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Biodegradation of the organochlorine insecticide, endosulfan, and the toxic metabolite, endosulfan sulfate, by *Klebsiella oxytoca* KE-8

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Abstract Biodegradation of endosulfan, a chlorinated cyclodiene insecticide, is generally accompanied by production of the more toxic and more persistent metabolite, endosulfan sulfate. Since our reported endosulfan degrader, *Klebsiella pneumoniae* KE-1, failed to degrade endosulfan sulfate, we tried to isolate an endosulfan sulfate degrader from endosulfan-polluted soils. Through repetitive enrichment and successive subculture using mineral salt medium containing endosulfan or endosulfan sulfate as the sole source of carbon and energy, we isolated a bacterium capable of degrading endosulfan sulfate as well as endosulfan. The bacterium KE-8 was identified as *Klebsiella oxytoca* from the results of 16S rDNA sequence analysis. In biodegradation assays with KE-8 using mineral salt medium containing endosulfan (150 mg l⁻¹) or endosulfan sulfate (173 mg l⁻¹), the biomass was rapidly increased to an optical density at 550 nm of 1.9 in 4 days and the deg-

radation constants for α - and β -endosulfan, and endosulfan sulfate were 0.3084, 0.2983 and 0.2465 day⁻¹, respectively. Analysis of the metabolites further suggested that *K. oxytoca* KE-8 has high potential as a biocatalyst for bioremediation of endosulfan and/or endosulfan sulfate.

Introduction

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9a-hexahydro-6,9-methano-2,3,4-benzodioxanthiepin-3-oxide) is a chlorinated cyclodiene insecticide currently used throughout the world for the control of numerous pests in a wide variety of food and non-food crops. This broad-spectrum insecticide comprises two parent stereoisomers— α -endosulfan and β -endosulfan; the α to β ratio is about 2.33:4 (Goebel et al. 1982; Sutherland et al. 2002c), and both isomers are extremely toxic to aquatic organisms. In comparison to the more recalcitrant chlorinated cyclodiene insecticides, endosulfan is relatively labile in the environment (Maier-Bode 1968); reported half-life values of α -endosulfan range from 43 days to several months (Stewart and Cairns 1974; Rao and Murty 1980; Sethunathan et al. 2004), and endosulfan is easily degraded at alkaline pH (Sutherland et al. 2000) and/or photo-oxidized by UV light in aqueous medium (Knoevenagel and Himmelreich 1976). However, natural biodegradation alone is still insufficient to remove endosulfan and endosulfan derivatives in on-farm wastewater or during soil bioremediation.

Several intensive studies on the biodegradation of endosulfan in soils or in water environments have been conducted using pure- or mixed-cultures of microorganisms (Awasthi et al. 2003, 1997; Siddique et al. 2003; Lee et al. 2003; Sethunathan et al. 2004; Sutherland et al. 2002b,c; Kullman and Matsumura 1996) (Table 1). Since the major biotransformation products of endosulfan include endosulfan sulfate and endosulfan diol, and endosulfan sulfate is more toxic and more persistent than the parent endosulfan (Kaur et al. 1998), recent research has focused on degradation of endosulfan sulfate (Sutherland et al. 2002c; Sethunathan et al. 2004).

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Table 1 Microbial degradation of endosulfan isomer and its toxic metabolite, endosulfan sulfate. α -ES α -Endosulfan, β -ES β -endosulfan, ESS endosulfan sulfate

Microorganism	Compound	Initial concentration (mg l ⁻¹)	Degradation rate (mg l ⁻¹ day ⁻¹)	Cell density	References
<i>Klebsiella pneumoniae</i> KE-1	α -ES	65	6.17	0.9 ^b	Kwon et al. 2002
	β -ES	28	2.55		
<i>Fusarium ventricosum</i>	α -ES	70	14.2	ND ^c	Siddique et al. 2003
	β -ES	30	6.6		
<i>Pandoraea</i> sp	α -ES	70	8.19	0.52 ^d	Siddique et al. 2003
	β -ES	30	3.18		
<i>Chlorococcum</i> sp	α -ES	4.24	0.13	ND ^c	Sethunathan et al. 2004
	ESS	8.44	0.12		
<i>Scenedesmus</i> sp	α -ES	4.24	0.14	ND ^c	Sethunathan et al. 2004
	ESS	8.44	0.05		
<i>Anabaena flosaquae</i>	α -ES	10	2.25 (4.0) ^a	50 ^e	Lee et al. 2003
<i>Anabaena</i> sp. PCC7120	α -ES	10	2.25	50 ^e	
<i>Bacillus</i> sp. co-culture	α -ES	388	29.7	2×10 ⁶ (cfu) ^f	Awasthi et al. 2003
	β -ES	386	27.7		
<i>Mycobacterium</i> sp. ESD Enriched culture	β -ES	125	<30	0.78 ^g	Sutherland et al. 2002b
	ESS	125	25	0.4 ^g	Sutherland et al. 2002c
	α -ES	125	17.8		
	β -ES	125	12.5		
<i>Phanerochaete chrysosporium</i>	α -ES	1	0.48	ND ^c	Kullman and Matsumura 1996

^apH controlled culture (pH 7.2)^bOD_{550 nm}^cNot determined^dOD_{600 nm}^eChlorophyll a concentration (mg l⁻¹)^fInitial cell concentration^gOD_{595 nm}

Recently, our group reported a potential endosulfan degrader, *Klebsiella pneumoniae* KE-1 (Kwon et al. 2002), and that the toxicity of endosulfan is closely associated with the oxidative stress via endosulfan-dependent reactive oxygen species generation (Sohn et al. 2004). Although *K. pneumoniae* KE-1 did not produce the toxic endosulfan sulfate during endosulfan degradation, this strain failed to degrade endosulfan sulfate. For practical applications, a bacterium capable of degrading endosulfan sulfate as well as endosulfan is necessary. In this study, we isolated an endosulfan sulfate degrader, *Klebsiella oxytoca* KE-8, from endosulfan-polluted soils through repetitive enrichment culture and successive subculture using mineral salt medium containing endosulfan or endosulfan sulfate as the sole source of carbon and energy. Biodegradation assays showed that *K. oxytoca* KE-8 has a high potential as a biocatalyst for bioremediation of endosulfan and/or endosulfan sulfate.

Materials and methods

Media and chemicals

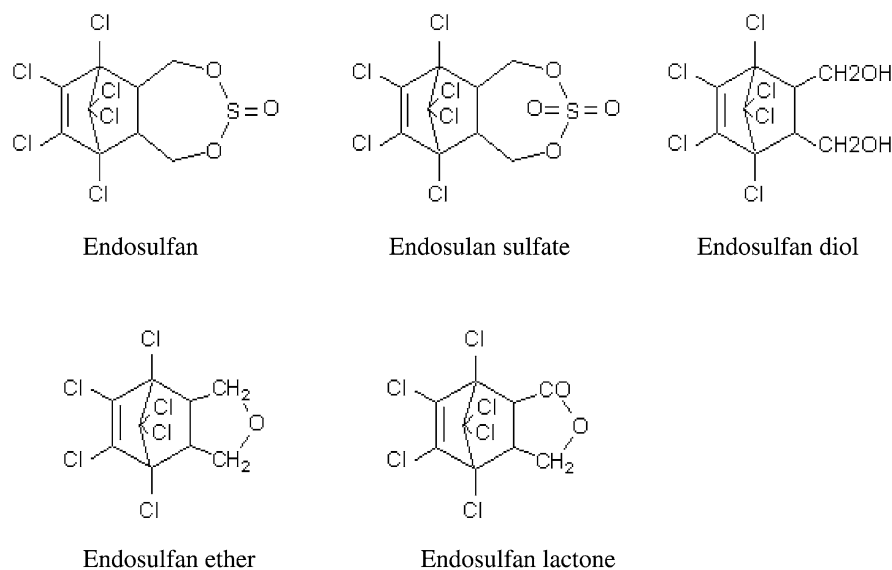
The organic pesticide endosulfan and its metabolites, such as endosulfan sulfate, endosulfan diol, endosulfan lactone

and endosulfan ether (Fig. 1) were purchased from Aldrich (Milwaukee, Wis.). Dichloromethane and acetone used in extraction and gas chromatography analysis were purchased from Junsei (Tokyo, Japan). The liquid mineral salt medium (MSM) used contained 1 g NH₄NO₃, 1 g K₂HPO₄, 0.5 g MgSO₄·7H₂O, 0.2 g CaCO₃ in 1 l distilled water as previously reported (Kwon et al. 2002). Bacto agar (Difco, Detroit, Mich.) was added to the above MSM at a concentration of 1.5% (w/v) to prepare solid medium. Endosulfan and endosulfan sulfate were dissolved in methanol at a concentration of 10 g/l respectively and added to MSM at appropriate concentrations after sterilization.

Isolation of an endosulfan-, and endosulfan sulfate-degrading microorganism

Soil samples were collected from ginseng and pepper fields near Andong, Kyungpook, Korea. The field had generally received several applications of endosulfan in the summer months for 5 years. Soil samples (2 g) were suspended in 50 ml liquid MSM containing 150 mg l⁻¹ endosulfan (endosulfan-MSM) in a 250 ml Erlenmeyer flask, and incubated at 30°C with shaking (70 rpm). After 7 days, 5 ml of each culture was re-inoculated into new endosulfan-MSM medium and further incubated at 30°C for 7 days.

Fig. 1 Structures of endosulfan, endosulfan sulfate and metabolites produced in microbial culture



This subculture was repeated under the same culture conditions, and then an aliquot (0.2 ml) from each culture was applied to solid endosulfan-MSM for isolation of single colonies. After 4–5 days, a single colony in endosulfan-MSM was transferred to liquid endosulfan-MSM or methanol-MSM, respectively, to exclude methanol-dependent strains. To isolate an endosulfan sulfate degrader, two newly selected strains, together with the previously isolated nine bacterial strains capable of degrading endosulfan (Kwon et al. 2002), were cultured in liquid MSM containing 173 mg l⁻¹ endosulfan sulfate. The strain named KE-8, which grows on endosulfan sulfate-MSM as well as endosulfan-MSM, has been deposited in the Korean Type Culture Collection under accession No. KCTC 10551BP.

Identification of the endosulfan degrader

To identify the KE-8 bacterium, a Vitek Gram-negative identification (+) kit (Biomérieux, Marcy L'Etoile, France) and a 16S rDNA sequence analysis were used. For sequence analysis, the same instrumentation and analytical techniques as previously reported (Kwon et al. 2002) were used. A 508 base segment of the 16S rDNA region was amplified with a forward primer, 9F [5'-GAGTTTGATCCTGGCT CAG; positions 9–27 (*Escherichia coli* 16S rDNA numbering)] and a reverse primer, 1542R (5'-AGAAAGGAGG TGATCCAGCC; positions 1542–1525) using an MJ Research (Waltham, Mass.) thermal cycler (Yoon et al. 1997). PCR was run for 35 cycles with the following thermal profile: denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s and extension at 72°C for 45 s. The final cycle included extension for 10 min at 72°C. The PCR product was purified using a QIAquick PCR product purification kit (Qiagen, Hilden, Germany) and the purified 16S rDNA was sequenced using a cycle sequencing ready reaction kit, ABI Prism BigDye Terminator (Applied Biosystems, Foster City, Calif.) and an Applied Biosystems model 310 sequencer (Perkin Elmer, Boston, Mass.). The primer for the

sequencing reaction was 536R (5'-GWATTACCGCGGCK GCTG-3'; positions: 536–519). The 16S partial sequence of isolated strain KE-8 was deposited in the GenBank database under accession no. AF440521.

Biodegradation of endosulfan and endosulfan sulfate

Pure culture experiments were performed in 250 ml flasks containing 50 ml MSM, supplemented with 150 mg l⁻¹ endosulfan. After 5 days, the cells were collected by centrifugation at 8,000 g for 15 min, washed twice with 0.015 M phosphate buffer (pH 7.0), and suspended in MSM. The washed cells were inoculated into MSM containing endosulfan (154 mg l⁻¹) or endosulfan sulfate (173 mg l⁻¹) and cultured at 30°C in a rotary shaker (130 rpm) for 6 days. The initial pH was set to 7.2 to prevent chemical hydrolysis of endosulfan at alkaline pH; alkaline hydrolysis of endosulfan formed endosulfan diol, with approximately 10-fold increases in hydrolysis occurring with each increase in pH unit (Sutherland et al. 2000). Endosulfan and its metabolites, culture pH and biomass were determined at 1-day intervals. Endosulfan-free- or endosulfan sulfate-free MSM served as negative controls, and non-inoculated flasks were also run as a control. All data are represented as the mean value of triplicate experiments. To investigate the effect of pH on endosulfan degradation and cell growth, the initial pH of the MSM was adjusted with 1 N NaOH or 1 N HCl. The degradation constant (k_1) was calculated using the following equation based on the first order kinetics derived from data from the exponential phase of degradation (Strotmann 2000):

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

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

Endosulfan and its metabolites were analyzed using a gas chromatography-ECD system as previously reported (Kwon et al. 2002).

Results

Isolation of endosulfan and endosulfan sulfate-degrading bacteria

From 30 soil samples, 16 different bacteria were isolated through repetitive enrichment culture and successive transfer using endosulfan-MSM. Among the different 16 strains, 14 showed more active growth in methanol-MSM medium compared to growth in endosulfan-MSM (results not shown). However, two strains, YS3-3 and KE-8, grew well in endosulfan-MSM, yielding an optical density at 550 nm of 0.6 in 7 days (Table 2), whereas these two strains had very poor growth in methanol-MSM. Strain KE-2 showed strong endosulfan degradation activity, and the average degradation rates for α -endosulfan and β -endosulfan were 13 and 2.4 mg l⁻¹ day⁻¹, respectively.

The two isolated bacterial strains, together with nine strains previously collected based on their ability to grow in endosulfan-MSM (Kwon et al. 2002), were transferred to solid endosulfan sulfate-MSM, and cultured at 30°C for 7 days. Among the 11 bacterial strains tested, only 1 bacterium, named KE-8, was able to grow on endosulfan sulfate-MSM. After several subcultures, KE-8 was finally selected as an endosulfan sulfate degrader. The previously selected endosulfan degrader, *Klebsiella pneumoniae* KE-1 (Kwon et al. 2002) failed to grow on endosulfan sulfate-MSM.

Identification of the endosulfan degrader

A partial 16S rDNA sequence was used to establish the identity of the isolated bacterial strain KE-8, the sequence being most similar to that of *Klebsiella oxytoca* type strain (ATCC 13182^T; sequence similarity 98%). Thus, strain KE-8 was identified as *Klebsiella oxytoca*.

Table 2 Degradation of endosulfan by two isolated strains grown in rotary shaking culture for 7 days using minimal salt medium (MSM) containing 150 mg l⁻¹ endosulfan

Strain	Remaining ES (mg l ⁻¹)			Ratio of isomers	Cell density (OD _{550 nm})	Final pH
	Total ES	α -ES	β -ES			
Control ^a	150	121	29	81 : 19	–	7.2
YS3-3	118	92	26	78 : 22	0.61	6.8
KE-8	60	43	17	72 : 28	0.86	6.2

^aWithout inoculation

K. oxytoca KE-8 was cultivated in endosulfan-MSM or endosulfan sulfate-MSM for 6 days. When endosulfan or endosulfan sulfate was omitted from the medium, no growth was observed and the pH remained at 7.2. In endosulfan-MSM, the cell density of KE-8 reached a maximum on day 4, and slightly decreased thereafter (Fig. 2a). The culture pH decreased to 5.6 from 7.2 on day 4 concomitant with cell density, and increased to 6.2 at the end of stationary phase. Endosulfan was degraded from 154 to 26 mg l⁻¹ in 6 days (a decrease of 83%), and degradation of α -endosulfan was more evident than that of β -endosulfan; the degradation constants for α - and β -endosulfan were 0.3084 day⁻¹ ($R^2=0.99$) and 0.2983 day⁻¹ ($R^2=0.962$), re-

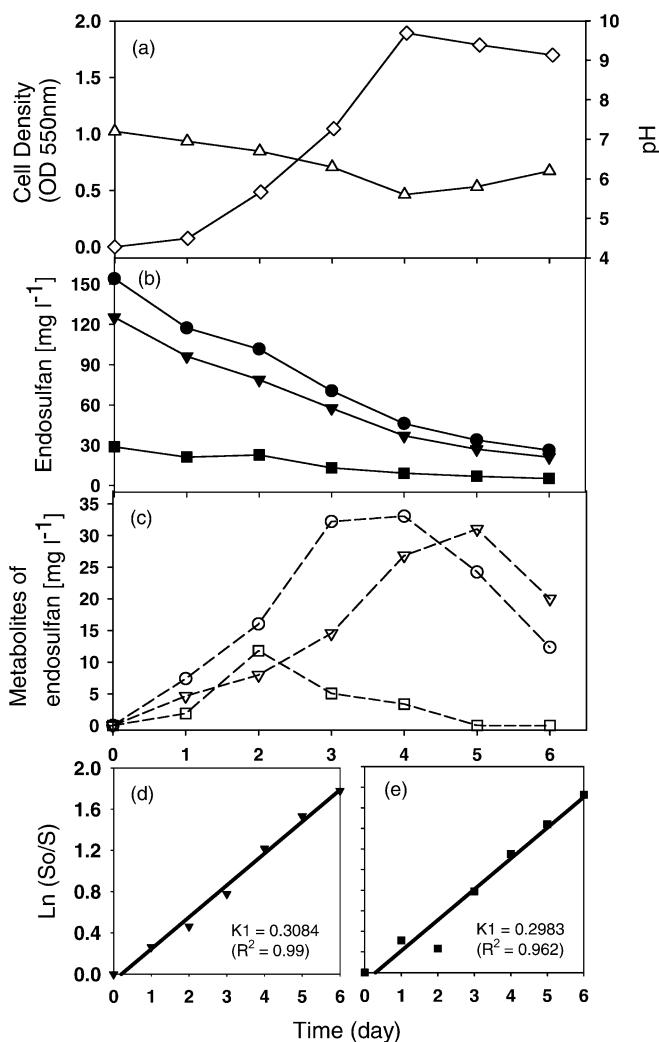
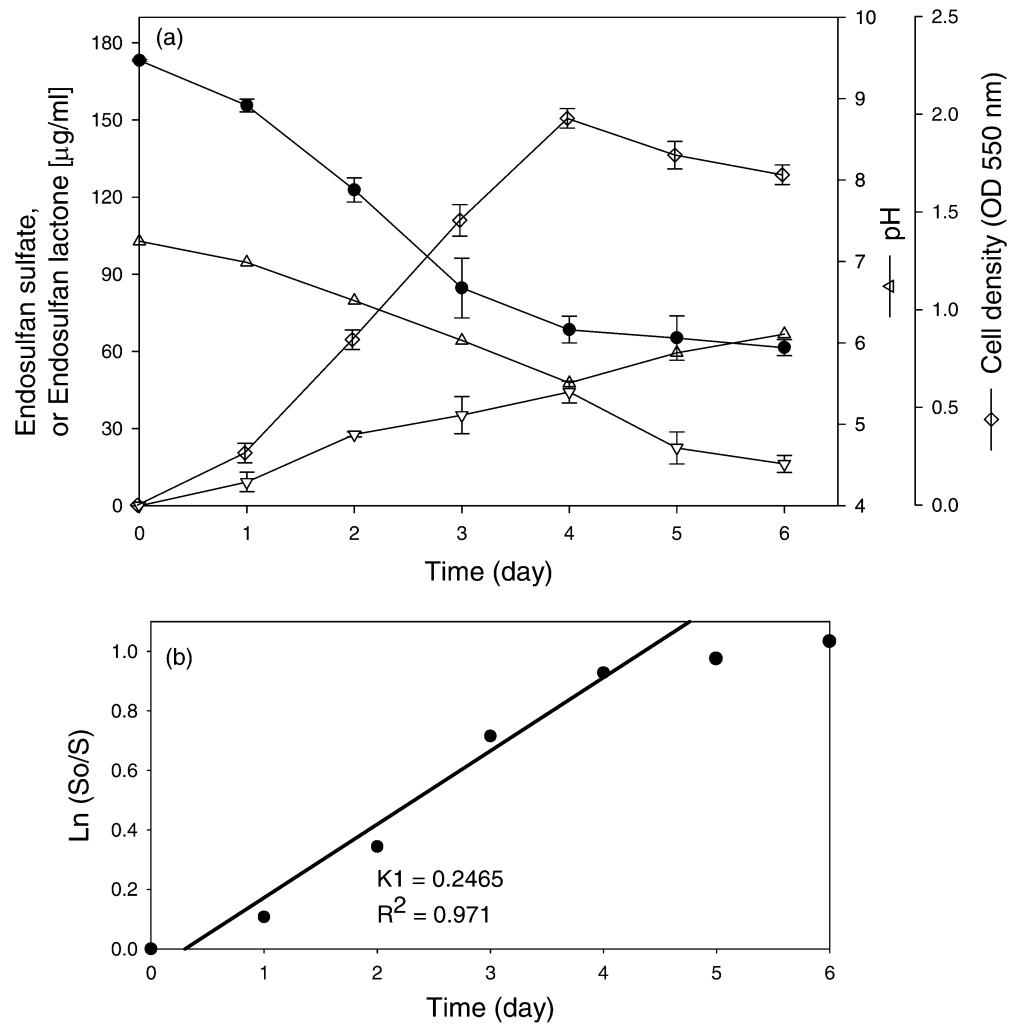


Fig. 2a-e Biodegradation of endosulfan by *Klebsiella oxytoca* KE-8. **a** Cell density (\diamond) of *K. oxytoca* KE-8 and culture pH (\triangle) in endosulfan-MSM over 6 days. **b** Degradation of endosulfan isomers [α -endosulfan (\blacktriangledown), β -endosulfan (\blacksquare), sum of α -endosulfan and β -endosulfan (\bullet)] by *K. oxytoca* KE-8 in endosulfan-MSM. **c** Concentration changes of endosulfan diol (\circ), endosulfan lactone (∇) and endosulfan sulfate (\square) in culture during endosulfan degradation. **d**, **e** Degradation constants for α -endosulfan (**d**) and β -endosulfan (**e**) based on first order kinetics from the data of the exponential phase of degradation

Fig. 3 Biodegradation of endosulfan sulfate by *K. oxytoca* KE-8. **a** Changes of endosulfan sulfate concentration (●), culture pH (△), cell density (◇) and endosulfan lactone (▽) over 6 days cultivation in endosulfan sulfate-MSM. **b** The degradation constant for endosulfan sulfate based on the first order kinetics of data from the exponential phase of degradation



spectively (Fig. 2b, d, e). A time-course of the metabolites showed that endosulfan diol and endosulfan lactone were major metabolites, their concentration increasing to 33 and 31 mg l^{-1} on days 4 and 5, respectively (Fig. 2c). At stationary phase, these concentrations were considerably decreased. Endosulfan sulfate was detected in the culture; the highest concentration was 11.8 mg l^{-1} on day 2, with the concentration slowly decreasing during the exponential and stationary phases. After 5 days, endosulfan sulfate was not detected. Other reported metabolites, such as endosulfan ether or endosulfan hydroxyether (Kwon et al. 2002; Siddique et al. 2003; Lee et al. 2003; Kullman and Matsumura 1996) were not detected. These results suggest that endosulfan sulfate is not the final metabolite in this culture, and that *K. oxytoca* KE-8 can degrade endosulfan sulfate as well as endosulfan.

To investigate the degradation of endosulfan sulfate, *K. oxytoca* KE-8 was cultivated in MSM supplemented with endosulfan sulfate (173 mg l^{-1}) as the sole source of carbon and energy. The cell density of KE-8 again reached a maximum on day 4 and afterwards slightly decreased (Fig. 3a). The culture pH decreased to 5.5 from 7.2 by day 4, concomitant with cell density, and increased to 6.1 at stationary

phase. The increases in culture pH during stationary phase are a prominent characteristic of KE-8. Endosulfan sulfate was degraded from 173 to 61 mg l^{-1} over 6 days (a decrease of 64%). In a prolonged culture over 12 days, only $58.2 \pm 2.53 \text{ mg l}^{-1}$ endosulfan sulfate remained (results not shown). About 94% of endosulfan sulfate metabolized was degraded in the first 4 days (degradation constant = 0.2465 day^{-1} , $R^2 = 0.971$) (Fig. 3b). Analysis of metabolites showed that endosulfan lactone is a major metabolite of endosulfan sulfate, and that its concentration slowly increased, reached a maximum (44 mg l^{-1}) on day 4, and then decreased rapidly. Only traces ($<1 \text{ mg l}^{-1}$) of endosulfan diol were detected, without concentration changes over 6 days, and endosulfan ether and endosulfan hydroxyether were not detected.

Discussion

Klebsiella oxytoca KE-8, a bacterium capable of degrading endosulfan sulfate as well as endosulfan, was isolated from the soil of a pepper field, Andong, Korea. The previously reported nine endosulfan degraders and a newly isolated

endosulfan degrader YS3-3 did not grow on endosulfan sulfate-MSM. These results suggest that endosulfan sulfate is the most persistent metabolite of endosulfan for these strains. However, our results do not represent the degradation efficiency of endosulfan sulfate in the environment, because endosulfan sulfate was used as sole carbon and energy source in this study and our culture medium includes MgSO_4 (0.05%). Supplementation of endosulfan sulfate as the sole sulfur source may be more effective in isolating endosulfan sulfate degraders, since it has a relatively reactive sulfite moiety (Sutherland et al. 2002c). In fact, an endosulfan-degrading *Mycobacterium* strain failed to metabolize endosulfan in sulfur-free medium containing 50 μM endosulfan upon supplementation of 200 μM MgSO_4 (Sutherland et al. 2002b). Although degradation of endosulfan sulfate by this strain was not reported, the presence of other carbon sources or inorganic sulfate in the environment may affect the biodegradation activity or degradation efficiency.

In the biodegradation assay with *K. oxytoca* KE-8, the concentrations of endosulfan and endosulfan sulfate in the culture decreased rapidly with concomitant increases in biomass. During the exponential degradation phase, the specific degradation constants (and half-life $t_{1/2}$) for α - and β -endosulfan, and endosulfan sulfate were 0.3084 day^{-1} (2.28 days), 0.2983 day^{-1} (2.41 days), 0.2465 day^{-1} (2.97 days), respectively (Figs. 2, 3). These results suggest that *K. oxytoca* KE-8 has similar degradation activity toward endosulfan isomers and endosulfan sulfate, and that *K. oxytoca* KE-8 could be successfully employed as a biocatalyst for bioremediation of endosulfan and endosulfan sulfate. This strain may provide a useful gene source to develop other strains for bioremediation (Sutherland et al. 2002a). The construction of a cosmid library from the KE-8 genome is in progress in our laboratory.

It is uncertain that endosulfan sulfate is converted to endosulfan diol as previously proposed (Verschuere 1999). In endosulfan sulfate-MSM culture, the concentration of endosulfan diol was maintained a very low level ($<1 \text{ mg l}^{-1}$), and endosulfan ether and endosulfan hydroxyether were not detected, whereas unidentified metabolites were detected by gas chromatography and HPLC. Therefore, we cannot exclude the possibility of direct desulfurization of endosulfan sulfate, or a novel degradation pathway in KE-8 as reported in enrichment culture of soil bacteria (Sutherland et al. 2002c). Further research on the metabolites of endosulfan sulfate, the degradation pathway of endosulfan sulfate, simultaneous degradation of endosulfan/endosulfan sulfate, and the effect of available sulfur compounds on degradation efficiency of endosulfan sulfate are necessary.

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