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QUALITATIVE DETECTION OF ENDOSULFAN DEGRADATION BY SOIL BACTERIA USING HPTLC AND GC-MS

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ABSTRACT

Pesticide poisoning is a very common phenomenon in India.Amongst many pesticides used, Endosulfan ($C_9H_6Cl_6O_3S$) - an organochlorine pesticide, is one of the common pesticides. It is a mixture of two isomers - α-endosulfan (70%) and β-endosulfan (30%). One of the common metabolites - endosulfan sulphate is considered to be highly toxic and persistent form of endosulfan. Hence in the current study an attempt was made to identify the metabolites that are derived from endosulfan on treating with soil bacterial isolates. In the present study, 03 bacterial isolates namely ES1(Paracoccus chinensis KS-11(T) - EU660389), ES2 (Planococcus rifietensis (T); M8- AJ493659) and ES3 (Pseudomonas aeruginosa PAL106- DQ464061) were used for determining the metabolites formed during endosulfan degradation. Each bacterial isolate was grown in presence of 500ppm of endosulfan for 28 days at 37^0C (at static and shaker conditions) and the metabolic end products were harvested by solvent extraction method (after 7, 14, 21 and 28 days interval). By using HPTLC technique, the time-wise chromatographic fingerprints these metabolic end products were developed. These harvested metabolic end products were further characterized by performing GC-MS analysis. From the results it was observed that during endosulfan degradation, the most commonly found intermediate metabolites (endosulfan sulphate, endosulfan lactone, endosulfan diol, endosulfan ether, endosulfan hydroxy ether and

endosulfan monoaldehyde) were not detected throughout the complete experimental duration. Absence of toxic endosulfan sulphate and formation of simpler benzene derivatives such as 1, 3, 5-trimethyl benzene suggested that all three bacterial isolates were transforming the toxic endosulfan to comparatively less toxic molecules thereby providing a possible treatment for removal of endosulfan from soil environment.

Keywords: Pesticide, Endosulfan sulphate, HPTLC, GC-MS.

Introduction:

Pesticides are the chemicals which play an important role in the availability of cheap and consistent supplies of food to the world population by preventing the pest attack (Akhtar and Ahmed, 2002). These pesticides reach soil either by direct application on soil surface or by injection into the upper layers of the soil (Akhtar and Solangi, 1990). However, from the aspect of environmental pollution, extensive use of pesticides not only limits plant growth but may also induce mutagenic and carcinogenic effects on non target organisms. Hence, biodegradation is considered to be a reliable and the cost-effective technique for pesticide abatement and a major factor determining the fate of pesticides in the environment (Munnecke and Heish, 1974). There are many pesticides and insecticides to which pests and insects are resistant. As a result they are not degraded in the environment by routine processes. These non-degradable compounds however are degradable by bacterial activity (Roberts *et al*., 1993). Hence, there is an increasing need for the development of new methods to detect, isolate and characterize the microbial strains playing a part in these degradation processes (Vallaeys *et al.*, 1996).

The current study involves use of the most widely used pesticide namely Endosulfan. It is one of the most common cyclodiene pesticides extensively used throughout the world as a broad-spectrum pesticide. Because of the abundant usage and the potential for environmental transport, endosulfan contamination is found very commonly in the environment. It is detected in the atmosphere, soil, sediments, surface water, rain water and food stuffs (U.S. Department of health and Human Services. 1990). As with most pesticides, the persistence and degradation of endosulfan is affected by the surrounding environmental conditions. The chemical and physical properties of endosulfan differ significantly from other cyclodiene insecticides that affect both, its environmental and biological fates (Sutherland *et al.*, 2000).

However, degradation rates are usually low and metabolism often results in the formation of endosulfan sulphate, an oxidative metabolite that is found to be as toxic and persistent as the parent compound, endosulfan (Martens, 1976). Hence, isolating the bacterial strains which are capable of degrading endosulfan into non-toxic compounds becomes an important need for safe environment.

Materials and methods:

The commercial grade pesticide namely Endosulfan (35% E.C.Bayer-Thiodan), was obtained from Pathare Nursery**,** Kalyan, Dist. Thane, Maharashtra. The commercial grade pesticide was used throughout the experimental work as it may closely resemble the active compound that the microbes are likely to be exposed to in soil environment.

03 bacterial isolates viz. *Paracoccus chinensis* KS-11(T) - EU660389 (ES1), *Planococcus rifietensis* (T); M8- AJ493659 (ES2) and *Pseudomonas aeruginosa* PAL106-DQ464061(ES3) showing presence of plasmid DNA were used in the present work to study degradation of endosulfan (Naphade *et al*., 2012). The isolates were maintained on sterile Nutrient agar slants as pure cultures at 10^0C.

Chromatographic analysis of pesticide degradation

Extraction of pesticide samples:

To analyse the products made by the bacterial isolates in presence of endosulfan, chromatography (HPTLC and GC-MS) was performed. The bacterial isolates were inoculated in sterile Nutrient broth with 500ppm of endosulfan and incubated at 37^0C for 28 days - at static and shaker condition. After every 07 days interval of incubation (*i.e*. 7, 14, 21 and 28 days), 10ml samples were withdrawn from each flask and residual pesticide with its metabolite was extracted using n-hexane as the organic solvent. The fractions were pooled together and used for HPTLC as well as sent to SAIF-IIT, Mumbai for GC-MS analysis.

HPTLC (High Performance Thin Layer Chromatography):

HPTLC was performed on aluminium HPTLC plates coated with 0.25 mm silica gel 60 F_{254} (Merck # 5554). 10 µl of samples were spotted by means of a CAMAG Linomat V sample applicator. The plates were developed in CAMAG-twin-trough chamber previously equilibrated with mobile phase for 30 minutes.After development, plates were dried undercurrent of air at room temperature and the bands were visualized at 254nm and 366nm

using CAMAG -UV cabinet. Further evaluation of the plates was performed with a CAMAG -Scanner III in conjunction with Cats 3 Version Software at 254nm and 366nm.

GC-MS (Gas Chromatography-Mass Spectrometry) analysis:

All fractions obtained during pesticide extraction were subjected to GC-MS analysis using the 1µl of the sample in n-hexane (solvent) and analysed at SAIF-IIT, Mumbai. The individual pesticide isomers and intermediate metabolites were identified by matching the retention times and mass spectrum with the authentic standards run previously.

Results and Discussion:

HPTLC Fingerprinting:

During HPTLC, the standard pesticide- endosulfan and the metabolic end products derived by the action of pesticide degrading bacteria were separated on silica gel 60 F254 plate and the separated spots were visualized at 254nm and 366nm using CAMAG -UV cabinet. Specific solvent system was standardised based on the structure and chemical properties of the pesticide. The solvent system used for Endosulfan was -Toluene: Diethyl ether (8:2).

On performing HPTLC, better results were shown at 366nm compared to 254nm. It was observed that under static condition, all the 03 isolates required minimum 07 days time to start degradation of endosulfan whereas under shaker condition, isolate ES3 gave an extra band indicating formation of a new metabolite in 07 days time of incubation (Figure 1). The same band was seen in L5 suggesting that isolate ES1 may have produced similar product in 14 days of incubation under shaker conditions. On comparing the results of HPTLC scan at static and shaker condition, it was observed that better results were obtained at 37^0 C/ static condition. No clear bands were seen under shaker condition. The Rf value of standard endosulfan I was found to be 0.71±0.05 and that of endosulfan II was 0.48±0.05 when the abovementioned solvent system was used (Siva *et al.,* 2013). The presence of endosulfan I was detected in all 12 samples used (all 03 isolates were grown in presence of endosulfan for different time duration). However, HPTLC fingerprinting did not give any clear idea about the pesticide degradation by microbes. Hence GC-MS analysis was carried out that gave a clue on the time required by microbes to lower the amount of pesticide added. The results also showed the disappearance of the pesticide and formation of the intermediate molecules suggesting the probability of pesticide degradation.

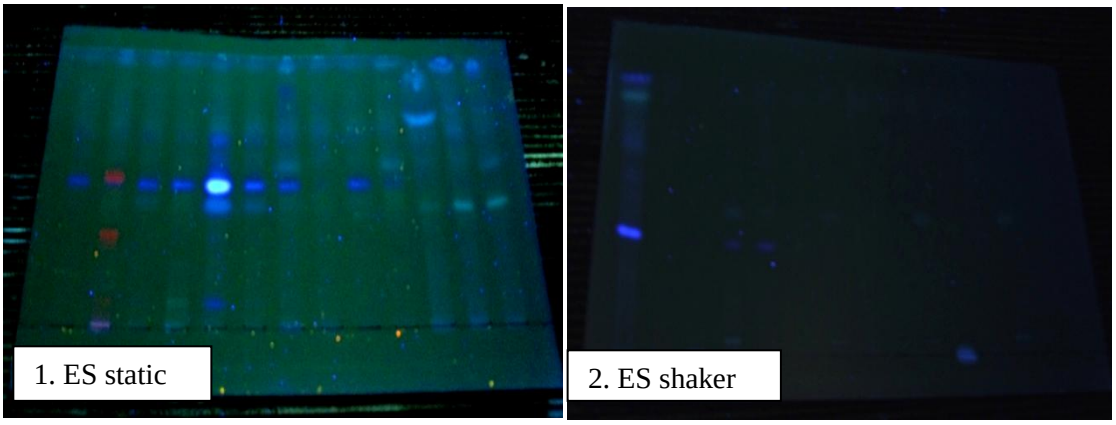

Figure 1: Results of HPTLC (at 366 nm)

Key: L1: Standard pesticide; L2: isolate ES1-07 days; L3: isolate ES2-07 days L4: isolate ES3-07 days; L5: isolate ES1- 14 days; L6: isolate ES2-14 days; L7: isolate ES3- 14 days; L8: isolate ES1-21 days; L9: isolate ES2-21 days; L10: isolate ES3-21 days; L11: isolate ES1-28 days; L12: isolate ES2-28 days; L13: isolate ES3- 28 days.

GC-MS analysis:

The identity of residual metabolic end products (analytes) derived from endosulfan was confirmed by GC-MS at SAIF-IIT, Mumbai that yielded satisfactory separation of the analytes. The retention time of the standard endosulfan (Figure 2) and the selected ions for quantification are summarised (Table 2 and Table 3). The selected ions are in agreement with those reported by other authors for the mass spectra of endosulfan (Paranthaman *et al.,* 2012; Kotonia *et al.,* 2007).

Throughout the present study, the intermediate metabolites (endosulfan sulphate, endosulfan lactone, endosulfan diol, endosulfan ether, endosulfan hydroxy ether and endosulfan monoaldehyde) reported by previous researchers were not found indicating that the 03 bacterial isolates used may be following a different metabolic pathway for degradation of endosulfan as was suggested in the study of Kumar and Philip, (2006). The absence of endosulfan sulphate - one of the common and toxic metabolites of endosulfan degradation suggests that the bacterial isolates under study were transforming the toxic pesticide to non-toxic metabolite (Table 2 and Table 3). It was seen that under static condition, endosulfan II was degraded by isolate ES2 in 21-28 days time whereas isolate ES3 could show the same degradation in 07 to 14 days. Endosulfan I was considerably rigid structure showing peak till

28th day of incubation for isolate ES1 and isolateES2. However isolate ES3 took 14-21 days time for degrading it (Table 1). Endosulfan II strongly adsorbs to microorganisms, with the majority of the insecticide being associated with the cell membrane rather than the growth medium (Sutherland *et al.,* 2000). Hence, degradation of endosulfan II presumably leads to an accumulation of products within the cell, facilitating their further degradation. Similarly under shaker condition, endosulfan I was present till the end of 28 days of incubation time when isolate ES1 and isolate ES2 were used as inoculum. However isolate ES3 could show degradation of endosulfan in 21-28 days of time. It was observed that isolate ES3 was probably the most suitable isolate for the degradation of endosulfan I and II (Table 1). In the present study it was seen thatprobably the microorganisms were using sulphite group of endosulfan as a source of sulphur for their growth.

Table 1: Time of degradation of endosulfan by bacterial isolates

Pesticide	**Bacterial Isolate**	**Time of degradation (days)**	
		Static	**Shaker**
Endosulfan-I	ES1	>28	>28
	ES2	>28	>28
	ES3	**14-21**	21-28
Endosulfan – II	ES1	>28	**21-28**
	ES2	**21-28**	**21-28**
	ES3	**7-14**	14-21

Figure 2: GC Result of Standard Endosulfan

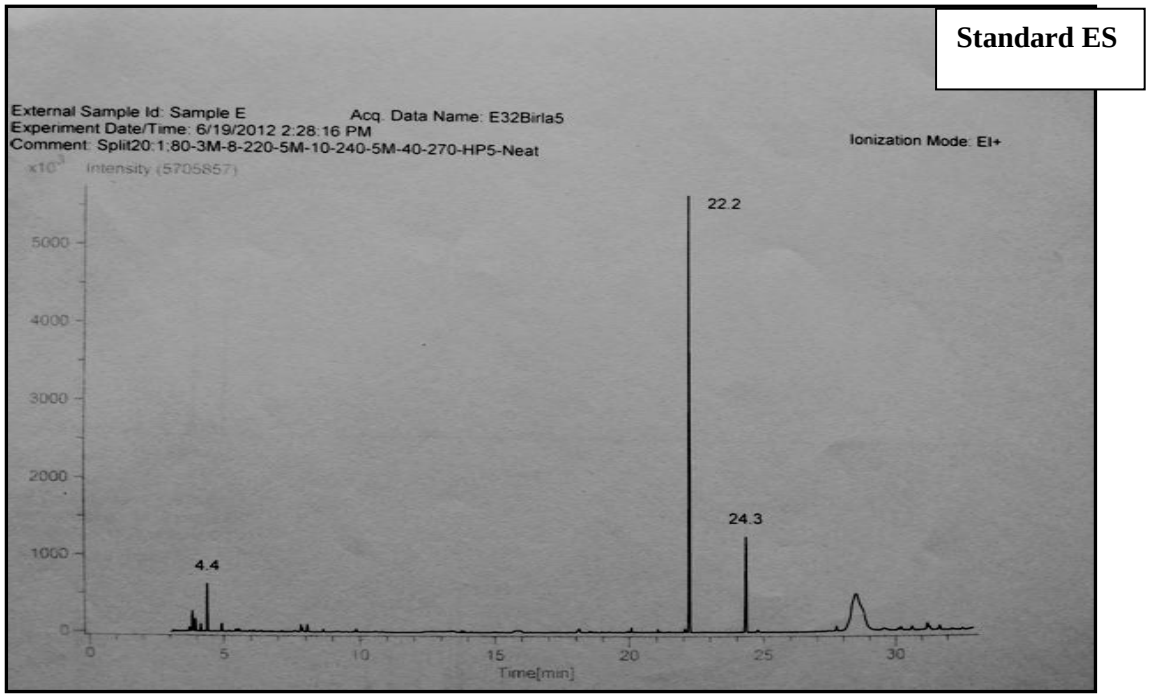

Table 2:MS results of metabolic end products derived from endosulfan under static condition

SAMPLE KEY	PRODUCTS	RETENTION TIME AND IONISATION PEAKS
Std. ES	1.Endosulfan I 2.Endosulfan II	Rt:22.2Minutes- 51,63,75,120,143,159,170,195,207,241,265,307,323,339,358,406 Rt:24.3Minutes- 50,63,75,85,102,143,159,170,195,207,241,277,307,323,339,358,406
ES1, 7days	1.Endosulfan I 2.Endosulfan II	Rt:22.2Minutes- 51,63,75,120,143,159,170,195,207,241,265,307,323,339,358,406 Rt:24.3Minutes- 50,63,75,85,102,143,159,170,195,207,241,277,307,323,339,358,406
ES1, 14days	1.Phenol,2,4-bis(1,1dimethylethyl)- 2. Endosulfan I 3. Endosulfan II	Rt 13.8Minutes- 51,57,74,91,107,128,147,163,191,206 Rt:22.2Minutes- 51,63,75,120,143,159,170,195,207,241,265,307,323,339,358,406 Rt:24.3Minutes- 50,63,75,85,102,143,159,170,195,207,241,277,307,323,339,358,406
ES1, 21days	1.Phenol,2,4-bis(1,1dimethylethyl)- 2. .Endosulfan I 3. .Endosulfan II	Rt:13.8Minutes- 51,57,74,91,107,128,147,163,191,206 Rt:22.2Minutes- 51,63,75,120,143,159,170,195,207,241,265,307,323,339,358,406 Rt:24.3Minutes- 50,63,75,85,102,143,159,170,195,207,241,277,307,323,339,358,406
ES1, 28days	1.Phenol,2,4-bis 1-methylproyl 2. Benzoic acid2-methoxy-3-4-	Rt:19.7Minutes- 57, 77,105,135,163,177,206. Rt:19.7Minutes- 59,77,91,119,147,207,245,277,308

	methoxy-2-methyl-4-oxobutanoyl-6-methyl-methyl ester 3. Endosulfan I 4.Endosulfan II	 Rt:20.4Minutes- 51,63,75,120,143,159,170,195,207,241,265,307,323,339,358,406 Rt:24.4Minutes- 50,63,75,85,102,143,159,170,195,207,241,277,307,323,339,358,406
ES2, 7days	1.Endosulfan I 2.Endosulfan II	Rt:22.2Minutes- 51,63,75,120,143,159,170,195,207,241,265,307,323,339,358,406 Rt:24.3Minutes- 50,63,75,85,102,143,159,170,195,207,241,277,307,323,339,358,406
ES2, 14days	1.5-hydroxy-2-methyl-3-hexanone 2. Endosulfan I 3. Endosulfan II	Rt:3.8Minutes- 71,86.92,102.97,156,241.12,280.66,340.98,430.15,504.10,564.22 Rt:22.22Minutes- 51,63,75,120,143,159,170,195,207,241,265,307,323,339,358,406 Rt:24.3Minutes- 50,63,75,85,102,143,159,170,195,207,241,277,307,323,339,358,406
ES2, 21days	1.Endosulfan I 2. Endosulfan II	Rt:22.22Minutes- 51,63,75,120,143,159,170,195,207,241,265,307,323,339,358,406 Rt:24.3Minutes- 50,63,75,85,102,143,159,170,195,207,241,277,307,323,339,358,406
ES2, 28days	1.Benzene,1,3,5-trimethyl- 2. Endosulfan I	Rt 3.8mis- 51,59,65,77,91,105,120 Rt:21.5Minutes- 51,63,75,120,143,159,170,195,207,241,265,307,323,339,358,406
ES3, 7days	1.Endosulfan I 2.Endosulfan II	Rt:22.2Minutes- 51,63,75,120,143,159,170,195,207,241,265,307,323,339,358,406 Rt:24.3Minutes- 50,63,75,85,102,143,159,170,195,207,241,277,307,323,339,358,406
ES3, 14days	1. Endosulfan I	Rt:20.4Minutes- 51,63,75,120,143,159,170,195,207,241,265,307,323,339,358,406
ES3, 21days	1.Benzenebutanoic acid α,α-dimethyloxo-	Rt: 3.4Minutes- 51,77,91,105,120,147,160,173,206
ES3, 28days	1. 3-hexanone,5-hydroxy-2-methyl- 2. Decane,5,6-bis92,2-dimethylpropylidene 3. Ethanol,2-(dodecycloxy) 4. 1,2-Benzene dicarboxylic acid,butyl2-ethylhexyl ester	Rt:3.71Minutes- 70.93,86.98,121,155.94,220.83,281.39,341.07,400.74,471.43,572.58 Rt:6.9Minutes- 55,69.03,97.04,111.05,125,196.11,266.99,355.22,428.60,526.90,573.5 Rt:7.08Minutes- 57.04,71.04,85.04,97.05,176.97,192.03,280.79,355.93,400.65,428.69, 574.8 Rt:9.53Minutes- 75.98,148.90,222.96,278.0,346.31,402.11,504.14,569.55

Table 3:MS results of metabolic end products derived from endosulfan under shaker condition

SAMPLE KEY	PRODUCTS	RETENTION TIME AND IONISATION PEAKS
ES1, 7days	1.Endosulfan I 2.Endosulfan II	Rt.22.2Minutes: 51,63,75,120,143,159,170,195,207,241,265,307,323,339,358,406 Rt. 24.3Minutes: 50,63,75,85,102,143,159,170,195,207,241,277,307,323,339,358,406
ES1, 14days	1.Phenol,2,4-bis(1,1 dimethylethyl) 2. .Endosulfan I 3. .Endosulfan II	Rt.13.8Minutes: 51,57,74,91,107,128,147,163,191,206 Rt.22.2Minutes: 51,63,75,120,143,159,170,195,207,241,265,307,323,339,358,406 Rt.24.3Minutes: 50,63,75,85,102,143,159,170,195,207,241,277,307,323,339,358,406
ES1, 21days	1.Phenol,2,4-bis(1,1 dimethylethyl) 2. .Endosulfan I 3. .Endosulfan II	Rt.13.8Minutes: 51,57,74,91,107,128,147,163,191,206 Rt.22.2Minutes: 51,63,75,120,143,159,170,195,207,241,265,307,323,339,358,406 Rt.24.3Minutes: 50,63,75,85,102,143,159,170,195,207,241,277,307,323,339,358,406
ES1, 28days	1.Phenol,2,4-bis 1-methylproyl 2. Benzoic acid2-methoxy-3-4-methoxy-2-methyl-4-oxobutanoyl-6-methyl-methyl ester 3.. Endosulfan I	Rt.19.7Minutes: 57, 77,105,135,163,177,206. Rt.19.7Minutes: 59,77,91,119,147,207,245,277,308 Rt.20.4Minutes: 51,63,75,120,143,159,170,195,207,241,265,307,323,339,358,406
ES2, 7days	1.Endosulfan I 2.Endosulfan II	Rt.22.2Minutes:51,63,75,120,143,159,170,195,207,241,265,307,323, 339,358,406 Rt..24.3Minutes: 50,63,75,85,102,143,159,170,195,207,241,277,307,323,339,358,406
ES2, 14days	1.Phenol,2,4-bis(1,1 dimethylethyl)- 2. .Endosulfan I 3. .Endosulfan II	Rt.13.8Minutes: 51,57,74,91,107,128,147,163,191,206 Rt.22.2Minutes: 51,63,75,120,143,159,170,195,207,241,265,307,323,339,358,406 Rt.24.3Minutes: 50,63,75,85,102,143,159,170,195,207,241,277,307,323,339,358,406
ES2, 21days	1.Phenol,2,4-bis(1,1 dimethylethyl)- 2. .Endosulfan I 3. .Endosulfan II	Rt.13.8Minutes: 51,57,74,91,107,128,147,163,191,206 Rt..22.2Minutes: 51,63,75,120,143,159,170,195,207,241,265,307,323,339,358,406 Rt.24.3Minutes: 50,63,75,85,102,143,159,170,195,207,241,277,307,323,339,358,406
ES2, 28days	1.Phenol,2,4-bis 1-methylproyl 2. Benzoic acid2-methoxy-3-4-methoxy-2-methyl-4-oxobutanoyl-6-methyl-methyl ester 3. Endosulfan I	Rt.19.7Minutes: 57, 77,105,135,163,177,206. Rt.19.7Minutes: 59,77,91,119,147,207,245,277,308 Rt.20.4Minutes: 51,63,75,120,143,159,170,195,207,241,265,307,323,339,358,406

ES3, 7days	1.Benzene-methanol,α,4-dimethyl- 2.Benzyl alcohol,2,3-dimethyl- 3.1-H-inden-1-one-2,3-dihydro- 4.Endouslufan I 5.Endosulfan II	Rt.4.1Minutes: 43,51,60,65,77,89,91,93,103,117,121,136 Rt.5.2Minutes: 51,65,77,91,118,136 Rt.5.6Minutes: 51,78, 104,132. Rt.18Minutes: 51,63,75,120,143,159,170,195,207,241,265,307,323,339,358,406 Rt.19.9Minutes: 50,63,75,85,102,143,159,170,195,207,241,277,307,323,339,358,406
ES3, 14days	1.Benzene methanol,α,4-dimethyl- 2. Endouslufan I 3.Endosulfan II	Rt.4.1Minutes: 43,51,60,65,77,89,91,93,103,117,121,136 Rt.18Minutes: 51,63,75,120,143,159,170,195,207,241,265,307,323,339,358,406 Rt.19.9Minutes: 50,63,75,85,102,143,159,170,195,207,241,277,307,323,339,358,406
ES3, 21days	1. 1-H-inden-1-one-2,3-dihydro- 2. Benzene-methanol,α,4-dimethyl- 3. Benzyl alcohol,2,3-dimethyl- 4. Endouslufan I	Rt.5.6Minutes: 51,78, 104,132. Rt.4.1Minutes: 43,51,60,65,77,89,91,93,103,117,121,136 Rt.5.2Minutes: 51,65,77,91,118,136 Rt.18Minutes: 51,63,75,120,143,159,170,195,207,241,265,307,323,339358,406
ES3, 28days	1. 3-hexanone,5-hydroxy-2-methyl- 2. Decane,5,6-bis2,2-di methylpropylidene 3. Ethanol,2-(dodecycloxy) 4. 1,2-Benzene dicarboxylic acid,butyl2-ethylhexyl ester	Rt.3.71Minutes: 70.93,86.98,121,155.94,220.83,281.39,341.07,400.74,471.43,572.58 Rt.6.9Minutes: 55,69.03,97.04,111.05,125.06,196.11,266.99,355.22,428.60,526.90,573.5 Rt.7.08Minutes: 57.04,71.04,85.04,97.05,176.97,192.03,280.79,355.93,400.65,528.69,574.80 Rt.9.53Minutes: 75.98,148.90,222.96,278.0,346.31,402.11,504.14,569.55

Summary and Conclusion:

From the recent study it is identified that endosulfan is capable of long range environmental transport since it is highly volatile and shows relative persistence in the atmosphere. The residues of endosulfan are potentially hazardous to the living system because of their inclination to bio accumulate in the lipid component of biological species and their resistance to degradation (Sarat and Barati, 2013). Endosulfan persists in the environment and bio-

accumulates in animals and plants, leading to instances of food contamination and eventually dietary exposure in humans. Even though the use of endosulfan is being banned, it is still used in large quantities especially in the developing countries like India.

Hence in the current study the main approach was to study the degradation ability of soil bacteria for endosulfan. The chromatography analysis helped in identifying the intermediate metabolites formed during endosulfan degradation by the bacterial isolates. The results showed absence of toxic endosulfan sulphate and formation of simpler benzene derivatives such as 1, 3, 5-trimethyl benzene suggesting that all three bacterial isolates were transforming the toxic endosulfan to comparatively less toxic molecules.Further studies may help in using the present data for creation of an important pesticide bioremediation system.

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