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Biodegradation of Organochlorine Insecticide Endosulfan by the Fungus *Eutypella* sp. KEF-1

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Abstract

BACKGROUND: α - and β - Endosulfan isomers of endosulfan, an endocrine disrupting chemical, are widely used cyclodiene organochlorine pesticide in worldwide, and it has widespread application in agriculture and can contaminate river-system as runoff from soil or aerial deposition
METHOD AND RESULTS: In this study, an attempt was made to isolate an endosulfan degrading fungus from endosulfan-polluted agricultural soil. Through repetitive enrichment and successive subculture in media containing endosulfan and its metabolites as the sole carbon source, a fungus designated KEF-1 was isolated. Based on phylogenetic analysis, strain KEF-1 was assigned to the genus *Eutypella*. Also, the ITS (internal transcribed spacer) sequences of KEF-1 were submitted to GenBank under accession number EF581006. In potato dextrose broth containing 8 $\mu\text{g/mL}$ endosulfan, strain KEF-1 completely degraded the endosulfan in 12 days.

CONCLUSION: These results suggested that *Eutypella* sp. KEF-1 has potential as a biocatalyst for endosulfan bioremediation

Key Words: Biodegradation, Bioremediation, Detoxification, Endosulfan, Endosulfan sulfate, *Eutypella* sp.

Introduction

Endosulfan is a cyclodiene insecticide that exhibits a relatively broad spectrum of activity. It is one of the few organochlorine insecticides, which is used extensively all over the world. Commercial formulations of endosulfan, a sulphurous acid ester of a chlorinated cyclic diol, consist of a mixture of two stereo isomers α - and β - endosulfan in a ratio of approximately 7:3 and registered under several trademarks, Thiodan, Cyclodan, Thimol, Thiofar and Malix (Kimber *et al.*, 1994). This insecticide has been used extensively for over 30 years on a variety of vegetables, fruits, cereal-grains, cotton as well as shrubs, trees, vines, and ornamentals. Endosulfan has been commonly detected in the atmosphere, soils, sediments, surface water,

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ground waters, and foodstuffs (Turner *et al.*, 1997; Kullman and Matsumura 1996; Siddique *et al.*, 2003a). Endosulfan could persist in soil and water environments for 3 to 6 months or more (Sethunathan *et al.*, 2002). However, endosulfan is extremely toxic to fish and aquatic organisms. It can affect the central nervous system, kidney, liver, and parathyroid gland and has adverse reproductive, teratogenic, and mutagenic effects (Sutherland *et al.*, 2002; Paul and Balasubramaniam 1997; Lu *et al.*, 2000; Sohn *et al.*, 2004; Shivaramaiah *et al.*, 2005). And, Karatas *et al.* (2006) reported 23 cases of endosulfan poisoning.

Since endosulfan and its metabolites breakdown products (endosulfan sulfate, endosulfan ether, endosulfan lacton etc.) are persistent in the environment with an estimated half-life of about 6 years and the rate of endosulfan degradation in soil is dependent on pH, endosulfan can be a source of later contamination. Endosulfan has been shown to exhibit strong endocrine disrupting activity because of toxic effects to fish and aquatic invertebrates and these have been implicated in mammalian gonadal toxicity, genotoxicity and neurotoxicity (Turner *et al.*, 1997; Siddique *et al.*, 2003a; Jin *et al.*, 1997; Ramamoorthy *et al.*, 1997). Our group has been focusing on the degradation and detoxification of endosulfan (Kwon *et al.*, 2002; Kwon *et al.*, 2005; Lee *et al.*, 2003; Lee *et al.*, 2006). The sulfite group of both endosulfan isomers can be subject to either oxidation to form the toxic metabolite endosulfan sulfate or hydrolysis to form the non-toxic metabolite endosulfan diol (Kwon *et al.*, 2002; Kwon *et al.*, 2005; Lee *et al.*, 2006). The formation of endosulfan sulfate is only known to occur through biological transformation, whereas hydrolysis resulting in endosulfan diol occurs readily under alkaline conditions (Siddique *et al.*, 2003a; Martens, 1976). Kullman and Matsumura (1996) also reported that most soil fungi produce endosulfan sulfate as the major metabolite of endosulfan. Endosulfan diol (alcohol) is a non-toxic metabolite to fish and other organisms and can be further degraded to non-toxic endosulfan ether, endosulfan hydroxyether, and endosulfan lactone. Thus production of endosulfan diol via hydrolysis may be an important detoxification pathway of endosulfan. Endosulfan degradation studies have shown that the toxic metabolite endosulfan sulfate is the major residue detected in plant and animal tissue after exposure to endosulfan (Shivaramaiah *et al.*, 2005; Lee *et al.*, 2006; Beck *et al.*,

1996; Palma *et al.*, 2009).

Several studies have shown that many fungi have the ability to degrade endosulfan. These include strains of *Aspergillus niger* (Mukherjee and Gopal, 1994; Bhalerao and Puranik, 2003), *A. terreus*, *Cladosporium oxysporum* (Mukherjee and Mittal, 2005), *Mucor thermohyalospora* (Shetty *et al.*, 2000), *Fusarium ventricosum* (Siddique *et al.*, 2003a), and *Phanerochaete chrysosporium* (Kullman and Matsumura, 1996).

Recent researches has focused on the degradation of endosulfan sulfate (Sethunathan *et al.*, 2002; Sutherland *et al.*, 2002; Kwon *et al.*, 2005; Lee *et al.*, 2006; Kumar *et al.*, 2007). Our group also reported on potential endosulfan degrading microorganisms, *Klebsiella pneumoniae* KE-1 (Kwon *et al.*, 2002), *K. oxytoca* KE-8 (Kwon *et al.*, 2005) and *Pseudomonas* sp. KS-2P (Lee *et al.*, 2006).

In this study, a potent endosulfan degrader was isolated from endosulfan-polluted soils through repetitive enrichment and successive subcultures using endosulfan and endosulfan sulfate as the carbon source. Biodegradation assay revealed the effectiveness of the newly isolated fungus *Eutypella* sp. KEF-1 to be a potential biocatalyst for the bioremediation of endosulfan and its metabolites.

Materials and Methods

Chemicals and media

Endosulfan (purity 97.5%), endosulfan sulfate (purity 99.5%) and its metabolites (Endosulfan ether, endosulfan lacton, endosulfan diol) were purchased from Labor Dr. Ehrenstorfer Schäfers (Augsburg, Germany). The dichloromethane used in the extraction, and methanol used HPLC analysis were purchased from Junsei Co. (Tokyo, Japan). The isolated fungal media used were potato dextrose broth or potato dextrose agar (PDB or PDA) (Difco, Detroit, MI). The PDB/PDA were sterilized by autoclaving for 15 min at 121°C and the final pH adjusted to 4.8 before autoclaved. The endosulfan and endosulfan sulfate were dissolved in methanol at 10 mg/mL and used in the medium at the appropriate concentrations after sterilization.

Isolation and identification of fungal strain KEF-1

The endosulfan-degrading microorganisms were initially isolated from endosulfan-polluted cultivated soils in Gyungsan, Gyungpook, Korea, using an

enrichment culture in a PDB containing 50 µg/mL endosulfan. Two grams (w/w) of soil samples were put into a 250-mL Erlenmeyer flask containing 20 mL of liquid PDB with 50 µg/mL of endosulfan. The flask was incubated at 30°C with shaking (150 rpm). After 10 days, 5 mL of culture broth from the individual flask culture were re-inoculated into a fresh PDB containing 50 µg/mL endosulfan and further cultured at 30°C for 7 days. Then, the culture broth was applied to solid 50 µg/mL endosulfan add the after autoclaved PDA for isolating the individual fungal isolates. The degradation ability of the colonies was tested by streak-plating onto a solidified 50 µg/mL endosulfan-PDA. The strain named KEF-1 was grown on endosulfan-PDA. Fungal strain KEF-1 was routinely cultured on endosulfan-containing PDA and maintained at 4°C or as a glycerol suspension (20%, w/v) at -70°C.

The fungal strain KEF-1 that showed endosulfan degrading activity was identified based on analysis of the DNA sequences of the internal transcribed spacer regions (ITS1-5.8S-ITS2 ITS) of their rRNA gene. Genomic DNA was extracted from fungal mycelia grown on PDA (30°C for 24 h) using a DNeasy Plant minikit (Qiagen, Valencia, CA) according to the instructions of the manufacturer. The primers ITS1 (5'-TCCGTAGGTGAACCTGCGG) and ITS4 (5'-TCC-TCCGCTTATTGATATGC) used to amplify the ITS region are located in conserved regions of the 3' end of the 18S rRNA gene and the 5' end of the 28S rRNA gene (White *et al.*, 1990). PCR was performed using a DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad, San Diego, CA) with the following regime: 94°C for 2 min, 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, and a final extension at 72°C for 10 min. The amplified PCR fragment was purified with Wizard SV gel and PCR clean-up system (Promega, Madison, USA) and was sequenced with an ABI PRISM Dye Terminator Cycle sequencing Kit (Perkin Elmer, Waltham, USA) using the same primers as for amplification. A BLAST search was used to identify the closest matched sequences in the GenBank database. The fungal sequence and other related sequences were sequentially aligned using CLUSTAL W program (Thompson *et al.*, 1994) and the alignments were adjusted manually where necessary to maximize alignment. Phylogenetic relationships were estimated using a PHYLIP 3.5c package (Felsenstein, 1985).

Biodegradation of endosulfan

Endosulfan degradation was assessed in a 250-mL Erlenmeyer flask containing 20 mL of liquid PDB with 8 µg/mL of endosulfan, and cultivations were conducted at 30°C in a rotary shaker (150 rpm) for 16 days, while the endosulfan, its metabolites, culture pH, and biomass were determined at 48h intervals. The total culture broth was extracted with twice the volume of dichloromethane. After concentration with nitrogen gas, endosulfan and its metabolites in the extract were analyzed by the Sykam HPLC system (Gewerbering, Germany), S2100 Solvent delivery system and S3210 UV-visible detector. The analytical column was Mightysil RP-18 column (Kanto Chemical Co., Tokyo, Japan), and the mobile phase consisted of 70% acetonitrile (v/v) with a flow rate of 1 mL/min. The column temperature was maintained at 40°C. A 20 µL of the final extract was injected into the column and the UV absorption at 214 nm was recorded (Siddique *et al.*, 2003b). The degradation constant was calculated using the following equation based on first order kinetics derived from the data from the exponential phase of degradation, as previously reported (Lee *et al.*, 2006).

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

Where S_0 was the initial substrate concentration, S was the remaining substrate concentration and t is the time in days.

The biodegradation of endosulfan and its metabolites was analyzed using thin-layer chromatography (TLC), and the total cell culture medium extracted three times with twice the volume of dichloromethane (J. T. Baker Inc, Phillipsburg, NJ). The dichloromethane layer was separated, pooled, and analyzed by TLC. The TLC was developed in hexane:chloroform:acetone (9:3:1, v/v) on silica gel 60 F₂₅₄ plates (Merck, Whitehouse Station, USA), and the separated spots analyzed using an AgNO₃-chromogenic reagent (Kovacs, 1963). The analysis used for the degradation of endosulfan sulfate was similar to that used for the degradation of endosulfan.

Results and Discussion

Isolation and Identification of strain KEF-1

Different microbes were isolated from 20 soil samples based on repetitive enrichment cultures and successive transfers using the endosulfan-PDA. Based on rapid growth in endosulfan-PDB, the strain KEF-1

was finally selected. The strain KEF-1 could completely degrade 8 µg/mL endosulfan in PDB over 12 days; the concentration of α - and β -endosulfan decreased to zero from 4.5 and 3.5 µg/mL respectively.

The ITS rDNA gene sequence of strain KEF-1 was determined directly following PCR amplification and the 556bp ITS region sequence of KEF-1 was determined. The nearly full-length ITS sequence was compared to existing sequences in GenBank. A BLAST search showed that it had 100% similarities with the ITS and 5.8S sequences from *Eutypella scoparia* MUT 485 and *E. scoparia* isolate *E* L56C. No variations in the lengths of the ITS and 5.8S regions were found among them. Phylogenetic analysis of the ITS rRNA gene sequences revealed a clear affiliation between strain KEF-1 and members of the genus *Eutypella* and its closest relatives were *E. scoparia* MUT 485 and *E. scoparia* isolate EL56C. Thus, on the basis of the phylogenetic data, strain KEF-1 was assigned to the genus *Eutypella* (Fig. 1). The ITS sequence of KEF-1 was assigned to the GenBank under

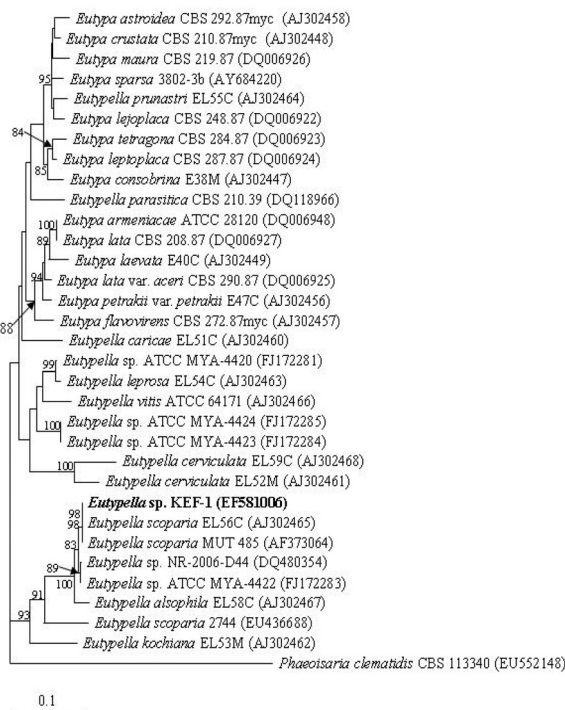


Fig. 1. Neighbour-joining tree based on ITS gene sequences showing the phylogenetic relationship between KEF-1 fungal strain and other related species. *Phaeoisaria clematidis* CBS 113340 was used as an outgroup. Bootstrap values greater than 1000 are given at the nodes; only values above 70% are given. Bar, 0.1 substitutions per nucleotide position.

accession number EF581006.

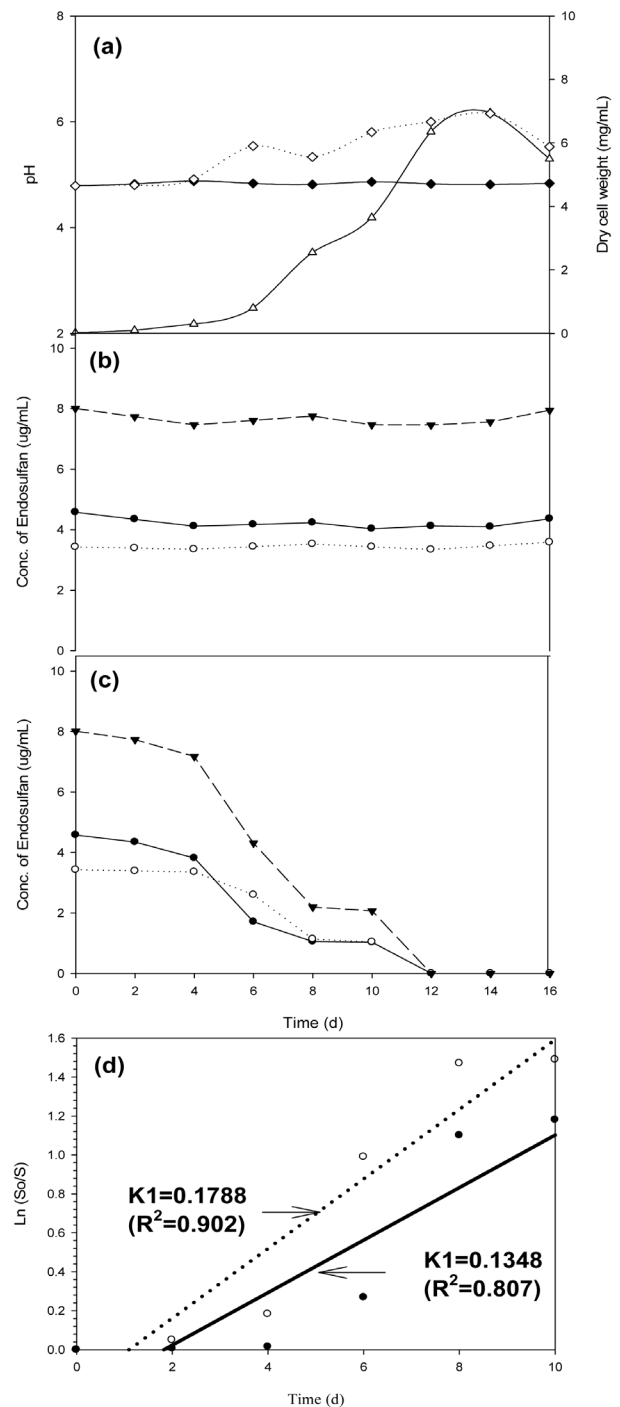


Fig. 2. Biodegradation of endosulfan by *Eutypella* sp. KEF-1. (a) KEF-1 growth and pH in endosulfan-PDB over 16 days, (b) Autoclaved KEF-1 on the basis of control concentration of endosulfan, (c) KEF-1 as degradation of endosulfan isomers and (d) degradation constants for endosulfan isomers on the basis of first-order kinetics from the data of the exponential phase of degradation. (control pH - \blacklozenge -, culture pH - \diamond -, cell growth - \triangle -, α -endosulfan - \bullet -, β -endosulfan - \circ -, sum of endosulfan - \blacktriangledown -)

Biodegradation of endosulfan and endosulfan sulfate by *Eutypella* sp. KEF-1

Eutypella sp. KEF-1 was cultivated in PDB containing endosulfan as the carbon and energy sources. In the endosulfan-PDB, cell growth was rapidly increased to 7 mg/mL (dry cell weight) during 16 days of cultivation. The culture pH increased to 4.8 for 1 day maintained its pH of 4.8 (Fig. 2a). The concentration of endosulfan was continuously decreased to 0 $\mu\text{g/mL}$ from 8 $\mu\text{g/mL}$ (Fig. 2c); the degradation constants of α - and β - endosulfan kinetics were 0.135 d^{-1} ($R^2=0.902$) and 0.179 d^{-1} ($R^2=0.807$), respectively (Fig.2d). It should be noted that the degradation of endosulfan was active after 4 days of cultivation (Fig. 2c). α - endosulfan activated degradation occurred after 4 days of cultivation. These results suggested that the mechanism responsible for α - endosulfan degradation may be different to that of β - endosulfan degradation. β - endosulfan degradation was observed to be stronger compared to α - endosulfan, which compares closely to the other report of Siddique *et al.* (2003a).

Endosulfan sulfate was detected after 2 days, but the cell growth of KEF-1 reached a maximum on day 8, then slightly decreased (Fig. 3). The concentration then decreased to 2 $\mu\text{g/mL}$ from 4 $\mu\text{g/mL}$ by day 12 (Fig. 3). The analysis showed that endosulfan lactone and endosulfan alcohol were also one of metabolites, respectively. Their concentrations were 1.8 and 0.5 $\mu\text{g/mL}$ respectively, after 2 days (Fig. 3). These results suggested that KEF-1 has the capability of

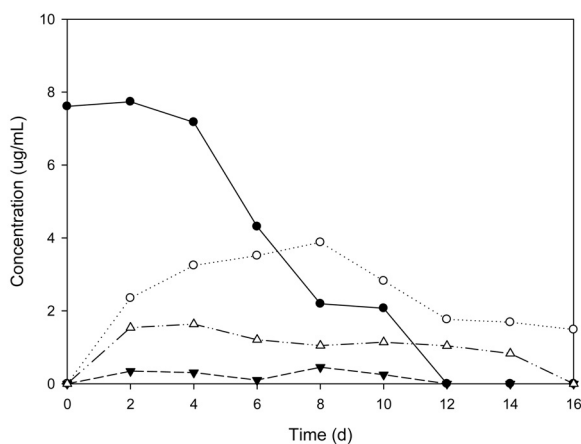


Fig. 3. Accumulation and biodegradation of endosulfan sulfate by *Eutypella* sp. KEF-1. Changes of endosulfan concentration (●), endosulfan sulfate (○), endosulfan alcohol (△) and endosulfan lactone (▼) over 16 days of cultivation in endosulfan-PDB.

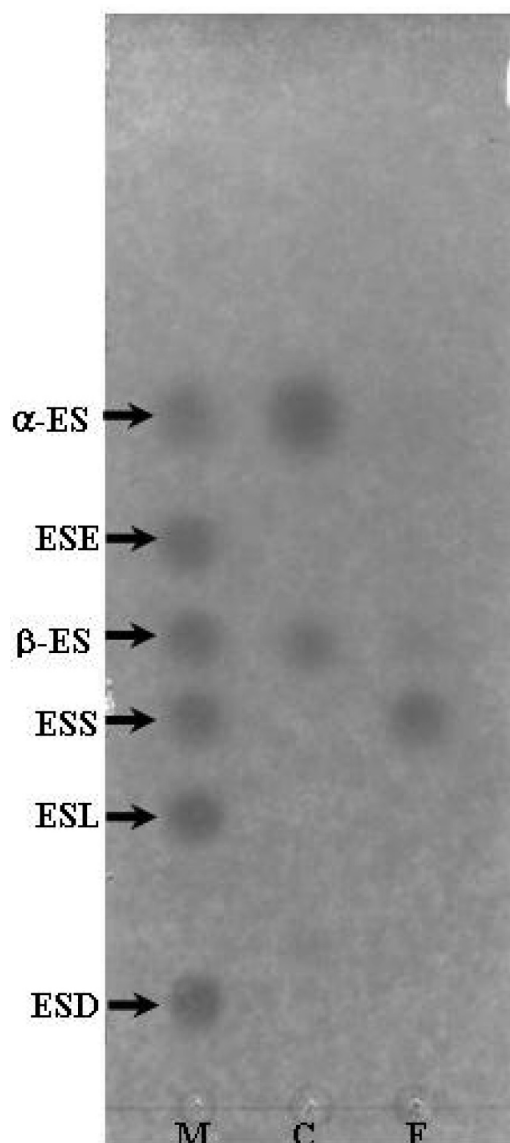


Fig. 4. Thin layer chromatogram showing metabolites formed during microbial metabolism of α - and β -endosulfan under uninoculated and inoculated conditions. (Standard loading: 4 μg) M: endosulfan and metabolites, C: endosulfan-control, F: fungi-12 day, ES: endosulfan, ESE: Endosulfan ether, ESS: Endosulfan sulfate, ESL: Endosulfan lactone, ESD: Endosulfan diol.

degrading endosulfan sulfate to yield endosulfan lactone and endosulfan alcohol. Other reported metabolites, such as endosulfan hydroxyether and/or endosulfan ether were not detected (Kullman and Matsumura 1996; Siddique *et al.*, 2003a; Kwon *et al.*, 2002; Lee *et al.*, 2003; Lee *et al.*, 2006). Our group previously reported that *K. oxytoca* KE-8 can degrade endosulfan to yield the metabolite endosulfan lactone (Kwon *et al.*, 2005).

The qualitative degradation of the endosulfan isomers by the KEF-1 was measured using TLC (Fig.

4). During the metabolism of α - and β -endosulfan, present either individually or together in PDB, endosulfan sulfate was accumulated and degraded (Fig. 3), while the amount of endosulfan decreased (Fig. 2C). Meanwhile, in the biodegradation assay with *Eutypella* sp. KEF-1, the concentrations of endosulfan and endosulfan sulfate in the culture decreased with an increase in the biomass, suggesting that *Eutypella* sp. KEF-1 exhibited a similar degradation activity toward the endosulfan isomers and endosulfan metabolites. Furthermore, *Eutypella* sp. KEF-1 is a useful gene source for developing other strains of mixed culture for bioremediation (Kwon *et al.*, 2002; Kwon *et al.*, 2005; Lee *et al.*, 2006; Kumar *et al.*, 2007). Thus, when examining the degradation activity of *Eutypella* sp. KEF-1 towards endosulfan and its metabolites, it was shown that strain KEF-1 was able to degrade both endosulfan isomers and its metabolites. Kullman and Matumura (1996) previously reported that most soil fungi produce endosulfan sulfate as the major metabolite of endosulfan through an oxidative pathway. The accumulated endosulfan sulfate, which is more toxic than endosulfan, was however degraded in this study. Therefore, the stain may prove to be useful in a mixed culture and as a gene source to develop other strains for bioremediation (Kwon *et al.*, 2002; Kwon *et al.*, 2005; Lee *et al.*, 2006).

According to an earlier report, endosulfan sulfate is more toxic and persistent than the parent compound. However, the formation of endosulfan diol and endosulfan hydroxy-ether constitutes detoxification. In the present study, *Eutypella* sp. KEF-1 was found to be efficient in transforming endosulfan in a PDB/A medium, resulting in the formation of non-toxic endosulfan diol as the metabolite, along with an insignificant amount of endosulfan sulfate that was degraded after 8 days.

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