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Enrichment and isolation of endosulfan degrading and detoxifying bacteria

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Abstract

In the present study, degradation of endosulfan by a mixed culture isolated from a pesticide-contaminated soil was studied in batch experiments. After two weeks of incubation, the mixed culture was able to degrade 73% and 81% of α and β endosulfan respectively. Endodiol was identified by GC/MS as degradation intermediate. The toxicity studies of endosulfan before and after degradation were carried out using micronucleus assay on human polymorphonuclear cells. The findings suggested that the metabolism of endosulfan isomers by the mixed culture was accompanied by significant reduction in the toxicity. Studies were also carried out to quantify the degradation potential of the individual species in the mixed bacterial culture. Two cultures identified by 16S rRNA as *Stenotrophomonas maltophilia* and *Rhodococcus erythropolis* were found to be responsible for majority of the degradation by the mixed culture. *S. maltophilia* showed better degradation efficiency compared to that by *R. erythropolis*. This is the first report of endosulfan degradation using the above-mentioned organisms.

Keywords: Endosulfan; Endodiol; Micronucleus (MN); *Stenotrophomonas maltophilia*; *Rhodococcus erythropolis*

1. Introduction

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,3,4-benzo-dioxathiepin-3-oxide) is a broad-spectrum cyclodiene insecticide (Fig. 1) that has been used extensively for over 30 y on a variety of crops (Sutherland et al., 2000). It is a mixture of two stereoisomers, α and β endosulfan, in a ratio of 7:3 and registered with several trademarks, Thiodan, Cyclodan, Thimol, Thiofar, Malix, etc. According to the Pesticide Manufacturers and Formulators Association of India, India is the world's leading manufacturer of endosulfan. In India, the National Institute of Occupational Health conclusively proved that endosulfan was a causative factor in the incidence of all crippling illness in the Kasargode area of Kerela where this insecticide was sprayed aerially in the cashew plantations (Devakumar, 2002).

It has been reported that endosulfan is genotoxic in mammalian cells (Chaudhuri et al., 1999; ASTDR, 2000). Due to the abundant usage and potential transport of endosulfan, contamination is frequently found in the environment at considerable distance from the point of application (Miles and Pfeuffer, 1997; Sethunathan et al., 2002). These health and environment concerns have led to an interest in degradation and detoxification of endosulfan.

Detoxification of endosulfan through biological means is receiving serious attention as compared to existing methods such as incineration and landfill (Siddique et al., 2003). Awasthi et al. (2003) reported the degradation and detoxification of endosulfan isomers by a defined co-culture of two Bacillus strains. The degradation of endosulfan and the metabolites formed during degradation by this bacterial co-culture were evaluated by the reduction in toxicity against the test organism *Tubifex tubifex*. A bacterial

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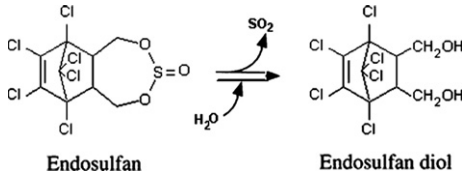

Fig. 1. Endosulfan and its metabolite endodiol.

strain, *Klebsiella oxytoca* KE8, was found to be capable of degrading endosulfan and endosulfan sulfate (Kwon et al., 2005). Also, a mixed bacterial culture of *Staphylococcus sp*, *Bacillus circulans*-I, and *Bacillus circulans*-II was able to mineralize 50 mg l^{-1} of endosulfan up to 72% and 76% in aerobic and facultative anaerobic conditions respectively (Kumar and Philip, 2006). Many researchers have reported isolation of pure and mixed cultures of bacteria and fungi capable of degrading endosulfan (Miles and Moy, 1979; Awasthi et al., 1997; Kullman and Matsumura, 1996; Sutherland et al., 2000). However, only few reports are available in the literature about complete mineralization of endosulfan and reduction in its toxicity. Research has been carried out on endosulfan genotoxicity using a hepatoma Hep G2 cell line (Lu et al., 2000). However, *in vitro* studies on normal human cells with these agents are still needed.

This paper presents the enrichment of endosulfan degrading mixed bacterial culture from pesticide-exposed soil. The toxicity was evaluated before and after degradation based on frequency of micronucleus (MN) emergence in polymorphonuclear leukocyte cells. The endosulfan degradation capacities of the pure cultures isolated from the consortium were also studied individually and the cultures responsible were identified.

2. Materials and methods

2.1. Reagents

Technical grade endosulfan (97% pure) was obtained from a pesticide manufacturer (Yawalkar Pesticides Ltd, India). High purity (99.4%) endosulfan for standard was obtained from sigma Aldrich. Endosulfan stock was prepared in acetone. All other chemicals and solvents used were of highest purity and chromatographic grade.

2.2. Enrichment and selection of microorganisms

Microorganisms used in this study were cultured from soil having several applications of different pesticides including endosulfan. Around 10 g of soil was added to 100 ml of basal medium (composition per litre: 5.8 g K_2HPO_4, 4.5 g KH_2PO_4, 2.0 g $(NH_4)_2SO_4$, 0.16 g $MgSO_4$, 0.02 g $CaCl_2$, 0.002 g Na_2MoO_4, 0.001 g $FeSO_4$ and 0.001 g $MnCl_2$) with 10 mg endosulfan and kept on an orbital shaker for 7 d at 150 rpm and ambient conditions. The microorganisms capable of growing in endosulfan containing basal salt medium were further enriched in enrichment media [The sulphur-free enrichment medium (pH 6.6–6.8) was prepared as in Sutherland et al. (2000)]. For this, about 10 ml of the soil suspension in basal medium was transferred into a fresh flask containing enrichment medium with 20 mg l^{-1} endosulfan. The cultures developed in the flask were successively transferred to fresh enrichment medium containing increasing concentrations of endosulfan. At about a final concentration of 100 mg l^{-1} of endosulfan, the endosulfan tolerant cultures were picked up for further degradation studies.

2.3. Isolation and identification of bacterial cultures

The bacterial cultures were isolated from the mixed culture by streaking them on enrichment medium agar plates. Six colonies showing different morphology were picked up and maintained on nutrient agar slants at 4 °C. The pure cultures thus selected, were grown separately in enrichment medium containing 100 mg l^{-1} of endosulfan at ambient conditions on a shaker at 150 rpm for 15 d. Two of the bacterial cultures, which were found to degrade endosulfan, were identified by 16S rRNA sequencing.

2.4. Experimental set-up

Batch experiments were performed in flasks. Hundred mg l^{-1} of endosulfan was added to enrichment medium inoculated with 0.10 OD (optical density at 600 nm) of culture. These flasks were incubated at ambient conditions on an orbital shaker at 150 rpm. A control (with endosulfan and without bacterial culture) was maintained under similar conditions to check the loss of endosulfan, if any, due to chemical hydrolysis or dissipation. Samples were collected at 0, 3, 7, 11 and 15th d and analyzed for growth of microorganisms and endosulfan levels by GC-ECD.

2.5. Analytical methods

2.5.1. Growth of microorganisms and pH

The increase in growth of microorganisms was monitored by measuring OD at 600 nm on a UV–Visible Spectrophotometer. The pH was measured as per standard method (APHA et al., 1998) using a pH meter.

2.5.2. Extraction and GC analysis

Endosulfan degradation was monitored by GC-ECD (Perkin Elmer Autosystems). 5 ml samples were taken at different intervals (0, 3, 7, 11 and 15 d), centrifuged and filtered through a 0.45 μm membrane filter (Millipore). The filtrate was extracted thrice with 3:1 hexane-acetone (v/v), flash evaporated at 45 °C water bath, and the residue was dissolved in 2 ml hexane.

GC-conditions: Extracted samples were analyzed by GC-ECD, using a pesticide specific column OV-101 set at 200 °C. Carrier gas used was nitrogen with a flow rate of 25 ml min^{-1}. Injector and detector temperatures were set at 220 °C and 275 °C respectively. Retention time for α