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Biodegradation of Endosulfan by a Bacterial Coculture

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Endosulfan, (1,2,3,4,7,7-Hexachlorobicyclo-2,2,1-heptene-2, 3-bis hydroxy methane-5,6-sulfite), a chlorinated pesticide of the cyclodiene group, is used extensively for the protection of cotton, tea and sugarcane crops. The insecticidal formulation contains two major stereoisomers i.e. alpha- and beta-endosulfan, in an approximate ratio of 70:30 (Maier- Bode, 1968, Goebel, 1983). It is extremely toxic to fish and aquatic invertebrates (Verschueren, 1983), and is a priority pollutant for international environmental agencies (Kieth et al, 1979). Bioremediation can lead to its elimination from industrial waste waters and contaminated sites. El Zorghani & Omer (1974) observed that isolated strains of Aspergillus niger can degrade endosulfan to endodiol. Martens (1976) studied the degradation of endosulfan by different bacterial and fungal cultures, and found that endodiol and endosulfate, respectively were the major metabolites accumulated. Besides, small amounts of endohydroxy ether and endolactone were also formed. Miles and Moy (1979) have proposed a pathway, wherein, the endosulfan is converted to endosulfate followed by endodiol, endohydroxy ether and endolactone. Formation of these metabolites has also been confirmed by other investigators (Kshemkalyani et al. 1987, Katayama and Matsumura 1993, Kullman and Matsumura 1996). However, information regarding further degradation is scanty.

We now describe the constitution of a two membered bacterial coculture that aerobically degrades endosulfan efficiently without accumulating any of its reported metabolites.

MATERIALS AND METHODS

Technical endosulfan, alpha-endosulfan, beta-endosulfan, endodiol, endosulfate and endohydroxyether were generous gifts from Farbwerke-Hoechst AG, Frankfurt, Germany. Thin layer plates (Silica gel 60, 0.2 mm thick) were purchased from E. Merck, Darsmstadt, Germany. All other reagents were of

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high purity and analytical grade. Bacterial coculture was made by selective enrichment method. To illustrate, soil samples were collected from a contaminated industrial area. A soil suspension (1 g/10 ml) was made in minimal medium (KH_2PO_4, 1.0 g; K_2HPO_4, l.0g; NH_4NO_3, 1.0g; $MgSO_4.7H_2O$, 0.2g; $CaCl_2$, 0.02 g; Fe $(SO_4)_3$, 0.01g; dissolved in distilled water and made up to 1 litre. The pH of the medium was 6.5). One ml of this suspension was inoculated into a flask precoated with 500 g of technical endosulfan and containing 10 ml of fresh minimal medium. The contents were incubated at 28°C for one week. Thereafter, 1 ml sample was drawn from this flask and reinoculated into a fresh flask as above. This process of growing bacteria was repeated for three more times. Finally, a loopful of this inoculum was streaked onto agar plates made with nutrient medium (Peptone, 1.0g; Yeast extract, 0.5 g; NaCl, 0.5 g; dissolved in distilled water and made up to 100 ml) and incubated for 24 hrs at 28°C. Two visibly distinct types of colonies were observed. Both the bacteria were grown in nutrient medium separately to mid log phase, then mixed together in equal amounts to make stock coculture, which was used for all further experiments.

To study the degradation of endosulfan and its metabolites, the stock coculture was grown in nutrient medium to mid log phase of growth. The cells were then centrifuged at 10,000 rpm for 10 min, washed twice and suspended in minimal medium. The suspension was then inoculated to 20 ml of minimal medium (Final O.D. - 0.1) containing 50 ppm of either technical endosulfan or alpha-endosulfan, beta-endosulfan, endodiol or endosulfate and incubated at 28°C for 3-15 days, with occasional shaking. Control flasks containing minimal medium and the pesticide or its metabolites, but without bacterial inoculation, were also included in the experiment for compensation of autodegradation, if any. After the incubation, 1.5 ml of samples were withdrawn from the flasks to estimate the released chloride ions by the colorimetric method (Bergmann and Sanik 1957). The remaining sample was acidified with 3-4 drops of conc. HCl and extracted with ethyl acetate for analysis of the unutilized pesticide by thin layer and gas chromatography. Thin layers of the silica gel were developed in Hexane:Chloroform:Acetone (9:3:1) and chlorinated compounds were detected by 2-phenoxy ethanol reagent (Kovacs, 1965). Gas chromatography was done on an Antek Gas chromatograph, equipped with Ni^{63} Electron Capture Detector at oven temp of 200°C using OV-17 as matrix and nitrogen gas as carrier.

The influence of auxillary carbon sources was examined with either glucose or succinate at 0.1 % final concentration, added in culture flasks containing 50 ppm of technical endosulfan in 20 ml minimal medium.

To analyze the degradation of endosulfan in soil samples, 5 g of air dried garden soil were uniformly mixed with 20 mg of endosulfan. 0.5 g of this soil was added to 20 ml minimal medium, inoculated with bacterial coculture (Final O.D. 0.1) and incubated at 28°C with occasional shaking. The reaction was stopped weekly up to four weeks by addition of 3-4 drops of conc. HCl. The contents were extracted with 20 ml of ethyl acetate by stirring on a rotary shaker at 200 rpm for 30 min. The ethyl acetate extract was analysed by thin layer and gas chromatography as described earlier.

RESULTS AND DISCUSSION

Microbial degradation of endosulfan was observed for 15 days. Substantial (nearly 50%) degradation of both the constituents was seen in seven days and almost all of the pesticide was degraded by the fifteenth day, with a simultaneous increase in bacterial mass (Fig. 2). Degradation of beta-endosulfan appears to be slower than the alpha-isomer. In a separate experiment concomitant to degradation, a release of chloride ion was also observed (inset to Fig. 2) indicating that dehalogenation is a part of degradative activity. Similarly about 50% degradation of alpha-endosulfan, beta-endosulfan and endodiol, individually, was observed in 7 days. Endosulfate, however, was very stable and no significant degradation of this metabolite was observed up to 3 weeks of incubation, under similar conditions. The reported (Miles, and Moy, 1979) interconversion between the isomers of endosulfan, or formation of endodiol or endosulfate was not observed during the degradation of alpha- or beta-isomers, in our study. A UV positive metabolite with an RF value of 0.35, not corresponding to any of the reported metabolites of endosulfan, was visualized during the first 3 days of incubation which later was (10 days) converted to another metabolite that has no mobility in TLC solvent system used (data not given). It is a possibility that during the degradation of endosulfan, endodiol is either formed but degraded rapidly or is not formed at all. On the other hand, the degradation of endosulfate is extremely slow and is probably not formed.

On the influence of auxiliary carbon sources, the presence of glucose in the medium did not have any significant effect, while succinate inhibited the degradation by nearly 50% (Fig.3). The inhibition of degradation by supplementary carbon sources has also been observed in many other cases (Watanabe, 1973, Sahu et al., 1993).

The degradation of soil bound endosulfan was slower by nearly four fold than in culture medium, as only 50% of the pesticide was degraded in four weeks (Fig. 4). The slow degradation could either be due to the adsorption of endosulfan to soil particles or because of the presence of other carbonaceous materials

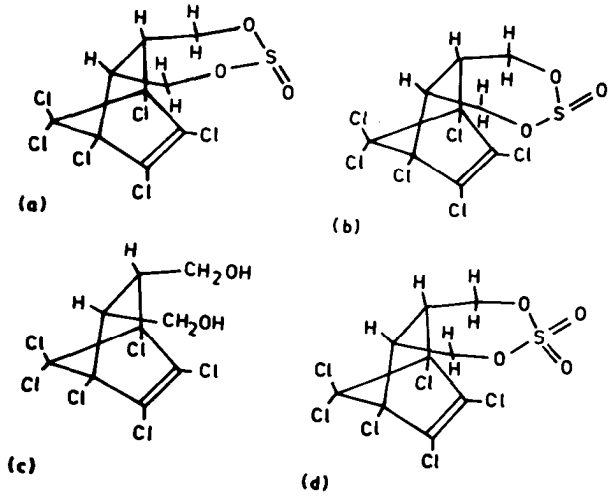


Figure 1. Endosulfan isomers and its metabolites (a) alpha- Endosulfan, (b) beta-Endosulfan, (c) Endodiol and (d) Endosulfate.

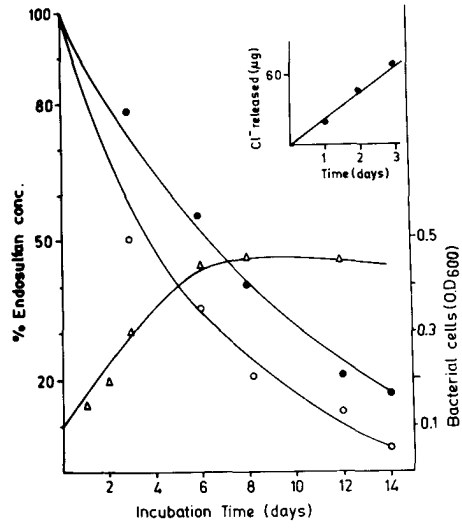


Figure 2. Degradation of technical Endosulfan by the bacterial coculture. Symbols -O- and -●- represent alpha- and beta-endosulfan recovered after different incubation times. -Δ - represents growth of the bacterial coculture during the incubation. Release of chloride ions is presented in the inset.

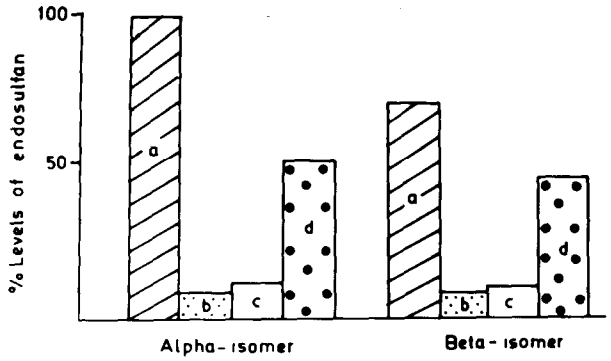
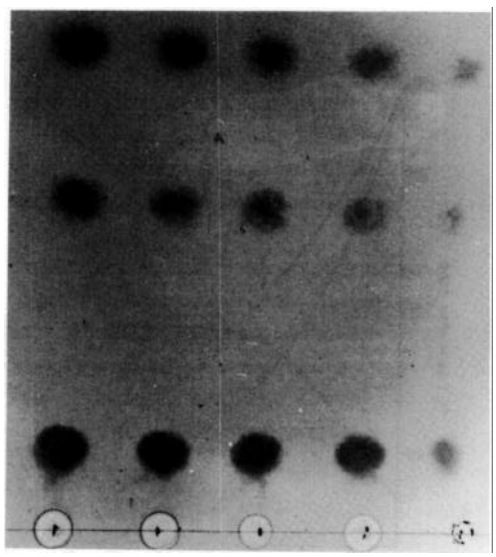


Figure 3. Influence of auxiliary carbon sources on the degradation of endosulfan by bacterial coculture. Uninoculated (a), Inoculated (b), Inoculated in presence of glucose (c) and Inoculated in presence of Succinate (d).



INCUBATION TIME (WEEKS)

Figure 4. Thin layer chromatograph of soil bound endosulfan, before (0 day) and after incubation (1-4 weeks) with bacterial coculture. aE, bE and ED represents alpha- endosulfan, beta-endosulfan and endodiol respectively.

in the soil. These findings demonstrate that our coculture extensively degrades endosulfan, both in culture media and in soil, through a distinct pathway wherein the previously reported metabolites do not accumulate. As such this coculture could be useful in detoxification of endosulfan containing effluents and in bio-remediation of damaged habitat.

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