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RESEARCH ARTICLE

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BIOREMEDIATION OF ENDOSULFAN BY BACTERIA ISOLATED FROM THE AGRICULTURAL FIELDS

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ABSTRACT

In the present study, endosulfan degrading capability of four bacterial monocultures (*RCE-2, GCE-4, GCE-5* and *JCE-4*) and two bacterial mixed-cultures (*GCE345* and *GCC134*) was investigated in terms of treatment duration and culture volume, using soil slurry medium. Among the bacterial mono-cultures, *JCE-4* was found to be most efficient with 16, 33, 60 and 65% of endosulfan (α + β-isomers) degradation in 5, 10, 15 and 30 days of treatment, respectively. Out of two bacterial mixed-cultures, *GCE345* was more efficient and resulted in 20, 39, 57 and 73% degradation of endosulfan (α + β-isomers) in 5, 10, 15 and 30 days of treatment, respectively. The fraction of endosulfan degraded was found to be higher with the increase in treatment duration till 30 days. The endosulfan degradation was also elevated by increasing the culture volume of respective bacterial cultures from 10% to 25% (v/v), for 10 days of treatment at room temperature.

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KEYWORDS

Bioremediation, Endosulfan, Mono-cultures, Mixed-cultures, Degradation

INTRODUCTION

Chlorinated organochlorine pesticides are one of the major groups of chemicals responsible for environmental contamination. Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepine-3-oxide), is a type of organochlorine insecticide. It is a contact and stomach poison, used to control chewing and sucking insects, such as Colorado beetle, flea beetle, cabbage worm aphids and leaf hopper. This insecticide is used to control insect pests on a wide range of crops, including cereals, cotton, coffee, fruits, oil seeds and vegetables[1,2,3].

Technical-grade endosulfan is a mixture of two stereo isomers, alpha and beta endosulfan, in a ratio of 7:3. Both the isomers are extremely toxic to aquatic organisms. Because of its widespread usage and potential transport, endosulfan contamination is frequently found in the environment at considerable distances from the point of its original applications. Many countries imposed ban on endosulfan production and/or usage, whereas in India it is still one of the priority pollutant for pest control. Because of its abundant usage and potential transport, endosulfan has been detected in the soil, sediments, atmosphere, surface and rain waters, and foods. It is extremely toxic to fish and aquatic invertebrates (Sunderam et. al., 1992) and has been implicated in mammalian gonadal toxicity (Sinha et. al., 1997), genotoxicity (Chaudhari et. al. 1999), and neurotoxicity (Paul & Balasubramaniam, 1997). The health and environmental concerns have led to an interest in degradation and detoxification of endosulfan in the environment[4,5].

Detoxification of pesticides through biological means is receiving serious attention over the existing methods of incineration and landfill. During the bioremediation process, heterotrophic microorganisms break down substrates (hazardous compounds) to obtain chemical energy, therefore, organic pollutants can serve as sources of carbon, energy and nutrients for microbial growth. Endosulfan degradation is affected by environmental conditions. Properties of pollutants and the characteristics of the soil like clay content, organic matter content and soil texture have influence on the degradation process[6,7]. Several studies have reported the isolation of bacterial co-culture (Awasthi et. al., 1997) and mixed-cultures (Sutherland et. al., 2000) capable of degrading endosulfan. Many bacteria and fungi including *Cornybacterium sp., Nocardia sp., Mycobacterium sp., Pseudomonas fluorescens, Penicillium sp., Aspergillus sp., Phanerochaete chrysosporium* have been reported to be endosulfan degraders (Kullmann and Matsumura, 1996). Endosulfan could be degraded by attack on the sulfite group by oxidation and or hydrolysis to form the toxic endosulfan sulfate and the nontoxic endosulfan diol respectively (Baar and Aust, 1987). Endosulfan can be used as a source of carbon and sulphur for many heterotrophic microorganisms[8,9]. Endosulfan diol, endosulfan sulfate, endosulfan ether, endosulfan hydroxyether, endosulfan lactone and endosulfan aldehyde have been reported as the major metabolites formed during biotransformation of endosulfan. Under soil conditions, endosulfan sulfate, which is more toxic than the parent compound itself, has been reported to be the major metabolite and which increases in the environment due to very low degradation rates. Therefore, it is important to develop economical and efficient strategy for bioremediation of endosulfan contaminated soil and water[10,11].

The objective of the present study was to investigate the endosulfan bioremediation capability of bacterial mono- and mixed-cultures in terms of treatment duration and culture volume. The degradation and metabolism of endosulfan by the bacterial cultures were examined using gas chromatography-mass spectrometry (GC-MS) for identification of breakdown products.

MATERIALS AND METHODS

Soil used for degradation study:

The soils used for the biodegradation experiments were collected from the agricultural fields (cotton, groundnut and vegetables cultivating fields) in Rajkot district of Gujarat State. The collected soils were sieved through 2 mm mesh. The sieved fraction was collected and preserved in air tight plastic containers for biodegradation studies.

Pesticide, chemicals and media:

The technical grade of endosulfan (Endoin, EC 35%), a type of cyclodiene organochlorine was selected for the present study. The pesticide was purchased from the local pesticide supplier. This commercial formulation of endosulfan was dissolved in sterile distilled water for amendments to soil samples. The analytical grade chemicals and reagents used in this work were purchased from Hi-media and Qualigens. The culture media used were Nutrient Broth (NB), Luria Bertani (LB) and M9.

Sterilization of media, solutions and apparatus:

All the media and solutions used were sterilized by autoclaving at 121^0C temperature, 15 psi pressure for 20 minutes. The glassware and other apparatus were sterilized in an oven at 180^0C for an hour. After sterilization, the media and solutions were cooled to room temperature and then stored under refrigeration for their subsequent use. The sterilized glassware were stored separately in an oven at 60^oC and cooled to room temperature before their subsequent use.

Bacterial cultures used:

Four bacterial mono-cultures viz. *RCE-2* (*Bacillus subtilis*), *GCE-4* (*Pseudomonas putida*), *GCE-5* (*Bacillus pumulus*) and *JCE-4* (*Arthrobacter sp.*) were used in this study. All these four bacterial cultures were obtained from the previous work on isolation, characterization and identification of endosulfan degrading bacteria from the cultivated soil (details not shown). Each of the two mixed-cultures, viz. *GCE345* and *GCC134* were prepared by mixing three bacterial monocultures in equal proportion.

Degradation studies:

The bacterial mono- and mixed-cultures to be assayed for their degradation of endosulfan were first sub-cultured in Luria broth nutrient medium and then transferred into soil slurry medium. Erlenmeyer flasks (250 mL) in triplicate containing 100 mL soil slurry medium were stoppered with cotton wool plugs and autoclaved before use. For determining the effect of treatment duration, 10 mL of the full grown bacterial cultures in Luria broth was transferred into flasks. To study the effect of culture volume on endosulfan degradation, 10, 15, 20 and 25 mL of respective cultures were transferred into the labeled flasks. The degradation assays were performed in sealed flasks spiked with 20 mg/L of endosulfan (α + β-isomers). Uninoculated flasks were used as controls. The flasks were incubated at room temperature and samples for pesticide residue analysis were taken aseptically.

Extraction of endosulfan from samples:

The recovery of endosulfan from soil slurry is important to determine the extent of bioremediation by the selected bacterial cultures. The extraction of endosulfan was carried out using chloroform as a solvent. The autoclaved soil slurry medium containing 10g soil per 100 mL of double distilled water in a sterile flask was mixed vigorously to secure homogeneous medium. The medium was supplemented with 20 mg/L of endosulfan for degradation assay. After incubation period, 10

mL of soil slurry was taken in a centrifuge tube and centrifuged at 4000 rpm for 20 minutes. After this, 5 mL of supernatant was transferred into another tube and an equal volume of chloroform was added, shaken gently and allowed to settle for 30 minutes. The upper water layer was discarded and 2 mL of bottom layer containing the pesticide (endosulfan) was taken in an eppendorf tube. The sample was stored at 4^{o}C till analyzed by GC-MS.

GC-MS analysis:

A GC-MS-QP2010 system equipped electron capture detector (ECD) was used to analyze the residual endosulfan in the sample after bacterial degradation. One micro-litre of each chloroform extract was manually injected. BPX5 column (30m, 0.25mm) was fitted and a temperature program (180^{o}C for 1.5 min, 260^{o}C for 20 min, at the rate of 10^{o}C/min) used. Nitrogen was used as carrier gas at a column head pressure of 95.6 kpa giving a linear carrier flow of 36.4 cm/s and column flow was 0.90 mL/min. The injection mode was split, injector temperature was 260^{o}C and detector temperature was 280^{o}C. The chromatographic analysis was done for 30 minutes. The chromatograms were recorded and integrated using a computer and Wiley Registry of Mass Spectral Data version-7 software. External standards were used for the quantification of sample concentrations.

Effect of treatment duration and culture volume:

For the determination of bioremediation capability of bacterial mono- and mixed-cultures in terms of treatment duration, the experimental system involved 10 mL of respective active bacterial culture per 100 mL of soil slurry containing 20 mg/L of endosulfan. The experimental system was incubated at room temperature for 5, 10, 15 and 30 days respectively. For determining the bioremediation capability of bacterial cultures in terms of culture volume, the experimental system was prepared using 100 mL of soil slurry containing 20 mg/L of endosulfan and inoculated with 10, 15, 20 and 25 mL of respective bacterial culture separately, and incubated at room temperature for 10 days. In both the cases, 10 mL broth was taken out in the sterile tube after incubation period and used for the estimation of residual endosulfan concentration using GC-MS.

Statistical analysis:

The data obtained from the study of samples were analyzed using statistical features available with MS-Excel worksheet. All the data were average of three replicates. The analyzed data were used to prepare result table and graphs. The useful inferences were derived from the interpretation of such result table and graphs.

RESULTS AND DISCUSSION

Degradation of endosulfan by bacterial mono-cultures:

The degradation of endosulfan was 12, 25, 52 and 59% after incubation period of 5, 10, 15 and 30 days, respectively, at room temperature, in soil slurry medium containing 20 mg/L of endosulfan and inoculated with 10% (v/v) active bacterial mono-culture *RCE-2*. Under the similar experimental conditions, the endosulfan degradation was 13, 22, 49 and 53% after incubation period of 5, 10, 15 and 30 days, respectively, by culture *GCE-4*. When soil slurry medium was inoculated with culture *GCE-5*, the degradation of endosulfan was 15, 25, 57 and 61% after incubation period of 5, 10, 15 and 30 days, respectively. The bacterial mono-culture *JCE-4* was most efficient with 16, 33, 60 and 65% degradation of endosulfan under similar incubation conditions, after incubation period of 5, 10, 15 and 30 days, respectively (**Figure-1**). The value of Pearson correlation between treatment duration and % degradation of endosulfan by bacterial mono-cultures (r = 0.872) was very significant (**Table-1**). The positive value of correlation coefficient indicated that the degradation of endosulfan in soil slurry medium by bacterial mono-cultures increases with increase in treatment duration. Therefore, the proper optimization of treatment duration

could play an important role in bioremediation of endosulfan contaminated soil and water.

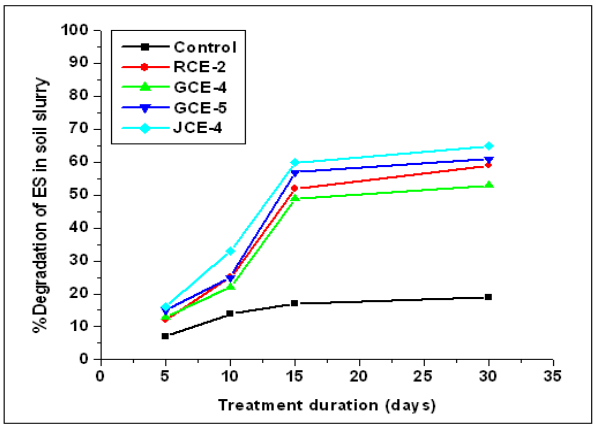

Figure-1

Endosulfan (α + β -isomers) degradation by bacterial mono-cultures in response to treatment duration

When soil slurry medium containing 20 mg/L of endosulfan was separately inoculated with 10, 15, 20 and 25 mL of respective bacterial mono-cultures per 100mL of medium and incubated at room temperature for 10 days, then the degradation of endosulfan was found to be 22, 35, 62 and 69%, respectively, by isolate *RCE-2*. The degradation was 23, 32, 59 and 63%, respectively, by isolate *GCE-4*. In case of isolate *GCE-5*, the degradation was 25, 35, 67 and 72%, respectively. The endosulfan degradation was 26, 43, 70 and 75%, when inoculated separately with 10, 15, 20 and 25 mL of *JCE-4* mono-culture and incubated at room temperature for 10 days (**Figure-2**). The value of Pearson correlation between culture volume and % degradation of endosulfan by bacterial mono-cultures (r = 0.968) was extremely significant (**Table-1**). The positive value of correlation coefficient indicated that the degradation of endosulfan in soil slurry medium by bacterial mono-cultures increases by increasing the culture volume of bacterial mono-cultures. Therefore, it important to optimize the culture volume of respective bacterial mono-cultures, prior to bioremediation of endosulfan contaminated soil and water.

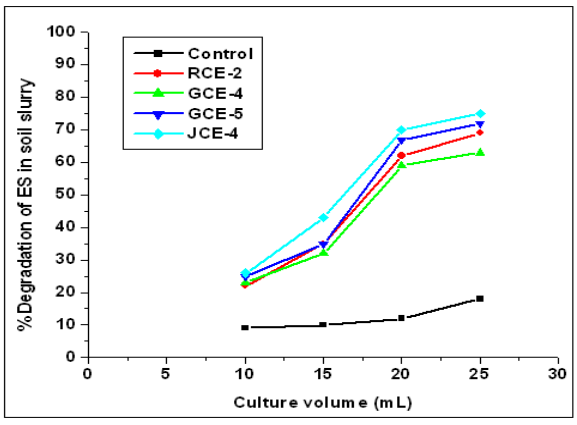

Figure-2

Endosulfan (α + β -isomers) degradation by bacterial mono-culturesin response to culture volume

Table-1

Linear regression analysis between degradation parameters and % degradation of endosulfan (α + β -isomers) by bacterial mono- and mixed-cultures

Degradation parameters		Degradation of endosulfan (%)	
		Pearson correlation	R-square
Treatment duration (days)	Mono-cultures	0.872	0.760
	Mixed-cultures	0.952	0.906
Culture volume (mL)	Mono-cultures	0.968	0.936
	Mixed-cultures	0.974	0.949

Degradation of endosulfan by bacterial mixed-cultures:

The degradation of endosulfan was 20, 39, 57 and 73% after incubation period of 5, 10, 15 and 30 days, respectively, at room temperature, in soil slurry medium containing 20 mg/L of endosulfan and inoculated with 10% (v/v) active bacterial mixed-culture G*CE345*. With mixed-culture *GCC134*, endosulfan degradation was 15, 33, 37 and 55% after incubation duration of 5, 10, 15 and 30 days, respectively, under the similar experimental conditions (**Figure-3**). The value of Pearson correlation between treatment duration and % degradation of endosulfan by bacterial mixed-cultures (r = 0.952) was very significant (**Table-1**). The positive value of correlation coefficient indicated that the degradation of endosulfan in soil slurry medium by bacterial mixed-cultures increases with the increase in treatment duration. The results showed that proper optimization of treatment duration could play a significant role in bioremediation process of endosulfan contaminated soil and water.

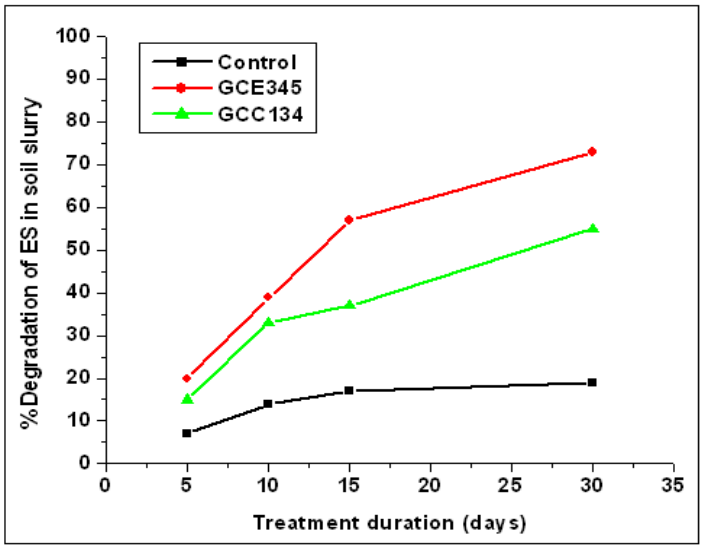

Figure-3

Endosulfan (α + β -isomers) degradation by bacterial mixed-cultures in response to treatment duration

When soil slurry medium, containing endosulfan (20 mg/L), was inoculated separately with 10, 15, 20 and 25 mL of respective bacterial mixed-cultures per 100 mL of medium and incubated at room temperature for 10 days, the degradation of endosulfan was found to be 30, 41, 77 and 83%, respectively, by mixed-culture *GCE345*. The degradation was 25, 36, 42 and 57%, respectively, by mixed-culture *GCC134*, under the similar experimental and incubation conditions (**Figure-4**). The value of Pearson correlation between culture volume and % degradation of endosulfan by bacterial mixed-cultures (r = 0.974) was extremely significant (**Table-1**). The positive value of correlation coefficient showed that the degradation of endosulfan in soil slurry medium by bacterial mixed-cultures enhanced by increasing the culture volume of respective bacterial mixed-cultures. Therefore, prior to bioremediation of endosulfan contaminated soil and water, the optimization of culture volume of bacterial mixed-cultures could yield better results.

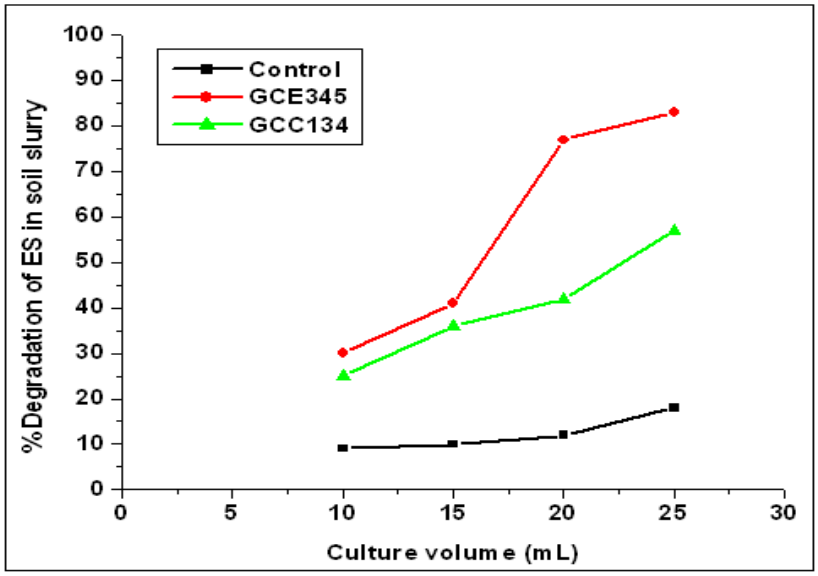

Figure-4

Endosulfan (α + β -isomers) degradation by bacterial mixed-cultures in response to culture volume

GC-MS analysis for toxic intermediates:

The GC-MS analysis of samples after degradation studies and comparison with standard library of Wiley Registry of Mass Spectral Data Version-7 confirmed the matching of mass/charge ratio v/s relative intensity of the samples to standard spectra of endosulfan. The results showed that the numbers of metabolic intermediates formed during the degradation of endosulfan by bacterial mixed-culture were relatively more in number than by bacterial mono-culture. The mass spectra of endosulfan containing samples treated with bacterial mono- and mixed-culture showed no any known toxic intermediates. The results suggested that the bacterial isolates were not forming any toxic

intermediates during the degradation of endosulfan and thus could be utilized for the bioremediation process of endosulfan contaminated soil and water.

CONCLUSION

Based on results of this study, it can be concluded that endosulfan bioremediation could be relatively more effective with bacterial mixed-cultures than mono-cultures. The positive and

extremely significant value of correlation coefficient between treatment duration or culture volume and % degradation of endosulfan showed that proper optimization of treatment duration and culture volume of bacteria mono- and mixed-cultures prior to bioremediation of endosulfan contaminated soil and water, could yield better results. Since, toxic intermediates were not detected, hence the present bacterial isolates could be effectively utilized for bioremediation process of endosulfan contaminated soil and water. Among the four mono-cultures, the isolate *JCE-4* was most efficient, while out of two mixed-cultures, culture *GCE345* was more effective in terms of degradation of endosulfan.

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REFERENCES

[1] Shivaramaiah H.M. & Kennedy I.R., Biodegradation of Endosulfan by a Soil Bacterium: J. of Env. Science & Health Part B, 41, 895-905 (2006)

[2] Goebel H., Chemical and physical properties of endosulfan and its degradation products: Residue Review, *83*, 8–28 (1982).

[3] Rainer Martens, Degradation of [8,9-^{14}C]Endosulfan by Soil Microorganisms: Applied & Env. Microbiol., 31 (6), 853-858 (1976).

[4] Mathava Kumar and Ligy Philip, Enrichment and Isolation of a Mixed Bacterial Culture for Complete Mineralization of Endosulfan: J. of Env. Sc. & Health Part B, 41, 81-96 (2006)

[5] Mathava Kumar and Ligy Philip, Endosulfan Mineralization by Bacterial Isolates and Possible Degradation Pathway Identification: Bioremediation Journal, 10(4):179–190, 2006

[6] Tariq Siddique, Benedict C. Okeke, et. al., Enrichment & Isolation of Endosulfan-Degrading Microorganisms: J. Environ. Qual. 32, 47–54 (2003)

[7] Mukherjee I. & Gopal M.: Toxicol Environ Chem, 46, 217-221 (1994).

[8] Sutherland T.D., Horne I., et. al., Enrichment of an Endosulfan-Degrading Mixed Bacterial Culture: Appl. Environ. Microbiol., *66*, 2822–2828 (2000).

[9] R. Jayashree & N. Vasudevan, Effect of tween 80 added to the soil on the degradation of endosulfan by *Pseudomonas aeruginosa*: Int. J. Environ. Sci. Tech., 4 (2), 203-210 (2007)

[10] Mohit Kumar, et. al., Microbial biodiversity and in situ bioremediation of endosulfan contaminated soil: Indian J. Microbiol., 48, 128–133 (2008)

[11] Mathava kumar & Ligy Philip, Bioremediation of endosulfan contaminated soil and water - optimization of operating conditions in laboratory scale reactors: Journal of Hazardous Materials: B136, 354–364 (2006).