Combined Bioaugmentation and Biostimulation - To Cleanup Endosulfan Contaminated Soil

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ABSTRACT— This study investigated bioaugmentation and biostimulation separately and at combine status as a strategy for removing soil bound endosulfan. The performance of each treatment was examined by monitoring estimation of free chlorides, endosulfan degradation by HPLC and its effects on soil functionality by monitoring enzyme activities.Combination of bioaugmentation with biostimulation (*Aspergillus niger* ARIFCC 1053 and 1% glucose) was found to be the most efficient treatment strategy resulting in undetectable levels of endosulfan in just 11 days. Change in pH and increase in released chlorides demonstrate microbial transformation of endosulfan. In treatment 4 pH dropped from 6.9 ± 0.05 to 4.2 ± 0.05 while no significant change in soil pH with treatment 1,2 and 3. The released chlorides increased in accordance with endosulfan degradation in all four treatments but the rate of increase was higher in treatment 4. In treatment 4, endosulfan was at undetectable level on 11thday and in treatment. Increase in SDA and ARSA was not significant with separate supplementation. However, a combined treatment with *A, niger* ARIFCC 1053 and glucose proved to be the most efficient in increasing SDA and ARSA among all study. Increase in enzyme activities suggests the soil fertility and possible involvement of these enzymes in endosulfan degradation. Therefore, a combination of bioaugmentation and biostimulation can be used to develop a realistic technology for treatment of endosulfan contaminated soils

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1. INTRODUCTION

Ever increasing world population is continuously putting tremendous pressure on agriculture for fulfilling its basic needs like food, clothes and shelter. This always demands the increase in agriculture productivity, leaving no choice to the farmers but to use synthetic agrochemicals (fertilizers and pesticides). In anticipation of increased productivity million of tons of pesticides are applied annually in modern agriculture to increase the production through controlling harmful effects caused by the target organisms including insects, fungi, bacteria, viruses as well as grasses grown in between the economical crops (Liu and Xiong, 2001). The extensive utilization of synthetic pesticides and/ or agrochemicals is relatively in the accumulation of the residues of these toxic chemicals in soil. These accumulated residues demonstrate the overall quality of soil and the surrounding environment as well. Besides this Pesticide-formulating industries are also contaminating the environment through various activities (Qureshi *et.al*. 2009). Pesticide exposure inflicts chronic and acute threats to human health those are long term low dose exposure to pesticide causes immune suppression, hormonal disruption, diminished intelligence, reproductive abnormalities and carcinoma (Gupta, 2004).

Soil bioremediation is an option that offers the possibility to degrade or render various contaminants harmless using natural biological activity. Apparently, it is relatively cost effective and ecofriendly, techniques, which generally have a high public acceptance and can easily and repeatedly often be carried out on site. Two important approaches of bioremediation are bioaugmentation and biostimulation. Bioaugmentation is the inoculation of contaminated soil, sediment or sludge with isolated microorganisms or their consortia with specific organic compound degrading capabilities to enhance *in-situ* or *ex-situ* bioremediation applications where as biostimulation involves identifying and adjusting certain physical and chemical factors (such as soil temperature, pH, moisture content, nutrient content etc) that may be impeding the rate of biodegradation of the contaminants by the indigenous microorganisms in the affected site (Abdulsalam *et.al*., 2011).

Chlorinated organic pesticides are one of the major groups of chemicals, responsible for environmental pollution. Endosulfan (6,7,8,9,10,10-Hexa chloro – 1,5,5a, 6,9,9a – Hexa hydro – 6,9 – Methano – 2,4,3 – Benzodioxathiepine 3 - Oxide) is a broad-spectrum insecticide, extensively used in most of the world to control the insect pests of a wide range of crops. Endosulfan presents risk for water (Tapsoba and Bonzi, 2006) and soil (Savadogo *et al.* 2006) pollution. It can bind to soil particles and persist for a relatively long period with half-life of 60-800 days depending on the type of soil

(Siddique *et al.* 2003 Tariq *et al*., 2006). Generation of endosulfan sulfate is the major concern of endosulfan degradation research, as this metabolite is more toxic, persists longer in soils and has bioaccumulation potential (Sutherland *et al*., 2002). In past investigations the microbial degradation of endosulfan have revealed various intermediates of metabolism including endosulfan-sulfate, -diol, -ether, -lactone, -hydroxyether and -dialdehyde (Martens 1976; Kwon et al., 2005). When developing bioremediation method, it is important to select the microorganisms that degrade organic pollutants and to identify their metabolic products. The present study evaluated different bioaugmentation and biostimulation treatments for endosulfan degradation. The effects of this treatment on soil properties were also evaluated.

2. MATERIAL AND METHODS

Chemicals

Technical grade endosulfan, a 35% emulsified preparation (Excel Industries Ltd., Mumbai, India) was used for the experiments. Standards endosulfan sulfate was obtained from Sigma-Aldrich, USA. All other reagents were of high purity and analytical grade.

Microorganisms

Fungal culture *Aspergillus niger* (*A. niger)* ARIFCC 1053 capable of degrading 350 mg l^1 endosulfan, isolated during previous studies was used in present study (Bhalerao and Puranik, 2007). The culture was maintained on agar slopes of the modified Czapek's-Dox medium containing sucrose, 30 g; $NaNO_3$, 2 g; KCl, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; glucose, 10 g; $FeCl_3$, 10 mg; endosulfan, 0.5 g; and agar, 12 g l^1 distilled water at pH 6.8.

Experimental design

Black cotton soil collected locally (Jalgaon district, Maharashtra, India) with undetectable level of endosulfan was used for present study. The physico–chemical characteristics of the soil were texture clay loam, pH 6.9, Electrical conductivity 0.5 m mhos, organic matter 2.56%, Total nitrogen 0.08 % and maximum water holding capacity 0.42 ml g^{-1}. Finely sieved (24 mesh size) 200 g soil was moisten and taken in each pot for further experiments. The experimental design consisted of four pots in three replicated pots. Each pot was amended with 350 mg g^{-1} (w/w) endosulfan. Control 1 and 2 were run with and without endosulfan for comparison. The following treatments were carried out for experimentation.

Treatment 1 - Biostimulation with 1% yeast extract

Treatment 2 - Biostimulation with 1% glucose.

Treatment 3- Combined bioaugmentation and biostimulation with 20 ml of spore suspension (10^{-8} spores ml^{-1}) of fungal culture *A. niger* ARIFCC 1053 and 1% yeast extract.

Treatment 4 - Combined bioaugmentation and biostimulation with 20 ml of spore suspension (10^{-8} spores ml^{-1}) of fungal culture *A. niger* ARIFCC 1053 and 1% glucose.

All pots were watered daily to maintain moisture.

Endosulfan degradation studies

The samples were taken from each pot at every alternate day up to the process of complete degradation of endosulfan and processed for estimation of released chlorides.

The released chloride was estimated by mercuric thiocyanate method determined by (Bergmann and Sanik 1957). The standard graph of sodium chloride stock solution (0.014 N) was prepared by using series of standards to plot the concentration of released chloride during endosulfan degradation by *A. niger*. All experiments were performed in triplicates.

3. ANALYSIS OF ENDOSULFAN DEGRADATION BY HPLC

Five gram of the soil sample was withdrawn on every alternate day and residual endosulfan was extracted by shaking soil sample along with 5ml acetonitrile for 1 h using a shaker at 150 rpm. Acetonitrile fractions from each flask were pooled and aliquots were analyzed by HPLC for quantitative determination during degradation. HPLC analysis of samples was performed with LC-MAD pump and UV detector (Chemito LC 6600 series model, Japan).The analytical column C18 was used and mobile phase consisted of 70% acetonitrile (v/v) with a flow rate of 1 ml/ min. Twenty micro liters of the final extract was injected and UV absorption at 214 nm was recorded (Lee *et.al*., 2006).

4. MONITORING OF SOIL ACTIVITIES DURING ENDOSULFAN DEGRADATION

Soil deydrogenase activity (SDA)

Dehydrogenase activity was measured by triphenyl formazan (TPF) method (Casida *et al*., 1964). One gram of soil was taken in a screw cap vial and added with 3.0 ml of 2, 3, 5, triphenyl tetrazolium chloride solution (3% w/v) and 1.0 ml of distilled water forming a thin layer of water above the surface of soil. The tubes were incubated in dark at 30 ± 1^0C for 24 h. After appropriate incubation (24 h), 25 ml of methanol was added in each tube, mixed on a vortex and left to stand for some time. Then the mixture was filtered through Whatman filter paper (No. 42) and optical density of the filtrate was measured spectrophotometrically at 485 nm. Dehydrogenase activity was expressed in terms of triphenyl formazon (TPF) produced day^{-1} g^{-1} of soil with reference to a standard curve of TPF.

Aryl sulphatase activity (ARSA)

Assay of aryl sulphatase involved the colorimetric estimation of the p-nitro phenol released by sulphatase activity when soil was incubated with buffered (pH 5.8) sodium-p-nitrophenyl sulphate (PNS) solution and toluene at 37^{o}C for 1 h as described by Tabatabai and Bremner (1970). 4 ml of modified universal buffer (pH 5.8), 0.25 ml of toluene and 1 ml of PNS solution were added to one gram soil in 50ml Erlenmeyer flask. After mixing the contents, the flasks were stoppered and incubated at 37^0 C. After 1 h incubation, 1 ml of 0.5 M CaCl2 and 4 ml of 0.5 M NaOH were added to the reaction mixture. The flasks were swirled for few seconds and filtered through Whatman filter paper No. 42. The color intensity of the filtrate was spectrophotometrically measured at 420 nm. The standard curve was drawn with a range of 0–50 µg of PNS. Sulphatase activity was expressed in terms of µg of p nitrophenol released hr^{-1} g^{-1} of soil. All the experiments in the study were carried out in triplicate and the results are means of the three.

5. RESULTS AND DISCUSSION

Released chloride during endosulfan degradation

Chloride content was increased with endosulfan degradation in all four treatments. In combined treatment with glucose (treatment 4) chloride released was ranged between 14 µg ml^{-1} to110 µg ml^{-1} within fifteen days. The rate of released chloride was comparatively slower in both separate biostimulation treatments (Fig 1). In combined treatment with yeast extract (treatment 3) the rate of released chloride was quite slower as compare to combined treatment of glucose (treatment 4). Verma et.al. (2006) and Bhalerao (2012) demonstrated that the significant increase of free chloride from soil amended with endosulfan clearly indicates degradation of endosulfan. The released chlorides increased in accordance with endosulfan degradation in all four treatments but the rate of increase was higher in treatment 4 (A. *niger* and glucose). In both control, there was no significant change in released chlorides up to 15^{th} days.

Endosulfan degradation in soil as a function of different treatments

A HPLC method was established for endosulfan detection during the course of its degradation in soil. In all four treatments endosulfan was gradually decreased with time. The rate of endosulfan degradation varied with different treatments. Detection of endosulfan at regular intervals (every alternate day) using HPLC confirmed the degradation ability of different treatments (Fig 2). Highest degradation in shorter period was observed in treatment 4. On 7^{th}day 93 to17% endosulfan was remained in all three treatments. Endosulfan was remained detectable up to 15 days in treatment 1 2, and 3. In treatment 4, endosulfan was at undetectable level on 11^{th}day. Treatment 4 proved most effective and taking shorter period for endosulfan degradation, it is just 11 days. Arshad *et.al.* (2007) mentioned degradation of soil bounded endosulfan by *Pseudomonas aeruginosa* after 16 days, Goswami and sigh (2009) reported degradation 80% and 86% of α and β endosulfan in soil within 18 days. Mathava Kumar and Ligy Philip (2006) demonstrated 71.58±0.2% and 75.88±0.2% of endosulfan degradation after 3 weeks of incubation with mixed bacterial culture in aerobic and facultative anaerobic conditions via batch experiment in soil. As compare to these earlier reports the period of 11 days for degradation of soil bounded endosulfan was the shortest period by treatment 4 (*A. niger* and 1% glucose).

In present study we observed slower degradation of soil bound endosulfan than in culture medium. The complete degradation of endosulfan in soil was achieved in treatment 4 within 11days.While our earlier study reported that complete degradation of endosulfan (350mg l^{-1}) was occurred within five days in broth (Bhalerao and Puranik 2007). Awasthi *et. al*., (1997) also suggested the degradation of soil bound endosulfan was slower by nearly fourfold than in culture medium. The degradation of soil bound endosulfan was slow; the reason may be due to the adsorption of endosulfan to soil particles or because of the presence of other carbonaceous materials in the soil (Sutherland et.al. 2000). Soil bound endosulfan is associated with generation of toxic metabolite endosulfan sulfate which may perhaps is more toxic and more persistent than endosulfan itself. Endosulfan sulfate an intermediate metabolite was detected after two days in all four treatments. In treatment 1, 2 and 3 it was not degraded up to fifteen days (TLC data is not shown here).Endosulfan sulfate was degraded after seven days in treatment 4. In control 2 with endosulfan, there is a formation of endosulfan sulfate. This indicates that living organisms in the soil may be necessary to bring about the oxidation of

endosulfan-to-endosulfan sulfate (Schmidt *et.al*. 1997). Disappearance of endosulfan sulfate after seven days may be because of the fact that the direct desulfurization of endosulfan sulfate (Sutherland et al., 2002) or a novel pathway could be the mechanism of degradation of endosulfan and endosulfan sulfate in *A. niger* (Bhalerao and Puranik, 2007).

Endosulfan was degraded fast in treatment 4 (*A.niger* ARIFCC 1053 and 1% glucose), it could degrade 98±0.5% soil bound endosulfan in 11 days. Glucose seemed to have more synergistic effect on degradation of endosulfan than yeast extract in pot assay. The similar observation was also indicated by Gao *et al*. (1997) that the glucose is water soluble, easily metabolizable and non-toxic, It is therefore believed that this stimulant could serve as safer and more acceptable. In contrast Awasthi *et al*., (1997) observed that the presence of dextrose affects the degradation of endosulfan. The results of treatment 4 are in agreement with the study of Olaniran *et al*., (2000). It demonstrates significant increase in degradation of dichloroethane after using combination of bioaugmentation and biostimulation. Clausen *et.al*., (2002) also suggest that the additional energy source citrate or succinate has enhanced the degradation of pesticide by *Pseudomonas sp*. However, many studies have reported that the addition of auxiliary carbon to the system having xenobiotic compounds increased the biodegradation potential of bacterial and fungal cultures which is often because of increase in metabolic activity of the microbes involved (Kumar and Philip, 2006).

Soil deydrogenase activity (SDA) and Aryl sulphatase activity (ARSA)

Change in SDA as a function of endosulfan degradation in all four treatments and two controls were investigated on every alternate day up to 15th day. A comparative analysis of supplementation with yeast extract and glucose separately and in combination proved that the increase in SDA was not significant with separate supplementation. However, a combined treatment with *A, niger* ARIFCC 1053 and glucose proved to be the most efficient in increasing SDA among all study (Fig 3). On 1st day of treatment 1, SDA was 242 ± 1.0 µg g^{-1} which further increase to 310 ± 1.0 µg g^{-1}on 15thday. In treatment 2 which consisted of incorporation of glucose (1%) the SDA was 240 ± 0.5 µg g^{-1} which reached to 322 ± 0.5 µg g^{-1} on 15th day. No significant difference in activities of treatment 1 and 2. A combined treatment of yeast extract and *A.niger* ARIFCC 1053 presented an increase in SDA which was highest at 374 ± 1.1µg g^{-1} on 15th day of analysis. Combination of *A. niger* ARIFCC 1053 and 1% glucose (Treatment 4) showed 240 ± 1.0 µg g^{-1} SDA on day first of analysis. The increase was slow up to 7th day which was observed to be accelerated. The highest enzyme activity was 450 ± 1.1 µg g^{-1} on last day of analysis.

ARSA was gradually increased in all four treatments with time. ARSA was comparatively lower in treatment 1, 2 and 3 than treatment 4. The same trend was followed in ARSA by SDA (Fig 4). On 1st day in treatment 1 the activity was 1.1 ± 0.05µg PNPg^{-1} which was further increased up to 1.9 ± 0.05µg PNP g^{-1} on 15th day. The first day activity was 1.1 ± 0.05 µg PNP g^{-1} in treatment 2 and in treatment 3 ARSA activities was 1.2 ±0.05µg PNP g^{-1}. On 15th day the activity reached at 2.0 ± 0.1µg PNP g^{-1} and 3.3± 0.05µg PNP g^{-1} in treatment 2 and 3 respectively. On 15th day the ARSA was 4.4 0.1 µg PNP g^{-1} in treatment 4 (Fig 3). The highest activity was observed in treatment 4 followed by treatment 3. In control 1 all parameters were shown no more difference within 15 days and in control 2 both activities were primarily suppressed and did not recover up to 15 days.

The soil dehydrogenase (SDA) and aryl sulphatase (ARS) activities followed the increasing rate with endosulfan degradation in all four treatments. But rate of increase was higher in treatment 4 as compare to other three treatments. These enzyme activities were investigated to evaluate the efficiency of the *A. niger* ARIFCC 1053 along with microbial population in utilizing organic matter and effects of various treatments. Increase in SDA activity shows the possibility of active utilization (metabolism) of compound by microbes either as a nutrient source or for the detoxification of the compound. These results confirmed the findings of previous study (Kalyani *et.al*. 2010). Increase in SDA is the presence of viable cells and it is a useful indicator of overall microbial activity in soil (Trevors 1984; Wei-xiang *et al*., 2004). Increase in the ARSA suggests the mineralization of ester sulfate in soils (Tabatabai, 1994). All these activities indicate possible involvement of these enzymes in the endosulfan degradation and showing positive effect on soil functionality.

6. CONCLUSION

Overall results of this study demonstrate that it is possible to achieve effective biodegradation of soil bound endosulfan just in 11 days by combined application of bioaugmentation and biostimulation. All analyzed parameters were proved the active participation of *A. niger* ARIFCC 1053, soil microorganisms and of biostimulation in endosulfan degradation. Change in pH and free released chlorides indicate microbial transformation of endosulfan. Enzyme activities are an indicator of soil fertility and suggest favorable effect on soil functionality. Hence, combined treatment is a promising approach for remediation of endosulfan contaminated soils in shorter period. The future study is to isolate extracellular enzymes from *A. niger* and develop the strategy of cell-free enzymes to remove organoclorine pesticide endosulfan from soil and water.

7. REFERENCES

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Captions of figures

Figure no 1

Treatment 1: Biostimulation with Yeast extract.
Treatment 2: Biostimulation with glucose.
Treatment 3: Combined Bioaugmentation and Biostimulation with *A. niger* and yeast extract.
Treatment 4: Combined Bioaugmentation and Biostimulation with *A. niger* and glucose.

Figure no 2

Treatment 1: Biostimulation with yeast extract
Treatment 2: Biostimulation with glucose
Treatment 3: Combined Bioaugmentation and Biostimulation with *A. niger* and yeast extract
Treatment 4: Combined Bioaugmentation and Biostimulation with *A. niger* and glucose

Figure no 3

Treatment 1: Biostimulation with yeast extract
Treatment 2: Biostimulation with glucose
Treatment 3: Combined Bioaugmentation and Biostimulation with *A. niger* and glucose
Treatment 4: Combined Bioaugmentation and Biostimulation with *A. niger* and yeast extract

Figure no 4

Treatment 1: Biostimulation with yeast extract
Treatment 2: Biostimulation with glucose
Treatment 3: Combined Bioaugmentation and Biostimulation with *A. niger* and glucose
Treatment 4: Combined Bioaugmentation and Biostimulation with *A. niger* and yeast extract

Fig 1: Free released chlorides during endosulfan degradation under treatment 1 to 4

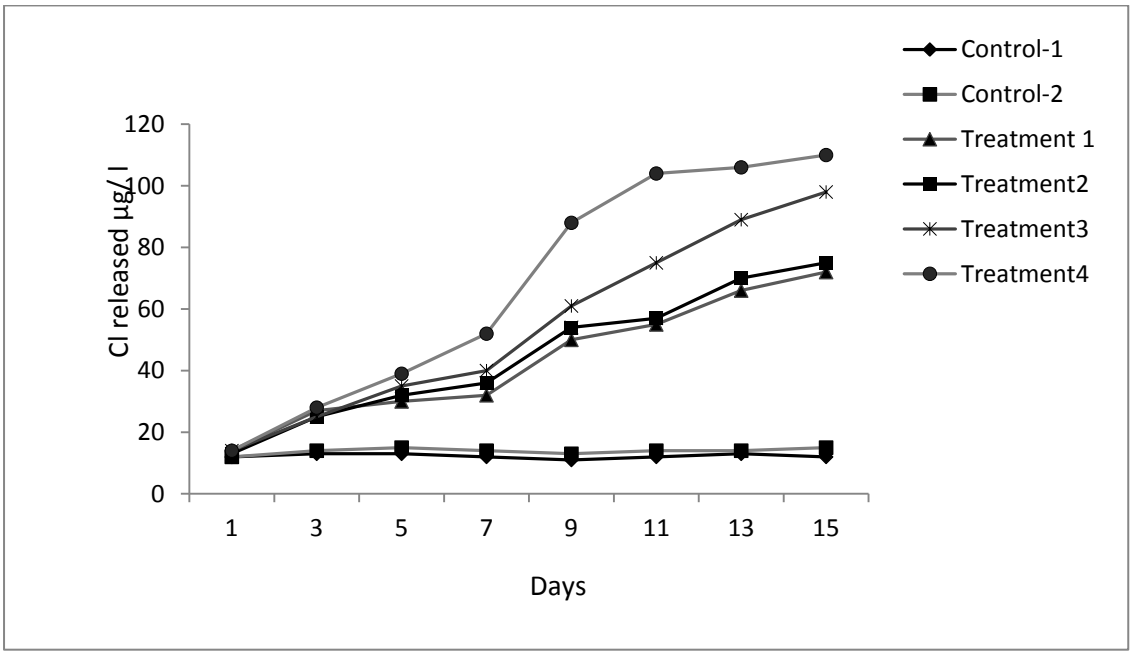

Fig 2: Effect of Treatment 1, 2, 3, and 4 on endosulfan degradation

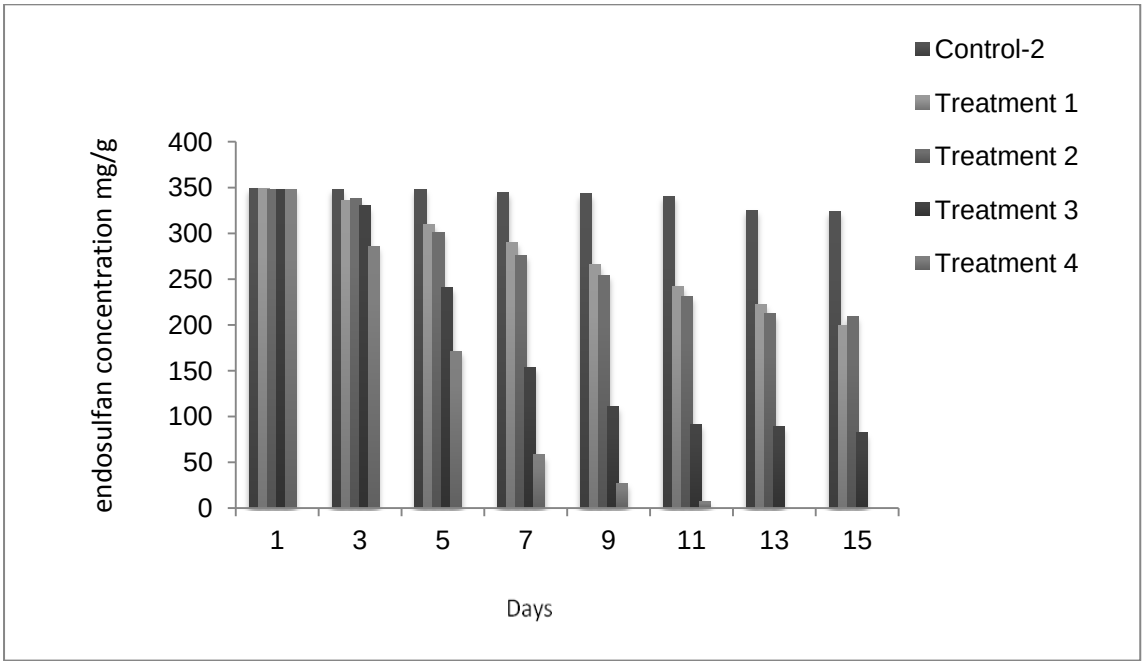

Fig 3: Dehydrogenase activity (SDA) during endosulfan degradation under treatment 1 to 4

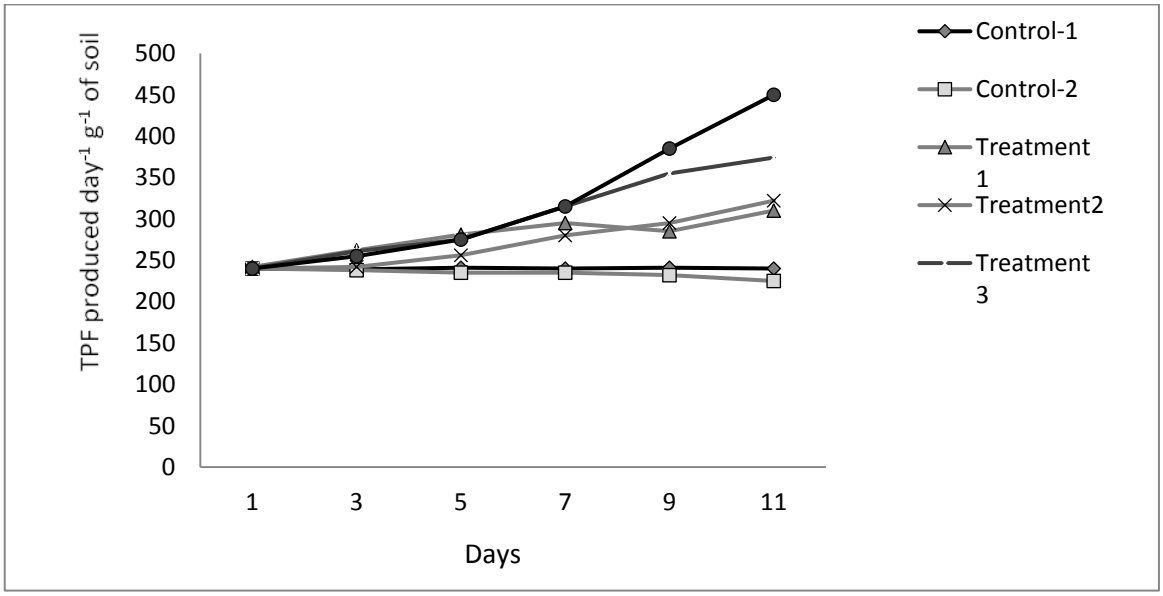

Fig 3: aryl Sulphatase activity (ARS) during endosulfan degradation under treatment 1 to 4