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Studies on endosulfan degradation by local isolate *Pseudomonas aeruginosa*

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ABSTRACT

A bacterium capable of tolerating high levels of endosulfan was isolated from soil of cotton field. The ability of the bacterium to degrade endosulfan was investigated by culturing it in minimal media and nutrient broth containing high levels of endosulfan. The 16S rRNA gene sequencing indicated the bacterium as *Pseudomonas aeruginosa*. Endosulfan degradation by the bacteria was found to occur more rapidly in media with supportive nutrients than the minimal media. *P. aeruginosa* was able to degrade 96% of endosulfan after 288 h under static conditions. The metabolites formed during endosulfan degradation were analyzed by using UV visible spectrophotometry and thin layer chromatography. The complete mineralization of endosulfan was found to occur after 28 days. The levels of dehydrogenase, arylsulfatase, dehalogenase enzymes and CO_2 were monitored to test the soil activity. The terminal products formed by bacteria during biodegradation were tested for phyto-toxicity. This study will be useful for the development of bioremediation strategies.

1. Introduction

Endosulfan is a chlorinated cyclodiene insecticide, which is one of the persistent organic pollutants. Endosulfan has less water solubility which adds up in its persistence in soil and water environments for 183 days or more (Rao and Murty, 1980; Kathpal et al., 1997). As a result endosulfan residues have been traced in surface and ground water and in soil (Sujatha et al., 1999; Berrakat et al., 2002; Cerejeira et al., 2003; Bhattacharya et al., 2003; Golfinopoulos et al., 2003). Presence of endosulfan residues environments have been reported to cause serious health hazards to human and environment. Therefore, removal of this pesticide from environment is very important. Incineration and landfill are existing practices but biological detoxification methods are emerging as an alternative due to their eco-friendly and cost effective nature (Siddique et al., 2003). Degradation of endosulfan involves oxidation or hydrolysis of sulphide group to form the toxic metabolite endosulfan sulphate and the less toxic endosulfan diol (Sutherland et al., 2000). Previously some microbial cultures have been investigated for their abilities to degrade endosulfan under aerobic and anaerobic conditions by different researchers (Katayama and Matsumura, 1993; Mukherjee and Gopal, 1994; Kullman and Matsumura, 1996; Awasthi et al., 1997; Shetty et al., 2000; Sutherland et al., 2000; Siddique et al., 2003). Bacterial cultures have been reported to have better promise to degrade endosulfan than fungal cultures (Siddique et al., 2003). Therefore, there is need to search bacteria with abilities to metabolize endosulfan. In this regard, screening and identification of the microorganisms with the ability to utilize pesticides as carbon and sulfur as nutrient sources is challenging task (Guerin, 1999; Sutherland et al., 2000). Bacteria with ability to metabolize high levels of endosulfan in liquid and soil can be good for bioremediation purpose. Investigations of the bacterial strains for the abilities to degrade endosulfan at static condition can be useful in devising new approaches of pesticide degradation for bioremediation of soils.This study reports for i) isolation, identification and characterization of a bacterium with ability to degrade Endosulfan, ii) biochemical changes occurred during interaction of the bacteria with endosulfan and iii) account of toxicity of the end products of endosulfan degradation on germination of seeds.

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2. Materials and methods

2.1. Materials

Technical grade endosulfan was purchased from Devisulfan (Endosulfan 35% EC), Dvidayal Agrochemicals Limited, Mumbai, India. Extrapure AR ethyl acetate, hexane, chloroform and acetone of HPLC grade were purchased from SD-Fine chemicals. Nutrient agar was purchased from HiMedia chemicals, India.

2.2. Enrichment, isolation and identification of the bacteria

The minimal agar media suggested by Tepper et al. (1994) with modifications containing following constituents in g/L Glucose 0.1, K_2HPO_4 1.0, $MgSO_4 \cdot 7H_2O$ 0.5, NaCl 0.5, $FeSO_4 \cdot 7H_2O$ 0.001, $MnSO_4 \cdot 4H_2O$ 0.01, $CaCO_3$ 0.05 and Agar–agar 30 g in 1000 ml distilled water containing 50–1000 ppm of endosulfan was prepared. The bacterium with ability to tolerate endosulfan was isolated from soil of cotton field of Jalgaon (latitude 20.901°N, longitude 76.017°E) contaminated with endosulfan. The bacterium was identified by 16S rRNA gene sequencing. For 16S rRNA gene sequencing, the bacterial culture was grown overnight on Luria Agar (LA) plates. Genomic DNA was extracted from a single colony of the bacteria using a phenol–chloroform method (Sambrook et al., 1989). Amplification of the DNA was carried out by polymerase chain reaction (Applied Biosystems) using universal bacterial 16S rRNA gene specific primers 27F/1525R and a region of the 16S rRNA gene was sequenced using Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems). Sequence was obtained using an automatic DNA sequencer (3730 DNA analyzer, ABI) and was deposited in the GenBank database. To construct phylogenetic tree, gene sequence generated in this study was aligned with homologous sequences deposited in GenBank for closely related bacteria using ClustalX (version 2.0.9) (Larkin et al., 2007). All sequences were manually edited using DAMBE (Xia and Xie, 2001). All uninformative sites were removed from further analysis. The phylogenetic tree was constructed by a neighbor-joining method for nucleotide sequences of each dataset using Molecular Evolutionary Genetic Analysis (MEGA) 4.1 (Tamura et al., 2007) with 1000 bootstrap replications and Kimura 2-parameter as a model of nucleotide substitution.

2.3. Effect of nutrient media on endosulfan degradation

The ability of isolate to degrade endosulfan at static condition in minimal broth and nutrient broth was tested up to 336 h. Growth of the bacteria was assessed by measuring the optical density at 600 nm.

2.4. Endosulfan degradation study at static and shaking conditions

The bacterial culture (5% v/v) displaying O.D of 0.15 a.u. at 600 nm was inoculated in 400 ml of nutrient medium in 1000 ml capacity flasks supplemented with 50 mg of endosulfan. The experiment was performed in duplicate. Two flasks containing minimal media inoculated with endosulfan and bacteria were kept at shaking at 120 rpm whereas other two flasks containing minimal media with endosulfan and bacteria were incubated at static conditions at 37 °C. Four uninoculated flasks containing minimal culture media with endosulfan were kept as controls in shaking and static conditions for 360 h. The samples from each flask were collected at the interval of every 48 h up to 360 h and concentration of endosulfan remained in the broth was determined by extraction. For this, equal volume of ethyl acetate was added to the broth, homogenized the mixture for 1 h and sonicated for 2 min and then centrifuged at 5000 rpm for 5 min. The supernatant or organic phase was collected and measured by UV–vis spectrophotometry. The change in the pH of the samples during incubation was monitored by pH meter (PICO pH meter, Lab India). Those conditions showing best microbial growth and high endosulfan degradation were used for the further experiments.

2.5. Effect of initial pH of media on endosulfan degradation

The pH value of medium is generally altered due to microbial metabolic activity or breakdown of chemicals by the microorganism. Therefore, the effect of initial pH on endosulfan degradation was investigated by setting pH of minimal media in the range of 4–9. The pH of the media was maintained using 0.1 N NaOH and 0.1 N HCl. The active bacterial culture (5% v/v) was inoculated in the 250 ml media in 500 ml capacity flasks and incubated at 37 °C using static conditions. The samples were taken out at regular 48 h time intervals from culture flasks and tested for growth, change in pH and endosulfan utilization.

2.6. Degradation of endosulfan in soil

Five kilogram of soil was collected from Jalgaon district for the experimental analysis of degradation of endosulfan in soil. The soil was sterilized at 121 °C at 15 psi for 1 h. Endosulfan concentration of 5000 ppm was achieved by mixing 5 g endosulfan in 1000 ml sterile water in 1 kg soil to make slurry in 2000 ml flask. The slurry was inoculated with 100 ml of 24 h old bacterial isolate culture and another soil slurry containing flask with endosulfan kept as control. The unsterilized soil mixed with 1000 ml distilled water, 10 ml sterile nutrient broth and endosulfan served as positive control, while this mixture without endosulfan served as negative control. 50 gm samples from each flask were collected at the every 48 h interval up to 360 h and concentration of endosulfan remained in the soil slurry was determined by extraction.

2.7. De-chlorination assay

The release of chloride ions during endosulfan degradation was estimated by the colorimetric method of Iwasaki et al. (1952). Soil and broth samples (1 g/ml) were collected at 48 h time intervals and diluted in 10 ml DW, filtered the diluted sample through Whatman filter paper no. 1. One milliliter filtrate was mixed with one milliliter mercury thiocyanate solution (95% alcohol) and incubated for 30 min to form mercury chloride. Then, 1 ml of ferrous alum reagent prepared in 6 N nitric acid was added. The orange red color developed after incubation of 5 min was measured at 460 nm colorimetrically. The released chloride was determined by using standard graph (Iwasaki et al., 1952).

2.8. Arylsulfatase activity

The aryl sulfatase assay was carried out using soil filtrate solution. 1 ml of soil filtrate was added with 1 ml sodium-p-nitrophenyl sulfate; 0.2 ml toluene and 1 ml acetate buffer (pH 5.8) and incubated at 37° C for 15 min. The reaction was terminated with addition of 0.5 ml of NaOH and the contents were centrifuged at 5000 rpm and the intensity of the supernatant was measured at 410 nm. PNS (p-nitrophenyl sulfate) sulfatase activity was expressed in terms of μm of p-nitrophenyl phosphate (PNP) released per minute as per Tabatabai (1994).

2.9. Dehydrogenase activity

Dehydrogenase activity was measured using the triphenyl formazan (TPF) method (Klein et al., 1971; Alef et al., 1995). 1 ml of filtered soil suspension at different time intervals was added with 1 ml of 2,3,5-triphenyl tetrazolium chloride and mixed for