Isolation and Characterization of a Novel Native Bacillus Spp. Capable Of Degrading Endosulfan

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Abstract – The ability of soil bacteria to utilize pesticide endosulfan as the sole carbon and energy source was investigated in this present research. Four bacterial strains were isolated with enrichment technique from the soil from natural habitats i.e. from the fields with a confirmed history of pesticide usage. The isolated bacterial colonies were subjected for the identification based on cultural and biochemical characterization. All the isolated bacteria were subjected for the tolerance limit test with the variable concentrations of endosulfan in vitro. The resistant strain to the high concentration of the pesticide was selected for further analysis. Based gram staining, biochemical tests and with specific media their morphological characters, this new bacterial isolate was identified as Bacillus spp. The biodegradation experiments were performed in high (100 ppm) concentration of endosulfan for 21 days. Bacillus spp. was able to efficiently degrade the persistant organic pollutant, endosulfan. At the end the degradation percentage was observed to be 87.00 % and, 79.60 % of endosulfan alpha and beta isomers, respectively. The results indicate that native strains have great potential for in situ remediation of pesticide-contaminated soils in highly pesticide polluted sites.

Keywords: Bacteria; Bacillus sp; Biodegradation; Endosulfan

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OVERVIEW

Currently India is one of the largest producers of pesticides in Asia and ranks 12th in world for application of pesticides. In spite of their enormous benefits the unregulated and indiscriminate application of pesticides has raised serious concerns about their impact on environment and human health (Tulsi Bhardwaj, 2013). Presently pesticides are known as common contaminants in the biosphere and on non-target organisms in our urban landscapes. Once they sprayed to the plantations, they remain persistent in the given site and may harm surrounding animals ranging from beneficial soil microorganisms, fish, birds, and other wildlife. There are several consequences being documented in several research studies. The devastating evidence that some of these chemicals caused so far to the human and other life forms and pose side effects to the environment (Forget, 1993)

Endosulfan, a highly toxic organochlorine pesticide is one of such devastating persistent organic pollutant, has emerged as a threat to a public health (U.S. EPA, 1996). Because of its threats to human health and the environment, a global ban on it has been implemented in several countries Endosulfan became a highly controversial agrichemical due to its potential for bioaccumulation, and role as an endocrine disruptor apart from its acute toxicity. Half-life of endosulfan and its metabolites vary from six months to six years as stated by Environment protection agency.

The natural degradation of this pesticide into its metabolite such as sulfate, diol, ether, and lactone is carried by chemical reactions either by hydrolysis or oxidation. This process is long-lasting and laborious. However, using alternative methods such as use of microbial consortia the process can result in accelerated rate of degradation.

Several reports on chlorinated pesticide degradation under aerobic and anaerobic conditions by microbial cultures of bacteria and fungus has been documented so far (Katayama and Matsumura,1993; Mukherjee and Gopal, 1994; Awasthi et al, 1997; Shetty , 2000) . Endosulfan can be utilized as a sole source of carbon and/or sulfur during biodegradation with specific in vitro conditions (Guerin, 1999; Sutherland et al ,2000).

The objective of the present study is to isolate and characterize endosulfan-degrading microbes and use them as bioremediation agents to decontaminate the pesticide contaminated soil thereby reducing the level of contamination in the environment.

MATERIALS AND METHODS

Technical grade endosulfan (under the trade name 'Endothion') EC 35% (emulsifiable concentrate) was purchased from local supplier which was manufactured by the Rallis India Ltd, India. The other chemicals hexane, conc. hydrochloric acid, acetone and distilled water were purchased from E Merck. Nutrient broth, Nutrient agar and other microbiological media including Minimal essential media were purchased from Himedia.

Soil sample collection

Soil samples were collected from natural habitats from the fields where pesticide usage confirmed. Sample was drawn between rows in line sown cropping area. The soil sample was collected from the 0-15 cm layer from the ground level. The collected samples were packed in ziploc airtight polythene bags. Each sample bag was well labelled with proper information. The collected samples were stored at 4^0 C till its further analysis in the laboratory.

Enrichment technique for isolation of Endosulfan degrading microbes

Soil Samples screened for their ability to grow with enriched media. The soil samples collected were air dried at room temperature and were passed through sieve with 2 mm mesh. Twenty grams of different soil samples were placed in Erlenmeyer flask. Each sample was further enriched by addition with endosulfan suspension to give final concentration of 20 ppm.

Flasks were shaken vigorously for uniform mixing of pesticide and incubated at room temperature and 37^0C for three weeks. The moisture content of the soil was kept constant by addition of distilled water in regular interval of one week along with regular addition of endosulfan to maintain the essential concentration 20ppm for the three weeks.

From the enriched soil samples, a stock culture media was prepared by transferring 5 g of the soil into a nutrient as described in earlier studies (Kumar and Philip 2006). Ten milliliters of the stock culture was transferred into a 100 ml Erlenmeyer flask containing fresh nutrient broth, and kept in shaking incubator for 24 hrs with 180 rpm speed at 37°C. Further, 1 ml of the culture from the previous flask was inoculated into a fresh Erlenmeyer flask containing nutrient broth spiked with 100 ppm of endosulfan and maintained at 37°C ± 2°C with shaking at 150 rpm for 1 week. After each week one ml of the culture suspension was transferred to a fresh Erlenmeyer flask and cultured as mentioned above. After six transfers, 1 ml of this inoculum spread on nutrient agar from an experimental flask. After incubation of 24 h the pure culture was maintained on slants and the identification was carried out for each of the isolated bacteria based on colony characteristics and biochemical tests.

Identification of the isolates

The major cultural characteristics and biochemical tests were performed as described in previous studies (Mata et al., 2002). Morphological and physiological characteristics of the best isolated strain were studied either on nutrient agar or in nutrient broth. Gram reaction, motility, shape and color of colony, catalase, urease, oxidase activities, nitrate reduction tween 20 and 80 hydrolyzes and indol productions were checked as recommended in literature (Smibert and Krieg 1994). Acid production from carbohydrates and sugars and utilization of carbon and nitrogen sources were evaluated as described earlier (Ventosa et al. 1982), respectively.

Determination of the endosulfan tolerance limit of bacterial Isolate

The obtained isolated pure strains of each of the bacteria were subjected for optimising their tolerance limit for various concentration of endosulfan. Strain was grown on nutrient agar slants. All the isolated bacteria with constant inoculums were subjected for assay with endosulfan concentration ranging from 20 to 1000ppm / 100 ml. Viability count was carried out after 48 h by growing the 100µl inoculums from the test broth onto a agar plates. After 24 h of incubation at 37°C ± 2°C results were documented under the result section. This test ensures the tolerance limit of each of the isolated bacterial strains and confirmed their application for further analysis

Biodegradation of endosulfan with pure culture

The pure cultures were grown in Burkes mineral medium as used in the earlier studies (Kathpal, T.S 1997) supplemented with defined concentrations of endosulfan as sole source. Totally three sets of Burkes mineral media in a 250 ml Erlenmeyer flask inoculated by pure culture were treated as follows (i) endosulfan alone (100 ppm), (ii) endosulfan (50 ppm), with inoculum (iii) endosulfan (100 ppm), with inoculums. Inoculated flasks were incubated in a rotary incubated shaker at 37^0 C for three weeks. After every week a constant test sample 15 ml was withdrawn aseptically and at the same volume withdrawn was

replaced with the equal volume of new media with inoculum on each test flask.

Endosulfan extraction and measurement

Ten milliliter of the each test samples (7^{th}, 14^{th} and 21^{st} day) and control samples (7^{th}, 14^{th} and 21^{st} day) were withdrawn aseptically and subjected for the extraction. Each sample first homogenized with a Teflon homogenizer which was followed by addition of 3 to 5 drops of conc. hydrochloric acid. The homogenized sample was extracted by adding equal volume (80:20 ml) of hexane: acetone mixture. This mixture was vortex for 10-15min thoroughly.

The same samples were centrifuged at 2000 rpm for 20 min. The organic layer was separated and dried over anhydrous sodium sulfate prior to injection for gas chromatographic analysis using electron capture detection (GC-ECD) (H. M. Shivaramaiah et al, 2006). The extracted sample was subjected to gas liquid chromatographic analysis.

Shimadzu Gas Chromatograph, GC-2010 PlusA equipped with Ni (550 MBq) ECD electron capture detector was used to analyze the endosulfan and its metabolites. The equipment had injector with capacity 1µl volume. Nitrogen was used as carrier gas at a column head pressure of 24 kpa giving a linear carrier flow of 4 cm with nitrogen as detector makeup gas (2 mL/min).Fused silica capillary column with column length 30 m, 0.32 mm internal diameter 0.25 µm thickness was fitted and a temperature program (55^0C for one min 180^0C at 40 min 240^0C at 20 min final time) used.

The injector temperature was 250^0C and detector temperature was 280^0C. Total chromatographic analysis run of each sample was for 40 min. The chromatograms were recorded and compared with standards to quantify sample concentrations.

RESULTS AND DISCUSSION

Soil collection and Analysis

The characteristics of the soil sample collected with the analysis were presented in Table 1. The soil profile

Table 1: Profile description of pesticide - contaminated soil

Test	Observations
Soil color	Dark brownish in color with a tint of black color in it
Soil texture	Clayey composition. Very fine granules and smooth texture
Water content	50-75% soil is moderately saturated with water
pH	6.5 – 6.7
Pesticide residue concentration/gm of soil	0.1 mg to 2.1 mg/Kg

Isolation Identification and selection of bacterial Isolates

The soil sample was subjected for the isolation on nutrient agar various colonies were observed after 24 h of incubation. Nearly four different bacterial colonies were observed. Based on the biochemical characterization the identification was of isolates was done. One of the bacterial isolates was identified as Enterobacter, Pseudomonas spp, and two from Bacillus spp respectively. Out of which two Bacillus spp one Bacillus strain was shortlisted as it was able to tolerate the high concentration (1000 ppm/100ml) of endosulfan. The biodegradation studies were carried out using the bacterial isolate.

Degradation of Endosulfan by Bacillus spp.

Biodegradation of endosulfan by soil bacteria is shown in Figures (1). Microbial degrading of endosulfan was observed in Brukes mineral media incubated for a period of 21 days. Evidence of metabolism of endosulfan is by measuring the substrate disappearance which is being calculated as a percentage reduction. Total degradation of endosulfan efficiency (alpha and beta isomers of endosulfan) was observed at the end of the 21 days of incubation with increase in bacterial mass. The percentage rate of degradation of parental molecules endosulfan alpha and beta respectively was determined by the result of GC ECD analysis showed the area covered by each peak of two isomers at the end of the 7 14 and 21 days of incubation. At the end of 21 days total of 87% degradation of alpha while 79% degradation of beta isomer was recorded.

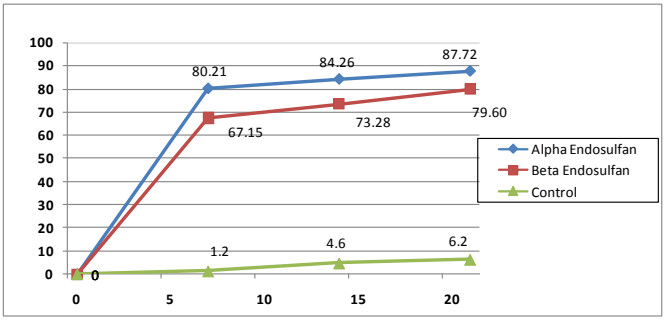

Fig1: Endosulfan Degradation of Alpha and Beta isomers by soil bacteria

Whereas, in controls (uninoculated with microbes) showed 94% of the parental molecule even after 21 days of incubation, indicating that minimal loss of pesticide could be due to natural chemical degradation, volatilization or extraction recovery. It was observed that degradation of beta endosulfan was slightly less compared to alpha endosulfan; reason behind this could be stereoisomerism of the molecules. Enzymes released by the bacteria could me more susceptible in degrading the alpha isomers. Our results observed correlates with the earlier findings (Siddique et al. 2003) which has reported the endosulfan degradation by microbes that bacteria have degraded more of alpha endosulfan than beta endosulfan.

Degradation pathway of endosulfan resulted in either the formation of endosulfan sulfate through oxidation or by formation of endosulfan diol with further breakdown products till endosulfan lactone. In our study the bacteria isolated from contaminated soil undergoes degradation but further studies need to be conducted for the identification of the end product. However, the earlier findings (Awasthi et al. 1997), have reported the only the formation of the endosulfan sulfate by bacterial coculture. Further investigation regarding the enzymes and genes responsible in degradation is in progress.

CONCLUSION

Results obtained in the present work indicate an important role of the isolated bacteria from Bacillus spp. in tolerating high concentrations of toxic chemicals and metabolic capability to degrade it. The isolated bacteria has a great potential to prevent the environment from the pesticide induced hazardous problems such as deterioration of soil quality, leaching, reduced biodiversity, disrupting the fragile ecosystem. To overcome several ecological problems, best remedy is to use the ecofriendly microbes to reduce the contamination. Our results obtained suggest that the Bacillus spp., which we have isolated, can be used to decontaminate the field area polluted with endosulfan.

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