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Effect of tween 80 added to the soil on the degradation of endosulfan by *Pseudomonas aeruginosa*

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ABSTRACT: Endosulfan, a chlorinated cyclodiene insecticide is of environmental concern because of its apparent persistence and toxicity to many non target organisms. Endosulfan is hydrophobic and persists in soil for more than a year. To overcome the problem of hydrophobic and limited availability, surfactants play a major role in soil remediation. In the present study, the effect of Tween 80 added to the soil on the degradation of endosulfan by *Pseudomonas aeruginosa* at different pH (7.0 and 8.5) was studied. The addition of synthetic surfactant Tween 80 enhanced the solubility and degradation of endosulfan. A significant degradation (94%) was observed in pH 8.5 and Tween 80 added soil; the bacterial population in the treatment unit T8 was 75 x 10^9 CFU/g of soil. The unit T4 inoculated at pH 8.5 showed 86 % alpha and 60 % beta endosulfan degradation, the bacterial population was 73 x 10^8 CFU / g of soil. The degradation of both the isomers were observed and accompanied with formation of endodiol and endosulfan sulfate.

Key words: *Tween 80, pH, endosulfan, degradation, Pseudomonas aeruginosa*

INTRODUCTION

Endosulfan is a chlorinated cyclodiene insecticide currently used throughout the world for the control of numerous insects in a wide variety of food and non-food crops. Endosulfan has been ubiquitously detected in the atmosphere, soils, sediments, surface waters, rain-waters and foodstuffs (Kwon, *et al.*, 2002). Endosulfan comprises two parent isomers alpha and beta endosulfan and the alpha to beta ratio of technical endosulfan is about 7:3 and both isomers are extremely toxic to aqueous organisms. 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 non-toxic endosulfan diol respectively. (Baar and Aust, 1987). The problem of endosulfan bioremediation is poor solubility and restriction of appropriate biocatalyst. The synthetic surfactant Tween 80 was non-toxic to soil microorganisms and inert to the soil matrix and had the additional benefit of causing an enhanced dissolution rate for single compounds. (Angela link, 2000). Despite the many advantages of surfactin over chemical surfactants for bioremediation, its use has been limited on account of the high production cost. Bury and Miller (1993) found that uptake of micellar n-decane and n-tetradecane was stimulated by a biodegradable synthetic surfactant resulting in higher growth rates. This was probably caused by direct uptake of the hydrocarbons along with the micelles. Aronstein and Alexander (1993) reported that synthetic surfactant at low concentrations may be useful for bioremediation of sites contaminated with hydrophobic pollutants. Singer and Finnerty (1984) reported that the addition of Tween 80 stimulated utilization of hexadecane by several strains of *Pseudomonas aeruginosa*. Wong, *et al.*, 2004 reported that *Pseudomonas aeruginosa* when combined with Tween 80 effectively enhanced the solubility and degradation of phenanthrene and they also reported that Tween 80 is biodegradable. Volkering, *et al.*, (1995) reported that the oxygen uptake rate of bacterial cells was not affected in the presence of surfactants. They also reported that surfactant concentrations up to 10g/L can be used without any toxicity effect on microorganisms. Soil pH also play a vital role in the degradation process. Some of the bacteria favour a pH near neutral and some are highly tolerant to alkaline conditions. The environmental fate of endosulfan in soils is influenced

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by the pH, texture, moisture content and also by the presence of organic matter and co-pollutants. The rate of degradation is a function of the prevailing temperature, moisture regime, the content, type of organic matter and clays present (Kookana, *et al.*, 1998).

In the present study we have isolated an endosulfan degrader, *Pseudomonas aeruginosa* through intensive screening from endosulfan polluted soil samples. In addition, Tween 80 was added to the bacterial culture at different pH (7 and 8.5) conditions to enhance the bioavailability and complete elimination of endosulfan from the contaminated soil.

MATERIALS AND METHODS

Chemicals

Endosulfan standards (a-endosulfan, b-endosulfan and its metabolites) were purchased from M/s. Chem Service Inc., West Chester, USA with a purity of 98%. A standard and stock solutions were prepared with n-hexane. The stock solution (1000 mg/mL) of each compound and stored in a refrigerator at 4 °C. All stock solutions were prepared and stored at -20 °C. Commercial endosulfan was purchased from Jayaprakash Fertilizers Agency, Thiruvallur district. The non-ionic surfactant Tween 80 and 2-phenoxy ethanol were obtained from Sigma Chemical Co., USA. All other chemicals, solvents and reagents used in the study were of analytical grade.

Enrichment and isolation of bacterial strain

Two grams of soil samples was taken in a 250 mL Erlenmeyer flask containing 50 mL of liquid mineral medium (Ammonium chloride 1.0 g, Potassium dihydrogen orthophosphate 2.0g, Dipotassium hydrogen orthophosphate 7.5 g, Magnesium sulphate 0.2 g, Sodium chloride 0.5g, Calcium carbonate 0.2 g, Glucose 1.0 g, Distilled water 1.0 L, pH 7.8 and agar 15% was added to solid medium) with 50 µg/mL of endosulfan and was incubated for 7 days in a rotary shaker (IKA, Germany) at 130 rpm. Five millilitres of culture broth from individual flask culture was re-inoculated to 50 mL of endosulfan mineral salt medium and further cultured at 30 °C for 7 days. Enrichment of the culture was done by repeated transfers. Then 0.1 mL of culture broth was plated on solid endosulfan mineral salt medium for isolating single colonies. The single colonies were characterized and identified as a *Pseudomonas aeruginosa* using biochemical tests (Table 1). The culture was maintained on mineral agar slants.

Growth studies

A loopfull of *P.aeruginosa* grown on mineral agar slant was inoculated into 250 mL conical flasks containinig 50 mL medium amended with endosulfan as substrate. The flasks were kept for 24 h on an orbital rotary shaker set at 130 rpm at 30 °C for the growth of the organism. Growth was measured at 24 h as viable cell count as well as turbidity at 550 nm. The cells were harvested by centrifugation at 5000 g for 10 min. The cell pellet was washed twice in 15 mM phosphate buffer, pH 7. The washed cells were used for degradation study ($2x10^5$ CFU).

Soil

The soil selected for this study was collected from Thiruvallur district had no previous exposure to pesticides and was classified as sandy loam. The soil was air dried and sieved through 2 mm sieve. The carbon content of the soil was estimated using TOC analyzer (Analytic gena, micro C). The pH of the sample was estimated as per the methods of Trivedy and Goel (1986) pH of the soil was 8.1, organic carbon 0.12%. The portions of soil were adjusted to pH values of 7.0 and 8.5 by the addition of 2 NHCl or 1N NaOH. Adjustments to desired pH were made three times over 10 days till the pH of the soil were stabilized. Excess liquid from each soil was drained after pH adjustment and commercial endosulfan 35EC was added to give a concentration of 2.35 mg/g active ingredient (compound that kills or controls the target pest). After air drying for 24 - 48 h, the soil was pulverized and used for degradation studies.

Degradation of endosulfan in pH adjusted soils

For each pH adjusted soil, 4 sets were made containing 20 g soil in each set's and Tween 80 was added at CMC (A phenomenon unique to surfactants is the self-assembly of molecules into dynamic clusters called micelles. Micelle formation occurs above a critical concentration of surfactant monomers referred to as the Critical Micelle Concentration (Miller and Zhang, 1997). Three replicates were maintained for each set. Experimental details were presented in Table 2. All the sets were received 6 mL of mineral medium without glucose. Distilled water was added to compensate the evaporational loss of water and soil was kept at 30 °C for 8 weeks and 1.2 g samples were taken at weekly intervals to quantify the residual endosulfan present in each soil sample.

Bacterial count

During the degradation process, the bacterial population (plate count technique) was monitored at weekly intervals. One gram of soil was taken and incubated for 30 min with 100 mL of sterile water at 30 °C at 150 rpm. A 100 mL sample of appropriate dilution of the soil suspension was inoculated onto nutrient agar plates. The plates were incubated at 30°C for 24 h and the number of colonies were counted.

Extraction of residual endosulfan from the soil

Approximately 1.2 g of wet soil was removed from the different treatments and air dried. One gram of dried soil was transferred to a test tube and extracted with 3 mL of ethyl acetate by vortexing. The ethyl acetate layer was decanted after 5 min. This extraction was repeated five times. The ethyl acetate fractions were pooled, passed through anhydrous sodium sulfate (5 g) and florosil (1 g) mixture and evaporated at room temperature. (Awasthi, *et al.*, 2000).

Chromatographic analysis

Pesticide residue was dissolved in acetone and an aliquot containing 5-10 mg of endosulfan was spotted on a silica gel plate and the chromatogram was developed in hexane: chloroform: acetone (9:3:1). The separated spots were visualized by spraying the chromogenic reagent ($AgNO_3$ in 2-phenoxy ethanol) (Kovacs, 1965). For gas chromatography, the residual pesticide was dissolved in 1 mL of acetone, diluted to 10^6 times with n-hexane, and analysed as per the above said procedure. One microlitre from each of the final residue solutions was injected into the GC (Chemito) model 1000 chromatography equipped with a packed glass column (4'x1/8", filled with 60-80 mesh coated over chromosorb with a mixture of 1.5% OV-17 and 1.95% QF) with ECD (Electron Capture Detector) for residue analysis in an injector temperature of 220 °C, oven temperature 190 °C, detector temperature 280 °C and nitrogen as carrier gas (27 mL/min). The qualitative identification of the endosulfan present in the samples was performed by comparing the relative retention times (RRT) with respect to the standard, for each peak in the real sample chromotogram to those RRT in the standard mixture chromotogram. The quantitative determination was performed by using the relative peak areas (RPA) and the relative concentrations (RC).

Table 1: Biochemical test for *Pseudomonas aeruginosa*

Biochemical test	Results
Indole	Negative
Citrate utilisation	Utilised
T. S. Iron Agar	k / k
Fermentative / Oxidative (Hugh Leifson)	Oxidative fermentation
Oxidase	Positive

Table 2: Experimental set up

T1	Soil (pH 7) + Endosulfan
T2	Soil (pH 7) + Endosulfan + *Pseudomonas aeruginosa*
T3	Soil (pH 8.5) + Endosulfan
T4	Soil (pH 8.5) + Endosulfan + *Pseudomonas aeruginosa*
T5	Soil (pH 7) + Endosulfan + Tween 80 (0.1 g/L)
T6	Soil (pH 7) + Endosulfan + Tween 80 (0.1 g/L) + *Pseudomonas aeruginosa*
T7	Soil (pH 8.5) + Endosulfan + Tween 80 (0.1 g/L)
T8	Soil (pH 8.5) + Endosulfan + Tween 80 (0.1 g/L) + *Pseudomonas aeruginosa*

Statistical analysis

The values from triplicate samples were analyzed using analysis of variance (ANOVA), and Fisher's LSD was used to compare means. *P* values less than 0.05 were considered significant.

RESULTS

Microbial methods to remediate hydrophobic organochlorines contaminated soils are often limited by low substrate solubilities which can reduce bioavailability to the degrading microorganisms. (Atlas and Cerniglia, 1995) Use of Tween 80 as a means of increased bioavailability of hydrophobic endosulfan to microorganisms under different pH (7.0 and 8.5) was studied.

Effect of pH on endosoulfan degradation

Treatment unit T2 at pH 7 showed 47% of alpha and 31% beta degradation, the bacterial population was 45 x 10^7 CFU/g of soil. The control unit T1 recorded 15% of alpha and 13% of beta degradation; the bacterial population was 73 x 10^5 CFU/g of soil (Fig. 1).

The unit T4 inoculated at pH 8.5 showed 86 % alpha and 60 % beta endosulfan degradation, the bacterial population was 73 x 10^8 CFU / g of soil. The control unit T3 (pH 8.5) showed 20 % of alpha endosulfan and 19% of beta endosulfan degradation where the bacterial population was 43 x 10^6 CFU/g of soil (Fig. 2). A significant degradation was observed at pH 8.5. The maximum degradation at pH 8.5 is mainly due to increased growth (73 x 10^8 CFU / g of soil) and activity of the bacteria in alkaline conditions.

This could be due to better bioavailability of endosulfan and optimal biotic activity of cells at this pH. At 8th. week of incubation showed faster disappearance of both the isomers with the formation of metabolites. TLC studies revealed the formation of two metabolites such as endosulfan diol and endosulfan sulfate at pH 7.0 and 8.5 (Fig. 3). Endosulfan was subjected to degradation by oxidation and hydrolysis conclusively, endosulfan sulfate formation was found to be favoured as oxidative production and a novel hydrolysis product was identified as endosulfan diol.

Effect of Tween 80 on endosulfan degradation

The unit T6 (pH 7 with Tween 80) recorded 62% of alpha and 58% of beta endosulfan degradation. The bacterial population in the soil was 36 x 10^8 CFU/g of soil. The control unit (T5) showed 12% of alpha endosulfan and 11 % of beta endosulfan degradation, the bacterial population in the soil was 5 x 10^6 CFU/g of soil (Fig. 4). When compared to Tween 80 with pH 7 and 8.5, pH 8.5 with Tween 80 recorded maximum significant degradation of endosulfan isomers (94% of alpha and 84% of beta), the bacterial population in the treatment unit T8 was 75 x 10^9 CFU/g of soil. The control unit (T7) showed 18% of alpha and 13% of beta endosulfan degradation. The cell growth was increased to 10x10^6 CFU/g of soil (Fig. 5). The unit T8 (*Pseudomonas aeruginosa* with Tween 80), showed optimum the degradation of endosulfan isomers. The release of endosulfan, which is a hydrophobic compound was dramatically enhanced by the aqueous concentration of Tween 80. Tween 80 might have reduced the interfacial tension of the soil organic matter and water, allowing to utilize the endosulfan by the microorganisms.

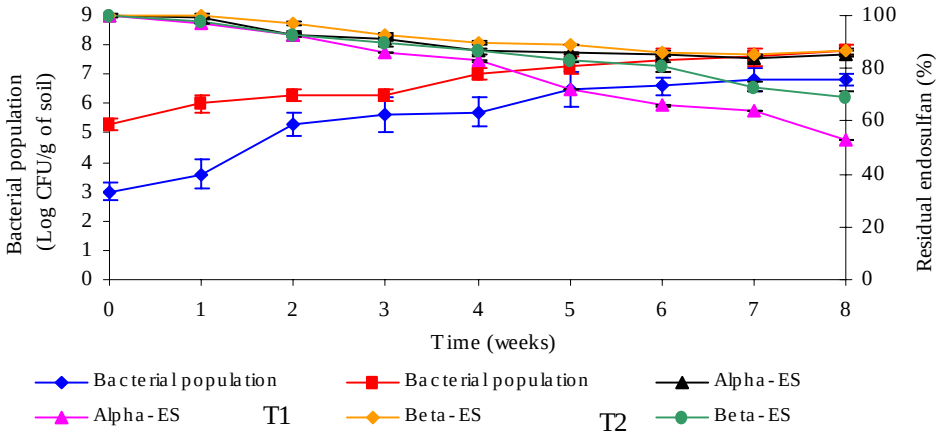

Fig. 1: Degradation of endosulfan (ES) at pH 7

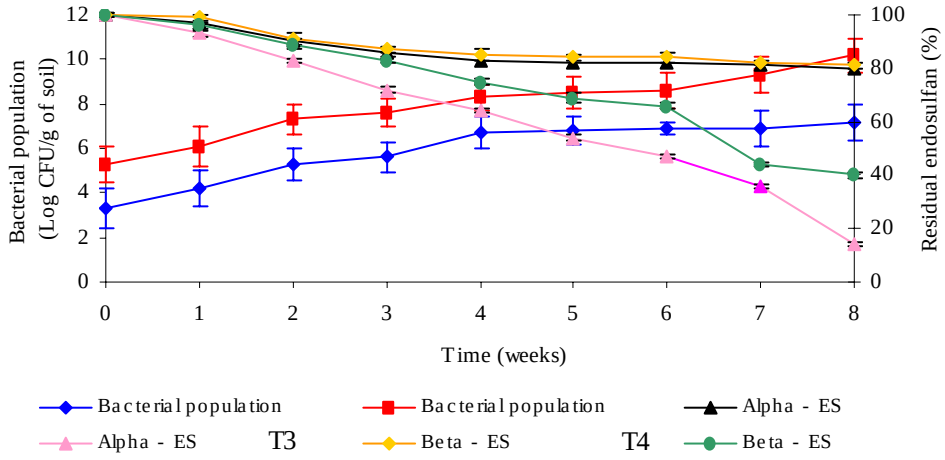

Fig. 2: Degradation of endosulfan (ES) at pH 8.5

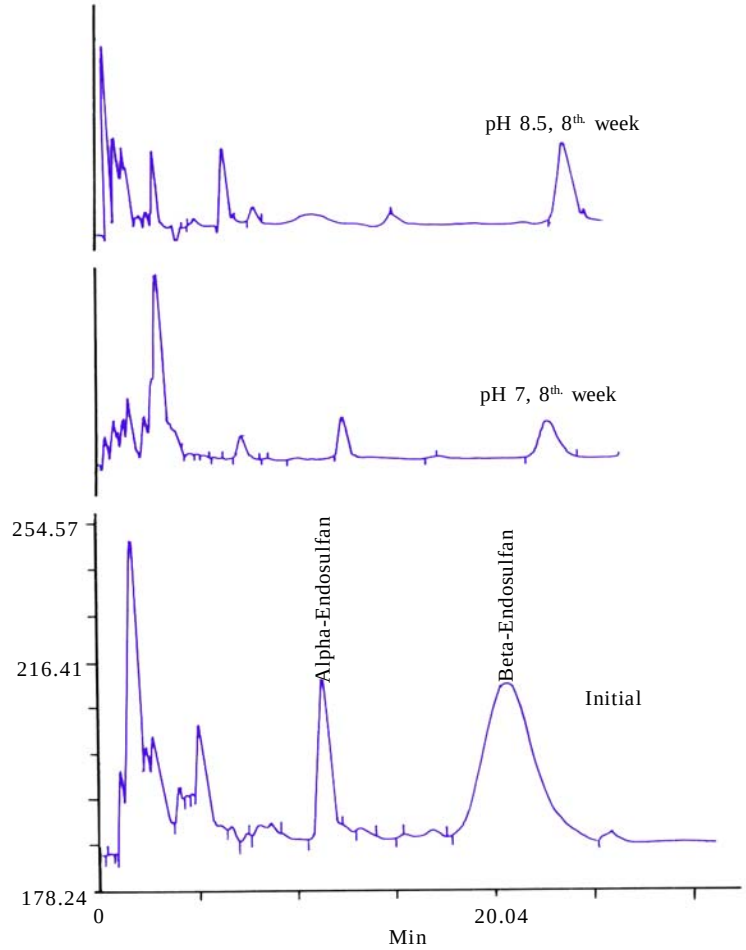

Fig. 3: Gas chromatographic profile of endosulfan degradation at pH 7.0 and 8.5

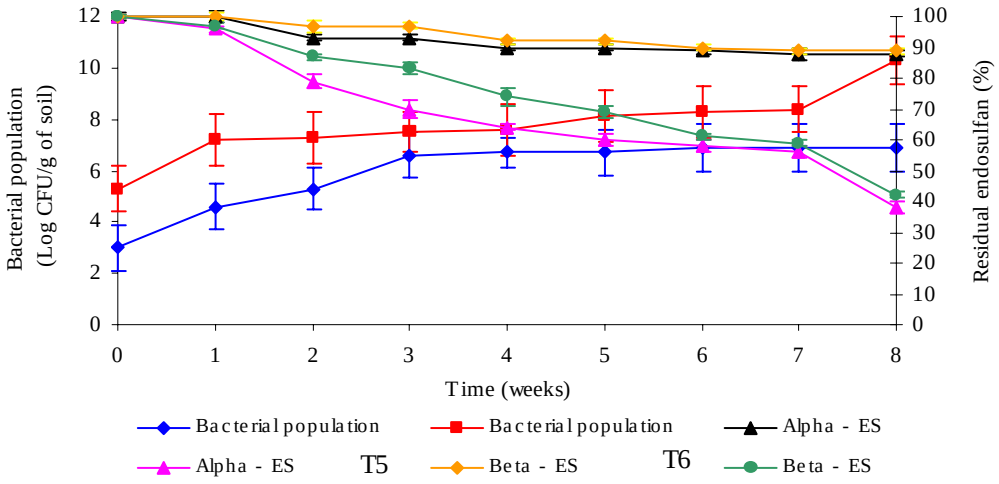

Fig. 4: Degradation of endosulfan (ES) with Tween 80 at pH 7

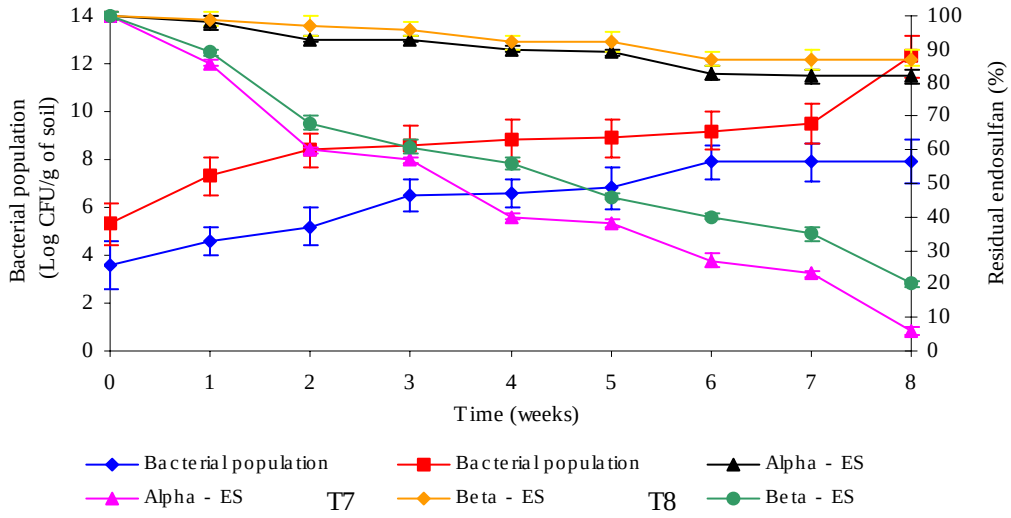

Fig. 5: Degradation of endosulfan (ES) with Tween 80 at pH 8.5

DISCUSSION AND CONCLUSION

The maximum degradation at pH 8.5 is mainly due to increased growth and activity of the bacteria in alkaline conditions. (Martens, 1976; Sutherland, *et al.*, 2002; Awasthi, *et al.*, 2002). This could be due to better bioavailability of endosulfan and optimal biotic activity of cells at this pH. Similarly Shalini Singh, *et al.*, (2000) reported that the degradation rate of endosulfan is dependent on the pH of the soil. When compared to acidic, alkaline conditions favor the maximum degradation. The breakdown product, endosulfan sulfate is more persistent than the parent compound, accounting for 90% of the residue in 11 weeks. Kumar and Philip (2006) isolated the highly efficient endosulfan degrading bacterial strains from soil. A total of 29 bacterial strains were isolated through enrichment technique from 15 specific sites using endosulfan as sole sulfur source. The three bacterial strains, *Pseudomonas spinosa, P. aeruginosa, and Burkholderia cepacia,* were the most efficient degraders of both alpha- and beta-endosulfan as they consumed more than 90% of the spiked amount. Maximum biodegradation by these three selected efficient bacterial strains was observed at an initial pH of 8.0 and at an incubation temperature of 30 ° C.

Kwon, *et al.*, (2002) reported that the endosulfan diol is the major metabolite as pH increases, while endosulfan sulfate is the major metabolite as pH decreases. Kumar, *et al.*, (2007) studied the degradation of endosulfan by a mixed culture isolated from a pesticide-contaminated soil was studied in batch experiments. After two weeks of incubation, the mixed culture was able to degrade 73% and 81% of alpha and beta endosulfan respectively. Two cultures identified by 16S rRNA as *Stenotrophomonas maltophilia* and *Rhodococcus erythropolis* were found to be responsible for majority of the degradation by the mixed culture. *S. maltophilia* showed better degradation efficiency compared to that by *R. erythropolis*.

Lee, *et al.*, (2006) isolated a bacterium through repetitive enrichment and successive subculture using endosulfan or endosulfan sulfate as the sole carbon source, The KS-2P was identified as *Pseudomonas sp.* on the basis of the results of a 16S rDNA sequencing analysis and MIDI test. The degradation ratios for endosulfan or endosulfan sulfate in minimal medium containing endosulfan (23.5 mug/mL) or endosulfan sulfate (21 mug/mL) were 52% and 71%, respectively. The results suggest that *Pseudomonas sp.* KS-2P has potential as a biocatalyst for endosulfan bioremediation.

Hussain, *et al.*, (2007) reported that the alpha endosulfan degraded more readily than beta endosulfan and endosulfan sulfate. The half life of alpha-endosulfan, beta-endosulfan and endosulphan sulfate was found to be 136.8, 273 and 301 days in sterilized Alfisol and 55, 256 and 277 days in non-sterilized Alfisol respectively. Shivaramaiah and Kennedy (2006) isolated three novel bacterial species, namely, *Staphylococcus sp.*, *Bacillus circulans*-I, and *Bacillus circulans*-II, from contaminated soil collected from the premises of

a pesticide manufacturing industry. Pure culture studies were revealed that the *Staphylococcus sp*. utilized more beta endosulfan compared to alpha endosulfan, whereas *Bacillus circulans*-I and *Bacillus circulans*-II utilized more alpha endosulfan compared to beta endosulfan.

The addition of Tween 80 (0.1g/L) in the treatment unit T8 might have emulsified the endosulfan, thereby increasing the amount of insecticide in contact with the soil bacteria. Surfactant even at very low concentration was shown to enhance the biodegradation of certain xenobiotics in soil (Haigh and Alcock, 1996). Aronstein and Alexander (1993) showed that nonionic surfactant (Novel 1111412-56) at 10 μg/mL added to the surface of lime silt loam soil enhanced the biodegradation of phenanthrene and biphenyl. The degradation of endosulfan predominantly describes the formation of endodiol and endosulfan sulfate. During the degradation process, hydrolysis takes place and endosulfan converted into endodiol. The endodiol is the major metabolite as pH increases. The formation of endodiol is the initial step in the degradation of endosulfan, further it may be converted into some other form. Endosulfan sulfate was the major metabolite at lower pH (pH 7). The metabolites Endosulfan sulfate formed indicated that the organism follows an oxidative pathway for metabolism of this pesticide (Kumar and Philip, 2006). Endosulfan sulfate appeared to be a terminal pathway product. Most of the research has concentrated on the degradation of endosulfan in liquid medium. (Sethunathan, *et al.*, 2002) A few studies carried out on the degradation of endosulfan in soil have been without surfactant amendment. (Trivedy and Goel,1986). This study proves that *Pseudomonas aeruginosa* combined with Tween 80 is able to achieve 94% degradation of endosulfan in contaminated soil. Hence, surfactant enhanced degradation studies is a promising approach for remediating endosulfan contaminated soils.

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