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ORIGINAL PAPER

Biodegradation of α and β endosulfan in broth medium and soil microcosm by bacterial strain *Bordetella* sp. B9

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Abstract Bacterial strains were isolated from endosulfan treated soil to study the microbial degradation of this pesticide in broth medium and soil microcosm. The isolates were grown in minimal medium and screened for endosulfan degradation. The strain, which utilized endosulfan and showed maximum growth, was selected for detail studies. Maximum degrading capability in shake flask culture was shown by *Bordetella* sp. B9 which degraded 80% of α endosulfan and 86% of β endosulfan in 18 days. Soil microcosm study was also carried out using this strain in six different treatments. Endosulfan ether and endosulfan lactone were the main metabolites in broth culture, while in soil microcosm endosulfan sulfate was also found along with endosulfan ether and endosulfan lactone. This bacterial strain has a potential to be used for bioremediation of the contaminated sites.

Keywords *Bordetella* sp. B9 · Biodegradation · Soil microcosm

Introduction

The chlorinated cyclodiene insecticide endosulfan (IUPAC name 6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,3,4-benzo(e)dioxathiepin-3-oxide) is a mixture of two stereo isomers i.e. α and β endosulfan in ratio of 70:30. It is moderately persistent in soil, where it is converted into sulfate, which is highly persistent. Endosulfan persists in the soil and water for 3–6 months or more (Awasthi et al. 2000; Kathpal et al. 1997) and has been detected in different components of the environment (U.S. Department of Health and Human Services 1990).

In soil, endosulfan can be utilized as a sole source of carbon and/or sulfur during biodegradation (Guerin 1999; Sutherland et al. 2000) and undergo degradation to non-toxic or less toxic endosulfan diol or oxidation to equally or more toxic endosulfan sulfate (Goebel et al. 1982). Formation of endosulfan sulfate is mediated essentially by microorganisms while hydrolysis of endosulfan to diol can be a microbial and/or chemical reaction, especially under alkaline conditions. Endosulfan diol can further be transformed by microorganisms to endosulfan ether, endosulfan hydroxyether, endosulfan dialdehyde and endosulfan lactone (Kullman and Matsumura 1996).

A number of studies have reported the degradation of endosulfan under aerobic and anaerobic conditions by different bacterial cultures. *Klebsiella pneumoniae* biologically degraded 8.72 μg endosulfan ml^{-1} day^{-1} when incubated with 93.9 μg ml^{-1} endosulfan for 10 days without formation of toxic endosulfan sulfate (Kwon et al. 2002). *Pandoraea* sp. degraded about 95 and 100% of α and β endosulfan respectively, in 18 days of incubation in flasks spiked with 100 mg l^{-1}

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of endosulfan, without forming endosulfan sulfate (Siddique et al. 2003b). *Klebsiella oxytoca* degraded 154–26 mg l^{-1} of endosulfan in 6 days, a decrease of 83% (Kwon et al. 2005). Endosulfan degradation has also been reported with mixed cultures (Miles and Moy 1979; Awasthi et al. 1997, 2003; Sutherland et al. 2000; Kumar and Philip 2006; Kumar et al. 2007). Weir et al. (2006) reported that in *Arthrobacter* sp. there is a single monooxygenase Ese gene encoding an enzyme capable of degrading both isomers of endosulfan and endosulfan sulfate.

Due to the persistence of pesticide and its metabolites, biological means for detoxification of endosulfan is receiving serious attention as an alternative to existing methods such as incineration and landfill (Siddique et al. 2003a). Keeping this in view, in the present study bacterial strain *Bordetella* sp. B9 was isolated using enrichment technique on endosulfan as a carbon source to search its potential to be used for bioremediation at the contaminated sites.

Materials and methods

Culture media and chemicals

Endosulfan (≤99% purity) was obtained from the Institute of Pesticide Formulation Technology, Gurgaon, India. Endosulfan sulfate, endosulfan diol, endosulfan ether, endosulfan hydroxyether and endosulfan lactone were obtained from Industrial Toxicological Research Centre, Lucknow, India. Ethyl acetate, hexane, acetone and ethanol used in extraction and gas chromatography were purchased locally. The composition of the different media used were, Broth Minimal medium: KH_2PO_4 1.0 g, K_2HPO_4 1.0 g, NH_4NO_3 1.0 g, $MgSO_4$ 0.2 g, $CaCl_2$ 0.02 g, $Fe(SO_4)$ 0.01 g, Double distilled water 1 l, pH 6.5; Nutrient medium agar plates: Peptone 10.0 g, Yeast extract 5.0 g, NaCl 5.0 g, Double distilled water 1 l, pH 6.5 (Awasthi et al. 1997).

Enrichment, isolation and identification of microorganisms

Surface soil (0-15 cm) from repeatedly endosulfan treated cotton fields was used for the isolation of bacteria. Soil samples (5 g) in triplicate were taken in 250 ml Erlenmeyer flasks containing 50 ml of the broth minimal medium and 50 μg ml^{-1} of technical endosulfan (stock 1×10^5 μg ml^{-1} in ethanol) and were aerobically incubated at 30°C with continuous shaking (150 rpm). After 10 days, 5 ml of broth culture from each flask was re-inoculated to 50 ml of fresh media with 50 μg ml^{-1} endosulfan and cultured under same conditions. This process was repeated three more times. After that 0.1 ml of culture broth was applied to agar plates for isolation of single colonies. Each colony was considered as a different strain and repeatedly streaked on agar plates. Pure strains were obtained by streaking for more than 20 times. Pure strains were further screened for endosulfan degradation before using it for subsequent studies.

To select the endosulfan degrading bacteria, the strains were grown in minimal medium with endosulfan. Nine strains of bacteria were initially isolated. These bacterial strains were identified in collaboration with the Department of Microbiology, University of Delhi, India, on the basis of various biochemical tests like gram staining, motility, production of acid from carbohydrate, urea utilization, gelatin hydrolysis, production of indole, citrate utilization, methyl red test, voges proskauer reaction, starch hydrolysis test, reduction of nitrate to nitrite, catalase test and 16S rRNA sequence analysis.

Isolated genomic DNA was used as the template for the PCR reaction for amplification of 1.2 Kb 16S rRNA gene. The forward and the reverse primer sequences used for the amplification were 5′ AGAGTTTGATCCTGGC TCAG 3′ (Forward Primer) and 5′ TACGGCTACCTTG TTACGACTT 3′ (Reverse Primer).

A PCR reaction mix containing 50 ng of template DNA, 75 p moles primer (forward and reverse),0.2 mM dNTP, 10× reaction buffer (pH 9), 2 U of Taq DNA polymerase (Banglore Genei). The thermocycler conditions were, initial denaturation at 95°C for 5 min, 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min and final extension at 72°C of 10 min.

Based on the growth in the endosulfan containing broth medium, three strains of bacteria i.e. *Bordetella* sp. B9, *Pseudomonas aeruginosa* S9 and *Pseudomonas aeruginosa* S2 were selected for further studies. In these three bacterial strains *Bordetella* sp. B9 degraded 80 ± 2% of α endosulfan and 86 ± 1% of β endosulfan in 18 days of incubation followed by strain *Pseudomonas aeruginosa* S9 (α 62 ± 0.2% and β 63 ± 0.5%) and *Pseudomonas aeruginosa* S2 (α 32 ± 1% and β 45 ± 1%). *Bordetella* sp. B9 which has highest degradation capability was taken for further studies.

Optimum concentration of endosulfan for *Bordetelle* sp. B9

Endosulfan ranging from 50 to 500 μg ml^{-1} was prepared in 100 ml minimal medium taken in 250 ml conical flasks to which 1 ml of bacterial inoculum (OD_{600} 0.5) was added. The flasks were shaken at 150 rpm at 30°C for 7 days to get optimum yield of bacterial isolate. Samples were taken after every 24 h and growth was measured at 600 nm spectrophotometrically.

Growth kinetics of *Bordetella* sp. B9

The growth was measured in terms of optical density and biomass. The study was performed in triplicate (from three flasks). Bacterial strain was grown in broth medium supplemented with 100 μg ml^{-1} of endosulfan. The flasks were then incubated at 30°C on a rotary shaker (150 rpm) for 18 days. The samples were taken at intervals of 0, 1, 4, 7, 10, 13 and 18 days. Growth was measured spectrophotometrically. For biomass study, 1 ml sample was taken from each interval and was centrifuged at 8,000 rpm (6,010×*g*) at 4°C in Sorvall centrifuge for 10 min. The supernatant was removed from the eppendorf tubes and these tubes were then air dried overnight and weighed to determine the biomass.

Biodegradation of endosulfan in broth culture

Bordetella sp. B9 was grown in broth medium with 100 μg ml^{-1} of endosulfan for 18 days. Uninoculated spiked flasks were also set up as a control. The samples taken at intervals of 0, 1, 4, 7, 13 and 18 days, were extracted by shaking with equal volume of ethyl acetate. The extraction procedure was repeated four times. The organic phases were pooled and evaporated to dryness in Univapo vaccum evaporator. Extracted residues were redissolved in 0.5 ml of ethyl acetate and analyzed by Gas Liquid Chromatography (Shimadzu chromatograph model GC-17 A) equipped with a ^{63}Ni Electron Capture Detector (ECD) and a SGE column, Code: 30 × 0.25 mm, ID-BP1, 0.25 μm. The oven temperature was programmed for an initial temperature of 180°C (hold 2 min) and raised to 250°C (hold 2 min) at 10°C min^{-1} and then finally raised to 270°C (hold 4 min) at 20°C min^{-1}. The temperature of the injector port and detector was maintained at 250 and 300°C, respectively.

Effect of supplementary carbon on the degradation of endosulfan

Growth of bacterial strain was measured by optical density and biomass in the presence of dextrose (1 g l^{-1}) as a supplementary carbon source in addition to endosulfan (100 μg ml^{-1}) in broth minimal medium for 18 days. Samples withdrawn at intervals of 0, 1, 4, 7, 10, 13, 18 days, were also analyzed for residual endosulfan concentration using gas liquid chromatography. Growth was also seen in medium supplemented with dextrose as a carbon source without endosulfan.

Biodegradation of endosulfan in soil microcosm

Microcosm study was carried out with isolated bacterium *Bordetella* sp. B9. Mass culturing of bacterium was done till late exponential phase. The culture medium was then centrifugated at 6,000 rpm (3,380×*g*) at 4°C for 15 min. The supernatant was removed and pellet was washed twice with double distilled water. Six microcosm treatments with different combinations were prepared using 1 l capacity cylindrical glass jars. Treatment 1 contained sterilized soil 250 g and endosulfan at a concentration of 50 mg kg^{-1} composite soil sample. Treatment 2 contained sterilized soil 250 g, bacterial inoculum at the concentration of 2×10^8 cells g^{-1} soil and endosulfan at a concentration of 50 mg kg^{-1} composite soil sample. Treatment 3 contained sterilized soil 250 g, bacterial inoculum at the concentration of 2×10^8 cells g^{-1} soil, endosulfan at a concentration of 50 mg kg^{-1} composite soil sample and cane molasses 2.5 g. Treatment 4 contained sterilized soil 250 g, bacterial inoculum at the concentration of 2×10^8 cells g^{-1} soil, endosulfan at a concentration of 50 mg kg^{-1} composite soil sample and dextrose (2.5 g). Treatment 5 contained natural soil 250 g, bacterial inoculum at the concentration of 2×10^8 cells g^{-1} soil and endosulfan at a concentration of 50 mg kg^{-1} composite soil sample and Treatment 6 contained natural soil 250 g and endosulfan at a concentration of 50 mg kg^{-1} composite soil sample. Adequate amount of water to yield a soil moisture content of the desired percent of water holding capacity upto 50% was also added. All the components in the six treatments were thoroughly mixed, the glass jars were covered with perforated aluminum foil and incubated at 30°C for 60 days

aerobically under sterile conditions in B.O.D incubator. Distilled water was added at regular intervals to compensate for loss of water. Experiment was done in triplicates and soil samples were taken out at 0, 1, 7, 10, 15, 20, 30 and 60 days interval for extraction of residual endosulfan. Colony count study was done for the soil samples taken at regular intervals.

Extraction of endosulfan from microcosm treatments

Endosulfan was extracted from each 5 g soil samples using 20 ml hexane and acetone mixture (8:2 v/v). The samples were shaken for 1 h on an orbital mechanical shaker and then filtered through Whattman filter paper. This procedure was repeated thrice. Collected solvent extract was dried up using rotary flash evaporator and redissolved in 3 ml ethyl acetate for Gas Chromatography analysis. The GC conditions were same as mentioned above.

Results

Bacterial strain used in the biodegradation study was Gram-negative and catalase-positive. *Bordetella* sp. type strain (HPC293), was identified from 371 base segment 16S r RNA analysis. The BLAST program was used for gene homology search with the standard program default.

Optimum concentration of endosulfan for each microbe

The lower concentrations (50, 100, 200 μg ml^{-1}) as well as higher concentrations (300, 400, 500 μg ml^{-1}) of the endosulfan were tried in order to check whether the supplemented concentration of endosulfan was either too low to thrive on or too high, to be toxic for the isolated microbe. The optimum concentration at which *Bordetella* sp. B9 showed the maximum growth was 100 μg ml^{-1}.

Growth kinetics of *Bordetella* sp. B9 in medium supplemented with endosulfan as a carbon source

Bordetella sp. B9 had no lag phase (OD 0.25 ± 0.13) because bacterium was collected from endosulfan repeatedly treated soil and isolated from culture

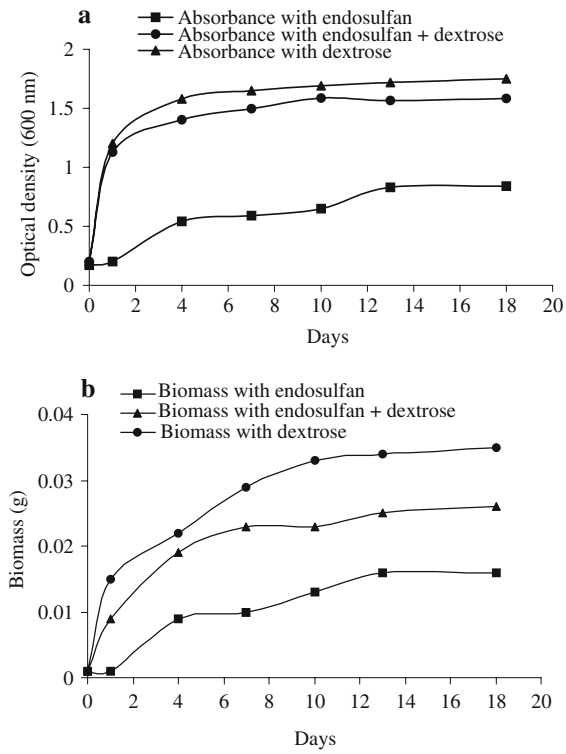

Fig. 1 Growth kinetics of *Bordetella* sp. B9 in broth culture (**a**) Optical density, (**b**) Biomass

medium containing endosulfan. It adapted itself in the medium quickly and utilized endosulfan as a carbon source. A sharp increase in growth was observed upto 4th day (OD 0.54 ± 0.05) after which a gradual increase was observed till 13th day (OD 0.83 ± 0.12). This is the period during which the strain has fully adapted itself to the medium. However, after that the growth was almost stagnant (OD 0.84 ± 0.05 after 18 days of incubation) (Fig. 1a). Biomass study showed similar pattern. The Biomass after 24 h of incubation was 0.001 ± 0.005 g, which increased to 0.009 ± 0.001 g after 4 days. An increase in weight was observed till 13th day (0.016 ± 0.001 g) after which no change in biomass was observed till 18th day (00.016 ± 0.001 g) (Fig. 1b).

Growth kinetics of *Bordetella* sp. B9 in medium supplemented with dextrose as a carbon source

In the presence of dextrose as a carbon source, *Bordetella* sp. B9 showed maximum growth, in exponential pattern. The initial optical density (600 nm) at 0 h was 0.20 ± 0.02, which increased to 1.2 ± 0.02

after 24 h of incubation and to 1.75 ± 0.01 after 18 days (Fig. 1a). Biomass at 0 h was 0.001 ± 0.001 g, after 24 h was 0.015 ± 0.001 g and after 18 days of incubation was 0.035 ± 0.002 g (Fig. 1b).

Degradation of endosulfan by *Bordetella* sp. B9 in culture

The degradation of α and β endosulfan was determined by monitoring their disappearance and the appearance of metabolites. Bacterial strain *Bordetella* sp. B9 degraded about 80 ± 2% of α endosulfan and 86 ± 1% of β endosulfan, in 18 days of incubation in flasks spiked with 100 μg ml^{-1} of endosulfan. This bacterium degraded about 10 ± 2% of α endosulfan and 13 ± 0.5% of β endosulfan in 24 h, which increased to 53 ± 0.9% (α endosulfan) and 59 ± 1.0% (β endosulfan) after 7 days of incubation. In the control flasks, after 18 days of incubation, degradation of about 11 ± 1% of endosulfan α and 5 ± 2% of endosulfan β was observed (Fig. 2). The pH of the culture varied in the range of 6.5 ± 0.01 to 6.2 ± 0.03 during the 18 days of incubation.

Effect of supplementary carbon source

The bacterial strain showed an exponential growth in the presence of dextrose in addition to endosulfan as a carbon source. The optical density (600 nm) after 24 h of incubation was 1.13 ± 0.03 which increased to 1.58 ± 0.05 after 18 days (Fig. 1a). Biomass of this strain at 0 h was 0.001 ± 0.002 g, after 24 h was 0.009 ± 0.003 g and after 18 days of incubation was 0.026 ± 0.001 g (Fig. 1b). Endosulfan degradation in the dextrose amended medium after 18 days of incubation was 53 ± 0.14% (α endosulfan) and 48 ± 0.0.08% of β endosulfan (Fig. 2).

Biodegradation of endosulfan in soil microcosm

The degradation of endosulfan in the different treatments is shown in Figs. 3 and 4. Treatment 1, control sample, contained sterilized soil and endosulfan. After 60 days of incubation about 40.4 ± 1.0% of α endosulfan and 22 ± 0.3% of β endosulfan disappeared from this treatment which might be due to chemical reactions at the set conditions. Treatment 2, which contained sterilized soil amended with bacterial inoculum and endosulfan showed highest degradation of 83.2 ± 2% of α endosulfan and 56.9 ± 1% of β endosulfan. Treatment 3 contained sterilized soil amended with bacterial inoculum, endosulfan and cane molasses as additional source of carbon. The percent degradation of endosulfan in this treatment was 70.4 ± 0.2% of α endosulfan and 36.1 ± 0.3% of β endosulfan. In Treatment 4 which contained sterilized soil amended with bacterial inoculum, endosulfan and dextrose as additional source of carbon, the percent degradation of endosulfan was 74.8 ± 0.5% of α endosulfan and 53.1 ± 0.2% of β endosulfan. Treatment 5 contained natural soil amended with bacterial inoculum and endosulfan showed a percent degradation of 60.5 ± 1.0% of α endosulfan and 35.4 ± 0.5% of β endosulfan. In

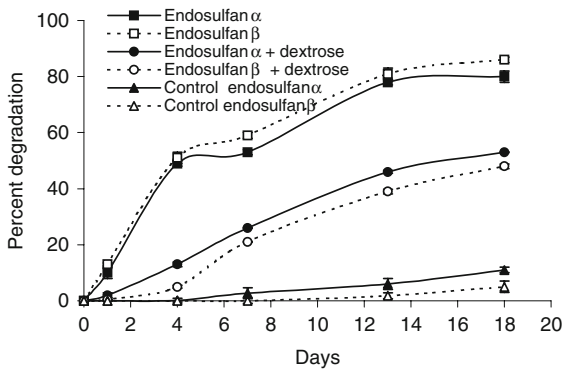

Fig. 2 Degradation of endosulfan by *Bordetella* sp. B9 in broth culture

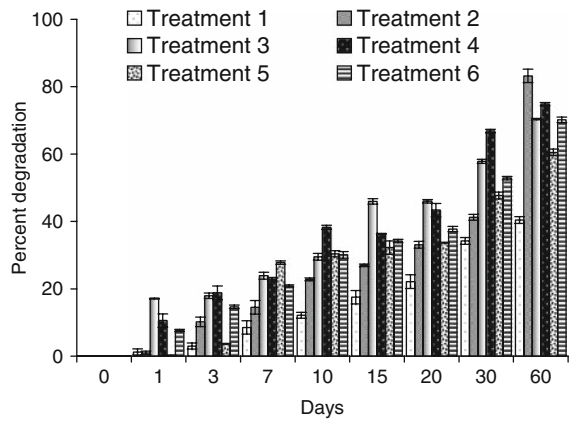

Fig. 3 Degradation of α endosulfan by *Bordetella* sp. B9 in soil microcosm in different treatments: Treatments 1–6

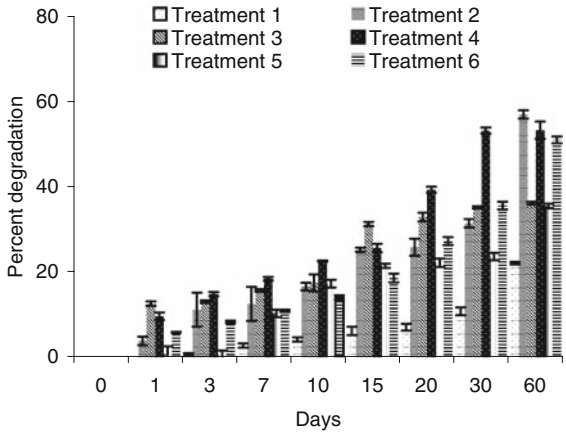

Fig. 4 Degradation of β endosulfan by *Bordetella* sp. B9 in soil microcosm in different treatments: Treatments 1–6

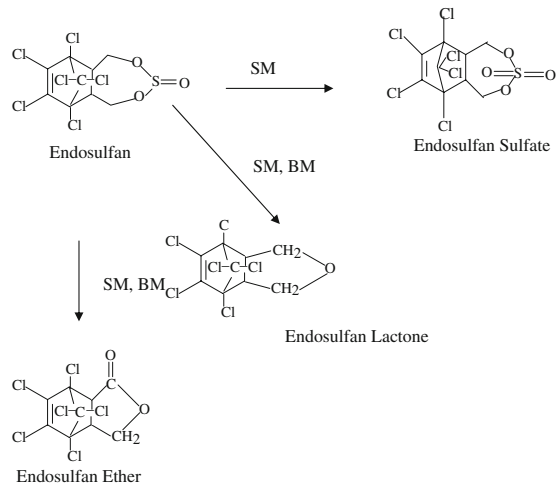

Fig. 5 Endosulfan degradation by *Bordetella* sp. B9 in broth culture and soil microcosm. SM-Soil microcosum, BM-Broth medium

Treatment 6, natural soil and endosulfan, a percent degradation of 70.2 ± 0.8% of α endosulfan and 50.9 ± 0.8% of β endosulfan was observed.

The variation in pH in different treatments during soil microcosm study was also observed. In Treatment 1 the initial pH at 0 day was 7.2 ± 0.1, which increased to 7.4 ± 0.5 after 60 days of incubation. In Treatments 2–4, the pH at 0 day were 7.1 ± 0.3, 6.9 ± 0.7 and 6.9 ± 0.2 which increased to 7.2 ± 0.07, 7.3 ± 0.5 and 7.0 ± 0.1, respectively, after 60 days of incubation. Slight increase in pH was observed in these treatments. However, in Treatments 5 and 6, the pH slightly decreased during 60 days of incubation. The pH in Treatments 5 and 6 at 0 day was 7.1 ± 0.3 and 7.2 ± 0.9 which decreased to 6.9 ± 0.4 and 7.0 ± 0.1, respectively, after 60 days.

Metabolites formed in the broth culture and soil microcosm study

GLC analysis indicated the degradation of endosulfan and formation of metabolites (Fig. 5). The main metabolites formed by *Bordetella* sp. B9 in broth culture were endosulfan ether and endosulfan lactone. No endosulfan sulfate residues were observed in the broth culture. The concentration of endosulfan ether after 6 days of incubation was 0.53 ± 0.2% which decreased to 0.41 ± 0.13% after 18 days. However, the concentration of endosulfan lactone was 0.24 ± 0.09% after 6 days which increased to 0.35 ± 0.07% after 18 days. In control sample, which did not have any bacteria, endosulfan ether (0.02 ± 0.01%) was the only metabolite observed after 18 days of incubation.

In the soil microcosm study the main metabolites formed were endosulfan ether, endosulfan lactone and endosulfan sulfate. In Treatment 1, which acted as a control, endosulfan ether appeared on 10th day with a concentration of 0.1 ± 0.09% that increased to 3.32 ± 0.3% after 60 days of incubation. Endosulfan lactone was found on 30th day with a concentration of 0.34 ± 0.1% which increased to 1.4 ± 0.1% after 60 days of incubation. Small amount of endosulfan sulfate was also found after 60 days of incubation (0.82 ± 0.2%) In Treatments 2–6, the metabolite endosulfan ether was formed on day one with a concentration of 0.6, 0.55, 0.46, 0.81 and 1.13%, respectively Endosulfan lactone was formed on 3rd day in Treatment 3 (1.6%), Treatment 5 (4.7%) and Treatment 6 (0.97%) and on 15th day in Treatment 2 (0.5%) and Treatment 4 (1.55%). Endosulfan sulfate appeared on 3rd day in Treatment 5 (9.7%) and Treatment 6 (3%) and on 7th day in Treatment 3 (0.54%) and on 10th day in Treatment 2 (0.4%) and Treatment 4 (0.3%). However accumulation of endosulfan sulfate was observed in all the treatments. The concentration of endosulfan sulfate after 60 days of incubation in Treatments 2–6 was 6, 8.3, 4.9, 29, 21%, respectively (Table 1). Metabolites were confirmed by matching retention times with standards by GLC.

Table 1 Percentage of endosulfan metabolites formed by *Bordetella* sp. B9 in soil microcosm

DAYS	T1			T2			T3		
	EE	EL	ES	EE	EL	ES	EE	EL	ES
1	0	0	0	0.6 ± 0.03	0	0	0.55 ± 0.02	0	0
3	0	0	0	0.6 ± 0.03	0	0	0.5 ± 0.02	1.6 ± 0.06	0
7	0	0	0	0.8 ± 0.07	0	0	0.81 ± 0.04	2.2 ± 0.09	0.54 ± 0.08
10	0.1 ± 0.09	0	0	0.8 ± 0.06	0	0.4 ± 0.05	0.96 ± 0.03	3.6 ± 0.09	1.1 ± 0.07
15	0.13 ± 0.06	0	0	1.0 ± 0.11	0.5 ± 0.08	1.0 ± 0.06	0.95 ± 0.09	3.7 ± 0.06	2.1 ± 0.07
20	0.16 ± 0.06	0	0	1.0 ± 0.09	0.7 ± 0.09	1.4 ± 0.09	1.02 ± 0.07	1.8 ± 0.06	2.9 ± 0.15
30	1.17 ± 0.12	0.34 ± 0.11	0	1.3 ± 0.09	1.3 ± 0.09	2.3 ± 0.1	1.77 ± 0.12	3 ± 0.11	3.6 ± 0.2
60	3.32 ± 0.3	1.4 ± 0.12	0.82 ± 0.23	5.0 ± 0.2	5 ± 0.07	6 ± 0.09	3.21 ± 0.2	7.3 ± 0.31	8.3 ± 0.4

T4			T5			T6		
EE	EL	ES	EE	EL	ES	EE	EL	ES
0.46 ± 0.05	0	0	0.81 ± 0.04	0	0	1.13 ± 0.3	0	0
0.6 ± 0.05	0	0	1.28 ± 0.02	4.7 ± 0.06	9.7 ± 0.15	2.0 ± 0.07	0.97 ± 0.05	3 ± 0.28
0.45 ± 0.04	0	0	1.8 ± 0.08	4.3 ± 0.09	13 ± 0.5	2.2 ± 0.09	1.18 ± 0.09	7 ± 0.41
0.49 ± 0.08	0	0.3 ± 0.05	1.65 ± 0.08	4 ± 0.19	16 ± 0.91	2.0 ± 0.09	1.45 ± 0.05	11 ± 0.41
0.62 ± 0.08	1.55 ± 0.06	1.48 ± 0.07	2.11 ± 0.02	4.5 ± 0.11	20.8 ± 2	2.5 ± 0.33	1.44 ± 0.05	11.5 ± 0.25
0.74 ± 0.06	1.9 ± 0.06	2.2 ± 0.07	2.38 ± 0.17	3.7 ± 0.16	22 ± 0.09	2.7 ± 0.21	1.62 ± 0.14	16 ± 0.77
1.09 ± 0.09	1.05 ± 0.03	3.3 ± 0.09	2.84 ± 0.09	4.2 ± 0.21	25.1 ± 0.29	1.9 ± 0.09	1.66 ± 0.09	15.6 ± 0.26
1.86 ± 0.09	1.17 ± 0.01	4.9 ± 0.09	3.95 ± 0.26	5.2 ± 0.09	29 ± 0.6	2.2 ± 0.32	1.64 ± 0.14	21 ± 1

EE, Endosulfan ether; EL, endosulfan lactone; ES, Endosulfan sulfate

Discussion

Bordetella sp. B9 has been isolated from the endosulfan contaminated soil and cultured successively in broth medium containing endosulfan as the source of carbon. This strain showed maximum growth as observed by the biomass and optical density and was selected for different studies. In broth culture, an increase in optical density with disappearance of endosulfan demonstrated the consumption of endosulfan as a carbon and energy source. Awasthi et al. (1997), Sutherland et al. (2000) and Siddique et al. (2003b) had also observed a substantial disappearance of endosulfan with a simultaneous increase in bacterial biomass. No significant changes in pH were observed in the broth medium therefore the degradation of endosulfan was not because of the pH.

Uninoculated controls in broth culture retained 89% of α endosulfan and 95% of β endosulfan after 18 days of incubation, indicating that little chemical degradation or volatilization of endosulfan had occurred and degradation in treated broth culture was mainly due to isolated bacterium.

The endosulfan concentration was negligible in bacterial cell extract (0.1%). This result shows that endosulfan was used almost completely by the microbial isolates for their metabolic activities and so the chance of bioaccumulation of endosulfan as the reason for its disappearance can be ruled out.

Exponential increase in microbial growth was observed in the presence of dextrose in the growth medium. Significant differences were observed while comparing the bacterial density between the mediums i.e. containing only endosulfan, endosulfan in addition to dextrose and only dextrose. The optical density of broth medium after 18 days of incubation, containing *Bordetella* sp. B9 with only endosulfan was 0.84, increase upto 1.58 when dextrose was supplemented in addition to endosulfan and to 1.75 when only dextrose was present in the medium was observed. Endosulfan degradation efficiency of the bacterial strain in the absence of dextrose was 80% of α endosulfan and 86% of β endosulfan after 18 days of incubation in broth medium. Addition of dextrose decreased the endosulfan degradation efficiency to 53% of α endosulfan and 48% of β endosulfan. This

shows that in the presence of dextrose the degradation of endosulfan was declined. This might be because dextrose being the easily available carbon source as compared to endosulfan and therefore, the bacterium preferred dextrose over endosulfan as an energy source. Awasthi et al. (1997) also observed that there was no increase in degradation efficiency of endosulfan with the addition of glucose. However, many studies have reported that the addition of auxiliary carbon to the system having xenobiotic compounds increased the biodegradation potential of bacterial and fungal cultures (Kumar and Philip 2006) which is often because of increase in metabolic activity of the microbes involved.

In Treatments 2–4 different carbon sources (endosulfan, cane molasses and dextrose, respectively) were used. In soil microcosm, *Bordetella* sp. B9 degraded endosulfan most efficiently in Treatment 2, utilizing endosulfan as a sole source of carbon and energy. Initially, the degradation was more prominent in Treatments 3 and 4 as compared to Treatment 2, because bacteria could derive energy more easily from molasses and dextrose as it is an easily available carbon source as compared to endosulfan (Kumar and Philip 2006). However, in cultures over 60 days where the bacterial strain already adapted to given environment, maximum degradation was observed in Treatment 2 because here no other energy source was present other than endosulfan.

The degradation potential of *Bordetella* sp. B9 decreased when it was augmented in natural soil where indigenous microbes were present. Also the degradation of endosulfan (50 mg kg^{-1} soil) was more in natural soil than in natural soil augmented with *Bordetella* sp. B9. This could be because of the natural bioprocess in Treatment 6.

Endosulfan ether and endosulfan lactone were the main metabolites formed and no residue of endosulfan sulfate was found in the broth medium (Kwon et al. 2002; Siddique et al. 2003b; Kumar et al. 2007).

The main metabolites formed in soil microcosm were endosulfan ether, endosulfan lactone and endosulfan sulfate showing that both oxidative and hydrolytic pathways were utilized by the bacterium to degrade endosulfan (Sutherland et al. 2000, 2002; Kwon et al. 2005; Weir et al. 2006; Shivaramaiah and Kennedy 2006).

Degradation of α endosulfan was more evident than that of β endosulfan in soil microcosm. Similar observations that β isomer was found to persist more than the α isomer were reported by Rao and Murty (1980); Kwon et al. (2002). However in broth culture, the bacterial strain degraded more β endosulfan as compared to α endosulfan (Siddique et al. 2003b; Kumar et al. 2007). The difference in the persistence of the two isomers has been attributed to various factors such as their differential volatilization, photodecomposition, alkaline hydrolysis as well as their biotic metabolism (Cotham and Bidleman 1989). This may also be due to stereoisomerism, where the enzymes released from bacterial system may be active towards one of the stereo isomers (Kumar and Philip 2006).

Based on the present studies, we can conclude that our bacterial strain has a potential to be used for bioremediation of the contaminated sites. Our bacteria which degraded 80% of α endosulfan and 86% of β endosulfan in 18 days in broth culture behaved differently in microcosm. In soil microcosm, *Bordetella* sp. B9 degraded about 83% of α endosulfan and 60% of β endosulfan in sterile soil but when the same bacteria is augmented in natural soil its degradation efficiency is reduced to 60.5% of α endosulfan and 35.4% of β endosulfan. Where as in natural soil without *Bordetella* sp. B9, the indigenous microbial population degrades endosulfan to 70.2% of α endosulfan and 50.9% of β endosulfan. These observations from soil microcosm studies undoubtedly indicate that the use of microbial isolates *Bordetella* sp. B9 for decontamination requires further intensive and extensive studies on its population dynamics and physiology in the presence of indigenous population for better development of bioremediation technology.

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