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Isolation of a Soil Bacterium Capable of Biodegradation and Detoxification of Endosulfan and Endosulfan Sulfate

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Endosulfan, an endocrine disrupting chemical, is a widely used cyclodiene organochlorine pesticide worldwide, and it blocks neuronal $GABA_A$-gated chloride channels in mammals and aquatic organisms. Endosulfan and its metabolites, such as endosulfan sulfate, are persistent in environments and are considered as toxic chemicals. For bioremediation of endosulfan, in this study, an attempt was made to isolate an endosulfan and endosulfan sulfate degrading bacterium from endosulfan-polluted agricultural soil. Through repetitive enrichment and successive subculture using endosulfan or endosulfan sulfate as the sole carbon source, a bacterium KS-2P was isolated. The KS-2P was identified as *Pseudomonas* sp. on the basis of the results of a 16S rDNA sequencing analysis and MIDI test. The degradation ratios for endosulfan or endosulfan sulfate in minimal medium containing endosulfan (23.5 μg mL^{-1}) or endosulfan sulfate (21 μg mL^{-1}) were 52% and 71%, respectively. Our results suggest that *Pseudomonas* sp. KS-2P has potential as a biocatalyst for endosulfan bioremediation.

KEYWORDS: Biodegradation; cyclodienes; detoxification; endosulfan; endosulfan sulfate; *Pseudomonas* sp.

INTRODUCTION

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,a-hexahydro-6,9-methano-2,3,4-benzodioxya nthiepin-3,-oxid) is a cyclodiene insecticide that exhibits a relatively broad spectrum of activity. This insecticide has been used extensively for over 30 years on a variety of vegetables, fruits, cereal grains, and cotton as well as shrubs, trees, vines, and ornamentals (*1*, *2*). Since endosulfan and its breakdown products are persistent in the environment with an estimated half-life of 0.7−6 years (*1*), endosulfan has been commonly detected in the atmosphere, soils, sediments, surface, groundwaters, and foodstuffs (*1*, *3*). However, endosulfan is extremely toxic to fish and aquatic organisms and affects the central nervous system, kidney, liver, blood chemistry, and parathyroid gland and has reproductive, teratogenic, and mutagenic effects (*4*−*7*). Also, endosulfan has a strong endocrine disrupting activity (*8*, *9*); our group has been focused on the degradation and detoxification of endosulfan (*10*−*13*).

Technical-grade endosulfan is a mixture of two stereoisomers, α- and β-endosulfan, in a ratio of 7:3. Generally, both isomers can be degraded by attacking the sulfite group via either oxidation to form the toxic metabolite endosulfan sulfate or hydrolysis to form the nontoxic metabolite endosulfan diol (*2*, *10*, *11*). The formation of endosulfan sulfate is only known to occur through biological transformation, whereas hydrolysis resulting in endosulfan diol occurs readily at an alkaline pH (*14*). Endosulfan diol is a nontoxic metabolite to fish and other organisms and can be further degraded to nontoxic endosulfan ether, endosulfan hydroxyether, and endosulfan lactone; thus, production of endosulfan diol via hydrolysis may be an important detoxification pathway of endosulfan (*7*, *15*).

Incubation of endosulfan with soil under aerobic conditions resulted in the formation of endosulfan sulfate as the major metabolite. Moreover, metabolism studies have shown that the toxic metabolite endosulfan sulfate is the major residue detected in plant and animal tissue after exposure (*15*−*17*). Therefore, recent researches have focused on the degradation of endosulfan sulfate (*18*, *19*). Our group also reported on potential endosulfan degraders, *Klebsiella pneumoniae* KE-1 (*10*) and *K. oxytoca* KE-8 (*11*), where *K. pneumoniae* KE-1 does not produce any toxic endosulfan sulfate during endosulfan degradation and *K.*

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oxytoca KE-8 could degrade endosulfan sulfate when the endosulfan sulfate is used as a sole carbon source.

In this study, a potent endosulfan sulfate degrader was isolated from endosulfan-polluted soils through repetitive enrichment and successive subculture using endosulfan or endosulfan sulfate as the sole carbon source. The biodegradation assay with newly selected bacterium revealed the effectiveness of *Pseudomonas* sp. KS-2P as a potential biocatalyst for the bioremediation of endosulfan or endosulfan sulfate.

MATERIALS AND METHODS

Chemicals and Media. Endosulfan (purity 97.5%) and endosulfan sulfate (purity 99.5%) were purchased from Labor Dr. Ehrenstorfer Schäfers (Germany). The dichloromethane used in the extraction and the methanol used in the HPLC analysis were purchased from Junsei Co. (Tokyo, Japan). The liquid mineral salt medium (MSM) contained 32 mM KH_2PO_4, 10 mM K_2HPO_4, 12 mM NH_4NO_3, 0.8 mM $MgSO_4$, 0.2 mM $MnSO_4$, and 0.035 mM $FeSO_4$ (Junsei Co. Japan). The MSM was sterilized by autoclaving for 15 min at 121 °C, and the final pH was adjusted to 7.4. For the solid medium, a 1.8% (w/v) Bacto agar (Difco, United States) was added to the liquid MSM. The endosulfan and endosulfan sulfate were dissolved in methanol at 10 mg mL^{-1} and were used in the medium at the appropriate concentration after sterilization.

Isolation and Culture Conditions. The endosulfan-degrading bacteria were initially isolated from endosulfan-polluted cultivated soils in Kyungsan, Kyungpook, Korea, using an enrichment culture in an MSM containing 100 μg mL^{-1} endosulfan. Two grams of soil samples was put into a 250-mL Erlenmeyer flask containing 20 mL of liquid MSM with 100 μg mL^{-1} of endosulfan and was incubated at 30 °C for 7 days with shaking (120 rpm). After 7 days, 5 mL of culture broth from the individual flask culture was reinoculated into a fresh MSM containing 100 μg mL^{-1} endosulfan and was further cultured at 30 °C for 7 days. Then, the culture broth was applied to solid endosulfan MSM for isolating single colonies. The biodegradation ability of colonies was tested by streak-plating onto a solidified endosulfan and endosulfan sulfate-MSM. The strain named KS-2P, which grew on both the endosulfan-MM and the endosulfan sulfate-MM, was deposited in the Korea Type Culture Collection under accession no. KCTC 10874 BP.

Identification of Endosulfan and Endosulfan Sulfate Degrader. The bacterial strain KS-2P was characterized using its physiological and biochemical features, fatty acid profile, and phylogenetic position on the basis of a 16S rDNA sequence analysis. The phenotypic profile of strain KS-2P was also investigated in duplicate using BIOLOG GN2 kits, following the manufacturer's instructions (*20*). The strain KS-2P was grown on tryptic soy agar plate (Difco Co., United States) at 30 °C for 24 h, and fatty acid methyl esters were obtained from freshly grown cultures by saponification, methylation, extraction, and washing according to the instructions of the MIDI system (Microbial ID). The fatty acid profiles were then compared with the profiles in the MIDI MIS library (21). For the sequence analysis, cells were harvested from a culture grown in an LB medium at 30 °C for 3 days, and then the isolation of the chromosomal DNA, PCR amplification, and direct sequencing of the purified products of strain KS-2P were carried out as described by Yoon et al. (*22*).

Biodegradation of Endosulfan and Endosulfan Sulfate. The cells were cultured in liquid MSM containing endosulfan (100 μg mL^{-1}) for 3 days, were collected by centrifugation, and were washed twice in a 0.1 M phosphate buffer (pH 7.0). The washed cells were then inoculated into MSM containing 23.5 μg mL^{-1} endosulfan or 21 μg mL^{-1} endosulfan sulfate. The cultivations were conducted at 30 °C in a rotary shaker (120 rpm) for 7 days, while the endosulfan, its metabolites, culture pH, and biomass were determined at 24-h intervals. The total culture broth was extracted with twice the volume of dichloromethane to prevent any extraction errors. After concentration with nitrogen gas, endosulfan and its metabolites in the extract were analyzed by HPLC system comprising an SCL-10A system controller, LC-10AD pump, and SPD-10A UV detector (Shimadzu, Japan). The analytical column was Mightysil RP-18 column (Kanto Chemical Co., Japan), and the mobile phase consisted of 70% acetonitrile (v/v) with a flow rate of 1 mL min^{-1}. The column temperature was maintained at 40 °C. Twenty microliters of the final extract was injected, and the UV absorption at 214 nm was recorded (*23*). The degradation constant (k_1) was calculated using the following equation on the basis of first-order kinetics derived from the data from the exponential phase of degradation, as previously reported (*11*).

Table 1. Composition of Cellular Fatty Acid of *Pseudomonas* sp. KS-2P[a]

saturated straight chain	%
$C_{12:0}$	6.43
$C_{14:0}$	0.61
$C_{15:0}$	tr
$C_{16:0}$	25.73
$C_{17:0}$	tr
$C_{18:0}$	1.22
unsaturated straight chain	**%**
$C_{18:1}\,\omega 7c$	15.53
hydroxy acids	**%**
$C_{10:0}$ 3OH	4.84
$C_{12:0}$ 2OH	3.30
$C_{12:1}$ 3OH	0.73
$C_{12:0}$ 3OH	4.26
saturated branched chain	**%**
iso- $C_{17:0}$	tr
$C_{17:0}$ cyclo	8.02
$C_{19:0}$ cyclo $\omega 8c$	tr
summed feature 2	tr
summed feature 3	28.23
others	tr

[a] Values are percentages of total fatty acids. tr, trace amount (<0.5%). Summed feature 2, $C_{12:0}$ ALDE; summed feature 3, iso-$C_{16:1}$ $\omega 7c$/$C_{15:0}$ iso 2OH fatty acid methyl ester.

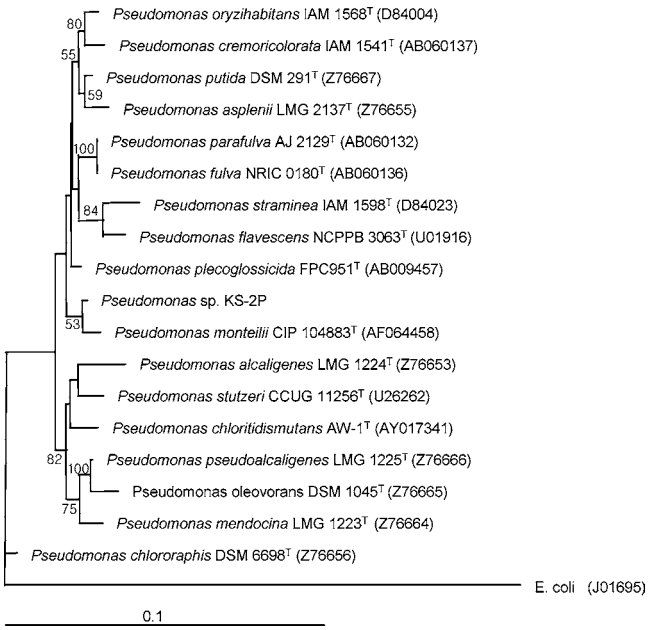

Figure 1. Phylogenetic tree for strain KS-2P and related organisms on the basis of 16S rDNA sequences. The distances were calculated using the neighbor-joining method. The numbers at the branch points are bootstrap values (on the basis of 1000 samplings), and only values greater than 50% are shown. The GenBank accession numbers are given. *Escherichia coli* was used as the outgroup.

$$S = S_0 e^{-k1t} \quad k_1 = \ln[S_0/S]/t$$

where S_0 is the initial substrate concentration, S is the remaining substrate concentration, and t is the time in days. After treatment of KS-2P, the toxicity of culture broth was evaluated by yeast-based cell growth inhibition (spot test). The yeast, *Saccharomyces cerevisiae*, was cultured in YPD (1% yeast extract, 1% polypeptone, 2% glucose) medium, and the cells were collected and were washed with 0.1 M phosphate buffer (pH 7.0). The cell concentration was adjusted to 10^8 cfu mL^{-1} using distilled water. As a control experiment, endosulfan was added to MSM and was extracted and concentrated, as described. The extract was added to the 1 mL of yeast suspension and was cultured at 30 °C. After 6 h, the 10 μL of treated yeast suspension was spotted on the YPD solid medium. The growth spot of yeast was determined after 24 h cultivation. In case of KS-2P treatment, endosulfan was added to MSM and then the KS-2P was inoculated. After 6 days, the culture broth was extracted and the growth inhibition activity of extract was determined using the same methods.

RESULTS AND DISCUSSION

Isolation and Identification of Strain KS-2P. From 20 endosulfan-polluted soil samples, different bacteria were isolated. On the basis of rapid growth in endosulfan- or endosulfan sulfate-MSM, the strain KS-2P was finally selected (results not shown). The strain KS-2P could degrade 44.5 μg mL^{-1}endosulfan in MSM containing 87.8 μg mL^{-1}endosulfan for 7 days; the concentration of α- and β-endosulfan was decreased to 31.3 and 12.0 μg mL^{-1} from 61.9 and 25.9 μg mL^{-1}, respectively.

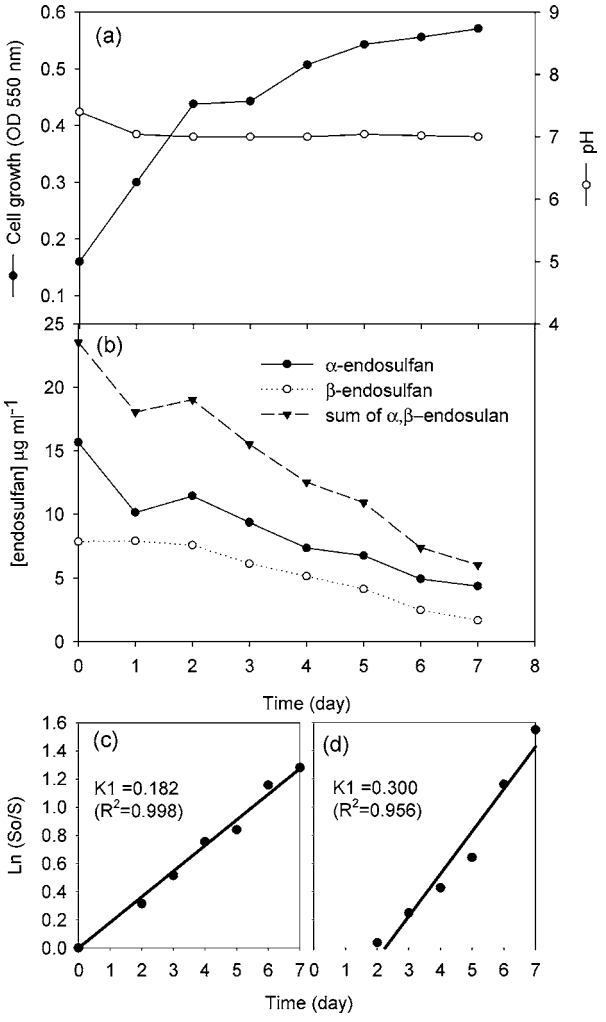

Figure 2. Biodegradation of endosulfan by *Pseudomonas* sp. KS-2P. (a) Cell growth and culture pH in endosulfan-MSM over 7 days. (b) Degradation of endosulfan isomers and (c) degradation constants for α- and (d) β-endosulfanon the basis of first-order kinetics from the data of the exponential phase of degradation.

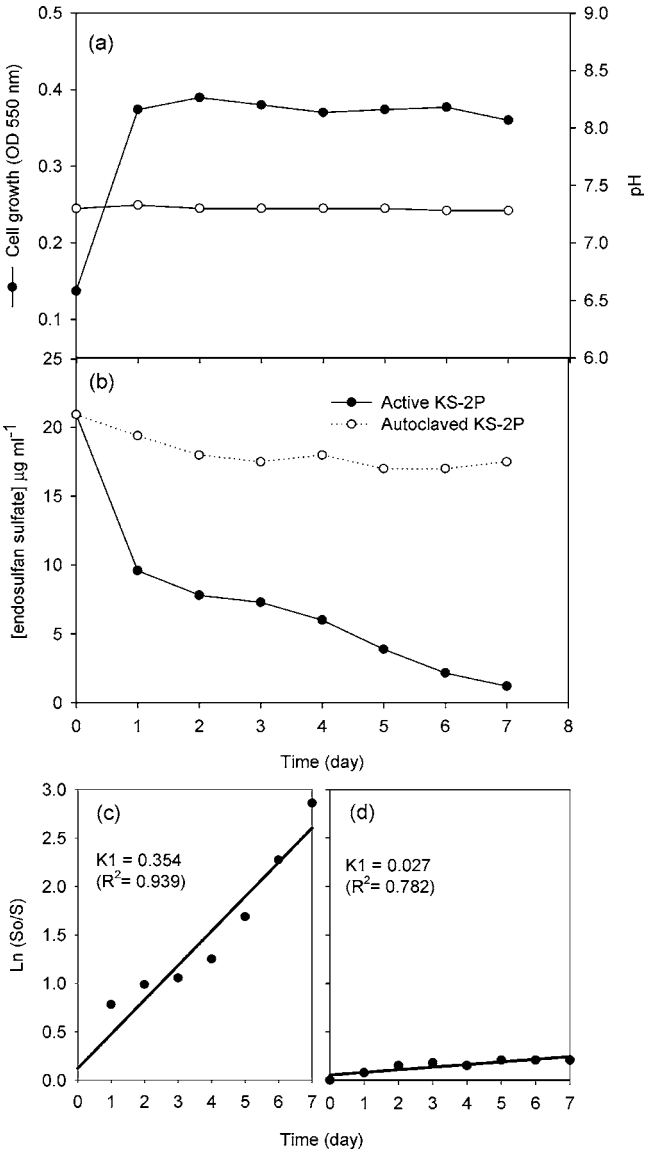

Figure 3. Biodegradation of endosulfan sulfate by *Pseudomonas* sp. KS-2P. (a) Cell growth and culture pH in endosulfan sulfate-MSM over 7 days. (b) Degradation of endosulfan sulfate and (c) degradation constants for endosulfan sulfate in the culture of active KS-2P and (d) autoclaved KS-2P on the basis of first-order kinetics from the data of the exponential phase of degradation.

The strain KS-2P was gram-negative and was a rod-shaped organism that occurred as a single cell. It was identified using BIOLOG GN microplates and Microlog release 3.5 software. The strain KS-2P was found to utilize the following substrates as carbon and energy sources: L-histidine, L-phenylalanine, and L-serine, whereas KS-2P did not utilize L-threonine, L-rhamnose, D-mannitol, D-trehalose, erythritol, and D-sorbitol as carbon and energy sources.

The cellular fatty acid analysis revealed that the predominant fatty acids were 16:0, 16:1ω7*c*/15 iso2OH, 18:1ω7*c*, 17.0 CYCLO, and 12:0. Other fatty acids present in smaller amounts were 18:0, 12:1 3OH, 14:0, 19:0 CYCLO ω8*c*, and 15:0 (**Table 1**). The almost complete 16S rDNA sequence of the strain KS-2P was determined following PCR amplification. Phylogenetic analysis of the 16S rDNA gene sequences revealed a clear

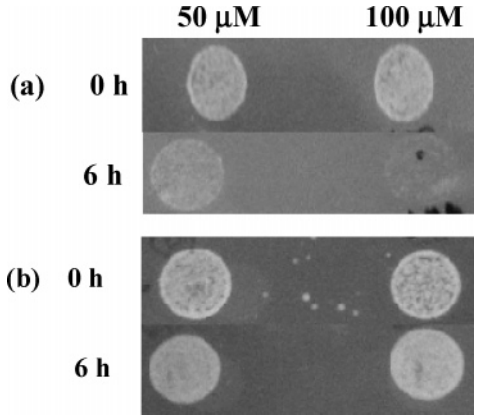

Figure 4. Toxicity determination of culture broth containing endosulfan on the basis of yeast-based cell growth inhibition (spot test). The yeast, *Saccahromyces cerevisiae*, was mixed with the extract of culture broth containing toxic endosulfan and its metabolites for 6 h, and then 10 μL of treated yeast suspension was spotted on the YPD solid medium. The growth spot of yeast was determined after 24 h cultivation. The toxicity of endosulfan was measured using medium extract containing different concentration of endosulfan.

affiliation between strain KS-2P and members of the genus *Pseudomonas*, where the closest sequence similarity (99.29%) was observed with *Pseudomonas monteilii* CIP 104883^T (**Figure 1**). Therefore, the strain KS-2P was identified as *Pseudomonas* sp.

Biodegradation of Endosulfan and Endosulfan Sulfate by *Pseudomonas* sp. KS-2P. *Pseudomonas* sp. KS-2P was cultivated in MSM containing endosulfan or endosulfan sulfate as sole carbon and energy sources. When endosulfan or endosulfan sulfate was omitted from the medium, no growth was observed and the pH remained at 7.4. In the endosulfan-MSM, the cell growth was rapidly increased to 0.44 (OD 550 nm) during 2 days cultivation and was slowly increased. The culture pH was decreased to 7.0 for 1 day and was maintained without apparent changes (**Figure 2a**). The concentration of endosulfan was continuously decreased to 6.0 μg mL^{-1} from 23.5 μg mL^{-1} (**Figure 2b**); the degradation constants of α- and β-endosulfan were 0.182 and 0.3 day^{-1}, respectively (**Figure 2c, 2d**). The degradation of β-endosulfan was active after 2 days cultivation (**Figure 2d**). These results suggested that the mechanism responsible for β-endosulfan degradation may be different from that of α-endosulfan degradation.

In the endosulfan sulfate-MSM, the cell growth of KS-2P reached a maximum on 2 days and then slightly decreased (**Figure 3a**). However, the pH was maintained at 7.3 without changes. The concentration of endosulfan sulfate was rapidly decreased to 9.6 μg mL^{-1} from 21.5 μg mL^{-1} for 1 day and decreased to 1.2 μg mL^{-1} after 7 days (**Figure 3b**). In the case of inoculation of autoclaved KS-2P, the concentration of endosulfan sulfate showed very low decreases; the degradation constants of active KS-2P treatment and dead KS-2P treatment were 0.354 and 0.027 day^{-1}, respectively (**Figure 3c, 3d**). These results suggested that the strain KS-2P has higher degradation activity for endosulfan sulfate than endosulfan, and KS-2P has advantage of our previously reported *K. oxytoca* KE-8 (*11*).

Biodegradation and Bio-Detoxification of Endosulfan by *Pseudomonas* KS-2P. Our group had reported the toxic mechanism of endosulfan using a unicellular higher eukaryote, *S. cerevisiae* (*12*). Since endosulfan resulted in oxidative stress in a concentration-dependent manner in *S. cerevisiae*, treatment of endosulfan and measurement of yeast viability may be useful to determine the toxicity of culture broth containing endosulfan. Moreover, the spot test used in this study is useful to evaluate the toxicity of culture broth, which involves the residual endosulfan and various possible toxic metabolites.

Spot test against 50 μM of endosulfan treatment for 6 h showed that the yeast had minor cell damages, whereas spot test against KS-2P treated-endosulfan (50 μM) did not show cell growth inhibition (**Figure 4a**). The severe cell growth inhibition by 100 μM endosulfan treatment was observed. However, in spot test against KS-2P treated-endosulfan (100 μM), the cell growth was similar to nontreated control. (**Figure 4b**). Our results suggested that the *Pseudomonas* sp. KS-2P has potential as a biocatalyst for endosulfan bioremediation.

LITERATURE CITED

(1) Siddique, T.; Benedict, C. O.; Muhammad, A.; Frankenberger, W. T., Jr. Biodegradation kinetics of endosulfan *Fusarium ventricosum* and a *Pandoraea* Species. *J. Agric. Food Chem.* **2003**, *51*, 8015−8019.

(2) Kullman, S. W.; Matsumura, F. Metabolic pathways utilized by *Phanerochaete chrysoporium* for degradation of the cyclodiene pesticide endosulfan. *Appl. Environ. Microbiol.* **1996**, *62*, 593−600.

(3) Turner, K. O.; Syvanen, M.; Meizrl, S. The human acrosome reaction in highly sensitive to inhibition by cyclodiene insecticides. *J. Androl.* **1997**, *18*, 571−575.

(4) Keith, G. R.; Drake, H. L. Priority pollutants I: A perspective view. *Environ. Sci. Technol.* **1979**, *13*, 416−423.

(5) Paul, V.; Balasubramaniam, E. Effect of single and repeated administration of endosulfan on behavior and its interaction with centrally acting drugs in experimental animals: a mini review. *Environ. Toxicol. Pharmacol.* **1997**, *3*, 151−157.

(6) Lu, Y.; Morimoto, K.; Takechita, T.; Takeuchi, T.; Saito, T. Genotoxic effects of α-endosulfan and β- endosulfan on human HepG2 cells. *Environ. Health Perspect.* **2000**, *108*, 559−561.

(7) Shivaramaiah, H. M.; Sanchez-Bayo, F.; Al-Rifai, J.; Kennedy, I. R. The fate of endosulfan in water. *J. Environ. Sci. Health, Part B* **2005**, *40*, 711−720.

(8) Ramamoorthy, K.; Wang, F.; Chen, I. C.; Norris, J. D.; McDonnell, D. P.; Leonard, L. S.; Gaido, K. W.; Bocchinfuso, W. P.; Korach, K. S.; Safe, S. Estrogenic activity of a dieldrin/toxaphene mixture in the mouse uterus, MCF-7 human breast cancer cells, and yeast-based estrogen receptor assays: no apparent synergism. *Endocrinology* **1997**, *138*, 1520−1527.

(9) Jin, L.; Tran, D. Q.; Ide, C. F.; McLachlan, J. A.; Arnold, S. F. Several synthetic chemicals inhibit progesterone receptor-mediated transactivation in yeast. *Biochem. Biophy. Res. Commun.* **1997**, *233*, 139−146.

(10) Kwon, G.-S.; Kim, J.-E.; Kim, T.-K.; Sohn, H.-Y.; Koh, S.-C.; Shin, K.-S.; Kim, D.-G. *Kebsiella pneumoniae* KE-1 degrades endosulfan without formation of the toxic metabolite, endosulfan sulfate. *FEMS Microbiol. Lett.* **2002**, *215*, 255−259.

(11) Kwon, G.-S.; Sohn, H.-Y.; Shin, K.-S.; Kim, E.; Seo, B.-I. Biodegradation of the organochlorine insecticide, endosulfan, and the toxic metabolite, endosulfan sulfate, by *Kelbsiella oxytoca* KE-8. *Appl. Microbiol. Biotechnol.* **2005**, *67*, 845−850.

(12) Sohn, H.-Y.; Kwon, C.-S.; Kwon, G.-S.; Lee, J.-B.; Kim, E. Induction of oxidative stress by endosulfan and protective effect of lipid-soluble antioxidants against endosulfan-induced oxidative damage. *Toxicol. Lett.* **2004**, *151*, 357−365.

(13) Lee, S. E.; Kim, J. S.; Kennedy, I. R.; Park, J. W.; Kwon, G. S.; Koh, S. C.; Kim, J. E., Biotransformation of an organochlorine insecticide, endosulfan, by *Anabaena* species. *J. Agric. Food Chem.* **2003**, *51*, 1336−1340.

(14) Martens, R. Degradation of [8,9-14C] endosulfan by soil microorganisms. *Appl. Environ. Microbiol.* **1976**, *6*, 853−858.

(15) Beck, E. W.; Johnson, J. C.; Soodham, D. W.; Leuck, D. B.; Dsway, L. H.; Robbins, J. E.; Bowman, M. C. Residues of endosulfan in meat and milk of cattle fed treated forages. *J. Econ. Entomol.* **1966**, *17*, 216−220.

(16) Maier-Bode, H. Properties effect, residues, and analytics of the insecticide endosulfan. *Residue Rev.* **1968**, *22*, 1−44.

(17) Beard, J. E.; Wara, G. W. Fate of endosulfan on plants and glass. *J. Agric. Food Chem.* **1969**, *17*, 216−220.

(18) Sutherland, T. D.; Weir, K. M.; Lacey, M. J.; Horne, I.; Russell, R. J.; Oakeshott, J. G. Enrichment of microbial culture capable of degrading endosulphate, the toxic metabolite of endosulfan. *J. Agric. Food Chem.* **2002**, *92*, 541−548.

(19) Sethunathan, N.; Megharaj, M.; Chen, Z. L.; Williams, B. D.; Lewis, G.; Naidu, R. Algal degradation of a known endocrine disrupting insecticide, α-endosulfan, and its metabolite, endosulfan sulfate of liquid medium and soil. *J. Agric. Food Chem.* **2004**, *52*, 3030−3035.

(20) Rüger, H.-J.; Krambeck, H.-J. Evaluation of the BIOLOG substrate metabolism system for classification of marine bacteria. *Syst. Appl. Microbiol.* **1994**, *17*, 281−288.

(21) Sasser, M. *Identification of Bacteria by Gas Chromatography of Cellular Fatty Acids;* MIDI Technical Note 101; MIDI: Newark, DE, 1990.

(22) Yoon, J.-H.; Lee, S. T.; Park, Y.−H. Inter- and intra specific phylogenetic analysis of the genus *Nocardioides* and related taxa based on 16S rDNA sequences. *Int. J. Syst. Bacteriol.* **1998**, *48*, 187−194.

(23) Siddique, T.; Zahir, A. Z.; Frankenberger, W. T., Jr. Reversed-phase liquid chromatographic method for analysis of endosulfan and its major metabolites. *J. Liq. Chromatogr. Relat. Technol.* **2003**, *26*, 1069−1082.

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