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Influence of isolated bacterial strains on the *in situ* biodegradation of endosulfan and the reduction of endosulfan- contaminated soil toxicity

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ABSTRACT

The recently discovered endosulfan-degrading bacterial strains *Pusillimonas* sp. JW2 and *Bordetella petrii* NS were isolated from endosulfan-polluted water and soil environments. The optimal conditions for the growth and biodegradation activity of the strains JW2 and NS were studied in detail. In addition, the ability of the strains JW2 and NS to biodegrade endosulfan in soils during *in situ* bioremediation experiments was investigated. At a concentration of 2 mg of endosulfan per kilogram of soil, both JW2 and NS had positive effects on the degradation of endosulfan; JW2 degraded 100% and 91.5% of α- and β-endosulfan, respectively, and NS degraded 95.1% and 90.3% of α- and β-endosulfan, respectively. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) of soil samples showed the successful colonization of JW2 and NS, and the toxicity of the soil decreased, as determined by single-cell gel electrophoresis (SCGE) assays of *Eiseniafetida* and micronucleus (MN) assays of *Viciafaba* root tip cells. Furthermore, the metabolic products of the bacterially degraded endosulfan from the *in situ* experiments were identified as endosulfan ether and lactone. This study provided potentially foundational backgrounds information for the remediation of endosulfan-contaminated soil.

1. Introduction

Endosulfan is a mixture of α- and β- isomers (7:3) and has been extensively applied to control a variety of crops including cotton, tea, and tobacco (Singh and Singh, 2014). Although endosulfan has been categorized as a persistent organic pollutant (POP) since 2011 (Chakrabarty et al., 2012), it is still being used in many countries (Zhao et al., 2014). Because of its wide use and persistence, endosulfan is often detected in the soil (Connolly et al., 2001), in rivers (Broomhall, 2002), in the air and in humans (Arrebola et al., 2001) worldwide. Previous studies have suggested that endosulfan is toxic to microbes, plants, animals, the environment and humans (Shao et al., 2012; Svartz et al., 2014). Therefore, studying the remediation and detoxification of endosulfan-contaminated soil is imperative.

The removal of endosulfan is influenced by several processes, such as soil infiltration, runoff, sorption and biodegradation, under field conditions (Antonious and Byers, 1997). Biodegradation is important because microbial activity results in the irreversible transformation of endosulfan (Parkpian et al., 1998). To remove this pollutant from the environment, the utilization of microbial degradation is an efficient approach that is also environmentally sound and cost effective (Kataoka and Takagi, 2013). In previous publications, we investigated the bioremediation and toxicity of endosulfan (Kong et al., 2013, 2014; Zhang et al., 2016), and several endosulfan-degrading microorganisms discussed in other studies, including *Staphylococcus* (Kumar and Philip, 2006), *Aspergillus* (Goswami et al., 2009), *Agrobacterium tumefaciens* (Thangadurai and Suresh, 2014), *Bacillus* (Ishag et al., 2016) and *Pseudomonas* (Chauhan et al., 2016), were also able to degrade endosulfan in contaminated soils. In this study, two new strains of bacteria were isolated, *Pusillimonas sp.* JW2 and *Bordetella petrii* NS, both of which possess the ability to degrade endosulfan. We then conducted further tests to investigate the optimal conditions under which these two strains both grow and degrade endosulfan.

The biodegradation of endosulfan in the laboratory has been reported in the literature. However, the parameters from lab-scale tests are hard to replicate in field biodegradation experiments, primarily

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because conditions differ greatly between the lab and the field, resulting in lab-scale tests being less comprehensive. Field-scale tests for estimating the level of *in situ* biodegradation by bacteria in agricultural soil is of great importance for selecting the appropriate remediation strategy for endosulfan-contaminated field sites. Therefore, to expand our understanding of the bioremediation potential of JW2 and NS, *in situ* biodegradation tests were performed by spraying inoculants containing endosulfan, JW2, and NS- on the soil of bare agricultural fields.

Field conditions are very complex, and each field contains countless microorganisms. A key step in the present study was to examine whether JW2 and NS could colonize soils successfully and balance well with the *in situ* original inhabitants during endosulfan biodegradation tests. The study by Altenburger et al. (2010) showed, using polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE), that sterile soil could be colonized by protozoa and their bacterial prey. Our earlier studies (Kong et al., 2014; Zhang et al., 2016) also used PCR-DGGE to study the colonization of JBW4 in soil (added through a reagent bottle) during endosulfan degradation. Thus, we employed PCR-DGGE to investigate the presence of the inoculated bacteria in the present study.

Many reported endosulfan-degrading bacteria and fungi result in the formation of endosulfan sulfate, which is more toxic and persistent in the environment than endosulfan itself (Kataoka and Takagi, 2013; Martens, 1976). Thus, it is necessary to assess the presence of endosulfan metabolites to better understand the detoxification of the inoculated endosulfan-degrading bacteria. Here, micronucleus (MN) assays and alkaline single-cell gel electrophoresis (SCGE) were performed to measure changes in soil toxicity during endosulfan degradation. SCGE is reportedly sensitive to low endosulfan concentrations and can be used to test the genotoxicity of soil after endosulfan degradation by bacteria (Kong et al., 2014). Another common indicator, the MN assay, is regarded as a clastogenic endpoint, indicating exposure to mutagenic or carcinogenic agents (Hajjouji et al., 2008). MN assays on *Vicia faba* root tips are effective for assessing the ecotoxicity of environmental pollutants. For example, DNA damage caused by some chemicals can be detected using MN assays on *V.faba* root tips (Marcato-Romain et al., 2009).

The objectives of this study were as follows: (1) Two endosulfan-degrading bacterial strains (JW2 and NS) were isolated, and the optimal conditions for their growth and the biodegradation of endosulfan were investigated. (2) The ability of JW2 and NS to degrade endosulfan in *in situ* soils was evaluated. (3) The survival of JW2 and NS during endosulfan degradation was studied. (4) The changes changes in soil toxicity before and after endosulfan degradation were measured. (5) The metabolites of endosulfan degradation under field conditions were detected.

2. Materials and methods

2.1. Chemicals and reagents

Emulsifiable endosulfan concentrates (96% purity) used in this study were purchased from the Shandong Rainbow Chemical Limited Company (Weifang, China). Endosulfan sulfate (purity: 97.7%), endosulfan lactone (purity: 99.0%), endosulfan diol (purity: 99.4%) and endosulfan ether (purity: 99.0%) were obtained from Dr. Ehrenstorfer (GmbH, Augsburg, Germany). Chemicals, such as ethanol, petroleum, ether and acetone, were graded as guaranteed reagents. The endosulfan was completely solubilized in acetone as a stock solution (1.005×10^4 mg/L) and diluted with medium to the required concentrations before use (Wen et al., 2009). Luria-Bertani medium (LB medium) was composed of the following: 5 g NaCl, 5 g yeast extract and 10 g peptone in 1 L of μltrapure water. Basal medium was composed of the following: 5 g peptone, 0.02 g $CaCl_2$, 4.5 g KH_2PO_4, 2.0 g $(NH4)_2SO_4$, 5.8 g K_2HPO_4, 0.001 g $MnCl_2$, 0.16 g $MgSO_4$, 0.001 g $FeSO_4$ and 0.002 g Na_2MoO_4 in 1 L of μltrapure water (Kumar et al., 2007).

2.2. Isolation and identification of endosulfan-degrading bacteria

The bacteria were cultured from activated sludge samples, which were collected from an endosulfan company (Shandong Chambroad Petrochemicals Co., Ltd). Approximately 10 g of sludge was mixed into 100 mL of basal medium containing 200 μg/mL of endosulfan in a 250 mL Erlenmeyer flask, in triplicate, and then incubated under aerobic conditions with continuous shaking (130 r/min) for 7 days at 30 ℃. Subsequently, 10 mL of medium from each culture were incubated as described above and this process was repeated five times. Finally, to isolate the single strains, 0.1 mL of each culture was inoculated on agar plates, followed by the aerobic incubation of the plates at 30 ℃ for 48 h. The strains with the best growth were then selected and streaked onto new agar plates for further purification. The inoculation of these selected species was finally conducted to measure the degradation of 100 μg/mL endosulfan over 5 days.

After adding an equal volume of hexane to the bacterial culture, the residual endosulfan was isolated to analyze the degradation levels of endosulfan. Using anhydrous Na_2SO_4, water was removed from the hexane, 2 μL of which was then analyzed using a gas chromatography (GC) system (Shimadzu GC-14C, Japan) equipped with a hydrogen flame ionization detector (FID) and an OV-1701 column set at 240 ˚C. The carrier gas was nitrogen at a flow rate of 25 mL/min. The temperatures of the injector and the detector were both set at 260 ˚C.

A 16 S rDNA sequence analysis was performed on strains with the ability to degrade endosulfan. Using polymerase chain reaction (PCR), the 16 S rDNA genes were amplified from the isolated genomic DNA using universal primers (27F, 5′-AGAGTTTGATCMTGGCTCAG-3′; 1492R, 5′-CGGYTACCTTGTTACGACTT-3′). PCR was performed with the following program: 28 cycles of 94 ˚C for 1 min, 58 ˚C for 1 min and 72 ˚C for 2 min. An additional elongation step of 10 min at 72 ℃ was included.

2.3. Optimization of conditions for maximum bacterial growth and endosulfan degradation

We tested the effects of different temperatures, pH values, endosulfan concentrations and bacterial strain concentrations on endosulfan degradation and bacteria growth over5 days by performing sacrificial flask experiments. The experimental procedures were similar to the reported method described by Kong et al. (2013). However, in this study, more parameters were added: pH values of 4, 5, 6, 7, 8, 9 and 10; different concentrations of endosulfan (10, 20, 50, 100 and 200 μg/mL); different inoculum sizes (200, 400, 600, 800 and 1000 μL suspensions of bacteria, OD600 = 1.0); and different temperatures (10, 20, 30, 35, 40 and 45 ˚C). Each incubation parameter was tested in triplicate. The increase in microorganism growth was measured at OD 600 nm using a UV–visible spectrophotometer.

Endosulfan biodegradation in liquid culture was also measured, using the optimal conditions determined in the experiments described above. To determine the growth of bacteria and endosulfan degradation, samples were collected at intervals of 0, 3, 5, 7, 10, 14 and 21 days. Uninoculated flasks were set as controls, and these experiments were performed in triplicate.

2.4. Degradation of endosulfan in situ by JW2 and NS

The *in situ* experiments were conducted in an agricultural field (0–40 cm of soil) at the Shandong Agricultural University in Tai'an (N 36, E 117), China. The physicochemical properties of the brown soil were measured as follows: organic matter 17.6 g/kg, available potassium 125.7 mg/kg, available nitrogen 132.3 mg/kg, available phosphorus 18.4 mg/kg, water holding capacity 18.5%, pH 7.6, silt 10.4%, sand 31.9%, and clay 57.7%. Endosulfan had not been used in the field, and endosulfan residue levels were below the detection limit. The field soil was sprayed with 3 mL of emulsifiable endosulfan concentrates,