Journal of Academia and Industrial Research (JAIR)
Volume 2, Issue 3 August 2013

ISSN: 2278-5213

RESEARCH ARTICLE

Degradation of Endosulfan using *Pseudomonas* sp. ED1 isolated from pesticide contaminated soil

G. Vijaiyan Siva and A. Rajam*
Dept. of Biotechnology, University of Madras, Maraimalai Campus, Chennai-600025, India
rajamashok@gmail.com*; +91 9841957782

Abstract

Bacterium capable of degrading endosulfan was isolated and found to be tolerant to (250 ppm) endosulfan than the rest of the isolates obtained from the soil contaminated with endosulfan. The 16s rRNA sequence analysis indicated that bacterium belonged to *Pseudomonas* sp. Thin layer chromatography (TLC) analysis showed the disappearance of β-endosulfan and formation of a new metabolite in the medium inoculated with *Pseudomonas* sp. ED1. Gas chromatography-mass spectrum (GC-MS) analysis showed the presence of unidentified end product as endosulfan monoaldehyde. To identify the responsive gene for degradation of endosulfan; endosulfan degrading Esd expression construct in pET14b (pET14b-esd- plasmid vector) was used as positive control for detection of the specific gene in the strain ED1. Dot blot showed the positive expression of endosulfan degrading genes in the strain ED1.

Keywords: Endosulfan, biodegradation, *Pseudomonas* sp., TLC, GC-MS, endosulfan monoaldehyde**.**

Introduction

Endosulfan is an organochlorine (OC) pesticide that has widespread use in many parts of the world, including European Union, India, Indonesia, Australia, Canada, United States, Mexico and Central America, Brazil and China (Aktar *et al*., 2009). Endosulfan has been in use for more than 5 decades and is effective against a broad number of insect pests and mites. Organochlorine pesticide residues are capable to affect the soil fertility, crop productivity, ecological imbalance and wide range of human health problems (Verma *et al*., 2011). In addition, endosulfan is extremely toxic to fish and aquatic invertebrates, moderately toxic to bird species and has also been shown to be mutagenic to bacteria, yeast and mammalian cells (Awasthi *et al*., 2003).

There is an increasing interest in microorganisms that can adapt and survive in the presence of endosulfan for their possible applications in bioremediation. Several studies have reported the isolation of bacterial coculture (Awasthi *et al*., 1997) and mixed co-culture (Sutherland *et al.*, 2000) capable of degrading endosulfan. Kwon *et al*. (2002) reported the degradation of endosulfan without forming toxic metabolite by *Klebsiella pneumoniae* KE-1. Bioaugmentation of endosulfan contaminated soil by fungal inoculant *Aspergillus niger* ARIFCC 1053 was reported by Bhalerao (2012).

The present study was aimed to degrade endosulfan from the soil using bacterium without forming toxic end product and also to detect the gene involved in the degradation process of endosulfan.

Materials and methods

Chemicals: Technical grade endosulfan (99% pure, Sun Agro Chemicals Pvt. Ltd.) was used throughout the study, which comprises of two isomers α and β-endosulfan in the ratio of 7:3. Esd expression construct in pET14b (pET14b-esd) was a gift from CSIRO Australia.

Microorganism: Soil samples used in the study were collected from agricultural fields contaminated with pesticides. The bacterial cultures capable of degrading endosulfan were isolated from the soil by enrichment technique. One gram of soil sample was diluted in 100mL of distilled water and serially diluted. The sample 0.1 mL was spread plated on nutrient agar with endosulfan at 37ºC. The tolerant strains were isolated and treated with different concentration of endosulfan ranging from 50-225 ppm to determine the tolerance level of each strain. The highly tolerant strain was isolated and characterized. This strain was repeatedly inoculated in minimal medium induced with technical grade endosulfan and maintained in the minimal medium as described by Sutherland *et al*. (2000).

Identification and characterization: The morphological, biochemical, and physiological characteristics of the bacterial isolates were used to identify the strain according to Bergey's Manual of Systematic Bacteriology (1985) and the Manual Methods of General Bacteriology (1994) and for further confirmation, 16S (ribosomal DNA) rDNA sequence comparison was done.

For rDNA analysis, DNA was isolated from the bacterial strain which was used as target for amplification using eubacterial primers:

fD1: 5′AGAGTTTGATCCTGGCTCAG3′ (*Escherichia coli* positions 8 to 27) and
rP2: 5′ACGGCTACCTTGTTACGACTT3′ (*Escherichia coli* positions 1494 to 1513).

Sequencing was carried out using an automated ABI 310 sequencer. The amplified rDNA sequences were compared directly with sequences in the EMBL and GenBank non-redundant nucleotide database using BLAST (Altschul *et al*., 1997). The sequence of this gene was compared against the sequence available from Genbank using the BLAST program (Altschul *et al*., 1997). Database sequences with high similarity were then directly aligned over equalized lengths with the isolate sequences and used in phylogenetic analysis.

Analysis of endosulfan in the medium: A separate experiment was conducted to detect the amount of endosulfan in the medium. Endosulfan (150 µg/mL) was amended in minimal medium along with *Pseudomonas* sp. and a separate set of flasks with uninoculated sample was maintained in parallel as reference. These samples were extracted three times with an equal volume of ethyl acetate. The ethyl acetate fractions from each flask were pooled and the aliquots were analyzed by gas chromatography (GC).

Analysis of endosulfan metabolites using TLC and GC-MS: Cultures (15 mL) were extracted with Dichloromethane (DCM 10 mL) and the organic phase was dried with $MgSO_4$ as described by Sutherland *et al*. (2000). The solution of endosulfan and its lipophilic metabolites was diluted with hexane. TLC was developed in hexane: chloroform: acetone; 9:3:1, on silica gel 60 F254 plates and the separated spots were visualized under UV (Awasthi *et al*., 2003). Under these conditions, the Rf values for a-endosulfan, b-endosulfan, endosulfan sulfate, endosulfan lactone, and endosulfan diol were 0.67, 0.45, 0.36, 0.27, and 0.1 respectively.

For gas chromatography and 63Ni electron-capture detection, a Nucon gas chromatograph- 5765, fitted with a 6'x1/8'' glass column, filled with 80-100 mesh, gas coated with a mixture of 1.5% OV-17 and 1.95% OV-210 was used earlier (Awasthi *et al*., 2003). GC-MS analysis was carried out on a Shimadzu QP5000 GC-MS, equipped with a DB-1 (100% dimethylpolysiloxane) capillary column and a quadrapole mass filter (Samanta *et al*., 1999). Briefly, the injector temperature was kept at 200ºC and the oven temperature was programmed for 120ºC for 5 min, followed by an increase of 10ºC min^{-1} to the final temperature of 200ºC (Awasthi *et al*., 2003).

Identification of gene responsible for degradation of endosulfan:
Dot blot analysis: The isolated genomic DNA was blotted on nitrocellulose membrane without treating it with restriction enzymes. The membrane was dried and soaked in 5% BSA in TBS-T (0.5-1 h, RT). Incubated with probe dissolved in BSA/TBS-T for 30 min at RT. The membrane was washed three times with TBS-T (3 x 5 min). The membrane was carefully removed and cross-linked in UV for 2 min. The X-ray film was exposed in the dark room.

Southern blot: The genomic DNA was isolated from the *Pseudomonas* sp. DNA was digested and the desired DNA region was amplified from 20 ng of genomic DNA using specific primers. The PCR conditions are 1′ pre-amplification at 94°C, 28 PCR cycles of 30 sec at 94°C, 30 sec at X°C* (*varies according to the annealing temperature of the primer set), 1′ and 30 sec at 72°C, and a final extension of 10′ at 72°C. The PCR products were electrophoresed on 1% agarose gels (0.5 X TBE) along with DNA size markers and transferred to nylon membranes (Hybond-N, Amersham, UK) using the capillary transfer method (Sambrook *et al*., 1989). After electrophoresis, the gel was treated with denaturation/alkaline transfer solution for 20′ with gentle rocking. After 20′ the solution was discarded; fresh solution was added and the procedure was repeated. The nylon membrane was initially wetted in water followed by wetting in denaturation solution and the DNA was transferred from gel to membrane using the capillary transfer method. After transfer; the blot was neutralized in the neutralization solution for 20′ and cross-linked by baking at 80°C for 2 h in an oven; followed by UV-cross linking (UVC500 UV-cross linker, Hoefer). The membrane was hybridized for 1 h in southern hybridization solution at 56°C. Thereafter, the solution was decanted and the denatured radio-labeled probe was added along with fresh southern hybridization solution. After 16-18 h of incubation at 56°C, the membrane was sequentially washed with 2X SSC, 0.1% SDS; 1X SSC, 0.1% SDS, and 0.5X SSC, 0.1% SDS at 56°C for 15′ each. The blot was placed on a moist 3 mm Whatmann paper, covered with cling film and exposed to a Kodak BioMax MS film at −70°C.

Results and discussion

Mixed bacterial cultures were isolated from the soil sample and were maintained in minimal medium. Pure cultures were tested for tolerance to different concentration of endosulfan. One particular strain ED1 was able to grow efficiently in higher concentration of endosulfan up to 250 ppm as sole source of sulfur. After 36 h of incubation, the endosulfan concentration was reduced to a negligible amount, whereas in the uninoculated sample the amount of endosulfan was found to be constant (Fig. 1 and 2).

Fig 1. Endosulfan in control condition at 36th h.

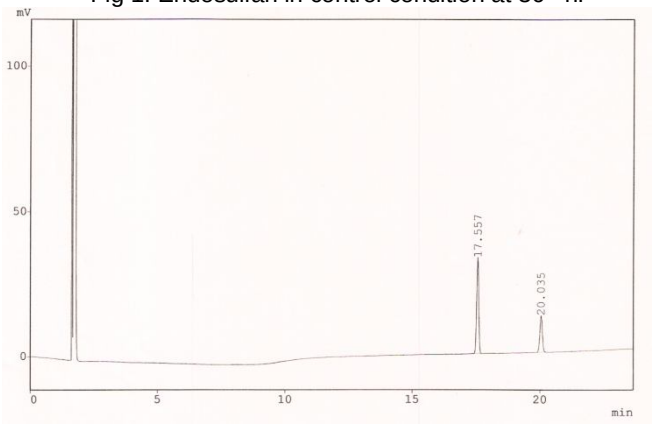
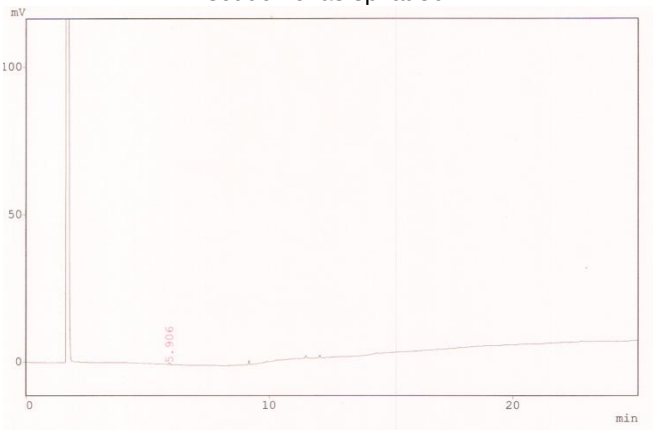


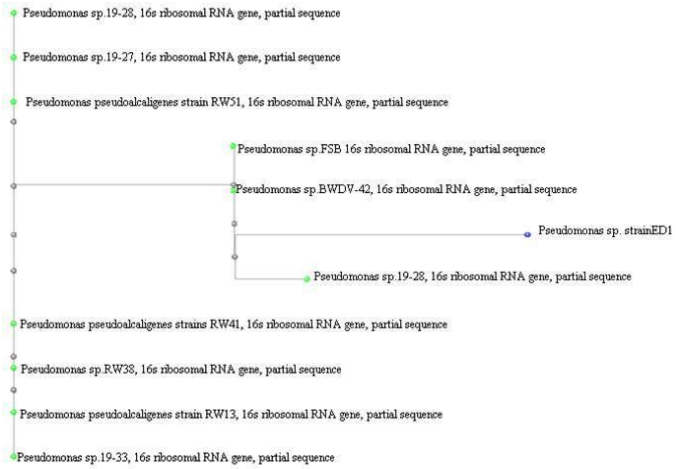
Fig. 2. Endosulfan in medium inoculated with *Pseudomonas* sp. at 36th h.



This shows that the endosulfan has been metabolized or has undergone enzymatic degradation. This bacterium has been found to be efficient in the removal of endosulfan. Kumar and Philip (2006) reported that after four weeks of incubation; mixed bacterial culture was able to degrade 71.82 ± 0.2% and 76.04 ± 0.2% of endosulfan in aerobic and facultative anaerobic conditions respectively. Kumar *et al*. (2008) reported that among the three mixed bacterial culture AE, BE, and CE, developed by enrichment technique with endosulfan as sole carbon source, consortium CE was found to be the most efficient with 72% and 87% degradation of a-endosulfan and b-endosulfan respectively in 20 d.

Characterization of endosulfan degrading bacteria: The physical characteristics of the strain ED1 was determined as a gram negative rod shaped bacterium. Biochemical tests included fermentation patterns of the sugars (arabinose, glucose, galactose, lactose, maltose, manitol, raffinose, sucrose and xylose), capability of starch, gelatin, and indole production, catalase and oxidase positive, VP and MR positive and being homofermentative or heterofermentative. 16s rDNA sequence analysis revealed that the bacterium belongs to *Pseudomonas* sp. (Fig. 3).

Fig. 3. Dendogram of *Pseudomonas* sp. isolated from soil contaminated with pesticides.



Pseudomonas spinosa, P. aeruginosa, (Hussain *et al.,* 2007), *Pseudomonas KS-2P* (Lee *et al*., 2006) and *Pseudomonas* IITR01 (Bajaj *et al*., 2010) are some of the *Pseudomonas* strains that have been reported so far, which can metabolize endosulfan (Sarah *et al*., 2012). Some of the strains which were reported to degrade endosulfan sulphate are *Arthrobacter* sp. (Weir *et al*., 2006), *Pseudomonas* KS-2P (Lee *et al*., 2006), *Klebsiella oxytoca* KE-8 (Kwon *et al*., 2002) and *Pseudomonas* IITR01. Both *Pseudomonas* KS-2P and *Pseudomonas* IITR01 was capable of degrading 70% of endosulfan sulphate.

Analysis of endosulfan metabolites: The extracts obtained from inoculated and uninoculated samples were developed in hexane: chloroform: acetonitrile in the ratio 4:0.5:0.5 on silica gel 60 F_{254}. Thin layer chromatography analysis indicated the formation of three spots (A, B, D) (Fig. 4) in the inoculated sample whereas the uninoculated sample showed two bright spots (C, D) and one mild spot (A) of endosulfan and the concomitant formation of endosulfan metabolites. Spot A (endosulfan diol) and D (α-endosulfan) were seen in both inoculated and uninoculated samples. Metabolite B was formed only in the inoculated sample. This metabolite was extracted and purified. This sample was collected and run on GC-MS to identify the end product. The GC-MS of metabolite B showed a peak with m/z of 342 followed by m/z of 307, 271.24, 241.12, 206.7, 193.89, and 170.223, these peaks were similar to the peaks of endosulfan monoaldehyde (Fig. 5 and 6). The GC-MS of the control (without *Pseudomonas* sp. ED1) showed peaks with m/z of 334 and 272.7506; these peaks were found to be similar to that of endosulfan sulfate. This result concludes that the endosulfan was degraded to endosulfan monoaldehyde within 36 h when inoculated with *Pseudomonas* sp. ED1 whereas in control (absences of the bacterial culture), endosulfan must have undergone oxidation to endosulfan sulfate.

Fig. 4. Thin layer chromatogram showing the metabolites formed during the microbial metabolism of a and b-endosulfan under uninoculated (UI) and inoculated (I) conditions. The separated spots 1-7 represent the authentic compounds a-endosulfan (ES), ES-ether, b-ES, ES sulfate, ES lactone, ES hydroxyether and ES diol, respectively. The spots A, B (both visualized by chromogenic $AgNO_3$ reagent) and C (visible under UV254) represent the position of the formed metabolites.

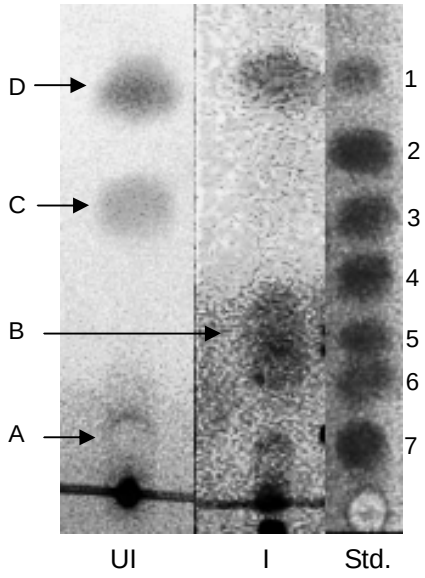


Fig. 5. Mass spectrum of endosulfan metabolite after 36th h of incubation of *Pseudomonas* sp.

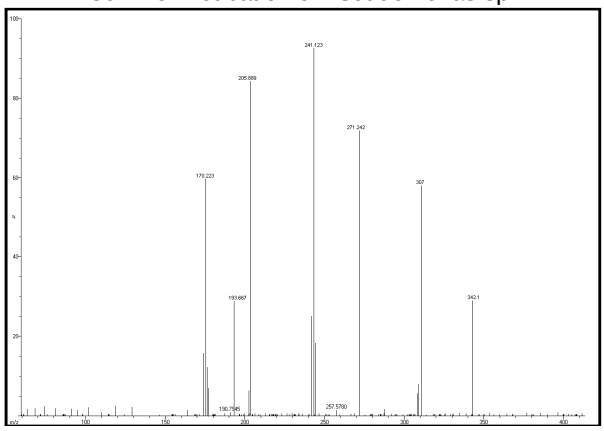
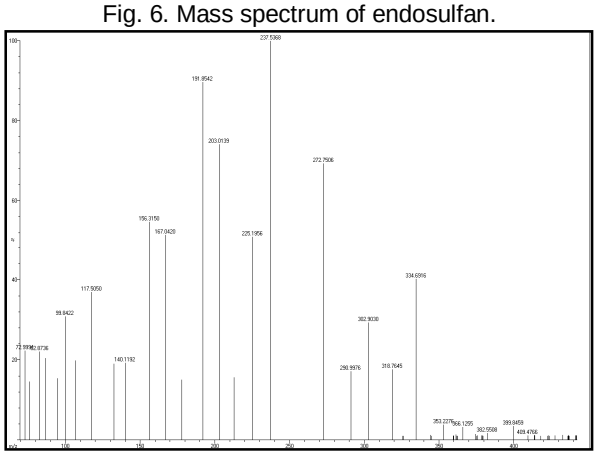


Fig. 6. Mass spectrum of endosulfan.



Similar result was reported by Sutherland *et al*. (2000) where a mixed culture degraded β-endosulfan to three metabolites of which putative monoaldheyde was the major metabolite formed and the other two were endosulfan hydroxyether and endodiol. Aswathi *et al*. (2003) reported that the degradation of both the isomers was accompanied by the formation of metabolites, enosulfan diol, endosulfan lactone, and an uncharacterized metabolite. However, the accumulation of metabolite endosulfan sulfate, which is more persistent and is nearly as toxic as α and β-isomers of endosulfan, was not observed in this study. Weir *et al*. (2006) showed that the metabolites found after degradation of endosulfan and endosulfate by *Arthrobacter* sp. strain KW were characterized by TLC and GC-MS analysis. Three metabolites of endosulfate were observed when *Arthrobacter* sp. strain KW was grown with endosulfate as the sole sulfur source. The TLC and GC analysis and their mass spectra suggest that these metabolites were endosulfan monoalcohol, endosulfan dimethylene and endosulfan hydroxymethylene; the three metabolites found in the mixed soil microbial culture from which *Arthrobacter* sp. strain KW was isolated.

Detection of gene in the strain: Dot blotting is a classic, simple assay for detection and quantification of a specific nucleic acid sequence (mRNA, rRNA and DNA) in a complex mixture (Thomas, 1980). Here, the DNA sample was fixed on a membrane and hybridized with an oligonucleotide or polynucleotide probe labeled radioactively. Positive sample (the esd gene from plasmid vector) and negative control (water) along with the test sample i.e. DNA from *Pseudomonas* sp. was placed on nitrocellulose membrane. Figure 7 shows a positive result, a dark spot was observed in the positive control and the test sample. This indicates that the biomolecule similar to that of esd gene was identified through the Dot blot technique. Dot blotting has been used to detect or quantify tfdA DNA (gene tfdA is involved in degradation of phenoxyacetic acids such as 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA)) and mRNA in environmental samples enriched on 2, 4-D (Holben *et al*., 1988; Xia *et al*., 1995; de Lipthay *et al*., 2002). However, due to reduced sensitivity compared to PCR techniques (Brockman, 1995; Takai and Horikoshi, 2000; De Lipthay *et al*., 2002), dot blotting is less suitable for oligotrophic environments associated with low target gene copy numbers (the detection limit of tfdA genes in a pure culture of *Ralstonia eutropha* JMP134 by dot blotting corresponded to 106 colony forming units (De Lipthay *et al*., 2002) but is valuable for the rapid quantification of nucleic acids in enriched microbial communities. A southern blot is a method routinely used for detection of a specific DNA sequence in DNA samples. Southern blotting combines transfer of electrophoresis-separated DNA fragments to a filter membrane and subsequent fragment detection by probe hybridization.

Fig. 7. Dot-Blot: A-DNA from *Pseudomonas* sp.; B-negative control (water); C-positive control (esd gene from the vector).

A B C

In this study, restriction analysis was initiated by digesting the DNA with various restriction endonucleases. DNA fragments were then separated electrophoretically in 0.8% agarose gels (DNA sample from *Pseudomonas* sp. which was digested and the positive esd gene which was isolated from *E. coli* BL21), blotted onto nitrocellulose membranes and fixed by standard procedures according to Sambrook *et al*. (1989). Southern blotting was performed with a Rediprime, Amersham blotter. Hybridizations with esd probes were carried out as described above. This showed a strong signal nearly at 1.5 kb in the positive esd gene loaded lane whereas no signal was obtained in the *Pseudomonas* DNA. Since the results of the Dot blot showed a positive signal, this confirms that the biomolecule similar to that of esd gene is present in the strain ED1. The southern blot analysis of a methyl parathion-degrading strain of a *Pseudomonas* sp. against α-^{32}P-labeled 1.2-kb probe containing an *opd* gene from *P. diminuta* GM showed that its methyl parathion hydrolase gene had homology to the *opd* gene (Chaudhry *et al*., 1988). Zhongli *et al*. (2001) isolated mpd gene from methyl parathion degrading gene from soil. This was confirmed by southern blot technique. Southern hybridizations with fragments of the *linA*, *linB*, *linC* and *linD* genes of *Sphingomonas paucimobilis* UT26 were carried out by Kumari *et al*. (2002) and these genes were capable of degrading hexachlorocyclohexane.

Conclusion

The endosulfan is persistent in soil for a longer period. The removal of this toxic pesticide was the main aim of this study. From the results we conclude that the bacterium *Pseudomonas* sp. was highly efficient bacterium to degrade toxic endosulfan. The product obtained was found to be as endosulfan monoaldheyde a non-toxic product. The release of this non-toxic product proves it to be a beneficial bacterium in bioremediation of endosulfan. Further experimentation was aimed to detect the responsive gene for degrading endosulfan in this strain. The pET14 b-esd was used as a positive control to find the same signal as that in the DNA isolated from *Pseudomonas* sp. ED1 DOT blot analysis showed a strong signal as that in the positive control. A specific restriction fragment in a Southern blot was unable to detect with the probe which had a similar sequence with the positive control. Hence further experiments should be conducted to identify specific gene or amino acid sequencing might reveal the exact gene for degradation. From these results we conclude that a similar gene might be responsible for degradation of endosulfan.

Acknowledgements

Author Rajam Ashok thanks Dr. H.D. Subashini for providing technical support for the paper work.

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