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Isolation and Characterization of an Endosulfan-Degrading Strain, *Stenotrophomonas* sp. LD-6, and its Potential in Soil Bioremediation

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Abstract

Aerobic bacteria degrading endosulfan were isolated from contaminated sludge. One of the isolates, LD-6, was identified as *Stenotrophomonas* sp. The bacterium could utilize endosulfan as the sole source of carbon and sulfur. 100 mg/l endosulfan was completely degraded within 10 days, and endosulfan diol and endosulfan ether were detected as major metabolites with a slight decrease in culture pH. The results indicated that *Stenotrophomonas.* sp. LD-6 might degrade endosulfan by a non-oxidative pathway. Biodegradation of both isomers was relatively better at a temperature range of 25–35°C, with a maximum at 30°C. In addition, cell crude extract of strain LD-6 could metabolize endosulfan rapidly, and degradative enzymes were intracellular distributed and constitutively expressed. Besides, application of the strain was found to promote the removal of endosulfan in soil. This study might help with the future research in better understanding of the biodegradation.

Key words: *Stenotrophomonas* sp., biodegradation, endosulfan

Introduction

Organochlorine pesticides are one of the major groups of chemicals being extensively used in agriculture and responsible for environmental contamination and ecological imbalance (Tiemann, 2008). Endosulfan is a mixture of two stereoisomers (α- and β-endosulfan) which differ dramatically in their physicochemical and environmental properties, in a ratio of 7:3 and registered with several trademarks, such as Thimol, Cyclodan, Thiodan, Malix, and *etc*. It is extensively used throughout the world to control insect pests and mites of different crops, such as cereals, cotton, tea, fruits and vegetables (Weber *et al*., 2009). Due to its hydrophobic nature, abundant application and environmental transportation, endosulfan contamination has been frequently detected in soils, sediments, waters, air, food products and even the environment at considerable distance from the application point such as the Arctic (Kaushik *et al*., 2010). Furthermore, it is extremely toxic to aquatic fauna, and its acute and chronic toxicity is well known in a variety of mammals including human beings (Weber *et al*., 2009). These health and environment concerns have led to an interest in degradation and detoxification of endosulfan.

Natural degradation of endosulfan under alkaline conditions or photooxidation by UV light is not sufficient for removal of endosulfan and its derivatives from the environment (Kwon *et al*., 2005). The environmental degrading pathways include hydrolysis of the sulfur moiety to nontoxic endosulfan diol and oxidation to endosulfan sulfate. Endosulfan diol can be further transformed to less or non-toxic metabolites, such as endosulfan ether, endosulfan hydroxyether and endosulfan lactone. Theoretically, formation of endosulfan diol *via* hydrolysis might be an important detoxification way (Shivaramaiah *et al*., 2005). In contrast, endosulfan sulfate is produced only through biological transformation, and it is more toxic and persists longer than the parent isomers. Consequently, production of endosulfan sulfate becomes the major concern of endosulfan degradation.

Biodegradation is recognized as a promising and attractive operational means to strengthen remediation performance and has been demonstrated to successfully enhance the degradation and removal of endosulfan

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(Kwon *et al*., 2005; Kumar and Philip, 2006; Li *et al*., 2009; Goswami *et al*., 2009). However, it does not always work, and application effects are less predictable. To achieve successful bioaugmentation, it is still necessary to select suitable microorganisms. In this study, an endosulfan degrader, *Stenotrophomonas* sp. LD-6, was isolated through repetitive enrichment culture and successive screening. The newly selected bacterium could completely degrade test endosulfan within 10 days through a non-oxidative pathway. Moreover, degrading characteristics, distribution and expression of degradative enzymes and biodegradation of endosulfan by the strain in soil were systematically investigated.

Experimental

Materials and Methods

Chemicals and medium. The organic pesticide endosulfan (99.5%) and its metabolites were purchased from Sigma Aldrich (Shanghai, China). Dichloromethane and acetone used in extraction and gas chromatography analysis were purchased from Zhuoyue Chemical Co. (Jiangsu, China). The compositions of the medium used were as follows – minimal salts medium (MSM, g/l): $MgCl_2$ 0.2, NH_4NO_3 1, KH_2PO_4 2, K_2HPO_4 7.5, NaCl 1, pH 6.8. For solid medium, 1.5% (w/v) agar was added to the liquid MSM. Endosulfan was dissolved in acetone at a concentration of 10^4 mg/l and added to the medium at appropriate concentration after sterilization.

Enrichment and isolation of endosulfan-degrading bacteria. Sludge sample was collected from a sewage outfall in Tonglu Pesticides Company, Zhejiang Province. 5 g sample was taken in a 250 ml Erlenmeyer flask containing 50 ml of the MSM and 50 mg/l of endosulfan and was incubated at 28°C with continuous shaking (160 rpm). After 7 d, 5 ml of culture was re-inoculated into 50 ml of fresh medium with 100 mg/l endosulfan and was cultured under same conditions. This process was repeated three more times. Four weeks later, dilution of the consortia was spread onto the selective MSM agar plates containing 100 mg/l of endosulfan and incubated at 28°C for 72 h. Well separated colonies were picked and repeatedly transferred onto the same solid media until obtaining pure culture.

Characterization of isolates. Phenotypic and biochemical characterization was performed according to *Bergey's Manual of Determinative Bacteriology* (Holt *et al*., 1994). The 16S rRNA genes of bacteria were amplified and analyzed as described by Yu *et al.* (2008). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0 (Tamura *et al*., 2007), and the dataset was bootstrapped 1000 times.

Degradation of endosulfan by *S.* sp. LD-6. Cells were grown in 50 ml of Luria-Bertani (LB) broth at 30°C, harvested by centrifugation at 10,000 g for 8 min, and washed twice with 10 mM phosphate buffer (pH 6.8). The washed cells were inoculated into the MSM containing 100 mg/l of endosulfan, which resulted in an optical density of 0.3–0.5 at 600 nm. Cultivation was conducted at 30°C in a rotary shaker (160 rpm) for 10 days, and endosulfan and its metabolites were determined at 2-day intervals. pH was measured according to the standard method (APHA, 2005). To evaluate the effect of adsorption, the experiments were repeated with heat-killed cells as described by Kwon *et al.* (2002). Effects of incubation conditions (shaking versus static) and temperature on biodegradation were examined according to the methods described by Hussain *et al.* (2007). Above experiments were carried out in triplicate.

Gas chromatographic (GC) and GC-MS analysis. Endosulfan and metabolites were analyzed by gas chromatography (GC-14B, Shimadzu) equipped with electron capture detector. The supernatant was saturated with NaCl and extracted three times with an equal volume of dichloromethane. The organic phases were combined and dehydrated by addition of anhydrous Na_2SO_4. After drying under a stream of N_2, the sample was redissolved in acetone and stored at 4°C before analysis. The experiment was carried out in triplicate. Samples (1 µl) were injected in splitless mode into a capillary column (DB-5, length 30 m, ID 0.2 mm, film thickness 0.25 µm). The carrier gas was N_2. Injector and detector temperatures were 300°C and 320°C, respectively. The temperature program used was 150°C for 1 min, then 300°C at 10°C min^{-1} and a 1-min hold at 300°C. All GC-MS analyses were conducted with a gas chromatograph (Shimadzu GC-2010) equipped with mass spectrometer (GCMSQP2010), auto injector (AOC-20i) and a DB-5MS capillary column (ID 0.25 mm, film thickness 0.25 µm), coupled to MS *via* direct interface. Helium was used as carrier gas with a flow rate of 1.0 ml/min. GC injector temperature was held at 300°C, and the column temperature was programmed as of GC. The electron impact mass spectra were obtained at 70 eV and monitored in the range of 50 to 400 *m*/*z*.

Enzymatic degradation. Bacterial cells grew in LB media were harvested at log phase by centrifugation at 12,000 g for 15 min at 4°C. The bacterial pellets were washed twice with distilled water and then suspended in 3 ml of phosphate buffer (0.05 M, pH 6.8). Localization of degrading enzymes was conducted by the method of osmotic shock (Huang *et al*., 2007). To determine whether enzymes responsible for endosulfan biodegradation were inducible or constitutive, the method described by Yu *et al.* (2008) was used. The reaction mixture (1.0 ml) contained phosphate buffer (0.05 M, pH 6.8), endosulfan (50 mg/l) and cell crude extract. Reactions were performed at 30°C for 45 min

without shaking, and the residual endosulfan was quantified by the GC method. All experiments were performed in triplicate.

Biodegradation of endosulfan in soil. Biodegradation of endosulfan in soil by *S.* sp. LD-6 was examined as described by Huang *et al.* (2007). Soil sample (5–15 cm soil layer) was obtained from a local farm, Linan. The soil was a sandy loam (sand 47%, silt 35%, clay 18%) with organic matter 4.74%, and a pH of 5.4. The soil has never been treated with endosulfan. The solution of endosulfan was added into the samples to give a concentration of 50 mg/kg. One set of fresh soil and sterile soil were inoculated with the strain (1×10^8 cells/g). Another uninoculated set was kept as a control. The inocula were thoroughly mixed with the soils under sterile conditions, and soil moisture was adjusted to 60% (w/w). Each sample was incubated under aerobic conditions at 25°C in the dark, and 5 g soil was collected at 7-day intervals and further analyzed as reported by Li *et al.* (2009). The experiment was carried out in triplicate.

Nucleotide sequence accession number. The 16S partial sequence of the isolated strain LD-6 was deposited in the GenBank database under accession number JQ670922.

Results and Discussion

Identification of endosulfan-degrading isolate. After selective enrichment, a total of 17 isolates were selected. They all exhibited similar characteristics: smooth and yellowish color on nutrient agar; short rod-shaped morphology; negative for Gram stain, gelatin hydrolysis, starch hydrolysis, H_2S, oxidase and decarboxylases; while positive for catalase. One representative strain, designated as LD-6, from among those with the best endosulfan metabolism (data not shown) was chosen for further characterization as follows: growth on glucose without acidification, using xylose as a carbon source, reduction of nitrate to nitrite, positive for growth at 4°C, methyl red and citrate but negative for growth at 40°C, indole, and the Voges-Proskauer test. The 16S rDNA sequence alignment and phylogenetic analysis (Fig. 1) revealed that strain LD-6 was homologous with *Stenotrophomonas* spp. The organism that exhibited the highest level of homology (99%) was *S. rhizophila*. From these results, LD-6 was identified as *Stenotrophomonas* sp.

Degradation of endosulfan by *S.* sp. LD-6. The degradation assay revealed the bacterium could utilize endosulfan as the sole source of carbon and sulfur. Endosulfan was rapidly degraded from 100 to 28.2 mg/l (decrease of 71.8%) during the initial 4 days with increase of biomass (data not shown). By day 10, the substrate was completely degraded with an overall degradation rate of 10.0 mg/(l · day) (Fig. 2). Furthermore, degradation rates for α- and β-endosulfan were 6.8 and 3.2 mg/(l · day), respectively. This is in accordance with previous studies which revealed the degradation of α-endosulfan is faster than that of β-endosulfan (Kwon *et al.*, 2002). The reason for above observations is still not well understood. Our finding that strain LD-6

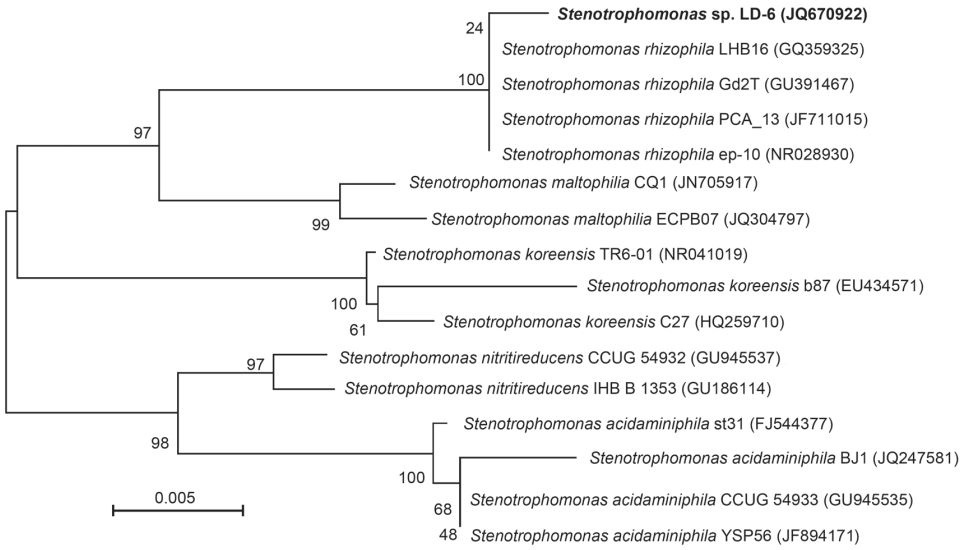

Fig. 1. Evolutionary distance tree based on the 16S rDNA sequence of *S.* sp. LD-6 with references.
The numbers at the forks indicate the bootstrap values in percentage. Bar, nucleotide difference per sequence position.
The accession numbers of the sequences are given in parentheses.

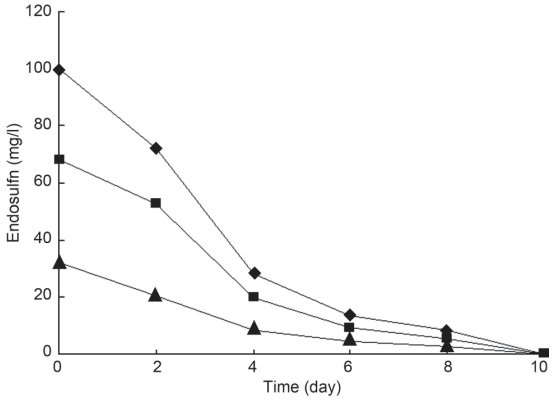

Fig. 2. Biodegradation of endosulfan by *S.* sp. LD-6 (◆: sum of *α*- and β-endosulfan; ■: *α*-endosulfan; ▲: β-endosulfan). The data were reported as means, and the error bars show standard errors (the same below).

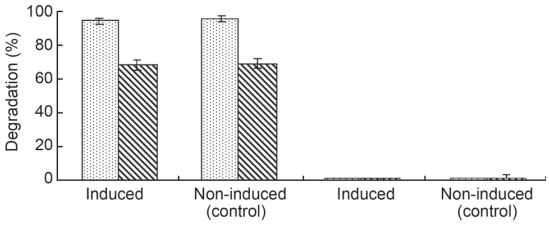

Fig. 3. Enzymatic degradation of endosulfan by cell crude extract of *S.* sp. LD-6 (▨: α-endosulfan; ▨: *β*-endosulfan)

is capable of degrading endosulfan is another instance of the genus *Stenotrophomonas* with regard to endosulfan biodegradation. Moreover, heat killed LD-6 did not have any effect on the degradation of endosulfan (data not shown), suggesting that the decrease was not due to adsorption.

Accumulation of endosulfan sulfate was not detected in this work, but endosulfan diol and endosulfan ether appeared as detected by the GC analysis using authentic standards and matching the retention times. During the degradation, the culture pH decreased to 6.5. These results are in accordance with previous studies which revealed that some bacteria, such as *K. pneumoniae*, *Pseudomonas fluorescens*, *Arthrobacter* sp., and other Gram negative rods, form endosulfan diol, not endosulfan sulfate, while the pH value decreased (Kwon *et al.*, 2002). Furthermore, these results suggested that the metabolites of endosulfan and the degrading mechanism might be independent of culture pH.

A significant difference between biodegradation of both α- and *β*-endosulfan by the strain was observed under static versus shaking incubation (Table I). Maximum biodegradation of α- and *β*-endosulfan (up to 98.7%) was recorded under shaking conditions, however, the counterpart was only 66.9% as found under static conditions. Better bioavailability of endosulfan to the microbes coupled with physiochemical degradation might explain the phenomenon to some extent. Furthermore, non-biological degradation could not be neglected and was more obvious under shaking conditions, which implied that aerobic conditions are relatively more conducive for abiotic degradation.

Biodegradation of α- and *β*-endosulfan by the bacterium was also investigated at different incubation temperatures. Biodegradation of both isomers of endosulfan was relatively greater at an incubation temperature range of 25–35°C, with a maximum at 30°C (data not shown). This was logical, because the optimum growth temperature of the strain is around 30°C. The minimum degradation of spiked endosulfan was recorded at 40°C. Besides, abiotic degradation was more pronounced at higher incubation temperatures.

Enzymatic degradation. Results of GC analysis indicated that no objective substance could be detected in the reaction mixture containing the intracellular fraction solution taken at 10 h. Furthermore, there was no downtrend of endosulfan content in the mixture containing the extracellular and membrane fraction solutions. These results suggested that the enzymes involved in the initial degradation of endosulfan in LD-6 were intracellular. As shown in Fig. 3, 94.1% of the α-endosulfan and 68.1% *β*-endosulfan were degraded by cell crude extract with induction, respectively. Similarly, 95.4% of the α-endosulfan and 69.0% *β*-endosulfan were degraded without induction. In controls, biodegradation was negligible. These results showed that there was no significant discrepancy in endosulfan degradation between induced and

Table I
Effect of incubation conditions on biodegradation of α- and *β*-endosulfan by strain LD-6 (*T* = 30°C, 10 days)

Treatment	α-endosulfan (%)		*β*-endosulfan (%)	
	Shaking[a]	Static	Shaking	Static
LD-6	98.7 (1.4)	66.9 (2.6)	52.8 (3.8)	34.4 (2.6)
Uninoculated control	9.8 (2.7)	6.3 (1.1)	8.5 (0.6)	5.3 (0.3)

The values are the average of three independent experiments, and the numbers in parentheses indicate the standard deviation (the same below).
[a] Shaking at 160 rpm.

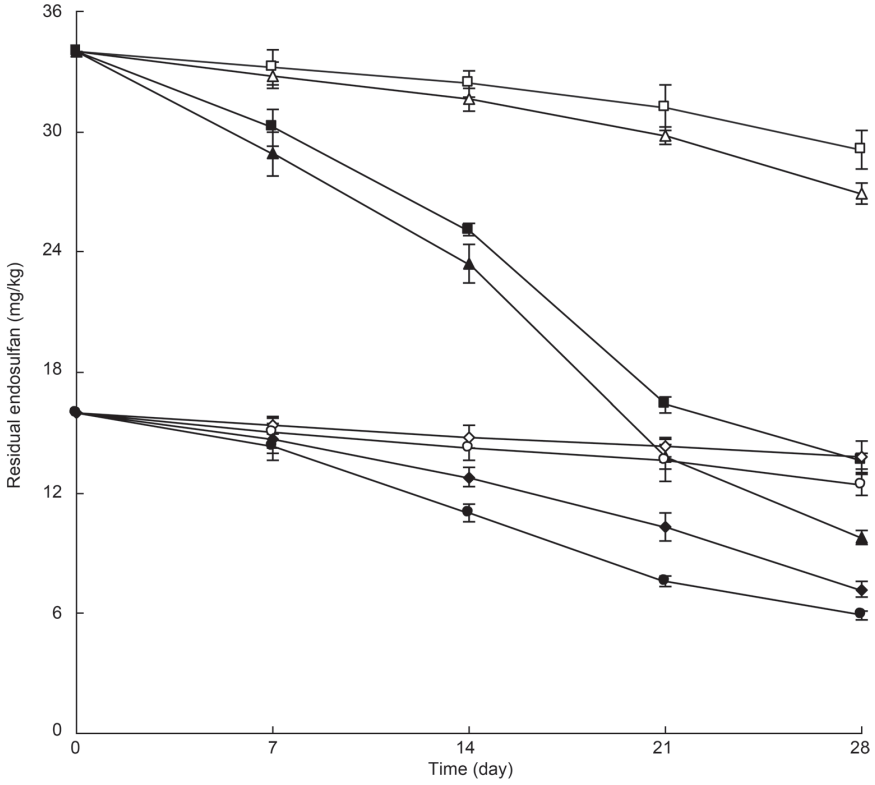

Fig. 4. Biodegradation of endosulfan by *S.* sp. LD-6 in soil.
□: alpha in sterilized soil (control); ■: alpha in sterilized soil + LD-6; Δ: alpha in fresh soil (control); ▲: alpha in fresh soil + LD-6; ◇: Beta in sterilized soil (control); ◆: Beta in sterilized soil + LD-6; ○: Beta in fresh soil (control); ●: Beta in fresh soil + LD-6.

non-induced cells, indicating that enzymes responsible for endosulfan biodegradation might be constitutively expressed. Moreover, the optimum degrading temperature and pH value were 30°C and 6.6, respectively (data not shown).

Biodegradation of endosulfan in soil. The *in situ* endosulfan-degrading ability of LD-6 was tested, and degradation patterns of α- and *β*-endosulfan in soil are presented in Fig. 4. Only 7.1 mg/kg α-endosulfan and 3.6 mg/kg *β*-endosulfan were removed in uninoculated fresh soil after 28 days of incubation. In contrast, 24.2 mg/kg α-endosulfan and 10.1 mg/kg *β*-endosulfan were removed after 28 days for fresh soil inoculated with strain LD-6 (Fig. 4). Obviously, endosulfan removal was enhanced while the bacterium was inoculated. Moreover, 20.4 mg/kg α- endosulfan and 8.8 mg/kg *β*-endosulfan were removed from sterilized soils. Similarly, the removal in fresh uninoculated soil was superior to that in the uninoculated sterile soil. Successful biodegradation of endosulfan from soil by isolated bacteria has been reported previously. For example, a strain, *Achromobacter xylosoxidans* CS5, originally isolated from activated sludge was examined for biodegradation. Inoculation of the strain was found to promote the removal of endosulfan in soil as described by Li *et al.* (2009). In the case of the experiment done by Arshad *et al.* (2008), *P. aeruginosa* degraded more than 85% of spiked α-endosulfan and *β*-endosulfan (100 mg/l) after 16 days in loam soil. Kumar and Philip reported that endosulfan was effectively degraded both in miniature and bench scale soil reactors (Kumar and Philip, 2006). In this study, addition of strain LD-6 to soil supplemented with endosulfan resulted in a higher removal rate than that observed in uninoculated soils. Meanwhile, endosulfan removal was slightly better in fresh soil inoculated with the bacteria than in inoculated sterilized soil suggesting a contribution of the indigenous flora to endosulfan removal. The data generated from this study could improve current understanding on endosulfan biodegradation.

Conclusions. In general, 17 isolates were obtained from the enrichment culture. One highly efficient aerobic degrader, strain LD-6, was selected for further examination and identified as *Stenotrophomonas* sp. The strain could utilize endosulfan as the sole source of carbon and sulfur, and 100 mg/l endosulfan could be fully

degraded within 10 days. With GC-MS analysis, endosulfan diol and endosulfan ether were detected as major metabolites. The data indicates that the bacterium might degrade endosulfan by a non-oxidative pathway. Biodegradation of both isomers was relatively better at a temperature range of 25–35°C, with a maximum at 30°C. Cell crude extract of LD-6 could metabolize endosulfan rapidly. Enzyme distribution experiment showed that degradative enzymes in the strain were endoenzymes and constitutively expressed. Furthermore, inoculation of strain LD-6 was found to accelerate the removal of endosulfan *in situ*. The data generated from this study could improve current understandings on endosulfan biodegradation. However, further researches are still needed before practical application, such as influence of multiple parameters on bioremediation and toxicological assessment of the bacterium.

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