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Halophilic bacterium JAS4 in biomineralisation of endosulfan and its metabolites isolated from *Gossypium herbaceum* rhizosphere soil

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ABSTRACT

Bacterial strain, *Halophilic* bacterium JAS4, capable of degrading endosulfan and its metabolites was isolated from *Gossypium herbaceum* rhizosphere soil by enrichment technique, considering the fact that the microorganism had adapted to exposure in pesticide after having been in contact with pesticide contaminated soil. The JAS4 isolate had remarkable potential to degrade 1000 mg/l of endosulfan by catabolic activity and transform them into simpler compounds. The biodegradation experiments showed that α,β-endosulfan and endosulfan sulfate in the aqueous medium was degraded by JAS4 strain which was characterized by the rate constant (k) of 0.017 d^{-1}, 0.003 d^{-1}, and 1.219 d^{-1}, respectively. The period within which the initial pesticide concentration was reduced by 50% (DT_{50}) was 40.7 d (α-endosulfan), 231 d (β-endosulfan) and 0.5 d (endosulfan sulfate). Inoculation of sterile soil with *Halophilic* bacterium JAS4 and nutrients enhanced the disappearance rate of pesticide, and DT_{50} for α,β-endosulfan and endosulfan sulfate was 0.01 d, 346.5 d and 1.07 d, respectively. In the present study powder formulations were prepared by two methods; they are less expensive and handling is also easy.

1. Introduction

Over the past decades, the extensive use of pesticide to enhance the agricultural production has increased tremendously all around the world. There are about numerous of commercial formulation available as pesticides, herbicides and fungicides. Though the environmental implication of pesticide usage is well documented, pesticide usage is still in practice, there are areas where pesticides have been still employed to a larger extent thus resulting in widespread distribution of pesticide. Endosulfan is one of the controversial pesticide which has been extensively used in agriculture and is banned from 2011 in India due to the toxic effects incurred by it in Kasaragod district of Kerala, India. One of the world's worst pesticide disaster endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,3,4-benzo-dioxathiepin-3-oxide, CAS No. 115-29-7) is an organochlorine pesticide, a derivative of hexachlorocyclopentadiene and is a mixture of α and β endosulfan at a ratio of 70:30 [1]. It is used against aphids, cabbage worms, white flies, leafhoppers and found to be neurotoxic to both insects and mammals, which acts as an endocrine disruptor. Endosulfan is hydrophobic, and persists in soil for more than a year [2]. However, bioremediation of the pesticide by bacteria and fungi are gaining interest as they are ecofriendly, economical when compared to other treatment methods such as incineration and landfill. The microorganisms especially bacteria and fungi possessing unique capability to degrade xenobiotic contaminants have been isolated from different part of the world. Much research has been conducted on pesticide degrading microorganisms. Endosulfan degrading ability was recorded in organisms like *Klebsiella oxytoca* [3], *Bacillus stearothermophilus* [4], *Mortierella* sp. strains W8 and Cm1-45 [5], *Trichoderma harzianum* [6], *Bacillus* sp., [7], *Aspergillus niger* [8], *Stenotrophomonas* sp. LD-6 [9], *Achromobacter xylosoxidans* CS5 [10], *Pseudomonas putida* and *Pseudomonas aeruginosa* [11], *Aspergillus sydoni* [12] and *Phanerochaete chrysosporium* [13].

In the present study, JAS4 bacterial strain was isolated using enrichment method which was able to degrade α-endosulfan, β-endosulfan and major toxic metabolite endosulfan sulfate. To the best of our knowledge this is the first work reporting on endosulfan degradation by *Halophilic* bacterium. As bioremediation studies will be incomplete without application part, in the present work emphasis on employing this efficient strain in degradation has been achieved by preparing appropriate formulation with cost effective fly ash, soil and molasses.

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2. Materials and methods

2.1. Sample collection

The soil sample used in this study was collected from *Gossypium herbaceum* field located in Vellore district, Tamil Nadu, India (12.93° N 79.13° E). The field was previously exposed to endosulfan in the summer months over a period of 5 years. Topsoil was collected from 15 cm deep, air dried, and stored for further analysis. The chemical properties of the soil were analyzed from Shri A.M.M Murugappa Chettiar Research Centre, Taramani, Chennai, India (Table 1).

2.2. Chemicals

Analytical standards of α-endosulfan (99% purity), β-endosulfan (99% purity) and endosulfan sulfate (99% purity) were purchased from Sigma Aldrich (St. Louis, MO, USA). Technical grade endosulfan of 35% emulsified preparation was used in this study was obtained from Hindustan insecticides limited, Kerala, India. Chromatographic grade acetonitrile and ethyl acetate were purchased from SD Fine Chem Limited (India). All other reagents used in this study were of analytical grade.

2.3. Enrichment and isolation of bacterial strain

Bacterial isolation was carried out in minimal salt medium (MSM). 20 g of soil sample was inoculated into 50 ml of MSM (g/l: Na_2HPO_4, 5.8; KH_2PO_4, 3.0; NaCl, 0.5; NH_4Cl, 1; $MgSO_4$, 0.25; distilled H_2O, 1000 ml and pH 6.8–7.0) and spiked with 35 mg/l endosulfan. The culture was incubated at room temperature at 100 rpm for 7 d. After a week, 5 ml of the sample was transferred into a freshly prepared MSM containing the same amount of endosulfan. Three successive transfers were carried out in fresh MSM containing endosulfan as the only carbon source. In the last transfer, 10-fold dilutions of cultures were prepared and 100 μl of sample was spread on nutrient agar plate containing 35 mg/l endosulfan. Isolated colonies were streaked onto nutrient agar plates containing endosulfan and purified by repeated streaking. Three morphologically different bacterial strains were isolated and these isolates were initially screened for endosulfan tolerance using minimum inhibitory concentration (MIC) method. Out of three isolates, strain JAS4 was selected for further study due to its ability to tolerate higher concentration of endosulfan (2100 mg/l).

Table 1
Chemical properties of the soil used in the experiment.

Properties	Soil sample
pH	7.84
EC	0.46
Organic carbon	0.46 kg/acre
Organic carbon	0.51%
Nitrogen	137.07 kg/acre
Phosphorus	8.16 kg/acre
Potassium	101.14 kg/acre
Calcium	314.19 mg/kg
Magnesium	155.4 mg/kg
Sodium	103.51 mg/kg
Iron	7.44 mg/kg
Manganese	6.72 mg/kg
Copper	1.01 mg/kg
Zinc	0.34 mg/kg
Sulfate	18.56 mg/kg
Humus	82.95 kg/acre
Total minerals	239.37 kg/acre

2.4. Identification of highly efficient bacterial strain

Strain JAS4 was identified according to the Bergey's Manual of Systematic Bacteriology [14] and sequence analysis of 16S rRNA gene was performed. Pure culture of strain JAS4 was grown in nutrient broth for 24 h, and genomic DNA was extracted using the AMpurE Bacterial gDNA Mini Spin kit (Amnion Biosciences Pvt. Ltd., Bangalore, India). For genetic affiliation analysis, 16S rRNA gene fragments were amplified by polymerase chain reaction (PCR) using the universal forward primer 5′-CWG RCC TAN CAC ATG SAA GTC-3′ and reverse primer 5′-GRC GGW GTG TAC NAG GC-3′. PCR reaction mix of 50 μl final volume contained: 50 ng sample gDNA, 100 ng forward primer, 100 ng reverse primer, 2 μl dNTP's mixture (10 mM), 5 μl 10× *Taq* polymerase buffer, 3 U *Taq* polymerase enzyme and PCR grade water to make up the volume. Amplified PCR product was sequenced by using ABI3730xl genetic analyzer (Amnion Biosciences Pvt. Ltd., Bangalore, India). The sequencing result was submitted to the GenBank National Center for Biotechnology Information (NCBI) database.

2.5. Growth kinetics of strain JAS4 in different media

Growth of strain JAS4 in different media, MSM and nutrient broth with endosulfan were studied in terms of optical density. To investigate the growth of strain JAS4 with endosulfan as sole source of carbon, 100 μl of strain JAS4 was inoculated into 20 ml of the MSM and 20 ml of nutrient broth medium with endosulfan (1000 mg/l) in 100 ml Erlenmeyer flask and the control flasks was maintained without endosulfan. The culture was incubated at 30 ± 2 °C on a rotary shaker at 120 rpm. The bacterial growth was regularly monitored by spectrophotometer at 600 nm.

2.6. Studies on degradation of endosulfan in MSM

The degradation studies were performed in 250 ml Erlenmeyer flasks containing 100 ml of sterile MSM supplemented with 1000 mg/l of endosulfan. One milliliter of bacterial suspension was transferred to the MSM to give a final concentration of approximately 3×10^6 cells ml^{-1}. All the flasks were incubated at 30 ± 2 °C on a rotary shaker at 120 rpm. Samples of MSM were periodically removed aseptically to determine endosulfan and its major metabolite concentration by high performance liquid chromatography (HPLC).

2.7. Studies on degradation of endosulfan in soil

The two soil microcosm treatments were carried out with isolated JAS4 strains: (1) addition of endosulfan, JAS4 and amended with nutrients (carbon, nitrogen and phosphorus) and (2) addition of endousulfan and JAS4 devoid of nutrients (control). Before using the soil for degradation studies, it was sterilized three fold by autoclaving for 30 min at 121 °C. 30 ml of solution containing JAS4 strain, nutrients and 1000 mg/kg of endosulfan were added to 250 ml Erlenmeyer flask which contained 100 g of sterilized soil. The sources of carbon, nitrogen and phosphorus were glucose, ammonium sulphate and dipotassium hydrogen phosphate, respectively. The amounts of carbon, nitrogen and phosphorus were calculated by the relationship C/N/P (100:10:1) [15,16]. All the flasks were incubated at 30 °C and soil samples were analyzed at one day interval regularly for the determination of endosulfan and its major metabolites.

2.8. Analytical methods

Endosulfan and its major metabolites in the aqueous medium was extracted by addition of equal volume of acetonitrile in a