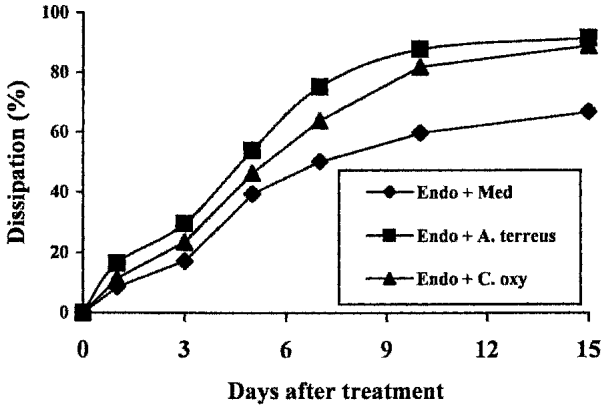

Fig 2. Effect of A. terreus and C. oxysporum inoculum on dissipation of endosulfan

Table 2. Regression equation and half-life.

Treatment	Regression Equation	Half life (days)	Correlation Coefficient (r)
Uninoculated Control Pesticide treatment-1	Y=2.24-0.03X	10.0	0.97
Inoculated Treatment- 2 Pesticide + *Aspergillus terreus*	Y=0.40-0.90X	4.2	0.96
Inoculated Treatment- 3 Pesticide + *Cladosporium oxysporum*	Y=2.28-0.06X	5.0	0.97

The present study indicates (Table 1 and Figure 2) that these soil fungi may be used to detoxify a persistent pesticide like endosulfan. The results of the present study suggest that the fungi are effective in detoxifying the pesticides when they are present in microgram amounts in the water and soil environment. Further, screenings of different fungal cultures need to be carried out to evolve a suitable technique for soil bioremediation.

Acknowledgments: We thank Dr. B.S.Parmar, Head, Division of Agricultural Chemicals for providing the facilities for work. Contribution No. 816 Division of Agricultural Chemicals.

REFERENCES

Canada National Research Council (CNRC, 1975) Endosulfan: its effects on environmental quality (NRC Associate Committee on Scientific Criteria for Environmental Quality, Report No. 11, Subcommittee of Pesticides on related compounds, Subcommittee Report No. 3, Publication No. NRCC 4098 of the Environmental Secretariat).

IPCS-Handbook on Endosulfan (1984) - Environmental Health Criteria 40, World Health Organisation Geneva .

Katayama A Matsumura F (1993) Degradation of organochlorine pesticides particularly endosulfan by *Trichoderma harzianum.* Environ Toxicol Chem 12: 10591065.

Kullman SW Matsumura F (1996) Identification of a novel cytochrome P-450 gene from the white rot fungus *Phanerochate chrysosporium.* Appl Environ Microbiol 62:59360.

Lee N Skerritt JH Mc Adam DP (1995) Hapten synthesis and development of ELISAs for detection of endosulfan in water and soil. J Agric Food Chem 43 6:1730-1739.

Martens R (1976) Degradation of (8, 9-14C) endosulfan by soil microorganisms. Appl Environ Microbiol 31: 853-858.

Miles JRW Moy P (1979) Degradation of endosulfan and its metabolites by a mixed culture of soil microorganisms. Bull Environ Contam Toxicol 23: 13-19.

Mukherjee I Gopal M. (1994) Degradation of beta-endosulfan by *Aspergillus niger.* Toxicol Environ Chem 46: 217-221.

NRCC 1975, Endosulfan and its effects on environmental Quality Publ. No. NRCC 14098, Environmental Secretariat National Research Council Canada, Ottawa, Canada, 73pp.

Peterson SM Batley G. E (1993) The fate of endosulfan in aquatic ecosystems. Environ Pollut 82: 143-152.

Shetty PK Mitra J Murthy K K Namitha K N Savitha K N Raghu K (2000) Biodegradation of cyclodiene insecticide by *Mucor thermo- hyalospora MTCC 1384* Curr Sci 79: 1381-1383.

Siddique T Benedict C Okekea Arshadb M Frankenberger W T Jr (2003) Enrichment and isolation of endosulfan – Degrading microorganisms. J Environ Quality 32: 47-54.

Verschueren K (1983) Endosulfan: Handbook of environmental data on organic chemicals Van Nostrand New York.