Enrichment and Isolation of Endosulfan-Degrading Microorganisms

Tariq Siddique, Benedict C. Okeke, Muhammad Arshad, and William T. Frankenberger, Jr.*

ABSTRACT

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,3,4-benzo-dioxathiepin-3-oxide) is a cyclodiene organochlorine currently used as an insecticide all over the world and its residues are posing a serious environmental threat. This study reports the isolation and identification of enriched microorganisms, capable of degrading endosulfan. Enrichment was achieved by using the insecticide as either the sole source of carbon or sulfur in parallel studies. Two strains each of fungi (F1 and F4) and bacteria (BF2 and B4) were selected using endosulfan as a sole carbon source. A *Pandoraea* species (Lin-3) previously isolated in our laboratory using lindane (γ-HCH) as a carbon source was also screened for endosulfan degradation. F1 and F4 (*Fusarium ventricosum*) degraded α-endosulfan by as much as 82.2 and 91.1% and β-endosulfan by 78.5 and 89.9%, respectively, within 15 d of incubation. Bacterial strains B4 and Lin-3 degraded α-endosulfan up to 79.6 and 81.8% and β-endosulfan up to 83.9 and 86.8%, respectively, in 15 d. Among the bacterial strains isolated by providing endosulfan as a sulfur source, B4s and F4t degraded α-endosulfan by as much as 70.4 and 68.5% and β-endosulfan by 70.4 and 70.8%, respectively, after 15 d. Degradation of the insecticide occurred concomitant with bacterial growth reaching an optical density (OD_{600}) of 0.366 and 0.322 for B4 and Lin-3, respectively. High OD_{600} was also noted with the other bacterial strains utilizing endosulfan as a sulfur source. Fungal and bacterial strains significantly decreased the pH of the nutrient culture media while growing on endosulfan. The results of this study suggest that these novel strains are a valuable source of potent endosulfan-degrading enzymes for use in enzymatic bioremediation.

THE CHLORINATED cyclic sulfite diester endosulfan is a cyclodiene insecticide possessing a relatively broad spectrum of activity. Technical-grade endosulfan is a mixture of two stereoisomers, α- and β-endosulfan, in a ratio of 7:3 (Fig. 1). It is used extensively throughout the world, including the USA, as a contact and stomach insecticide and as an acaricide on field crops (cotton [*Gossypium hirsutum* L.], paddy, sorghum [*Sorghum bicolor* (L.) Moench], oilseeds, and coffee [*Coffea arabica* L.]), vegetables, and fruit crops (Lee et al., 1995; Kullman and Matsumura, 1996). Because of its abundant usage and potential transport, endosulfan contamination is frequently found in the environment at considerable distances from the point of its original applications (Mansingh and Wilson, 1995; Miles and Pfeuffer, 1997). Endosulfan has been detected in the atmosphere, soils, sediments, surface and rain waters, and food stuffs (United States Department of Health and Human Services, 1990). It is extremely toxic to fish and aquatic invertebrates (Sunderam et al., 1992) and has been implicated in mammalian gonadal toxicity (Sinha et al., 1997), genotoxicity (Chaudhuri et al., 1999), and neurotoxicity (Paul and Balasubramaniam, 1997). These health and environmental concerns have led to an interest in detoxification of endosulfan in the environment.

Detoxification of pesticides through biological means is receiving serious attention as an alternative to existing methods, such as incineration and landfill. A preliminary step in the investigation of enzymatic technologies for endosulfan detoxification is the definitive identification of a biological source of endosulfan degrading activity. Microorganisms have increasingly been investigated as a source of xenobiotic-degrading enzymes (Chen and Mulchandani, 1998).

Several studies have reported the isolation of bacterial co-culture (Awasthi et al., 1997) and mixed cultures (Sutherland et al., 2000) capable of degrading endosulfan. Mukherjee and Gopal (1994) reported the degradation of β-endosulfan by *Aspergillus niger*. Although *Trichoderma harzianum* (Katayama and Matsumura, 1993), *Phanerochaete chrysosporium* (Kullman and Matsumura, 1996), and *Mucor thermohyalospora* MTCC 1384 (Shetty et al., 2000) have been examined for endosulfan degradation, these fungi were isolated for other degradative activities.

In a bioremediation process, heterotrophic microorganisms break down substrates (hazardous compounds) to obtain chemical energy, hence organic pollutants can serve as carbon, energy, and nutrient sources for microbial growth. Some studies have described endosulfan as a sulfur source for microbial growth and a poor biological energy source when used as a sole carbon source (Sutherland et al., 2000; Guerin, 1999). Sutherland et al. (2000) selected microorganisms for their ability to release the sulfite group from endosulfan and to use this insecticide as a source of sulfur for bacterial growth. Awasthi et al. (1997) isolated a bacterial co-culture using endosulfan as a sole carbon source.

In this study, microorganisms were isolated through enrichment on endosulfan as a carbon or sulfur source. We report bacterial and fungal strains that, to our knowledge, are the most active endosulfan-degrading single strains of microorganisms. These strains will be further investigated for their enzymatic reactions in detoxification of endosulfan.

MATERIALS AND METHODS

Reagents and Chemicals

Technical-grade endosulfan (99.5% pure) was purchased from Chem Services (West Chester, PA). Technical grade endosulfan (used commercially) is a mixture of two diastereoisomers, α-endosulfan and β-endosulfan, in a ratio of 7:3. Acetone, hexane (99.9% pure), ethanol, and a pesticide calibration

T. Siddique, B.C. Okeke, and W.T. Frankenberger, Jr., Dep. of Environmental Sciences, Univ. of California, Riverside, CA 92521. M. Arshad, Dep. of Soil Science, Univ. of Agriculture, Faisalabad, Pakistan. Received 31 Jan. 2002. *Corresponding author (william.frankenberger@ucr.edu).

Published in J. Environ. Qual. 32:47–54 (2003).

Abbreviations: FTW, nutrient culture medium; LSU, large subunit; NSM, nonsulfur nutrient culture medium; OD, optical density.

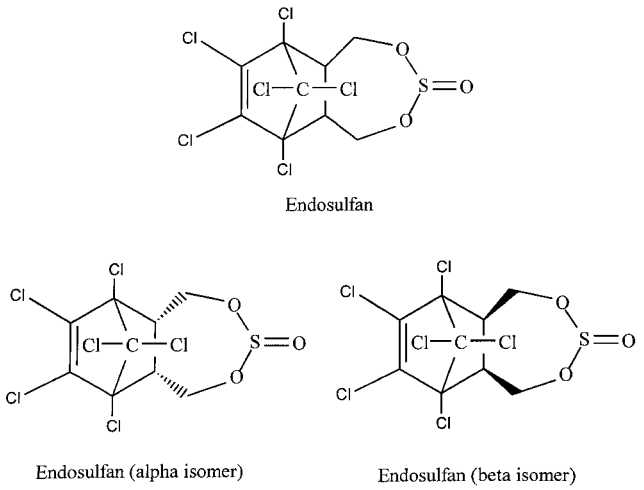

Fig. 1. Endosulfan and its two isomers.

standard mixture (608 calibration mixture) were purchased from VWR Scientific Products (San Diego, CA). Other chemicals were of analytical grade and purchased from commercial sources.

Sample Collection for Enrichment Studies

Twelve environmental samples were used in this enrichment study for the isolation of endosulfan-degrading microorganisms. Eight soil samples were collected from different sites with a history of pesticide applications (Table 1). Two sediment samples were collected from the Salton Sea, California. A high-density bacterial cocktail (HDBC) and a sample of food-processing waste (FPW) obtained from the Center for Environmental Microbiology (Riverside, CA) were also used for enrichment studies. The collected samples were stored at 4°C.

Enrichment of Microbial Communities

Microbial inocula for the enrichment studies were prepared by shaking 20 g of sample overnight in 100 mL nutrient culture medium (FTW) and nonsulfur nutrient culture medium (NSM) at 25°C and 160 rpm. The solid particles were allowed to settle for one hour and aliquots of the supernatant were used to inoculate the nutrient culture media. The NSM and FTW were prepared to assess endosulfan as a sulfur or carbon source, respectively, for microorganisms in the enrichment study. The FTW medium (Herman and Frankenberger, 1999) is comprised of (in g L^{-1}): K_2HPO_4, 0.225; KH_2PO_4, 0.225; $(NH_4)_2SO_4$, 0.225; $MgSO_4 \cdot 7H_2O$, 0.05; $CaCO_3$, 0.005; and $FeCl_2 \cdot 4H_2O$, 0.005 blended with 1 mL of trace elements solution (Focht, 1994). The Focht trace element solution contained (in mg L^{-1}): $MnSO_4 \cdot H_2O$, 169; $ZnSO_4 \cdot 7H_2O$, 288; $CuSO_4 \cdot 5H_2O$, 250; $NiSO_4 \cdot 6H_2O$, 26; $CoSO_4$, 28; and $Na_2MoO_4 \cdot 2H_2O$, 24. The NSM consisted of (in 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 (in 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. The pH of both FTW and NSM was adjusted at 7.2. Erlenmeyer flasks (50 mL) and nutrient culture media were autoclaved separately for 20 min at 121°C. Fifty microliters of acetone containing 0.5 mg endosulfan was aseptically added to each sterilized flask in a laminar flow hood allowing the acetone to evaporate. Nine milliliters of nutrient culture media were added to each flask. Then, 1 mL of supernatant solution from the source flasks was added to inoculate the spiked flasks. Uninoculated spiked flasks were also set up as a control to compensate for any chemical degradation. The aerobic culture was incubated at 30°C with orbital shaking (160 rpm) for two weeks (Round 1 enrichment culture). Thereafter, 0.1 mL of the culture was transferred onto 10 mL of fresh sterile FTW or NSM enrichment media containing 50 mg L^{-1} endosulfan and further incubated for two weeks (Round 2 enrichment culture).

Endosulfan-Degrading Monocultures

To obtain pure cultures of single strains, 1-mL aliquots of Round 2 enrichment cultures were centrifuged (8000 rpm, 10 min) with a Beckman (Fullerton, CA) Microfuge II. The supernatant was removed and cell residues were resuspended in 50 μL of sterile FTW and NSM nutrient culture media by vortexing. Aliquots of this suspension were plated on FTW–endosulfan or NSM–endosulfan agar media by streaking. This solid medium was prepared by adding 2% washed agar to the enrichment basal media followed by autoclaving (121°C, 15 min). Thereafter, endosulfan (100 mg L^{-1}) was aseptically added after cooling the molten agar to about 50°C. Agar plates were incubated under aerobic conditions at 30°C and discrete colonies were isolated. Isolates were purified further by streaking on fresh plates.

Screening of Isolates for Endosulfan Degradation in Liquid Culture Media

Two isolates each of fungi and bacteria exhibiting luxury growth on FTW–endosulfan agar, and five bacterial strains on NSM–endosulfan agar, were selected. The bacterial isolates were grown in nutrient culture media containing 100 mg L^{-1} endosulfan. Cultures were incubated (30°C, 160 rpm) for one week and cells were harvested by centrifugation (5000 rpm, 20 min) and washed twice in 40 mL of nutrient culture media. Cells were thereafter resuspended in the same media. Endosulfan was dissolved in acetone and ethanol (1:1) and 80 μL

Table 1. Source of environmental samples for enrichment and microbial enumeration studies.

Sample code	Type of material	Sample description
B1	soil	cotton-growing field, Nees Avenue, Firebaugh, CA
B2	soil	cotton-growing field, 7th Avenue, Firebaugh, CA
B3	soil	cotton-growing field, Fairfax Avenue, Firebaugh, CA
B4	soil	tomato-growing field, Fairfax Avenue, Firebaugh, CA
F1	soil	fallow field, agricultural operation, University of California, Riverside
F2	soil	maize-growing field, agricultural operation, University of California, Riverside
F3	soil	citrus-growing field, University of California, Riverside
F4	soil	citrus-growing field around Riverside
S1	sediment	Salton Sea, CA
S2	sediment	Salton Sea, CA
FPW	solid waste	food-processing waste
HDBC	liquid	high-density hydrocarbon-degrading bacterial cocktail

of acetone and ethanol were used to spike 250-mL Erlenmeyer flasks containing 50 mL of nutrient culture media to give a final concentration of 100 mg endosulfan L^{-1}. Inocula (2 mL) were added to each spiked flask except the control flasks after adjusting their optical densities and incubated (30°C, 160 rpm) for 14 d. Fungal isolates were grown on FTW–endosulfan agar. Two agar blocks (1 × 1 cm) bearing a confluent growth of the fungal isolates were used to inoculate each flask. The flasks were capped with sterile rubber stoppers and incubated (30°C, 160 rpm) for 14 d. This study was performed in triplicates.

Molecular Identification of Fungal Isolate

The best fungal isolate, tentatively designated F4, was identified by analysis of the large subunit (LSU) rRNA gene sequence (Midi Labs, Newark, DE). Briefly, primers corresponding to the LSU rRNA gene of *Schizosaccharomyces japonicus* positions 3334 and 3630, were used to amplify the LSU rRNA gene by PCR. The template was genomic DNA isolated from fungal biomass. Excess primers and dNTPs were removed from the PCR product with Microcon 100 molecular mass cutoff membranes (Millipore, Bedford, MA). Purity of the PCR product was checked by agarose gel electrophoresis. Cycle sequencing of the PCR product was performed with an AmpliTaq FS DNA polymerase and dRhodamine dye terminators (Applied Biosystems, Foster City, CA). The samples were electrophoresed on an ABI Prism 377 DNA sequencer (Applied Biosystems). Sequence data was analyzed using MicroSeq, a microbial analysis software and database (Applied Biosystems, 2001). Neighbor joining phylogenetic trees were generated (Saitou and Nei, 1987) with the top 10 alignment matches.

Analytical Procedures

Endosulfan in cultures was extracted by addition of equal volume of acetone and shaken for one hour with a reciprocating shaker. One milliliter was then transferred to 9 mL of hexane and further shaken for 30 min. The sample was dehydrated, by passing it through anhydrous Na_2SO_4, and concentrated with a rotary evaporator. Appropriate dilutions of the sample extract were then analyzed with a Hewlett-Packard (Palo Alto, CA) gas chromatograph equipped with an electron capture detector (GC–ECD), an autosampler, and a DB-5MS capillary column with a 0.25-mm i.d. and 0.25-μm film thickness (J&W Scientific, Folsom, CA). The oven temperature was programmed at 175°C for 1 min, followed by a linear increase of 2.43°C min^{-1} to 260°C, holding at 260°C for 5 min. The injector temperature was 250°C and the detector temperature was 300°C. Hydrogen was the carrier gas at a flow rate of 0.8 mL min^{-1}.

Bacterial densities in liquid cultures were determined spectrophotometrically by measuring the absorbance at 600 nm.

RESULTS

Endosulfan Degradation by Enriched Cultures

Substantial degradation of endosulfan isomers was observed in Round 1 and Round 2 enrichment cultures when samples were analyzed by GC. In Round 2 enrichment cultures after 15 d of incubation, endosulfan degradation ranged from 39 to 87% in the flasks spiked with endosulfan as a sulfur source. In enrichment cultures where endosulfan was added as a sole carbon source, endosulfan degradation ranged from 46% to a level beyond detection (100%) after 15 d of incubation (Table 2). The pH of the enrichment culture ranged from 3.50 to 6.90 in inoculated flasks as compared with pH 7.00 of uninoculated flasks serving as the control. Optical densities of the enrichment cultures measured at λ_{600} nm ranged between 0.08 to 0.53.

A number of pure cultures were isolated from the streaked plates prepared with agar–endosulfan as a carbon or sulfur source. Two fungal (F1, F4) and two bacterial (BF2, B4) strains were selected and isolated on the basis of growth on endosulfan as a sole carbon source. Five bacterial strains (B2r, B4s, F1p, F3w, F4t) were also selected and isolated from streaked plates containing endosulfan as a sulfur source.

Degradation of Endosulfan by Selected Strains of Bacteria and Fungi

Endosulfan as a Carbon Source

Two fungal (F1 and F4) and two bacterial strains (BF2 and B4), selected because of their prolific growth on streaked plates containing endosulfan as a sole carbon source, were further tested for their ability to degrade endosulfan. One bacterial strain, Lin-3 isolate (Okeke et al., 2002) active on lindane (a chlorinated pesticide), was also tested for endosulfan degradation. The pH of nutrient culture medium prepared for fungal strains was

Table 2. Residual α- and β-endosulfan in enrichment cultures after 15 d of incubation. Initial endosulfan concentration in the cultures was 50 mg L^{-1}.

	Nutrient culture medium (FTW)		Nonsulfur nutrient culture medium (NSM)	
Culture†	α-endosulfan	β-endosulfan	α-endosulfan	β-endosulfan
	mg L^{-1}			
B1	10.00	6.00	14.02	7.49
B2	9.89	6.38	13.56	8.48
B3	14.69	8.97	15.94	8.55
B4	7.20	3.29	13.28	7.60
F1	1.20	0.56	11.79	6.24
F2	ND‡	ND	3.80	0.98
F3	16.64	9.26	11.68	6.57
F4	4.44	1.50	7.93	4.68
S1	17.50	9.40	6.89	5.07
S2	14.20	8.00	9.60	5.64
FPW	ND	ND	9.83	5.53
HDBC	16.78	9.33	18.26	9.45
Control	32.28	17.04	30.30	15.14

† See Table 1 for descriptions.
‡ Not detected.

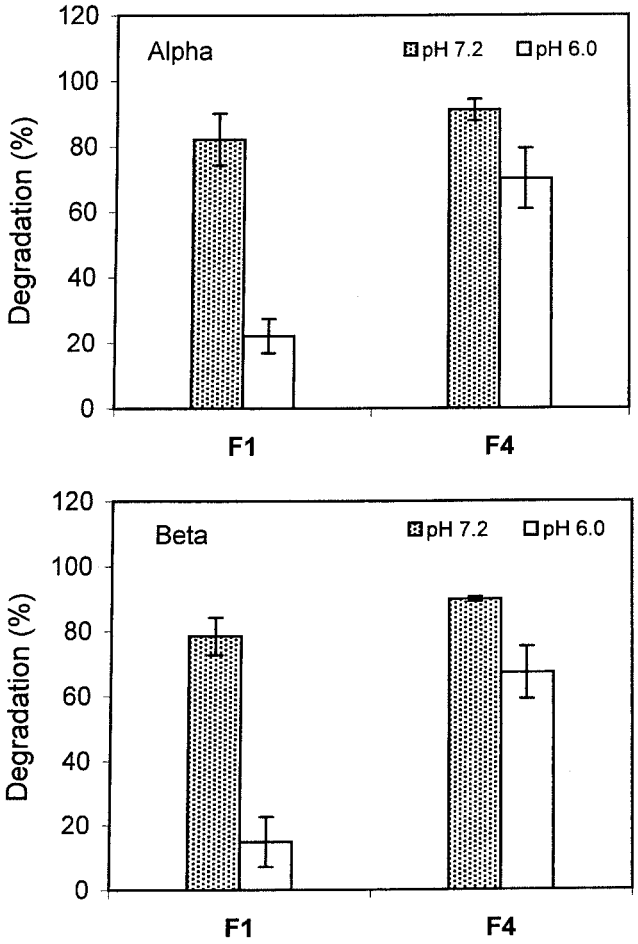

Fig. 2. Degradation of α- and β-endosulfan as a source of carbon in a liquid culture at two pH levels (7.2 and 6.0) by F1 and F4 fungal isolates after 15 d of incubation.

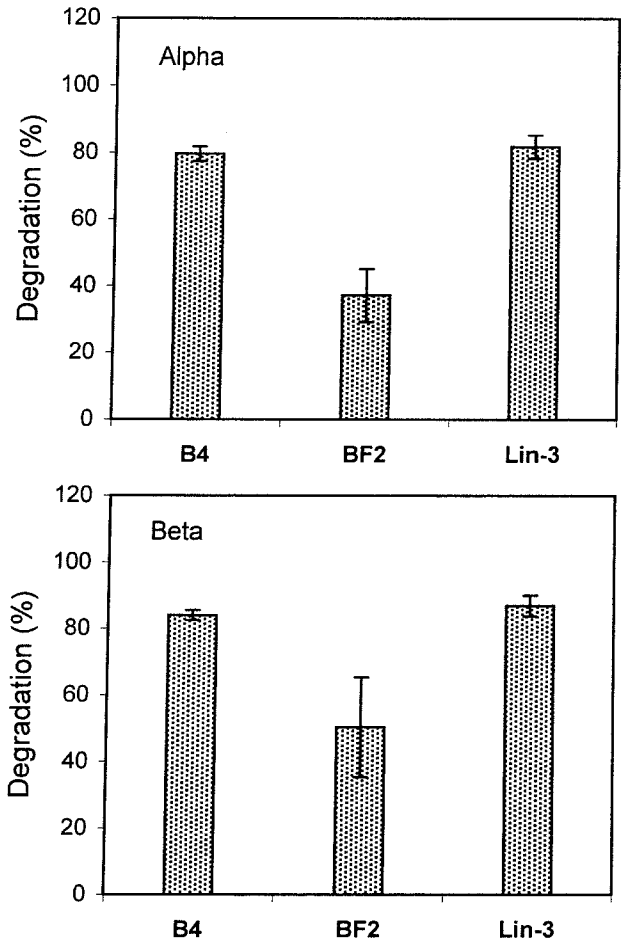

Fig. 3. Degradation of α- and β-endosulfan as a source of carbon in a liquid culture by BF2, B4, and Lin-3 bacterial strains after 15 d of incubation.

adjusted to 7.2 and 6.0 to evaluate the performance of fungal strains at neutral and slightly acidic pH, respectively. Data regarding α-endosulfan degradation in nutrient culture medium after 15 d of incubation revealed that fungal strains F1 and F4 depleted α-endosulfan up to 82.2 and 91.1%, respectively, at pH 7.2 (Fig. 2). Comparatively less degradation was observed in those flasks adjusted at pH 6.0. Strains F1 and F4 degraded 22.2 and 70.1% of α-endosulfan, respectively, in the nutrient culture medium at pH 6.0 after 15 d. A similar trend was noted in the case of β-endosulfan degradation by these strains (Fig. 2). After 15 d of incubation, 78.5 and 89.9% of β-endosulfan were removed by F1 and F4, respectively, in the medium adjusted to pH 7.2. At pH 6.0, F1 and F4 degraded 14.9 and 67.2% of β-endosulfan, respectively.

Three bacterial strains (BF2, B4, and Lin-3) degraded α-endosulfan up to 37.2, 79.6, and 81.8%, respectively, after 15 d of incubation (Fig. 3). β-Endosulfan was degraded (15 d) in the nutrient culture medium by as much as 50.3, 83.9, and 86.8% by BF2, B4, and Lin-3, respectively (Fig. 3). Strains B4 and Lin-3 emerged as the most capable of degrading almost the same amount of both α- and β-endosulfan in the nutrient culture medium.

Endosulfan as a Sulfur Source

Endosulfan was also tested as a source of sulfur for bacterial growth. Five strains (B2r, B4s, F1p, F3w, and F4t) were selected for further studies from the streaked plates. Data regarding α- and β-endosulfan degradation by these bacterial strains after 15 d of incubation are presented in Fig. 4. Concentration of α-endosulfan in the nutrient culture medium decreased to 13.4, 70.2, 33.4, 45.7, and 68.5% by B2r, B4s, F1p, F3w, and F4t, respectively. A similar trend was observed in the case of β-endosulfan degradation in the nutrient culture medium after 15 d of incubation. Degradation of β-endosulfan by B2r, B4s, F1p, F3w, and F4t was 5.8, 70.4, 34.4, 47.8, and 70.8%, respectively, after 15 d of incubation (Fig. 4).

Comparative efficiency of all fungal and bacterial strains degrading endosulfan in nutrient culture medium is summarized in Table 3, showing residual α- and β-endosulfan in culture medium after 15 d of incubation.

Bacterial Density and Culture pH

Optical density (λ_{600}) and pH of the nutrient culture media were also measured to assess the relationship between growth and metabolic activities of the microor-

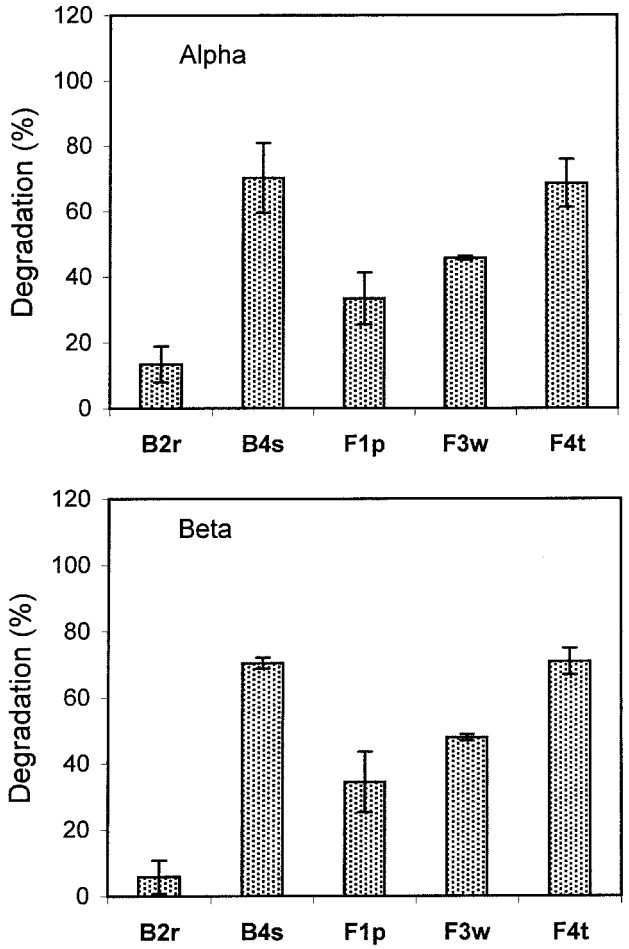

Fig. 4. Degradation of α- and β-endosulfan as a source of sulfur in a liquid culture by B2r, B4s, F1p, F3w, and F4t bacterial strains after 15 d of incubation.

ganisms (Table 4, Fig. 5). In observing bacterial growth amongst the three bacterial strains (BF2, B4, and Lin-3) grown in FTW nutrient culture medium in response to endosulfan supplied as a sole source of carbon, the highest OD_{600} was recorded in the case of B4 (0.366) followed by Lin-3 (0.322) and BF2 (0.296). Bacterial densities measured in the NSM containing endosulfan as a source of sulfur showed the highest OD_{600} (0.359) with strain B4s and the lowest (0.142) with F1p. Strains B2r, F4t, and F3w produced optical densities of 0.258, 0.180, and 0.148, respectively.

Change in pH of the nutrient culture media (FTW and NSM) after 15 d of incubation is shown in Fig. 5. Culture pH decreased to the acidic range due to metabolic activities of the growing microorganisms. Fungal isolates drastically decreased the pH of the culture medium (FTW). The F1 isolate initially grown at pH 7.2 decreased the pH of the culture medium to 3.68 after 15 d of incubation, while the F4 isolate decreased the pH to 3.30. When F1 and F4 isolates were grown in nutrient culture medium initially set at pH 6.0, both reduced the culture pH to 3.2.

All bacterial strains, grown on either nutrient culture media (FTW or NSM), significantly decreased the pH of the culture medium. Of the three bacterial strains tested on the consumption of endosulfan as a sole carbon source in FTW, Lin-3 produced the greatest acidity (pH 3.33) in the cultured flasks. Strains BF2 and B4 decreased the culture medium pH to 3.62 and 3.44, respectively. Five bacterial strains grown in NSM containing endosulfan as a sulfur source also significantly decreased the culture pH when compared with the pH (7.04) of uninoculated control flasks. Extreme reduction in pH (3.47) was also noted in flasks inoculated with the B4s strain. Strains B2r, F1p, F3w, and F4t decreased the pH to 3.61, 5.8, 5.93, and 5.84, respectively.

Phylogenetic Identity of Fungal F4 Strain

The endosulfan-degrading fungal isolate (F4), grown on endosulfan as the sole carbon source, was identified as *Fusarium ventricosum* by LSU rRNA gene sequence analysis. The gene bank accession number was AF513980. The percentage identity of isolate F4 was 100% (0.00% genetic difference [GD]). Based on the first 300 base pair of the variable D_2 region of the LSU rRNA gene, the isolate is most closely related to *Fusarium solani* (99.69% identity) followed by *Fusarium eumartii* (98.75% identity). The phylogenetic neighbor joining tree of *Fusarium ventricosum* is presented in Fig. 6.

DISCUSSION

The present study describes the enrichment, isolation, and identification of microbial strains capable of degrading endosulfan. Enrichment was achieved by providing

Table 3. Residual α- and β-endosulfan (mg L^{-1}) in flasks inoculated with isolated microbial strains after 15 d of incubation. Initial endosulfan concentration in the culture was 100 mg L^{-1}.

Isolate	Identity	Source	α-Endosulfan		β-Endosulfan		Endosulfan (carbon or sulfur source)
			Treated	Control	Treated	Control	
F1	fungi	F1	10.8 (±4.8)†	60.5 (±8.1)	5.65 (±1.5)	26.2 (±4.3)	carbon
F4	*Fusarium ventricosum*	F4	5.42 (±1.9)	60.5 (±8.1)	2.66 (±0.2)	26.2 (±4.3)	carbon
BF2	bacteria	F2	49.6 (±6.2)	78.9 (±12.9)	17.8 (±5.4)	35.8 (±6.5)	carbon
B4	bacteria	B4	16.1 (±1.7)	78.9 (±12.9)	5.76 (±0.6)	35.8 (±6.5)	carbon
Lin-3	*Pandoraea* sp.	Okeke et al. (2002)	14.4 (±2.7)	78.9 (±12.9)	4.74 (±1.2)	35.8 (±6.5)	carbon
B2r	bacteria	B2	52.4 (±3.3)	60.5 (±8.1)	24.7 (±1.3)	26.2 (±4.3)	sulfur
B4s	bacteria	B4	18.0 (±6.4)	60.5 (±8.1)	7.77 (±0.4)	26.2 (±4.3)	sulfur
F1p	bacteria	F1	40.3 (±4.8)	60.5 (±8.1)	17.2 (±2.4)	26.2 (±4.3)	sulfur
F3w	bacteria	F3	32.9 (±0.4)	60.5 (±8.1)	13.7 (±0.3)	26.3 (±4.3)	sulfur
F4t	bacteria	F4	19.1 (±4.5)	60.5 (±8.1)	7.65 (±1.1)	26.3 (±4.3)	sulfur

† Values in parentheses show standard deviation ($n - 1$).

Table 4. Optical density (OD_{600}) of bacterial isolates in nutrient culture medium (FTW) and nonsulfur nutrient culture medium (NSM) after 15 d of incubation.

FTW			NSM				
B4	BF2	Lin-3	B2r	B4s	F1p	F3w	F4t
0.366 (±0.009)†	0.296 (±0.011)	0.322 (±0.025)	0.258 (±0.009)	0.359 (±0.013)	0.142 (±0.014)	0.148 (±0.028)	0.180 (±0.011)

† Values in parentheses show standard deviation ($n - 1$).

endosulfan as the sole carbon or sulfur source in separate studies. Sutherland et al. (2000) and Guerin (1999) considered endosulfan as a poor biological energy source and attributed previous unsuccessful enrichment attempts to poor utilization of endosulfan as a sole source of carbon. However, Awasthi et al. (1997) successfully enriched a bacterial co-culture using endosulfan as a sole carbon source. In this enrichment study, endosulfan was used separately as a carbon or sulfur source to identify which microorganisms prefer endosulfan as a carbon or sulfur source and to what extent endosulfan is degraded when used as a carbon or sulfur source.

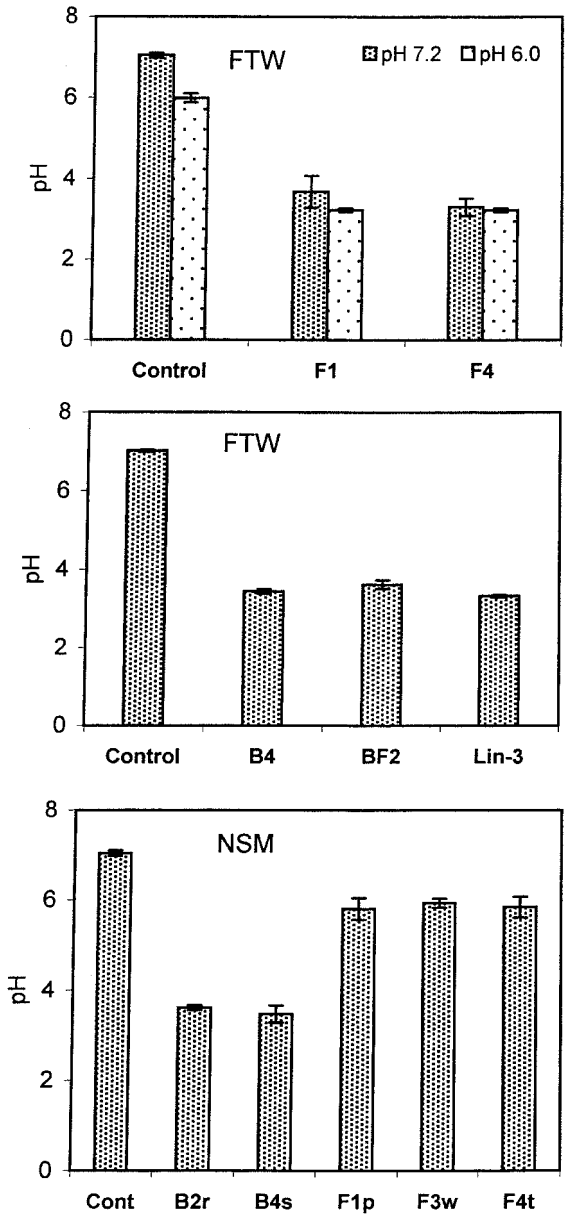

Fig. 5. pH of nutrient culture media after 15 d of incubation inoculated with different strains of bacteria and fungi.

Two fungal strains isolated through enrichment using endosulfan as a carbon source degraded both isomers of endosulfan in FTW. Strains F1 and F4 degraded α-endosulfan up to 82.2 and 91.1% and β-endosulfan up to 78.5 and 89.9%, respectively, in the flasks spiked at 100 mg L^{-1} endosulfan after 15 d of incubation. Similar findings on endosulfan degradation were reported by Shetty et al. (2000) who studied the ability of a fungus, *Mucor thermo-hyalospora* MTCC 1384, to degrade endosulfan in culture medium. Their results indicated that about 78% of the endosulfan isomers were degraded from the flasks spiked at 5 mg L^{-1} after 20 d of incubation. Kullman and Matsumura (1996) reported the disappearance of endosulfan in carbon-deficient medium by *Phanerochaete chrysosporium*. Mukherjee and Gopal (1994) reported that β-endosulfan dissipated in the presence of *Aspergillus niger* to 98.6% by Day 15 as compared with 78.4% in the control medium without *Aspergillus niger*. Isolate F4, which was identified as *Fusarium ventricosum* by LSU rRNA gene sequence analysis, supports the role of *Fusarium* spp. in bioremediation of xenobiotics. Mitra et al. (2001), Jirku et al. (2001), and Yagafarova et al. (2001) revealed that other *Fusarium* species (*Fusarium solani*, *Fusarium proliferatum*) are active in the bioremediation of DDT and hydrocarbons in oil contaminated soils as well as other xenobiotics in water.

Of the three bacterial strains in this study, B4 and Lin-3 showed almost equal capabilities of utilizing endosulfan as a sole carbon source in the culture medium. These results are in conformity with the work of Awasthi et al. (1997), who observed that nearly 50% degradation of both isomers of endosulfan was achieved in the flasks spiked at 50 mg L^{-1} in 7 d by a bacterial co-culture using endosulfan as a sole source of carbon.

Among the bacterial strains isolated through enrichment using endosulfan as a sulfur source, B4s and F4t degraded α-endosulfan up to 70.2 and 68.5% and β-endosulfan up to 70.4 and 70.9%, respectively, after 15 d of incubation. These results are in accordance with the work of Sutherland et al. (2000), who isolated a mixed bacterial culture capable of utilizing endosulfan as a sulfur source.

In our study it was observed that the amount of endosulfan degraded by bacteria as a sulfur source was lower than the amount removed as a sole carbon source. This is most likely attributed to the fact that carbon is required by bacterial cells in the greatest amount followed by nitrogen, phosphorus, and sulfur for the construction

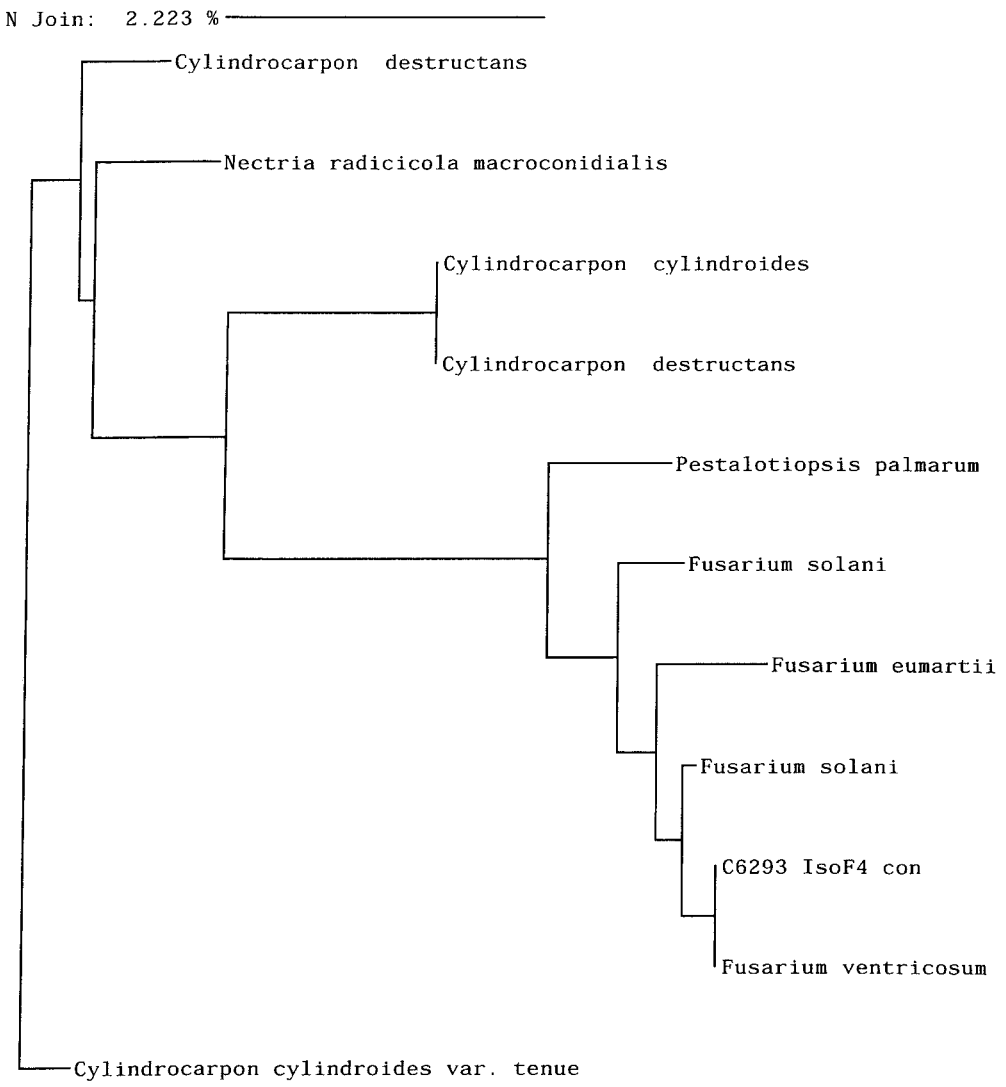

Fig. 6. Neighbor joining phylogenetic tree of *Fusarium ventricosum*.

of cell components as well as for obtaining energy (Alexander, 1998).

Degradation rates of α- and β-endosulfan by both fungal and bacterial strains were evaluated. The fungal isolates removed relatively more α-endosulfan than β-endosulfan, while bacteria degraded relatively more β- than α-endosulfan. Awasthi et al. (1997) reported that degradation of β-endosulfan appeared to be slower than α-endosulfan.

Bacterial strains degrading more endosulfan within the culture media showed higher bacterial densities. Bacterial strains B4 and Lin-3, which degraded the highest amount of α- and β-endosulfan in the nutrient culture media using endosulfan as a carbon source, produced the highest bacterial densities as compared with BF2, which degraded less endosulfan. The same trend was observed in those bacterial strains that depleted α- and β-endosulfan as a sulfur source except F4t, which degraded more α- and β-endosulfan, but showed less optical density when compared with B2r. Sutherland et al. (2000) and Awasthi et al. (1997) also observed substantial disappearance of endosulfan with a simultaneous increase in bacterial mass.

Isolated fungal and bacterial strains significantly decreased the pH of the culture media after 15 d of incubation. Strains degrading the highest concentration of endosulfan drastically increased acidity of the culture media to a pH level of 3.3. But these results are in contradiction to the findings of Miles and Moy (1979) and Martens (1976), who reported that microbial degradation of endosulfan increased the pH of the growth media. They reported that it is difficult to differentiate between chemical and biological degradation, as culturing often leads to an increase in pH, which favors chemical hydrolysis of endosulfan. With multiple experiments in our study, pH decreased with bacterial metabolism of α- and β-endosulfan. pH reduction in the culture medium may either be due to the dehalogenation of endosulfan resulting in the formation of HCl or organic acids produced by microorganisms during their metabolic activities. Sutherland et al. (2000) reported that the proton-transfer chemical ionization (PCI) mass spectrum of

the metabolite displayed fragment ions indicating consecutive losses of two molecules of HCl from the molecular parent ions $[M + H]^+$.

In summary, we have successfully enriched and isolated hyperactive endosulfan-degrading fungal and bacterial strains that can utilize endosulfan as a carbon or sulfur source. The results have valuable applications for endosulfan bioremediation in polluted sites. The use of microorganisms for bioremediation requires an understanding of all the physiological and biochemical aspects involved in chemical transformations. Future research will focus on identification and isolation of the enzymes involved, including a study of their regulation and optimization of conditions. Modern molecular approaches developed for remediation will be applied to soil and natural waters.

REFERENCES

Alexander, D.B. 1998. Bacteria and archaea. p. 44–71. *In* D.M. Sylvia et al. (ed.) Principles and applications of soil microbiology. Prentice Hall, Upper Saddle River, NJ.

Applied Biosystems. 2001. MicroSeq D2 fungal database. Applied Biosystems, Foster City, CA.

Awasthi, N., N. Manickam, and A. Kumar. 1997. Biodegradation of endosulfan by a bacterial coculture. Bull. Environ. Contam. Toxicol. 59:928–934.

Chaudhuri, K., S. Selvaraj, and A.K. Pal. 1999. Studies on the genotoxicology of endosulfan in bacterial system. Mutat. Res. 439:63–67.

Chen, W., and A. Mulchandani. 1998. The use of live biocatalysts for pesticide detoxification. Trends Biotechnol. 16:71–76.

Focht, D.D. 1994. Microbiological procedures for biodegradation research. p. 407–426. *In* R.W. Weaver et al. (ed.) Methods of soil analysis. Part 2. SSSA Book Ser. 5. SSSA, Madison, WI.

Guerin, T.F. 1999. The anaerobic degradation of endosulfan by indigenous microorganisms from low-oxygen soils and sediments. Environ. Pollut. 106:13–21.

Herman, D.C., and W.T. Frankenberger, Jr. 1999. Bacterial reduction of perchlorate and nitrate in water. J. Environ. Qual. 28:1018–1024.

Jirku, V., J. Masak, and A. Cejkova. 2001. Significance of physical attachment of fungi for bio-treatment of water. Microbiol. Res. 156:383–386.

Katayama, A., and F. Matsumura. 1993. Degradation of organochlorine pesticides, particularly endosulfan by *Trichoderma harzianum*. Environ. Toxicol. Chem. 12:1059–1065.

Kullman, S.W., and F. Matsumura. 1996. Metabolic pathways utilized by *Phanerochaete chrysosporium* for degradation of the cyclodiene pesticide endosulfan. Appl. Environ. Microbiol. 62:593–600.

Lee, N., J.H. Skerritt, and D.P. McAdam. 1995. Hapten synthesis and development of ELISAs for the detection of endosulfan in water and soil. J. Agric. Food Chem. 43:1730–1739.

Mansingh, A., and A. Wilson. 1995. Insecticide contamination of Jamaican environment. Baseline studies on the status of insecticidal pollution of Kingston Harbour. Mar. Pollut. Bull. 30:640–645.

Martens, R. 1976. Degradation of [8,9-^{14}C] endosulfan by soil microorganisms. Appl. Environ. Microbiol. 6:853–858.

Miles, C.J., and R.J. Pfeuffer. 1997. Pesticides in canals of South Florida. Arch. Environ. Contam. Toxicol. 32:337–345.

Miles, J.R.W., and P. Moy. 1979. Degradation of endosulfan and its metabolites by a mixed culture of soil microorganisms. Bull. Environ. Contam. Toxicol. 23:13–19.

Mitra, J., P.K. Mukherjee, S.P. Kale, and N.B.K. Murthy. 2001. Bioremediation of DDT in soil by genetically improved strains of soil fungus *Fusarium solani*. Biodegradation 12:235–245.

Mukherjee, I., and M. Gopal. 1994. Degradation of beta-endosulfan by *Aspergillus niger*. Toxicol. Environ. Chem. 46:217–221.

Okeke, B.C., T. Siddique, M.C. Arbestain, and W.T. Frankenberger, Jr. 2002. Biodegradation of γ-hexachlorocyclohexane (Lindane) and α-hexachlorocyclohexane in water and a soil slurry by a *Pandoraea* species. J. Agric. Food Chem. 50:2548–2555.

Paul, V., and E. Balasubramaniam. 1997. Effect of single and repeated administration of endosulfan on behaviour and its interaction with centrally acting drugs in experimental animals: A mini review. Environ. Toxicol. Pharmacol. 3:151–157.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406–425.

Shetty, P.K., J. Mitra, N.B.K. Murthy, K.K. Namitha, K.N. Savitha, and K. Raghu. 2000. Biodegradation of cyclodiene insecticide endosulfan by *Mucor thermo-hyalospora* MTCC 1384. Curr. Sci. 79: 1381–1383.

Sinha, N., R. Narayan, and D.K. Saxena. 1997. Effect of endosulfan on testis of growing rats. Bull. Environ. Contam. Toxicol. 58:79–86.

Sunderam, R.I.M., D.M.H. Cheng, and G.B. Thompson. 1992. Toxicity of endosulfan to native and introduced fish in Australia. Environ. Toxicol. Chem. 11:1469–1476.

Sutherland, T.D., I. Horne, M.J. Lacey, R.L. Harcourt, R.J. Russel, and J.G. Oakeshott. 2000. Enrichment of an endosulfan-degrading mixed bacterial culture. Appl. Environ. Microbiol. 66:2822–2828.

United States Department of Health and Human Services. 1990. Toxicological profile for endosulfan. Agency for Toxic Substances and Disease Registry, Atlanta, GA.

Yagafarova, G.G., E.M. Gataullina, V.B. Barakhnina, I.R. Yagafarov, and A.Kh. Safarov. 2001. A new oil-oxidizing micromycete *Fusarium* sp. Prikl. Biokhim. Mikrobiol. 37:77–79.