Microbial biodiversity and in situ bioremediation of endosulfan contaminated soil

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Abstract Molecular characterization based on 16S rDNA gene sequence analysis of bacterial colonies isolated from endosulfan contaminated soil showed the presence of *Ochrobacterum* sp, *Burkholderia* sp, *Pseudomonas alcaligenes*, *Pseudomonas* sp and *Arthrobacter* sp which degraded 57–90% of α -endosulfan and 74–94% of β -endosulfan after 7 days. Whole cells of *Pseudomonas* sp and *Pseudomonas alcaligenes* showed 94 and 89% uptake of α -isomer and 86 and 89% of β -endosulfan respectively in 120 min. In *Pseudomonas* sp, endosulfan sulfate was the major metabolite detected during the degradation of α -isomer, with minor amount of endosulfan diol while in *Pseudomonas alcaligenes* endosulfan diol was the only product during α -endosulfan degradation. Whole cells of *Pseudomonas* sp also utilized 83% of endosulfan sulfate in 120 min. *In situ* applications of the defined consortium consisting

of *Pseudomonas alcaligenes* and *Pseudomonas* sp (1:1) in plots contaminated with endosulfan showed that 80% of α -endosulfan and 65% of β -endosulfan was degraded after 12 weeks of incubation. Endosulfan sulfate formed during endosulfan degradation was subsequently degraded to unknown metabolites. ERIC-PCR analysis indicated 80% survival of introduced population of *Pseudomonas alcaligenes* and *Pseudomonas* sp in treated plots.

Keywords Endosulfan degradation · metabolites · endosulfan sulfate · endosulfan diol · *Pseudomonas* sp · *Pseudomonas alcaligenes* · ERIC-PCR

Introduction

Significant increases in agricultural productivity have resulted from control of agricultural pests with manmade chemical pesticides, but on the same hand there is widespread concern about the residues of these manmade chemicals in food and the environment. Endosulfan (1,2,3,4,7,7-hexachlorobicyclo-2,2,1-heptene-2,3-bis(hydroxy methane-5,6-sulfite)) is a mixture of two stereoisomers, α - and β -endosulfan, in a ratio of 7:3. Endosulfan is used extensively throughout the world to control the insect pests of a wide range of crops and most importantly, it has been used for the control of *Helicoverpa* sp. in cotton cropping. Endosulfan is extremely toxic to fish and aquatic invertebrates and has been implicated increasingly in mammalian gonadal toxicity, genotoxicity, and neurotoxicity [18]. It can bind to soil particles and persist for a relatively long period with half-life of 60–800 days [16]. The α -isomer has been found to persist more than β -isomer and the difference in the persistence of the two isomers has been attributed to

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various factors such as their differential volatilization, photodecomposition, alkaline hydrolysis as well as their biotic degradation.

Several microorganisms both bacteria and fungi, which degrade endosulfan have been isolated and characterized [17,1,3,18,12,21,10,15]. Endosulfan diol, endosulfan sulfate, endosulfan ether, endosulfan hydroxyether, endosulfan lactone and endosulfan aldehyde have been reported as the major metabolites formed during biotransformation of endosulfan. Under soil conditions, endosulfan sulfate, which is more toxic than the parent compound itself [13], has been reported to be the major metabolite and) which increases in the environment due to very low degradation rates [20].

Thus in the present study we report on the diversity of endosulfan degrading bacterial systems, evaluate the capability of the enriched bacterial isolates to *in situ* degrade endosulfan and endosulfan sulfate in the complex environment of soil and to assess the survival of the introduced population in the soil based on ERIC - PCR.

Materials and Methods

Isolation and screening of bacterial strains for endosulfan degradation

Soil samples used in the present study for the isolation of endosulfan-degrading microorganisms were collected from Cashew nut plantations in Kasargod District, Kerala, India and endosulfan concentration was between 2.64–34.86 (mg/kg soil). Soil (approximately 10 g) was enriched for endosulfan-degrading organisms by using endosulfan as the only added source of carbon in Bushnell Haas Broth (BHB) medium. Endosulfan was aseptically added to each sterilized flask in a laminar flow hood allowing the acetone (carrier) to evaporate till endosulfan particles floats on the surface. Bacterial strains were isolated from the mixed bacterial culture by dilution technique on agar plates containing endosulfan (50mg/l). Bacterial isolates were then picked and grown overnight in 2ml Luria Broth (LB) medium (2×10^8 /ml) and centrifuged. Pellets were washed with 10mM phosphate buffer (pH 6.8) and inoculated to 50ml BHB media in 250-ml flask containing 50-mg/kg endosulfan. The flasks were incubated on rotary shaker (New Brunswick) for 7 days (37°C, 120 rpm). The complete content (50ml) was extracted with 20ml dichloromethane thrice and the solvent was evaporated and redissolved in acetone. Residual endosulfan was determined by standard curve. Extraction efficiency was found to be $95\% \pm 3\%$.

Whole cells experiment

Cells were grown in 2L-shake flask containing 1L Luria Broth medium. After 24hrs, the cells were centrifuged (10,000 X g, 15min) and washed with 10 mM phosphate buffer (pH 6.8). The pellets were resuspended in 10 mM phosphate buffer (pH 6.8) and then starved for 15/16hr by incubating at 37°C. Protein was estimated by the method described by Itzhaki and Gill, 1964.

Reaction mixture consisted of cell suspension (2 mg protein/ml) in 20ml of 10 mM phosphate buffer (pH 6.8). Reaction was initiated by addition of 100 µg of endosulfan. Uptake of substrate was monitored every 15 min up to 2hrs. Uptake was stopped by adding 6N HCl and extracted with 2ml dichloromethane three times. Solvent was evaporated at room temperature, redissolved in acetone, and residual endosulfan was analyzed by gas chromatography.

Plot Studies

Randomized design was adopted to study the endosulfan degradation in plots (1X1m). Plots were contaminated with endosulfan to achieve 50mg/kg concentration. Cultures were grown in LB medium, harvested after 24 hours and washed. Pellets were resuspended in minimal salt medium and inoculated to plots to achieve 10×10^8 cfu/g soil. Control plot received only minimal salt medium without any cells. Moisture content was maintained at 30% and racking was done regularly. After regular intervals of time, soil samples were collected from five different points, mixed together and then three samples were taken to evaluate the population dynamics and level of endosulfan.

Extraction of endosulfan from soil

Soil (5g) was taken uniformly in a flask and extracted thrice with hexane: acetone (3:1) (5 ml each time) by placing in sonicator water bath (Power Sonic 510) at 50KHz and sonicated at interval of one minute after every 5 min thrice, after which the flasks were removed and kept on a stationary platform to allow the settling of soil. Endosulfan present in the supernatant was aspirated out into an evaporating disc, solvent was evaporated, and redissolved in acetone (2ml) and quantified using GC. The extraction efficiency of endosulfan from soil was always found to be between 80–90 % and of endosulfan sulfate was between 91–94%.

Gas Chromatographic (GC) and GC-MS analysis

Chlorinated pesticide endosulfan was analyzed by Gas chromatography (Autosystem XL, Perkin Elmer) with electron capture detector. Samples were injected and analyzed using capillary column (5% phenyl polymethylsilicone, PE-5) with temperature gradient of 150 °C (2 min) to 280 °C @ 8 °C/min and nitrogen as carrier gas @ 0.75ml/min. Compounds were separated on a PE-5 capillary column (Perkin-Elmer). Electron ionization (70 eV) was performed with a trap current of 100 mA and a source temperature of 200°C. Full-scan spectra were acquired over the ranges of *m/z* 50 to 500 (EI) at 2 s per scan. Helium was used as carrier gas with a flow rate of 1.0 ml/min. Temperature was programmed as of GC.

Molecular characterization of endosulfan degrading bacterial Isolates

Chromosomal DNA was extracted by a modified ROSE (rapid one step extraction) method [19]. Bacterial isolates were characterized and identified by amplifications of 16s rDNA gene sequence. PCR product was cloned into the pDrive-cloning vector and transformed into *E. Coli* DH5 [8]. 16s rDNA sequence of the bacterial isolates and similar sequences were aligned using the Clustal W and dendrograms were generated by the distance neighbour-joining method using MEGA3 software [11]. Numbers are percent gap values after 500 bootstrap replications. Only values above 75% were considered.

Development of DNA fingerprints

Genomic DNA of the bacterial colonies were diluted appropriately and used directly for PCR amplifications. PCR primer sequences [20], used to develop molecular marker, were

ERIC – IR (5' – 3') – ATG TAA GCT CCT GGG
GAA TCA C
ERIC – 2 (5' – 3') - AAG TAA GTG ACT GGG
GTG AGC G

Touch down PCR was used to generate complex DNA fingerprints with 20 cycles, annealing was done at 45 °C to 65 °C with increment of 1 °C/cycle (1.2min), followed by 15 cycles at 65 °C (1.2min). An aliquot of the amplification product was analyzed using 1.5% agarose electrophoresis.

Results and Discussion

Endosulfan contaminated soil samples were serially diluted on basal medium with endosulfan (50mg/l) as the sole carbon source. Five distinct colony types were able to grow. 16s rDNA analysis showed the presence of *Pseudomonas alcaligenes* and *Pseudomonas* sp along with *Ochrobacterium* sp, *Arthrobacter* sp and *Burkholderia* sp (Fig. 1).

Ochrobacterium sp, *Arthrobacter* sp, *Pseudomonas alcaligenes*, *Burkholderia* sp and *Pseudomonas* sp degraded -endosulfan to by 50, 45, 23, 32, and 64 % respectively after 3 days of incubation which increased to 57, 74, 57, 76 and 90% respectively as compared to 9% in control after 7 days (Fig. 2). Similarly, *Ochrobacterium* sp, *Arthrobacter* sp, *Burkholderia* sp, *Pseudomonas alcaligenes* and *Pseudomonas* sp degraded -endosulfan by 63, 54, 61, 37, and 80% respectively after 3 days which increased to 74, 88, 97, 88, and 94% respectively as compared to 7% in control after 7 days (Fig. 2). In endosulfan-contaminated soil, Gram-ve bacteria were predominant and amongst them, *Pseudomonas* sp and *P. alcaligenes* showed the maximum utilization of endosulfan. Therefore these were selected for further studies on the rate of endosulfan uptake and product formation.

Uptake of individual isomer of endosulfan as substrate by whole cells of *Pseudomonas* sp showed 73% uptake of - isomer in first 15 min and 94% after 120min as compared to 6% in control (Fig. 3). In *Pseudomonas alcaligenes*, it uptake was 30% after 15min, which enhanced to 89% after 120min (Fig. 3). Similarly *Pseudomonas* sp utilized 91% and *Pseudomonas alcaligenes* utilized 89% of -endosulfan after 120min as compared to 4% in control (Fig. 3). There was no change in the pH of the medium during degradation studies, it being stable at pH 6.8.

Product formation by *Pseudomonas* sp indicated that endosulfan sulfate was the major metabolite formed with minor amount of endosulfan diol (Fig. 4) due to the degradation of -isomer, as confirmed by GC-MS. Previous reports also indicated the formation of endosulfan sulfate as the major metabolite of endosulfan oxidation, which was not utilized by the majority of the Gram +ve bacteria [19,20] and so accumulated in the system [13,10,19]. Endosulfan sulfate has been found to be more toxic and persistent than endosulfan and might hinder the further degradation of endosulfan. In *Pseudomonas alcaligenes* during endosulfan degradation only endosulfan diol was detected indicating the absence of oxidation reaction. Thus in *Pseudomonas* sp oxidation along with hydrolysis was predominant while in *Pseudomonas alcaligenes* hydrolysis was the only mechanism of endosulfan degradation. As compared to our natural selection of microbes with hydrolytic mechanism, Kwon

Fig. 1 Dendrograms showing the phylogenetic position of enriched bacteria were based on 16S rDNA gene relatedness. Sequences were aligned using the ClustalW (WWW Service at the European Bioinformatics Institute) and the dendrograms were generated by the distance neighbour-joining method using MEGA3 software (Kumar et al, 2004). Numbers are percent gap values after 500 bootstrap replications. Only values above 75% are given.

Fig. 2 Degradation of α - and β -endosulfan (50mg/kg) in BHB medium as sole carbon source by enriched bacterial isolates after 3 and 7 days

et al (2002) specifically screened 40 isolates to select KE-1 that degrade endosulfan to diol. We did not detect endosulfan hydroxyether or endosulfan lactone during the degradation of endosulfan diol by *Pseudomonas alcaligenes*, which has been observed in many microorganisms [18]. Moreover *Pseudomonas alcaligenes* did not utilize endosulfan lactone, which suggested that initial reaction of hydrolysis of endosulfan generates diol but is not probably converted to lactone as has been reported earlier [13,10.]

In situ degradation of endosulfan was studied in plots contaminated with 50mg/kg endosulfan. Soil was sandy loam with pH 6.8. Organic carbon, available phosphorus and total nitrogen were 0.273%, 42.40mg/kg and 1552mg/

Fig. 3 Time course of uptake of α - and β -endosulfan (individual) by whole cells of *Pseudomonas sp* and *Pseudomonas alcaligenes*.

Fig. 4 Product formation during the uptake of α -endosulfan by whole cells of *Pseudomonas sp.*

kg respectively. Moisture content through out the experiment was maintained at 30%. Plots were inoculated with *Pseudomonas alcaligenes* and *Pseudomonas sp* (1:1) to achieve 10^8 cells/g of soil. Defined consortium enhanced the degradation of α - and β -endosulfan to 80% and 70% respectively as compared to uninoculated control, which showed the loss of 29% and 24% of α - and β -endosulfan respectively after 12 weeks of incubation (Fig. 5). Molecular markers based on molecular typing with repetitive sequence (Rep)-based PCR with primer sets ERIC (enterobacterial repetitive intergenic consensus), were generated and used to trace the introduced population into a contaminated soil environment. Population dynamic studies based on ERIC-PCR showed that introduced population (10×10^8) to contaminated plots declined to (3.6×10^8) till 4th week and after which it increased to 8.2×10^8 at 8th week and steadied afterwards, till the end of observation (Fig 5).

Sutherland *et al* (2000) have clearly doubted the ability of mixed culture to utilize endosulfan as sole sulfur source while present in the complex environment of soil. Though,

Awasthi *et al* (2000) evaluated the various environmental factors such as moisture content, soil pH, and substrate concentration etc along with the addition of bacterial co-culture on the degradation of endosulfan isomers in soil; but they failed to correlate the survival of the bacterial co-culture and endosulfan degradation in soil as they reported that alkaline hydrolysis was playing a major role in endosulfan degradation. Population dynamics studied by antibiotic plates in the present study indicated that these isolates are stable in contaminated soil.

Product formation during endosulfan degradation as measured by GC-MS showed the presence of endosulfan sulfate concomitant with the disappearance of endosulfan after one week of incubation (Fig. 6). Endosulfan sulfate continued to accumulate till 6th week and concentration increased to 17.6mg/kg of total endosulfan added (50mg/kg) as compared to 5mg/kg in control (Data not shown). After 6th week, the level of endosulfan sulfate started declining gradually and came down to 9mg/kg at 12th week of incubation.

Awasthi *et al* (2000) observed the formation of endosulfan diol as the major metabolite of endosulfan degradation, primarily by alkaline hydrolysis in soil with high pH value.

Fig. 5 *In situ* degradation of α - & β -endosulfan and survival of defined consortium in plot (1×1 m). *Pseudomonas alcaligenes* and *Pseudomonas sp* were grown in LB medium and mixed in equal proportion to develop defined consortium. Survival of defined consortia was based on ERIC-PCR.

Fig. 6 Formation and degradation of endosulfan sulfate by defined consortium in plots.

Sutherland *et al.*, (2000) has also postulated the formation and persistence of endosulfan sulfate during the biological degradation of endosulfan. We observed the formation of endosulfan sulfate as a major metabolite and it was subsequently utilized during endosulfan degradation. This suggested the active role of our isolates in the degradation of endosulfan isomers in soil. Moreover, for the first time, we have showed the degradation of endosulfan sulfate a persistent metabolite either by defined consortia or by pure bacterial isolates (unpublished data) in the soil. We were unable to detect any known metabolite during the degradation of endosulfan sulfate. This observation was well in accordance with the observation of Guerin (2005) who reported that major amount of endosulfan sulfate was converted to non-recoverable acidic forms, although he also observed the formation of a small proportion of endosulfan diol in endosulfan sulfate treated soil, which was not detected in our study.

Therefore, these bacterial strains either individually or as group have immense potential to act as a technology for the clean up of sites contaminated with either endosulfan or endosulfan sulfate under natural conditions.

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