MICROBIAL DEGRADATION OF ORGANOPHOSPHATE COMPOUND ENDOSULFAN BY SOIL BACTERIUM ISOLATES ED-P1

Prafulla Katkar[1] and Sanjeev Patankar[2]
1. Guru Nanak College of Science Ballarpur, Dist-Chandrapur, M.S. India-442701
Email-psk.zeal@gmail.com
2. SSJ college, Arjuni (Morgaon), Dist-Gondia M.S. India-441701

Abstract

The remediation of insecticides is acquiring a considerable attention, particularly since the extensive characterization of enzymes capable of detoxifying a range of organophosphate compounds. The basis of investigations for enzyme capable of detoxifying other classes of insecticides requires a source of enzyme for catalytic detoxification. This study describes the enrichment of a culture of soil bacteria capable of degrading endosulfan. However endosulfan has relatively reactive cyclic sulfite diester group70. In this study, microorganisms were selected for their ability to release the carbon group from endosulfan and to use this as source of carbon for the growth. The enrichment was achieved and maintained by providing endosulfan as the only carbon source. Endosulfan is poor biological energy source, as it contains only six potential reducing electrons and previous attempts to enrich for endosulfan degrading microorganism using the insecticide as sulfur source have been not very successful. An essential step in the investigation of an enzymatic method for endosulfan degradation is the definitive identification of a biological source of endosulfan degrading activity. This selection procedure enriches for a culture capable of either the direct hydrolysis of endosulfan or the oxidation of the insecticide followed by its hydrolysis. In particular, enzymatic insecticide bioremediation is the focus of extensive study after the isolation of enzymes capable of detoxifying a range of organophosphate compounds from several bacterial species.

Keywords: Endosulfan, Microorganisms, Gas chromatography, Degradation, Oxidation

Introduction

There are varieties of soil microorganism that have ability to degrade the endosulfan. The degradation of endosulfan by soil microorganism of family Pseudomonas Sp. was studied. In microbial degradation of endosulfan under aerobic condition, soil microorganism degrades the endosulfan and yielded the endosulfan sulphate (30-60%), with some endodiol (2.6%) and endolactone (1.2%). As with the most pesticides, the persistence of and degradation of endosulfan are affected by the environmental conditions in which it is found. Endosulfan does not undergo direct photolysis but is transformed by the chemical hydrolysis under alkaline condition such as in sea water (Armburst 1992). In soil, endosulfan has been shown to be degraded by a variety of microorganisms **(**Katayama *et al* 1991**)**. However degradation rates are usually low and metabolism often results in the formation of endosulfan sulfate, an oxidative metabolite shown to be equally as toxic and persistence as the parent compound, endosulfan. Because of its persistence and toxicity, endosulfan contamination poses a significant environmental concern. Microorganisms have increasingly been investigated as a source of xenobiotics-degrading enzymes (Chen *et al* 1998). We are interested in the isolation of endosulfan degrading bacterium for further investigation into enzymatic endosulfan bioremediation. The parenthetical numbers refers to the percentage of the applied endosulfan recovered as a metabolite. Sixteen

of 28 fungi, fifteen of 49 soil bacteria and three of 10 actinomycetes metabolized greater than 30% of the applied C-14 endosulfan. Endosulfan sulphate was the major metabolite formed by the fungi and endodiol was the predominant product of the bacteria (Maier-Bode 1968). Since the removal of carbon moiety dramatically decrease the vertebrate toxicity of endosulfan (Stewart *et al* 1974), this results in concurrent detoxification of the insecticide. Results suggest that while both isomers can be degraded by microbial organisms, the degradation materials released counteract the growth of the microorganisms. Only a small amount of C-14 labeled carbon dioxide was detected, indicating minimal mineralization. Using endosulfan as the only available carbon source, we can enrich soil inocula for microorganisms capable of releasing the sulfur from the endosulfan, thereby providing a source of carbon for growth (Wegman et al 1978).

We report here on the resultant bacterial culture that, the culture degrades endosulfan to produce a novel metabolite to occur as a result of chemical hydrolysis. These results suggest that the obtained bacterial isolates at optimized growth condition are a potential source of an enzymatic bioremediating agent. In this study, microorganisms were selected for their ability to release the carbon group from endosulfan and to use this as source of carbon for the growth. In this work we have studied the different process optimization parameters to obtain the maximum degradation.

Material and Methods

1. Materials and reagents

Technical grade endosulfan was supplied from Department of microbiology Guru Nanak College of science, Ballarpur (M.S.). Technical grade endosulfan (used commercially) is a mixture of two diastereomers, alpha - endosulfan and beta - endosulfan in a ratio of 7:3, hexane (HPLC grade), and acetone. Standard chemical were used for the preparation of nutrient media. For the chemical and instrumental analysis, spectrophotometric grade chemical were used.

2. Sample collection for isolation studies

The soil sample for the enrichment and the isolation of the microorganisms was collected from the cotton field near Gadchandur (M.S) India at the end of growing seasons. The field had generally received several application of endosulfan in the month of September to October for at least 2-3 times. The soil was fertile gray. The top soil collected from the upper layer (approximately 15 cm) and stored at 4ºC prior to the experimental studies.

3. Nutrient media for the enrichment of microorganisms

The endosulfan enrichment media for the isolation of microorganisms was prepared by the addition of following component (gm/lit). This media is actually a basal medium containing the endosulfan as a carbon source (Katkar *et al* 2015). KH_2PO_4-0.5, K_2HPO_4-0.5, NaCl-0.5, $MgSO_4$ $7H_2O$-0.5, $FeSO_4$ $7H_2O$, $CaCl_2$-0.002, $NaMI_4$-0.001, $CoNO_3$-0.0005, $ZnSO_4$-0.0005, $MnSO_4$-0.0005, Endosulphan-0.001, pH-7.2

4. Isolation of endosulfan degrading microorganisms

For the isolation of endosulfan degrading microorganisms, soil perfusion apparatus was designed. This work on the air pressure created by the vacuum. The small holes were made at the top and sand pebbles were kept over it for the support and slow perfusion of the soil sample to the medium which is kept at the bottom. The tap water is open to create air pressure; this air pressure is helpful for the aeration to the medium. The soil moistens with the media and perfused to the medium at the bottom. This process recycles continuously and microorganisms present in the soil enriched into the media. The endosulfan enrichment medium was added to the bottom. The sand pebbles were kept over the holes at the top. The fertile gray soil (approximately 10gm), and then the tap water is open such that the medium rises above the soil and soil sample slowly perfused to the medium. The apparatus were kept run for the 10 days. After the 10 days of incubation, the small alliqout of enriched soil inoculum were plated over the endosulfan enrichment agar. The different population of microorganisms on the endosulfan enrichment agar then achieved.

6. Identification of endosulfan degrading microorganisms

For the identification of single strain of isolates ED-P1 following microscopic, morphological and biochemical studies were been carried out.

6.1. Microscopic Studies

Microscopic details of the isolate ED-P1 have been done. The given isolates are whether Gram positive or Gram negative also been decided.

6.2. Morphological studies

Under the morphological studies, the various colonies characteristics like, shape color and growth pattern have been studied.

6.3. Biochemical studies

Following various biochemical tests have been carried out for each isolates; Indole, Methyl Red, Voges Prausker's and Citrate utilization test, Catalase test, Starch utilization test, Oxidase test, Nitrate reduction test, Urease test.

6.4. Sugar Fermentation Test

For the sugar fermentation test 0.5% NaCl. 0.5% peptone and 0.5% of the sugars were been added and incubated with the given isolate ED-P1. The tubes were observed for the production of acid gas after 24 hours.

7. Analytical Method

7.1 Optimization of bacterial density

Optical densities at λ600 of the endosulfan enrichment media incubated with the given isolates ED-P1 were measured to assess the relationship between growth and metabolic activities of microorganisms, the bacterial growth of the isolate ED-P1 were observed in response to endosulfan supplied as the source of carbon. The optical density of each isolates was measured with the interim of two days by the visible spectrophotometer and respective readings were recorded.

7.2 Optimization of pH of the Medium

The pH of the endosulfan enrichment media was measured in the order to assess the relationship between growth and metabolic activities of the microorganism. The change in the pH of the endosulfan enrichment media with interim of two days were recorded during the 10 days of incubation. The initial pH of the media was adjusted to 7.2.

7.3 Extraction of Endosulfan from the Media

Endosulfan was extracted from the enrichment media for the degradation studies. Approximately 25 ml culture media sample were taken out from the soil perfusion apparatus and equal volume of acetone (i.e. 25 ml) were added. The acetone - sample mixtures were shaken for 1 hr on the magnetic stirrer. 1ml of the mixture were taken out and transferred to 9 ml of hexane. These mixtures were then further shaken for 15 min **(**Siddique *et al* 2003). The sample was dehydrated by the addition of Na_2SO_4. The sample is then store in vials at 4ºC for the further analysis.

7.4 Quantitative Estimation of Endosulfan by Gas Chromatography

The quantitative analysis of endosulfan and its metabolite was done by gas chromatography-chemito model 1000 GC equipped with electron capture detector by using a glass column (8 inches length X 0.25 inch diameter). Nitrogen was used as carrier gas at the flow rate of 1.5 ml / min. The injected volume of sample in GC was 2 µl. The extracted endosulfan sample were been analyzed by Insecticide Residue Testing Laboratory, Nagpur.

Experimental results and discussion

1. Microscopic and Morphological characters

The isolate ED-P1 showed Gram Negative rod shaped cells arranged mostly separated. The colonies on the endosulphan enrichment media were red/pink colored, moist, pleomorphic with round shape.

2. Biochemical Test

The results of all biochemical test performed with isolate ED-P1 are given bellow

Sr. No.	Name of The Test	Inference
1.	Indole Test	Negative
2.	Methyl Red Test	Negative
3.	Voges Proskauer's Test	Negative
4.	Citrate Utilization Test	Positive
5.	Starch Hydrolysis Test	Negative
6.	Oxidase Test	Positive
7.	Catalase Test	Positive
8.	Urease Test	Negative
9.	Nitrate Reduction Test	Positive
10.	Gelatin Hydrolysis Test	Positive

Table No.1 – Biochemical characterization of the isolate ED-P1

3. Sugar Fermentation Test

The result of sugar fermentation test of isolate ED-P1 is given in the table below. From the result, it has been observed that all the isolate utilize the carbohydrate as a source of carbon and energy through enzymatic breakdown producing acid and gas (Sonnenwirth *et al* 1973).

Sr. No.	Sugars	Acid	Gas
1.	Glucose	-	-
2.	Manitol	+	+
3.	Lactose	-	-
4.	Maltose	-	-
5.	Ribose	-	-
6.	Sucrose	+	-
7.	Xylose	-	-
8.	Arabinose	-	-
9.	Mellibiose		-
10.	Raffinose	+	-
11.	Tetrahalose	-	-
12.	Cellobiose	+	

Table No.2 – Sugar fermentation test of isolate ED-P1

4. Identification of isolated strain of bacteria

From of the results of microscopic, morphological and biochemical test, the isolate ED-P1 has been identified as *Pseudomonas sp.* The obtained result were studied and compared with standard results of respective bacteria (Hugh *et al* 1973).

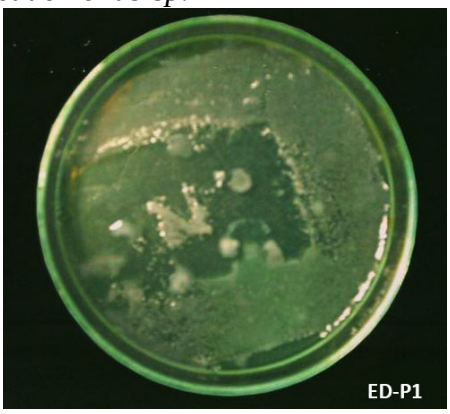

Fig. 1 – Pure culture of bacterial isolate ED-P1 grown on endosulphan enrichment media isolated from mixed culture

5. Measurement of bacterial density

Optical densities (λ600) of the respective isolate are represented in the figure.2. The highest OD_{600} recorded for ED-P1 was 0.33. As per the result, it has been found that the bacterial strain degrading more endosulfan within the culture media showed higher bacterial density.

Siddique *et al* (2003) was observed the same in that bacterial strain that depleted α and β endosulfan as a sulphur source. Sutherland *et al* (2000) and Awasthi *et al* (1997) who observed the substantial disappearance of the endosulfan with the simultaneous increase in the bacterial mass.

Bacterial density obtained with the isolate ED-P1 are quite higher in comparison to Kwon G. S. et al (2005) who were worked with Klebsiella Oxytoca. The utilization of endosulfan was accompanied by the increase optical density (OD595) of the culture media ranging from 0.51 to 0.89 as observed by Hussain et al (2007).

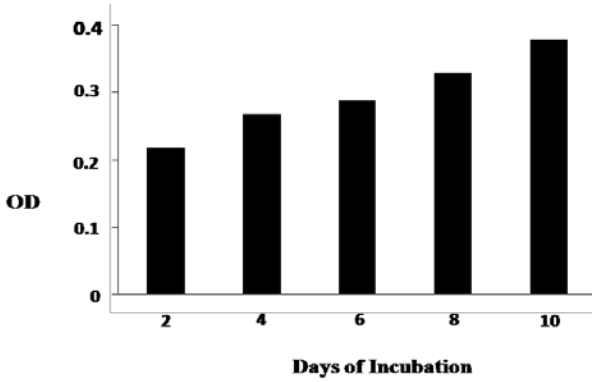

Fig. 2 - Variation in the O.D $_{600}$ of bacterial culture ED-P1 after 10 days of incubation.

6. Measurement of pH of medium

The change in the pH of the endosulfan enrichment media after 10 days of incubation is shown in the figure 3. The culture pH decreased to acidic range due to metabolic activities of the growing organism. The isolate ED-P1 showed the decreased pH of the medium to 3.79 after the 10 days of incubation. The results are very much similar to the work of Siddique *et al* (2003). It has been observed that the decreased in the pH of the medium was found to be associated with enhanced degradation of the endosulfan. With the interim of two day during each pH reading, pH decreased with the bacterial metabolism. The decrease in the pH may either be due to the dehalogenation of endosulfan resulting in the formation of the organic acid produced by microorganism during their metabolic activities.

Martens R. *et al* (1976) were observed that some of the bacteria which showed the pH value of 8.3 and 8.5 at the end of the experiment. This higher pH value was probably due to the chemical hydrolysis but some of the bacteria were having the low pH values which indicate that a large portion of degradation was enzymatic. Endosulfan is susceptible to alkaline hydrolysis occurring with approximately 10 fold increased in hydrolysis with each increased in pH unit. Many previous studies have been unable to differentiate between chemical and biological hydrolysis of endosulfan because microbial growth has led to the increased in alkalinity of the culture media (Guerin *et al* 1992).

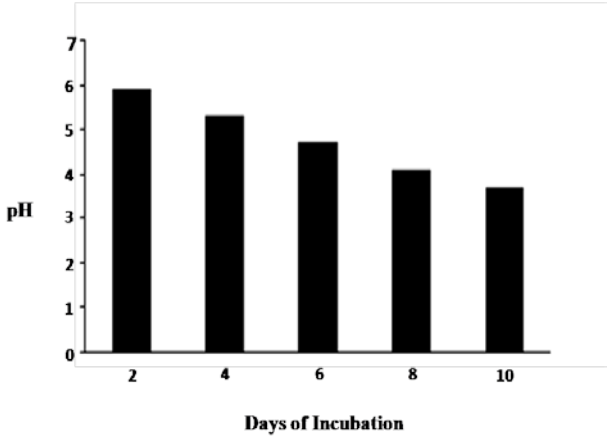

Fig. 3 - Variation in the pH of bacterial culture ED-P1 after 10 days of incubation.

7. Degradation of Endosulfan by the Bacterial Isolate ED-P1

The degradation of endosulfan was been confirmed by analyzing the sample by gas chromatography as shown in the figure 4. The degradation was determined by monitoring endosulfan disappearance by GLC-ECD detection. The bacterial isolate ED-P1 degraded 91.7% (0.083 ppm) endosulfan after the 10 days of incubation. The initial concentration of endosulfan in the culture media was 1 ppm. The isolate ED-P1 degraded 86% (0.140 ppm) of α-endosulfan and 97.2% (0.028ppm) of β-endosulfan. The degradation of β-endosulfan was found to be higher than that of α-endosulfan by ED-P1 isolates. The result of this study

suggests that the ED-P1 isolate are a valuable source of potent endosulfan degrading enzymes for use in enzymatic biodegradation.

The endosulfan was used separately as a carbon source to identify which microorganism prefers endosulfan as a carbon source and to what extent endosulfan is degraded when used as carbon source. The obtained results are much similar to findings of Siddique *et al* **(**2003) who had worked on *Fusarium ventricosum* which degraded α-endosulfan upto 82.2% and 89.0% of β-endosulfan when endosulfan supplied as carbon source. The bacterium *Pseudomonas Spinosa* and *Pseudomonas aeruginosa* were the most efficient degraders of both α-endosulfan and β-endosulfan as they consumed more than 90% of endosulfan (Hussain *et al* 2007), (Katkar *et al* 2018).

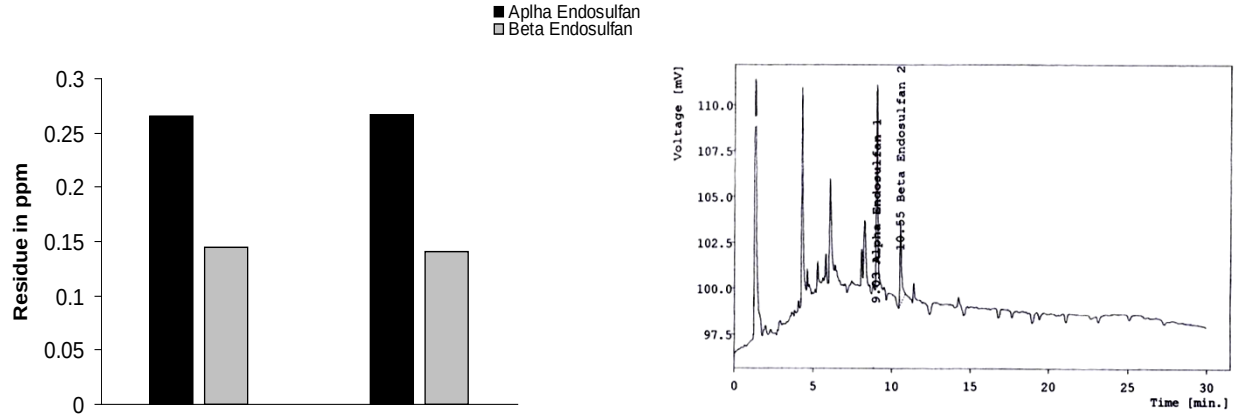

Figure 4- Gas chromatographic analysis of endosulphan degradation by isolate ED-P1.

8. Conclusion

Microorganisms have increasingly been investigated as a source of xenobiotics-degrading enzymes. We are interested in the isolation of endosulfan degrading bacterium for further investigation into enzymatic endosulfan bioremediation. Using endosulfan as the only available carbon source, we can enrich soil inocula for microorganisms capable of releasing the sulfur from the endosulfan, thereby providing a source of carbon for growth. Since the removal of carbon moiety dramatically decrease the vertebrate toxicity of endosulfan, this results in concurrent detoxification of the insecticide. We report here on the resultant bacterial culture that, the culture degrades endosulfan to produce a novel metabolite not reported to occur as a result of chemical hydrolysis. These results suggest that the obtained bacterial isolates are a potential source of an enzymatic bioremediating agent.

We are currently attempting to isolate a pure bacterium from the soil that is capable of detoxifying endosulfan. Such a bacterium would potentially be a valuable source of catalytic enzymes for the development of bioremediating agent to reduce endosulfan residue problems in run-off from irrigation waters.

9. References

1. Armburst, K. I. (1992). Fate of endosulfan, chlorthal-dimethyl and dichloran in the marine environ. PhD thesis. University of California, Davis.

2. Awasthi, N., Manickam, N., and Kumar, A., (1997). Biodegradation of the endosulfan by bacterial coculture. *Bull. Environ. Contam. Toxicol*. 59: 928-934.

3. Chen, W., and Mulchandani, A. (1998). The use of live biocatalyst for pesticide detoxifiacation. *Trends Biotechnol.* 16: 71-76.

4. Guerin, T. F. and Kennedy, I. R. (1992). Distribution and dissipation of endosulfan and related cyclodiens in sterile aqueous systems: Implication for studies on biodegradation. *J. Agric. Food. Chem.* 40: 2315-2323.

5. Hugh A and culardi, In: Lennette, Spaulding and Traunt (eds): *Manual of chemical microbiology* (2nd ed.) 1974.

6. Hussain, S., Arshad, M., Saleem, M., Khalid, A. (2007). Biodegradation of alpha and beta endosulfan by soil bacteria. *Biodegradation*. Jan 25.

7. Katayama, A. and Mastumura. (1991). Photochemical enhanced microbial degradation of environmental pollutants. *Environ. Sci. Technol*. 25: 1329-1353.

8. Katkar, P.S. Khobragade, H. and Patankar, S.D. (2015). Enrichment, isolation and identification of endosulfan degrading

microorganisms. *Int. J. res. In Biosci. Agri. And Technol*. Special Issue 89-94.

9. Katkar, P.S. and Patankar, S.D. (2018). Degradation of endosulphan by soil bacterium isolate ED-P1. *International Journal of Life Science*. Special Issue A12: March 2018 89-94.

10. Kwon, G. S., Sohn H. Y., Shin, K. S., Kim, E., Seo, B. I. (2005). Biodegradation of the organochlorin insecticide endosulfan and toxic metabolite endosulfan sulfate, by Klebsiella Oxytoca KE-8. *J. appl. Microbial Biotechnol.* 67 (6): 845-850.

11. Martens, R. (1976). Degradation of [8, 9-14C] Endosulfan by soil microorganisms. *App. Env. Microbio*. 31(6): 853-858.

12. Maier-Bode, H. (1968) Properties, effect, residues and analytics of the insecticide endosulfan. *Residue Rev*., 22: 1-44.

13. Siddiqui, T, Okeke, B. C., Arshad, M., and Franknberger, W. T. Jr. (2003). Enrichment and isolation of endosulfan microorganism. *Journal of Env. Quality*. 47-54.

14. Sonnenwirth, A. C. (1973). Data on enterobacteriacae from differentiation of enterobacteriacae by biochemical tests USPHS centre for disease control Atlanta.

15. Stewart, D. K. R., and Cairns, K. G. (1974). Endosulfan persistence in soil and uptake. *J. Agric. Food Chem*. 22: 984-986.

16. Sutherland, T, Herne, I, Lacey, M., Harcourt, R., Russel, R., and Okeshott, G. (2000). Enrichment of endosulfan degrading mixed bacterial culture. *Appl. Env. Microbiology*. 66 (7): 2822-2828.

17. Wegman, R. C. C. and Greve, P. A. (1978). Organochlorine, cholinesterase inhibitors and aromatic amines in Dutch water samples, Sept. 1969-Dec. 1975. *Pestic. Monit. J.,* 149-162.