IRIS
Journal for Young Scientists
ISSN 2278 – 618X **(Print)**
ISSN 2278 – 6384 **(Online)**

http://www.patnawomenscollege.in/journal

In vitro degradation of endosulfan by soil fungi

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Received : November 2011
Accepted : March 2012
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Abstract : *Endosulfan, a chlorinated cyclodiene insecticide is of environmental concern because of its apparent persistence and toxicity. This preliminary study was conducted to isolate efficient endosulfan degrading fungal strains from soil having history of repeated endosulfan exposure. Czapeks.dox nutrient media amended with endosulfan (100mg/L) was used for the isolation of fungi from the contaminated soil. Among the isolated fungal strains, four efficient fungal isolates were selected for the analysis of endosulfan degradation. The selected fungal isolates were identified as Aspergillus spp. and yeast-like organisms. Biodegradation of endosulfan by soil fungi was accompanied by a substantial decrease in pH of broth from 7.0 to 1.4. Effect of agitation on biodegradation was also studied. Degradation under agitation conditions was found to be more faster than that under the static conditions. Endosulfan and its metabolites were analyzed through thin layer chromatography. The major metabolites detected were endosulfan diol, endosulfan lactone and endosulfan ether. Interestingly, endosulfan sulfate; a persistent and toxic metabolite was not detected.*

Key words: *Biodegradation, soil fungi, endosulfan.*

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Introduction :

Endosulfan (bicyclo-[2.2.1]-2-heptene 5,6,-bioxy methylene sulfite) is a broad spectrum insecticide used throughout the world for the control of numerous insects in a wide variety of food and non food crops. The technical grade endosulfan is a mixture of two stereoisomers i.e. alpha and beta endosulfan in the ratio 7:3. The b-isomer is more persistant than a-isomer (Kumar and Philip 2006). As endosulfan is widely used in agriculture, its residues have been detected in the atmosphere, soil, sediment, surface and rain waters and food. Its persistence in soil and water environment has been widely reported by different researchers under different conditions. Fungi play

a key role in the conversion of cyclodiene insecticides in soil to non toxic products. The present study was aimed to isolate and characterize soil fungi capable of degrading endosulfan. The metabolites of endosulfan were examined using Thin Layer Chromatography. Degradation products included endosulfan lactone, endosulfan monoaldehyde and endosulfan diol.

Materials and Methods :

Four soil samples collected from different sites, having a history of repeated endosulfan exposure, were used in this study for the isolation of endosulfan degrading fungi. The soil was collected from a depth of 10-15 cm. The samples were air-dried, ground, sieved and stored at 4°C for further use.

Reagents and chemicals :

Endosulfan (35% EC) was procured from Excel Industries Limited, Bombay. Stock solution (0.3 mg/ ml) of the insecticide was prepared in sterile distilled water. The other chemicals and solvents used were of highest purity grade and purchased from the commercial sources.

Isolation of the fungal strains :

The enrichment culture technique was used for the isolation of fungal strains from the soil. The enrichment was done by incorporating the insecticide as the sole source of carbon in Czapeks-dox nutrient medium (sodium nitrate-3g; potassium chloride -0.5 g; Dipotassium hydrogen phosphate-1 g; magnesium sulfate-0.5 g; ferrous sulfate – 0.01 g and distilled water -1 L). The pH of the medium was adjusted to 7.0. The 250 ml Erlenmeyer flasks were autoclaved for 20 min at 121°C. Endosulfan was added aseptically to each flask to attain a final concentration of 100 mg/L. Microbial inocula was prepared by shaking 10 g of soil in 100 ml of distilled water. The inoculum was serially diluted to a concentration of 0.1 mg/ml and 1 ml of it was used for inoculation. The inoculated flasks were incubated for 5 days at 28±2°C on a Rivotek shaker incubator (100 rpm).

Purification and screening of fungal isolates:

The solid medium was prepared by adding 2% agar to the enrichment medium followed by autoclaving at 121°C for 20 min. Endosulfan was added to attain a final concentration of 100 mgL^{-1}. The molten endosulfan agar was poured out into petridishes and the culture plated on the solidified agar plates were incubated under aerobic conditions at 28±2 °C for 48 hours. Discrete colonies of fungi showing prolific growth were isolated. Isolates were purified further by streaking on fresh endosulfan amended agar plates. The selected fungal isolates were preserved on agar slants containing nutrient culture medium.

Characterization of selected fungal isolates:

Morphological and microscopic characteristics of the selected fungal isolates were noted. For identification, the techniques as described by Gilman (1975) and Onions et al (1981) were used.

Biodegradation of endosulfan by selected fungal isolates :

Four fungal isolates showing good growth on agar medium were investigated for their capability to degrade endosulfan between 3 and 9 days of incubation. For this purpose, Erlenmeyer flasks (250 ml) were taken for each fungal strain. The flasks contained 50 ml of Czapeks-dox nutrient medium amended with endosulfan to a concentration of 100 mg/l. Inoculum consisting of myecilial disc of 6 mm diameter were obtained from a 3 day old culture medium amended with endosulfan. These were aseptically transferred to each flask. The pH of the medium was adjusted to 7.0. Control flasks were also maintained. The flasks were incubated on orbital shaker incubator at

28±2°C temperature and 100 rpm. To minimize the error, the study was carried out in triplicate. After 3 days of incubation, contents of one set of flask marked for 3 days incubation were filtered in sterile vials using sterile Whatman filter paper no.1. Likewise, filtration was done for flasks after 6 days and 9 days of incubation period. The collected filtrates were stored in refrigerator for further analysis.

Effect of agitation on biodegradation of endosulfan by selected fungal isolates :

To assess the effect of agitation on biodegradation of endosulfan by the selected fungal strains, one set of flasks were incubated on orbital shaker incubator at 28±2°C temperature and 100 revolutions min^{-1} and another was incubated with similar parameters of temperature under stationary condition.

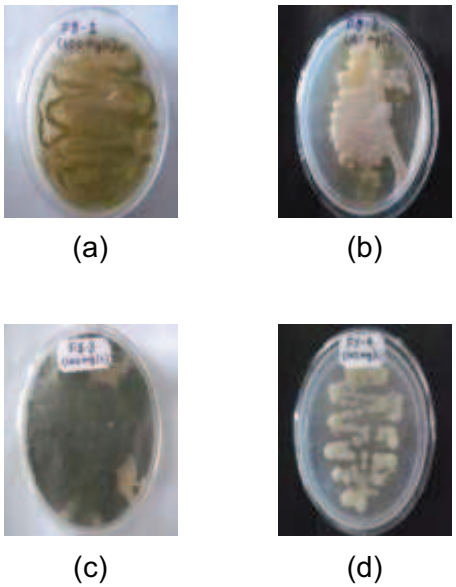
Analytical procedure for detection of endosulfan degradation :

Endosulfan degradation was observed by Thin Layer Chromatography. Different fractions were examined by using 0.25 mm silica G plates. Hexane, chloroform, acetone (9:3:1 v/v/v) was used as the mobile phase. The TLC plates were sprayed with ortho-toludine (3% solution in acetone) and exposed to sunlight for 15 minutes for the detection of endosulfan and its metabolites. The filtrates obtained for each isolate after 3, 6 and 9 days of incubation and pure Endosulfan, were run on the TLC plates. Rf value of endosulfan metabolites were calculated and compared with standard Rf values.

Results and Discussion :

Biodegradation of both isomers of endosulfan by four fungal isolates (FS1, FS2, FS3 and FS4) was studied over 9 days of incubation. There was no significant difference in degradation among the fungal isolates. During the first 3 days of incubation it was slow, it accelerated at day 6 and at the end of incubation (day 9) endosulfan was almost completely degraded. More biodegradation was observed under agitation and much less in static incubation. Three fungal strains exhibiting the highest ability to degrade endosulfan were identified as *Aspergillus sp.* (FS-1), *Aspergillus sp.,*(FS3) and a yeast - like organism (FS2). The fourth isolate FS4 was also a yeast - like organism (Fig.1). During the endosulfan degradation the major metabolic product detected through TLC were endosulfan diol and endosulfan lactone (Fig 2).

Biodegradation of endosulfan by fungi substantially decreased the pH of the broth. All the three efficient fungal strains showed similar trend in pH change during the period of incubation. However, a considerable difference in pH was noted between biodegradation of endosulfan under static condition versus incubation with agitation (Fig 3, 4 and 5).



(a) (b) (c) (d)

Fig 1. Four efficient fungal isolates. a: FS1 *(Aspergillus sp.),* b: FS2 (yeast like organism), c: FS-3 *Aspergillus sp.*(FS3), d: FS4 (yeast like organism)

(A) (B) (C)

Fig 2. Peaks obtained on TLC plates. (A) 3 days culture, (B) 6 days culture, (C) 9 days culture

Fig 3. pH status in fungal cultures after 3 days of incubation (agitation vs stationary)

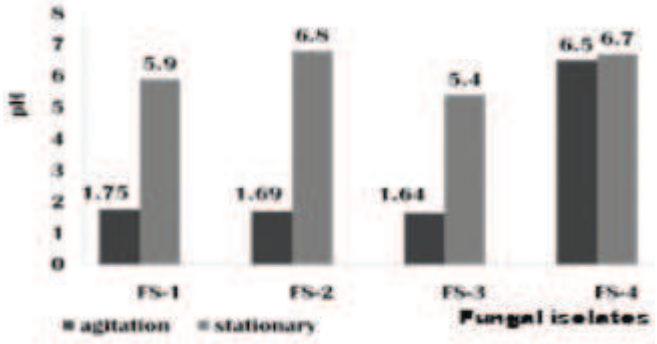


Fig 4. pH status in fungal cultures after 6 days of incubation (agitation vs stationary)

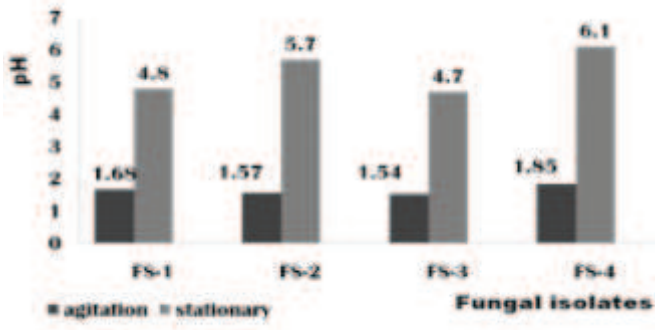


Fig 5. pH status in fungal cultures after 9 days of incubation (agitation vs stationary)

Bioremediation of pesticides containing soils and water environment has become one of the burning issues due to their injurious impact on public health and environment quality. This investigation represents the isolation and screening of efficient fungal cultures capable of degrading both the isomers of endosulfan. It was achieved by growing fungal cultures in a medium containing endosulfan as a sole carbon source. All the efficient isolates were from soils of long history of exposure to the insecticide. This is in agreement with Alexander (1977) who reported that longer usage of pesticides in soil increases the microbial tolerance which probably increases their capability to degrade pesticide contaminants.

All the fungal isolates selected for the analysis, observed change in pH from 7.0 to 1.4. More efficient strains, however, caused more fast and pronounced reductions in pH. Out of the four fungal isolates investigated, the isolates FS.1, FS.2, FS.3 were found to be more efficient than isolate FS.4 in an aqueous environment. No growth occurred in control cultures in the absence of endosulfan. The lowering of pH may be due to the formation of organic and inorganic acids as a result of dehalogenation of endosulfan during biodegradation (Sutherland et al 2000). Moreover biodegradation and lowering of pH was more under agitating condition than in the static condition which agrees with the findings of Hussain et al (2007). Agitation may have resulted in more bioavailability of substrate to the fungi or more O_2 in the medium for fungal activity. Growth in fungal isolates in media containing endosulfan and associated change in pH indicate that insecticide endosulfan is utilized by the microbial culture as a nutrient. These findings are in agreement with the findings of Sutherland et al (2002) and Siddique et al (2003).

Thin Layer Chromatographic analysis of the culture extract confirmed the gradual disappearance of both the isomers of endosulfan

and formation of its metabolites with a simultaneous decrease in pH. Two metabolites were identified as endosulfan lactone and endosulfan diol on the basis of co-migration with pure endosulfan on TLC plates. There is documented evidence of transformation of endosulfan to endosulfan diol by *Aspergillus niger* (El Zorgani and Omer1974). Interestingly, endosulfan sulfate, a persistent and toxic metabolite of endosulfan did not accumulate in the broth culture. This may imply that these fungi adopted a hydrolytic pathway of degradation of this compound instead of an oxidative one (Shetty et al 2000). Degradation of alpha isomer was found to be more fast than beta isomer. The result is in conformity with the result of Sutherland et al (2002). Nevertheless, further analysis of degraded metabolites is required by HPLC or GC .

Acknowledgements :

We are grateful to Dr. Sister Doris D'Souza A.C., Principal, Patna Women's College and the Research Committee for providing facilities and financial support under the Basic Scientific Research (BSR) Scheme.

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