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ORIGINAL PAPER

# Biodegradation of α- and β-endosulfan by soil bacteria

**Sarfraz Hussain · Muhammad Arshad · Muhammad Saleem · Azeem Khalid**

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**Abstract** Extensive applications of persistent organochlorine pesticides like endosulfan on cotton have led to the contamination of soil and water environments at several sites in Pakistan. Microbial degradation offers an effective approach to remove such toxicants from the environment. This study reports the isolation of highly efficient endosulfan degrading bacterial strains from soil. A total of 29 bacterial strains were isolated through enrichment technique from 15 specific sites using endosulfan as sole sulfur source. The strains differed substantially in their potential to degrade endosulfan in vitro ranging from 40 to 93% of the spiked amount (100 mg $l^{-1}$). During the initial 3 days of incubation, there was very little degradation but it got accelerated as the incubation period proceeded. Biodegradation of endosulfan by these bacteria also resulted in substantial decrease in pH of the broth from 8.2 to 3.7 within 14 days of incubation. The utilization of endosulfan was accompanied by increased optical densities ($OD_{595}$) of the broth ranging from 0.511 to 0.890. High performance liquid chromatography analyses revealed that endosulfan diol and endosulfan ether were among the products of endosulfan metabolism by these bacterial strains while endosulfan sulfate, a persistent and toxic metabolite of endosulfan, was not detected in any case. The presence of endosulfan diol and endosulfan ether in the bacterial metabolites was further confirmed by GC-MS. Abiotic degradation contributed up to 21% of the spiked amount. The three bacterial strains, *Pseudomonas spinosa*, *P. aeruginosa*, and *Burkholderia cepacia*, were the most efficient degraders of both α- and β-endosulfan as they consumed more than 90% of the spiked amount (100 mg $l^{-1}$) in the broth within 14 days of incubation. Maximum biodegradation by these three selected efficient bacterial strains was observed at an initial pH of 8.0 and at an incubation temperature of 30°C. The results of this study may imply that these bacterial strains could be employed for bioremediation of endosulfan polluted soil and water environments.

**Keywords** Bacterial biodegradation · Endosulfan · pH · Temperature

S. Hussain · M. Arshad (✉) · M. Saleem · A. Khalid
Institute of Soil & Environmental Sciences, University of Agriculture, Faisalabad 38040, Pakistan
e-mail: bio@fsd.comsats.net.pk

## Introduction

Endosulfan (6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9a-hexahydro-6, 9-methano-2, 3, 4-benzodioxyanthiepin-3-oxide) is widely employed as pesticide (insecticide and acaricide) in world agriculture. Technical grade endosulfan contains

two stereoisomers, α- and β-endosulfan in the ratio of 7: 3 respectively (Fig. 1). In the close vicinities of agricultural fields, the contamination of atmosphere, soils, sediments, surface and rain waters, and foodstuffs by endosulfan has been documented in numerous previous studies (United States Department of Health and Human Services 1990). The persistence of endosulfan in soil and water environments has been observed by different researchers under different conditions (Rao and Murty 1980; Guerin and Kennedy 1992). Its harmful impacts on aquatic fauna (Sunderam et al. 1992) and numerous mammalian species including human beings have been reported several times in literature (Paul and Balasubramaniam 1997; Sinha et al. 1997; Chaudhuri et al. 1999).

To date, some physicochemical and biological remedial strategies have been described by researchers which lead to degradation of endusulfan into both toxic and non-toxic metabolites (Knoevenagel and Himmelreich 1976; Kullman and Matsumura 1996; Sutherland et al. 2000; Siddique et al. 2003a). In Pakistan, endosulfan is applied to cotton and other crops as an insecticide. Its repeated applications resulted in the contamination of soil and water environments at several sites, particularly in the close vicinities of agricultural fields, which may play havoc with environmental quality and public health. Residues of this pesticide have been detected in cotton seed, cotton lint, milk, drinking water and other food stuffs (Masood and Hassan 1995; Ahad et al. 2000). That is why it is necessary to develop strategies primarily based on biological and biochemical means to detoxify the pesticide residues accumulated in soil and water environments. In this study, we isolated 29 strains of bacteria capable of degrading endosulfan from endosulfan-polluted soil and water environments through repetitive enrichment culture and successive subculture. Among these bacterial cultures, three highly efficient bacterial strains, namely *Pseudomonas spinosa*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia* were further tested for their potential to degrade endosulfan.

## Materials and methods

Fifteen soil samples collected from different sites (Table 1) having a history of repeated endosulfan applications were used in this enrichment study for the isolation of endosulfan-degrading bacteria. The collected samples were ground, passed through 2 mm sieve and stored at 4°C.

### Reagents and chemicals

Technical (95.5%) and analytical grade (99.5%) endosulfan, and its major metabolites were obtained from Bayer Crop Science, R-PT-Analytics, FFM Germany. Stock solutions of endosulfan and its metabolites were prepared in acetone. HPLC grade acetone and acetonitrile were purchased from J. T. Backers, Holland. All other chemicals were of analytical grade and purchased from commercial sources.

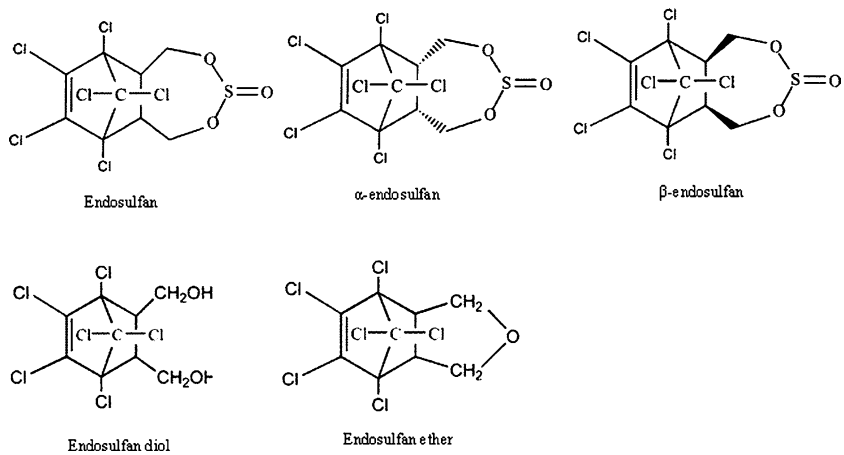


**Fig. 1** Endosulfan isomers and metabolites

**Table 1** Collection of soil samples from endosulfan contaminated sites for the isolation of bacteria

| Sample code | Sample description |
|---|---|
| MN1 | Cotton growing field, Multan. |
| MN2 | Cotton growing field, Multan |
| KH1 | Fallow Field, Khanewal |
| KH2 | Cotton growing field, Khanewal |
| KB1 | Tomato growing field, Kabirwala |
| KB2 | Cotton growing field, Kabirwala |
| KB3 | Fallow Field, Kabirwala |
| SL1 | Tomato growing field, Sahiwal |
| SL2 | Maize growing field, Sahiwal |
| SL3 | Tobacco harvested field, Sahiwal |
| SG1 | Sugar cane growing field, Sargodha |
| SG2 | Sugar cane growing field, Sargodha |
| SG3 | Citrus growing field, Sargodha |
| TJ1 | Melon growing field, Tandojam |
| TJ2 | Melon growing field, Tandojam |

## Isolation of bacterial strains

Enrichment culture technique was used for isolation of bacterial strains capable of utilizing endosulfan as a sole S source. Microbial inocula for enrichment studies were prepared by shaking 20 g of soil sample overnight in 100 ml nutrient culture medium at 30°C and 150 rpm. The solid particles were allowed to settle for 1 h and aliquots of supernatant were filtered and used for inoculation of nutrient culture medium. The isolated cultures were purified through non sulfur medium (NSM)/endosulfan enrichment medium followed by incubation at 30°C and orbital shaking at 150 rpm for 2 weeks. The NSM consisted of (g $l^{-1}$): $K_2HPO_4$, 0.225; $KH_2PO_4$, 0.225; $NH_4Cl$, 0.225; $MgCl_2{\cdot}6H_2O$, 0.845; $CaCO_3$, 0.005; $FeCl_2{\cdot}4H_2O$, 0.005; D-glucose, 1.0; and 1 ml of trace element solution per liter. The trace element solution prepared for NSM contained (mg $l^{-1}$): $MnCl_2{\cdot}4H_2O$, 198; $ZnCl_2$, 136; $CuCl_2{\cdot}2H_2O$, 171; $CoCl_2{\cdot}6H_2O$, 24; and $NiCl_2{\cdot}6H_2O$, 24 (Siddique et al. 2003a). The pH of NSM was adjusted at 7.2. Nutrient culture medium and Erlenmeyer flasks of 50 ml were autoclaved for 20 min at 121°C temperature separately. To each sterilized flask, already dissolved endosulfan in acetone was added aseptically to attain a final concentration of 100 mg $l^{-1}$ in a flow hood and waited for a while to allow acetone to evaporate. About 9 ml of nutrient culture medium and 1.0 ml of supernatant solution from source flask were added to spiked flasks. These flasks were incubated on an orbital shaker (150 rpm) at 30°C for 10 d. Thereafter, 0.1 ml of culture was transferred into 10 ml of fresh sterile NSM enrichment medium containing 100 mg $l^{-1}$ endosulfan and further incubated for 15 d.

The solid medium was prepared by adding 1.5% agar to the enrichment medium followed by autoclaving at 121°C for 20 min. Endosulfan was aseptically added to the medium in a laminar flow hood at about 50°C to get a concentration of 100 mg $l^{-1}$. The molten agar was plated out in Petri dishes and 0.5 ml of culture was spread on NSM/endosulfan agar. The plates were incubated under aerobic conditions at 30°C for 48 h. The discrete colonies of microbes showing luxurious growth were isolated. Isolates were purified further by streaking on fresh plates thrice and selected isolates of bacteria were preserved on nutrient agar medium for further experiments.

## Identification of highly efficient bacterial strains

The bacterial isolates exhibiting maximum biodegradation of endosulfan in enrichment medium were identified by using standard protocol (Prescot et al. 1993) and identification was further confirmed by Biolog® Identification System (Micrologᵀᴹ System Release 4.2, Hayward, CA, USA). Biolog® Identification System has been found equally reliable for identification as 16s rRNA (Flores-Vargas and O'Hara 2006). Three strains including TJ1.B1, TJ2.B6 and MN2.B14 were identified as *Pseudomonas spinosa*, *Burkholderia cepacia* and *Pseudomonas aeruginosa*.

## Biodegradation of endosulfan by bacterial isolates

The isolates demonstrating prolific growth were investigated for their capability to degrade endosulfan over time (at 3, 7 and 14 d incubation). For this purpose, 100 ml Erlenmeyer flasks and medium (NSM) were autoclaved at 121°C for 20 min separately. The nutrient medium was adjusted to pH of 8.2. 50 ml of autoclaved

medium were put into each flask. These flasks were spiked with endosulfan to a concentration of 100 mg $l^{-1}$. The flasks were inoculated with 800 µl bacterial inocula adjusted to a set optical density of ($OD_{595}$ = 0.81). These inoculated flasks were incubated at 30°C on an orbital shaker at 150 rpm. Uninoculated flasks (control) were also prepared to check the abiotic degradation under the same conditions. This procedure was carried out in triplicate and results are means of three.

### Effect of pH on biodegradation of endosulfan by selected bacterial isolates

To assess the comparative biodegradation of both α- and β-endosulfan by selected bacterial strains *P. spinosa*, *P. aeruginosa* and *B. cepacia* at different pH, 50 ml nutrient medium (NSM) (autoclaved at 121°C for 20 min) in 100 ml Erlenmeyer flasks adjusted to pH values of 4, 5, 6, 7, 8, 9 and 10 were used. These flasks were spiked with endosulfan to a concentration of 100 mg $l^{-1}$. The broth in the flasks was inoculated by adding 800 µl of bacterial inocula ($OD_{595}$ = 0.81). These inoculated flasks were incubated at 30°C on an orbital shaker at 150 rpm. Uninoculated flasks were also prepared to check abiotic degradation. This procedure was conducted in three repeats.

### Effect of incubation temperature on biodegradation of endosulfan by selected bacteria

About 50 ml autoclaved nutrient medium (NSM) adjusted to pH 8.0 and spiked with endosulfan to a concentration of 100 mg $l^{-1}$ was used. The flasks (100 ml capacity) containing spiked NSM broth were inoculated by adding 800 µl bacterial inocula ($OD_{595}$ = 0.81). These inoculated flasks were incubated at 20, 25, 30, 35, 40 and 45°C on an orbital shaker at 150 rpm. Uninoculated flasks were also prepared to check abiotic degradation. There were three repeats for each incubation temperature.

### Effect of shaking versus static conditions on biodegradation of endosulfan

Biodegradation of endosulfan in liquid culture was investigated under optimum conditions obtained from previous steps under static vs. shaking incubation. For this purpose, 50 ml of nutrient solution were put into the flasks. The medium was autoclaved at 121°C for 20 min. After cooling to about 50°C temperature, the flasks were spiked with endosulfan already dissolved in acetone to attain an optimum concentration of 100 mg $l^{-1}$ of each endosulfan isomer. The flasks were then inoculated with 800 µl of already prepared inoculum ($OD_{595}$ = 0.81). The same amount of nutrient solution was put into an uninoculated flask brought to pH 8.0 and spiked with endosulfan at 100 mg $l^{-1}$ as a control to check physiochemical degradation of endosulfan. One set of flasks was incubated on an orbital incubator (150 rpm) and another set was incubated at 30°C for 14 d. This procedure was done in triplicate.

### Extraction and analytical procedure for endosulfan detection

Endosulfan and its metabolites were extracted from the bacterial culture through addition of acetonitrile to the enrichment medium followed by shaking. For this purpose, 5 ml of each culture were taken in separate flasks and the same volume of acetonitrile was added to these flasks. The flasks were shaken for 1 h using a reciprocating shaker at 160 rpm. An appropriate amount of samples was filtered by passing through Gelman Acrodisc syringe filter of 0.2 µm GHP membrane. Samples were analyzed by HPLC using ODS Hypersil column (250 × 4.6 mm) as the stationary phase and acetonitrile: water (70: 30, v/v) as the mobile phase. The solutes were detected using UV-VIS detector at 214 nm (Siddique et al. 2003b). The optical densities of the cultured media were determined spectophotometrically by measuring the absorbance at 595 nm. The pH was measured with the Jenway 3510 pH meter.

### GC-MS analysis

The intermediates/products of the endosulfan degradation by selected strains of bacteria were further confirmed by GC-MS. Endosulfan and its intermediates in the bacterial cultures were

extracted by adding equal volume of acetone and shaking for 1 h with a reciprocating shaker. About 1 ml of the extract was then transferred to 9 ml of acetone and shaken for 30 min. The sample was dehydrated by passing it through anhydrous $Na_2SO_4$ and concentrated with the rotary evaporator. The extract was analyzed with a gas chromatograph (Shimadzu GC-2010) equipped with mass spectrometer (GCMS-QP2010), auto injector (AOC-20i) and a DB-5MS capillary column (internal diameter, 0.25 mm; film thickness, 0.25 μm), coupled to MS via direct interface. The injector and detector were operated at 260 and 280°C respectively. The initial oven temperature was 180°C and set for a linear increase of 5°C $min^{-1}$ to a final temperature 250°C. The helium was used as carrier gas at a flow rate of 1.0 ml $min^{-1}$. The ion source temperature was 230°C while interface temperature was 250°C. The electron impact (EI) mass spectra were obtained at 70 ev and monitored in the range of 50 to 400 m/z.

## Results

### Biodegradation of endosulfan by bacterial isolates

The disappearance of endosulfan from the spiked and inoculated broth varied substantially among the 29 bacterial strains (data not shown). During the first 3 days of incubation, biodegradation of both isomers of endosulfan was ≤ 10% of the spiked amount while in seven days incubation, the degradation ranged between 22–60% (α-endosulfan) and 18–57% (β-endosulfan). At the end of the incubation (14 d), the removal of both isomers of endosulfan by bacterial strains ranged between 43 and 93%. Three strains identified as *P. spinosa*, *P. aeruginosa* and *B. cepacia* showed the highest potential to degrade endosulfan (Table 2) and they were selected for further studies. Abiotic degradation was only recorded beyond the 7th day of incubation. The pH of the broth decreased in parallel with progressive degradation of endosulfan i.e., higher degradation resulted in a lower broth pH (Table 3). A highly significant negative linear relationship ($r = 0.54^{**}$) was found between percent degradation of endosulfan and resultant decrease in pH of all the cultures media. The largest decrease in pH from 7.2 to 3.6 was recorded in the case of *P. aeruginosa*, *B. cepacia* and *P. spinosa*, which degraded α- and β-endosulfan beyond 90% of the spiked amount. The optical density of the broth increased, ranging from 0.511 to 0.890, with the increased biodegradation of endosulfan (Table 3). A highly significant linear positive correlation ($r = 0.78^{**}$) was found between percent biodegradation of endosulfan and increase in optical density of the broth.

Interestingly, accumulation of endosulfan sulfate, a persistent metabolite of endosulfan, did not appear during the whole course of study; however, endosulfan diol and endosulfan ether appeared in little amounts as detected by HPLC analysis using authentic standards and matching the retention times of the respective peaks (data not shown). This was further confirmed by analyzing bacterial metabolites using GC-MS.

**Table 2** Biodegradation of spiked α-and β-endosulfan in the broth by three efficient strains of bacteria (the data are average of three repeats)

| Bacterial strain | % degradation of α –endosulfan | | | % degradation of β –endosulfan | | |
|---|---|---|---|---|---|---|
| | 3 d | 7 d | 14 d | 3 d | 7 d | 14 d |
| Uninoculated control (abiotic degradation) | – | 9.5 ± 1.5 | 21.4 ± 2.8 | – | 7.8 ± 1.4 | 19.4 ± 2.6 |
| *Pseudomonas spinosa* (TJ1.B1) | 9.3 ± 0.3 | 58.7 ± 4.2 | 92.9 ± 3.5 | 9.0 ± 0.5 | 56.3 ± 5.1 | 88.2 ± 4.8 |
| *Burkholderia cepacia* (TJ2.B6) | 10.7 ± 0.6 | 54.7 ± 3.8 | 93.3 ± 4.7 | 10.1 ± 0.4 | 49.4 ± 2.6 | 87.9 ± 4.6 |
| *Pseudomonas aeruginosa* (MN2B14) | 10.7 ± 0.4 | 60.7 ± 3.2 | 93.7 ± 3.5 | 9.4 ± 0.6 | 57.6 ± 4.4 | 87.2 ± 3.7 |

± Standard error

LSD = 4.12 (α–Endosulfan), 5.48 (β–endosulfan) at $P < 0.05$

**Table 3** Change in pH and optical density of the broth as a result of endosulfan degradation by three efficient strains of bacteria over a period of time (the data are average of three repeats)

| Bacterial strains | pH | | | Optical density | | |
|---|---|---|---|---|---|---|
| | 3 d | 7 d | 14 d | 3 d | 7 d | 14 d |
| Uninoculated control (abiotic degradation) | 8.11 ± 0.08 | 7.98 ± 0.13 | 7.77 ± 0.12 | – | – | – |
| *Pseudomonas spinosa* (TJ1.B1) | 7.83 ± 0.17 | 6.73 ± 0.11 | 3.66 ± 0.21 | 0.121 ± 0.011 | 0.378 ± 0.034 | 0.874 ± 0.047 |
| *Burkholderia cepacia* (TJ2.B6) | 7.79 ± 0.23 | 6.96 ± 0.16 | 3.63 ± 0.21 | 0.126 ± 0.014 | 0.361 ± 0.019 | 0.841 ± 0.031 |
| *Pseudomonas aeruginosa* (MN2B14) | 7.81 ± 0.12 | 6.63 ± 0.21 | 3.76 ± 0.18 | 0.127 ± 0.011 | 0.415 ± 0.027 | 0.890 ± 0.026 |

± Standard error

LSD = 0.26 (pH), 0.20 (optical density) at $P < 0.05$

## Effect of pH on biodegradation of endosulfan by selected bacterial strains

Biodegradation of α- and β-endosulfan by efficient bacterial strains (*P. spinosa*, *P. aeruginosa* and *B. cepacia*) was further investigated at different initial pH of broth culture (Fig. 2). Biodegradation of both isomers of endosulfan by these bacterial cultures was more pronounced at alkaline pH values of the broth but it was inhibited strongly at acidic pH values. Maximum biodegradation of both α-and β-endosulfan by bacterial strains was observed at an initial pH of 8.0 and minimum at an initial pH of 4.0. The biodegradation of endosulfan ranged from 26 to 95% as the broth pH increased from 4.0 to 8.0. All three selected bacterial strains behaved similarly for biodegradation of endosulfan in culture broths of different pH. There was a more abiotic degradation in alkaline ranges of pH than the acidic.

## Effect of incubation temperature on biodegradation of endosulfan by selected bacterial strains

Biodegradation of α- and β-endosulfan by *P. spinosa*, *P. aeruginosa* and *B. cepacia* was also investigated at different ranges of incubation temperature (Fig. 3). Biodegradation of both isomers of endosulfan was relatively greater at an incubation temperature of 25–35°C, with a maximum at 30°C. The minimum degradation of spiked endosulfan was recorded at 45°C. All the three strains responded similarly to change in temperature. Abiotic degradation was more pronounced at higher incubation temperature.

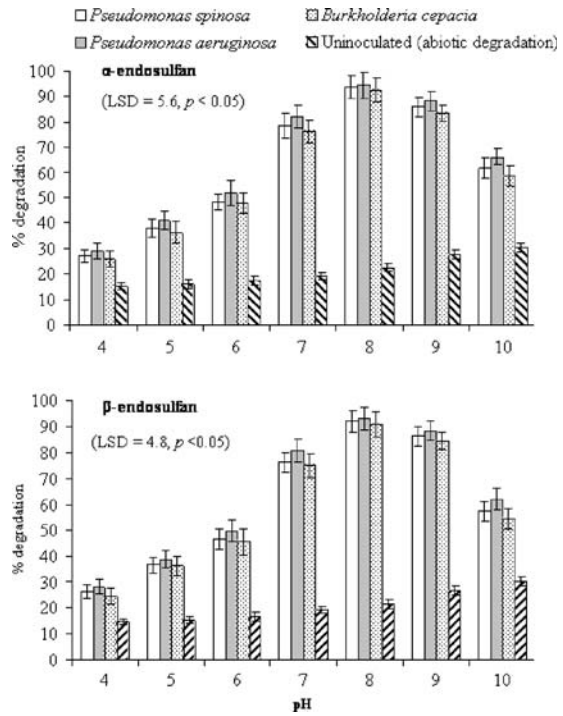


**Fig. 2** Biodegradation of α- and β-endosulfan by three efficient strains of bacteria at different ranges of pH (The error bars show standard errors)

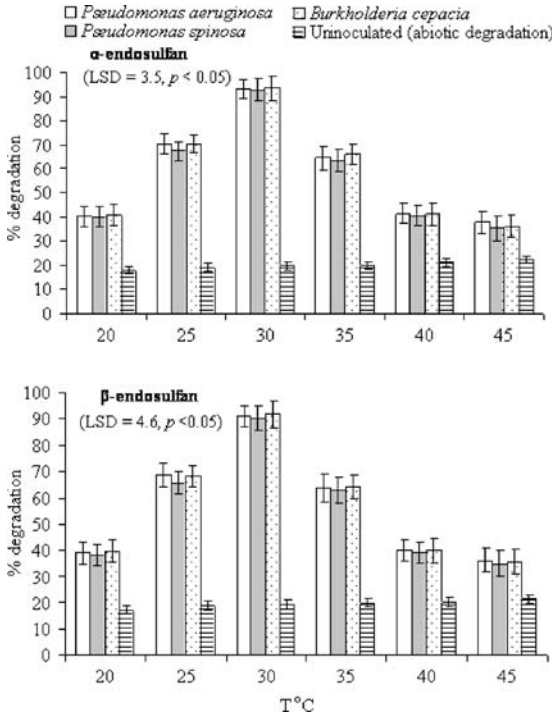


**Fig. 3** Biodegradation of α- and β-endosulfan by three efficient strains of bacteria at different incubation temperatures (The error bars show standard errors)

## Effect of shaking vs. static conditions on biodegradation of endosulfan

A significant difference between biodegradation of both α- and β-endosulfan by the selected strains of bacteria was observed under static vs. shaking incubation (Table 4). Maximum biodegradation of α- and β-endosulfan (up to 95%) in the broth inoculated with selected bacteria was recorded under shaking conditions. A maximum biodegradation of only 66% of the spiked amount of endosulfan was found under static condition. Likewise, abiotic degradation was more obvious under shaking conditions than under static incubation of the broth culture (Table 4).

## Discussion

This study presents the enrichment and isolation of highly efficient bacterial strains capable of degrading endosulfan into less toxic compounds. Among the 29 bacterial strains tested, there were great variations in their potential to degrade endosulfan (data not given). The highly efficient bacterial isolates were identified as *P. spinosa*, *P. aeruginosa and B. cepacia*. The bacteria belonging to *Pseudomonas* sp. *and Burkholderia* sp. are gram negative soil bacteria and have been previously documented as excellent degraders of a wide range of xenobiotics and recalcitrant compounds both in soil and water environment (Radehaus and Schmidt 1992; Filonov et al. 2006). These efficient bacterial strains degraded both isomers of endosulfan i.e., α- and β-endosulfan more than 90% within 14 days of incubation. The biodegradation rates observed in the case of these strains were much higher than of previously documented bacterial strains utilizing endosulfan either as sulfur or carbon source (Awasthi et al. 1997, 2003; Sutherland et al. 2002; Kumar and Philip 2006). This might be due to their prolific growth during the incubation period as evident from the higher optical densities or the presence of an efficient enzymatic system responsible for the degradation. There was a parallel decrease in pH of the cultured medium as the biodegradation

**Table 4** Effect of shaking vs. static conditions on biodegradation of α-and β-endosulfan by three efficient strains of bacteria

| Bacterial strains | % degradation of α- endosulfan | | % degradation of β- endosulfan | |
|---|---|---|---|---|
| | Shaking | Static | Shaking | Static |
| Uninoculated control (abiotic degradation) | 21.6 ± 2.3 | 14.5 ± 1.8 | 19.7 ± 1.9 | 13.3 ± 1.4 |
| *Pseudomonas spinosa* (TJ1.B1) | 92.6 ± 5.7 | 60.2 ± 3.7 | 88.4 ± 4.6 | 56.7 ± 3.3 |
| *Burkholderia cepacia* (TJ2.B6) | 91.5 ± 4.8 | 58.2 ± 3.2 | 86.6 ± 5.3 | 55.8 ± 3.7 |
| *Pseudomonas aeruginosa* (MN2B14) | 95.7 ± 5.4 | 65.6 ± 3.6 | 91.4 ± 5.2 | 62.4 ± 3.4 |

± Standard error

LSD = 3.16 (α–Endosulfan), 2.57 (β–endosulfan) at $P < 0.05$

**Table 5** Optical densities ($OD_{595}$) of three efficient bacterial strains at different incubation conditions

| Bacterial strains | PH | | | | | | | Temperature (°C) | | | | | | Shaking vs. Static | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 20 | 25 | 30 | 35 | 40 | 45 | Shaking | Static |
| *Pseudomonas spinosa* (TJ1.B1) | 0.27 ± 0.03 | 0.38 ± 0.04 | 0.51 ± 0.04 | 0.72 ± 0.04 | 0.86 ± 0.05 | 0.76 ± 0.04 | 0.46 ± 0.04 | 0.43 ± 0.03 | 0.76 ± 0.05 | 0.88 ± 0.05 | 0.71 ± 0.03 | 0.56 ± 0.03 | 0.38 ± 0.02 | 0.88 ± 0.04 | 0.61 ± 0.04 |
| *Burkholderia cepacia* (TJ2.B6) | 0.21 ± 0.02 | 0.37 ± 0.04 | 0.49 ± 0.03 | 0.74 ± 0.05 | 0.84 ± 0.05 | 0.79 ± 0.05 | 0.42 ± 0.03 | 0.45 ± 0.02 | 0.74 ± 0.05 | 0.88 ± 0.05 | 0.70 ± 0.04 | 0.54 ± 0.03 | 0.34 ± 0.02 | 0.85 ± 0.05 | 0.63 ± 0.04 |
| *Pseudomonas aeruginosa* (MN2B14) | 0.31 ± 0.03 | 0.42 ± 0.03 | 0.50 ± 0.04 | 0.77 ± 0.04 | 0.93 ± 0.06 | 0.81 ± 0.04 | 0.47 ± 0.03 | 0.45 ± 0.03 | 0.80 ± 0.04 | 0.96 ± 0.05 | 0.84 ± 0.04 | 0.56 ± 0.04 | 0.43 ± 0.03 | 0.90 ± 0.04 | 0.63 ± 0.03 |

± Standard error

LSD = 0.25 (pH), 0.24 (temperature), 0.28 (shaking vs. static) at $P < 0.05$

proceeded. This dramatic reduction in pH of the bacterial cultures might be due to dehalogenation of endosulfan and subsequent formation of acidic substances. These results confirmed the findings of previous studies (Sutherland et al. 2000, 2002; Kwon et al. 2002; Awasthi et al. 2003; Siddique et al. 2003a). They reported that decrease in pH might be due to the formation of HCl or organic acids by microorganisms.

By using authentic standards, HPLC analysis demonstrated the disappearance of both isomers of endosulfan with the formation of endosulfan diol and endosulfan ether as metabolites of endosulfan. The formation of these intermediates was confirmed by GC-MS. Endosulfan sulfate, a persistent metabolite of endosulfan, was not detected in the broth culture. These results revealed that these bacterial species adopted the hydrolytic pathway of endosulfan biodegradation (Kim et al. 2001), contrary to oxidative pathway of endosulfan biodegradation in which endosulfan sulfate is formed (Kullman and Matsmura 1996; Kown et al. 2002). The data recorded over the incubation time indicated that during the first 3 days, there was a very little biodegradation of both α- and β-endosulfan, which might represent a lag phase while it got accelerated as the incubation proceeded, most likely due to induction/activation of enzymes in the inoculated cultures. Finally, biodegradation of α-and β-endosulfan by three efficient strains *P. spinosa*, *P. aeruginosa* and *B. cepacia* was found maximum at an initial broth of pH 8.0, incubation temperature of 30°C and under shaking conditions. It is highly likely that these incubation conditions might be more conducive to bacterial growth as supported by OD values (Table 5). In general, neutral to slightly alkaline conditions are considered more favorable for bacterial growth than acidic conditions (Alexander 1977; Sylvia et al. 2005). It is very likely that the initial alkaline pH 8.0 changed to neutral during the initial period of incubation as evident from Table 3, favoring luxury growth of bacteria and degradation of endosulfan. A pH greater than 8.0 might have resulted in a shock for the bacteria at the start of incubation, which slowed down the proliferation of bacteria. The optimum biodegradation of endosulfan by bacterial isolates at 30°C and

medium pH of 8.0 is similar to previous findings (Sutherland et al. 2002; Siddique et al. 2003a). The biodegradation under shaking conditions was more pronounced over static conditions. That might be due to better bioavailability of endosulfan to the microbes coupled with physiochemical degradation (Nazli et al. 2003). The abiotic degradation was also greater under shaking conditions than under static conditions, which may imply that aerobic conditions are relatively more conducive for abiotic (chemical) degradation of endosulfan as well. Both $\alpha$-and $\beta$-endosulfan were degraded by soil bacteria at almost the same rates. This implies that either both the isomers are being metabolized by the same set of enzymes or the bacteria carry necessary enzyme(s) for the degradation of both the isomers. However, previous studies have shown that microbial species prefer $\alpha$-endosulfan for degradation over $\beta$-endosulfan (Kown et al. 2002; Siddique et al. 2003a).

## Conclusions

Some soil indigenous bacteria carry the ability to degrade endosulfan at accelerated rates. Such bacteria could be useful in framing a bioremediation strategy for pesticide contaminated soil and water environments.

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