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BIODEGRADATION OF ENDOSULFAN BY *ASPERGILLUS NIGER* ISOLATED FROM COTTON FIELDS OF PUNJAB, PAKISTAN

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Abstract

The study was designed to isolate an efficient fungal strain which can be used for biodegradation of the endosulfan in the soils and water bodies. Four fungal strains (AE, BE, CE, DE) were isolated from 150 soil samples collected from different cotton fields of Punjab and were screened further to check their potential for the biodegradation of endosulfan. The fungal strain AE showed highest degradation rate of endosulfan (98.6%) in four days of incubation at 30°C. The fungal strain AE was identified initially on morphological basis and was confirmed subsequently as *Aspergillus niger* on the basis of 18S rDNA technique. The conditions for the degradation of endosulfan by strain AE were optimized at the lab scale so that they can be further applied for field trials. It was notices that *Aspergillus niger* strain AE completely degraded 0.1% of endosulfan using 2mL of spore inoculum at pH 4 within four days of incubation at 30°C**.**

Key Words: Isolation, Screening, 18SrDNA, Morphology, Inoculum.

Introduction

Endosulfan is a pesticide which is used all over the globe on different food crops for the control of pests. It can be used for a large range of insects for example, aphids, beetles, thrips, caterpillars, borers, mites, bugs and cutworms etc. The crops on which endosulfan is most commonly sprayed for the control of insects include cotton, rice, soy and tea (Kidd & James, 1991). Endosulfan is the common name of the chemical compound 6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a hexahydro-6,9-methano-2,4,3 benzodioxa-thiepin,3-oxide that specifically acts as an insecticide. The chemical structure of endosulfan contains a reactive cyclic sulfite diester group that is actually responsible for its lower persistence in environment than other cyclodienes but surely still higher than other insecticides (Singh *et al*., 1990). United Nations Environmental Program (UNEP) has identified endosulfan as a persistent organic substance.

In Pakistan, endosulfan has been used widely for more than 35 years to control pests. In some areas of Punjab, its residues have also been found in the blood of the farmers working in the cotton field (Masood & Hassan, 1995). In cotton seeds, concentration of endosulfan was found to be 1.4 mg per kg and 0.023 mg per kg in cotton lint (Tariq *et al*., 2004).A range of chronic effects on hormonal system and acute diseases like cancer can occur because of the endosulfan exposure. It poses a threat to human life as it can easily contaminate breast milk, placental tissues, umbilical cord and adipose tissue. It can cause convulsions and hyper excitation, as well as have negative impact on heart and respiratory process (Soto *et al*., 1994). It promotes the allergic response and suppresses the immune system. Neurological anomalies e.g., epilepsy and Parkinson's disease can also occur while birth defects have also been observed in human population.

Microbial degradation is an efficient strategy to clean up the contaminated site without any damage to the environment (Hussain *et al*., 2007).Microbial degradation of endosulfan has resulted into various intermediate metabolites like endosulfan sulphate, diol, ether, lactone, hydroxyether and dialdehyde (Kwon *et al*., 2005).The dominant pathway for the degradation of endosulfan is hydrolysis as it produces endodiol which is non-toxic rather than the endosulfate which is itself quite toxic formed by the oxidation pathway (Kennedy *et al*., 2001). In comparison to alpha isomer, beta isomer hydrolyzes much more fast because of the less steric hindrance from the S=O bond of the beta isomer leading to more easy attack at the S atom. In addition, endosulfan can be degraded faster in aqueous phase than in the solid phase (Goebel *et al*., 1982).

Some soil microorganisms in a pure culture have the ability to degrade endosulfan to a non-toxic metabolite. Fungal strains like *Chaetosartorya stromatoides*, *Aspergillus terricola* and *Aspergillus terreus* were reported to be efficient strains degrading 70% of the alpha and beta endosulfan isomers (Hussain *et al*., 2007). Besides fungal strains, many bacterial strains e.g. *Klebsiella oxytoca*, *Bacillus sp., Pandoraea sp. and Micrococcus sp*. can degrade endosulfan in solutions and soil (Bhalerao & Puranik, 2007). Some photosynthetic microbes are also important in endosulfan degradation studies e.g. *Anabaena sp., Chlorococcum sp. and Scenedesmus sp*. (Lee *et al*., 2003).

The main objective of the present work was to isolate a fungal strain with high potential to degrade endosulfan and to optimize the condition of degradation, so that the strain can be used on large scale for the degradation of residual endosulfan in the soils of Pakistan.

Materials and Methods

Isolation of fungal strains: Soil samples were collected from 150 different cotton fields across the Punjab, Pakistan. A soil auger was used to randomly collect the soil samples from the depth of 10cm. The collected soil samples were placed in plastic bags, labeled and transferred to the research lab of Institute of Industrial Biotechnology, GCU, Lahore. Soil samples were enriched using enrichment medium and fungal strains were isolated on Czapek dox medium plates having endosulfan final concentration of 0.1% (1mg/mL).Plates were inoculated by adding 0.5mL of different serial dilutions of enriched culture and incubated at 30oC for 10-12 days.

Inoculum preparation: Spore suspension of the fungal strains was prepared by adding 10mL of sterilized saline water in the slants. Spores were scratched to have a homogenized mixture of spores. Vegetative inoculum was also used in the present study which was prepared by inoculating the Czapek dox media with spore suspension of the fungal strain to grow the culture for 48 hrs at 30^0C and 180 rpm.

Screening of fungal isolates: Fifty milliliter of Czapek dox media was prepared in a flask for each strain and spiked with 0.1% (1mg/mL) concentration of endosulfan and autoclaved. 1mL of the spore suspension was transferred into the medium. Inoculated the flasks with the isolated fungal strains separately and were placed in shaking incubator at 30ºC and 180 rpm for four days. Biodegradation of endosulfan by all the four strains was estimated.

Analysis of endosulfan: A spectrophotometric method was used for the estimation of endosulfan residues in the culture and absorbance was detected at 605 nm (Venugopal, 2011).

Identification of fungal strain: Identification of fungal strain was carried out by studying the morphological characteristics and using 18S rDNA technique after Haq *et al*., 2014.

Different parameters like rate of endosulfan degradation, adsorption of endosulfan, effect of endosulfan concentration on degradation, effect of inoculum type and size and effect of medium pH on the biodegradation of endosulfan by the selected fungal strain were studied. Computer software Costat, cs6204W.exe was used for the statistical analysis. Significance difference among replicates has been presented as Duncan's multiple range tests in the form of probability (p) values.

Results and Discussion

Four fungal strains (AE, BE, CE, DE) were isolated from 150 soil samples of the cotton field by using the serial dilution method. Aspergillus species have been reported for endosulfan degradation by Bhalrao & Puranik (2007) and Hussain *et al*., (2007). Degradation zones were observed on the plate around the fungal colonies. It was also observed that after the prolonged incubation period, crystalline structures were formed on the plate which may represent the degraded form of endosulfan. Screening (Fig. 1) showed that Strain AE degraded 98.6%, strain BE degraded 76.1%, strain CE degraded 86.3% and strain DE degraded 82.4% of endosulfan in 4 days of incubation at 30oC. It is clear that strain AE can degrade endosulfan more efficiently because of the action of certain enzymes in its metabolic pathway. Strain AE was further studied for the optimization of different parameters for endosulfan degradation.

Rate of degradation of endosulfan with all the fungal isolates was also analyzed separately as shown in Fig. 2 and was observed that the mycelial growth for all four strains was very less after 48 hrs of incubation but after next 48 hrs, strain AE showed more growth as compared to other three fungal strains. All the isolated fungal strains had the ability to completely degrade endosulfan in a specific time period. It was found that strain AE is more efficient in degrading endosulfan than other fungal isolates as it can degrade 99% while strain BE, CE and DE degraded endosulfan up to 78%, 89.3% and 84.9% endosulfan, respectively in four days of incubation at 30ºC. Endosulfan was completely degraded by strain AE in almost five days while strain BE and CE degraded it completely in 7 days and strain DE degraded endosulfan completely in 9 days of incubation. 58.9% abiotic degradation of endosulfan was also observed in four days. Endosulfan was completely degraded in approximately 14 days under specific conditions of temperature, pH and agitation. This shows that abiotic degradation is possible as it is also reported by Hussain *et al*., (2007) and can be rapid under controlled conditions but was rather slower than the biodegradation with fungal strains.

Strain AE was identified both morphologically and using molecular techniques. The colonies of strain AE was observed to change from white to black colonies having incubation time of 3 days at 30ºC. The diameter of colonies was calculated i.e. 17-21 mm. The conidia were observed under microscope at different magnification 10x, 40x and 100x. Hyphae were found to be septet. On the basis of all the observation strain AE was initially identified as Aspergillus specie. 18S rDNA was used to identify the organism. Isolate DNA was sequenced and was compared with already available sequences of Aspergillus sp. available at NCBI data base. It was found that our sequence was closely related to two species of *Aspergillus niger* i.e. TR-H and ETYB-13.

The effect of different concentrations of endosulfan on biodegradation rate of strain AE was studied and it was observed that the rate of degradation was inversely proportional to the amount of endosulfan. The rate of degradation was decreased with increasing the endosulfan concentrations in the flasks as shown in Fig. 3. Strain AE degraded 91.2% of 0.1% (1mg/mL) endosulfan and 91.7% of 0.2% (2mg/mL) endosulfan in 2 days while complete degradation of these amounts of endosulfan was observed in four and eight days, respectively. 85% of 0.3% (3mg/mL) endosulfan and 76% of 0.4 and 0.5% (4, 5 mg/mL) endosulfan was degraded by strain AE in 8 days of incubation indicating the decrease in rate of degradation with the increase in endosulfan concentration until the residual amount of endosulfan become constant and no further degradation was occurred. Elsaid *et al*., (2010) and Shivaramaiah & Kenedy (2006) also reported the degradation of endosulfan by providing 0.1 % endosulfan (maximum amount of spiked endosulfan used for the analysis of degradation). This showed that the fungal isolate (strain AE) had the ability to tolerate much high concentrations of endosulfan up to 0.5 %. As compared to the other reported concentrations of endosulfan. It must contain some effective enzyme systems that can efficiently metabolize the endosulfan. The studies showed that the strain AE can degrade high amount of endosulfan but also up to a certain limit. It may utilize the required amount of endosulfan by degrading it while the other amount remains constant

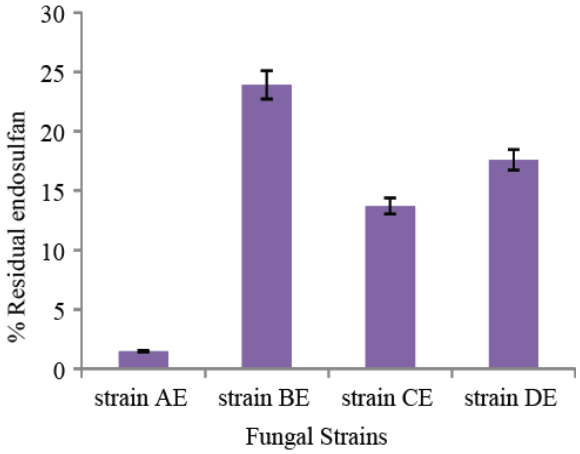

Fig. 1. Screening of fungal isolates for biodegradation of endosulfan. The standard deviation (SD≤±0.05) between the three replicates is represented by Y- error bars

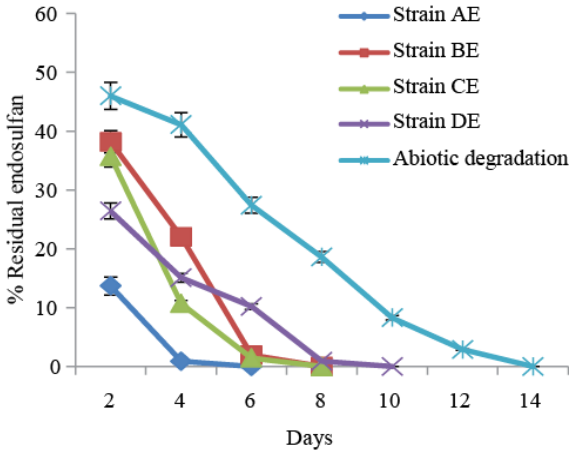

Fig. 2. Rate of biodegradation of endosulfan by isolated fungal strains. The standard deviation (SD≤±0.05) between the three replicates is represented by Y- error bars

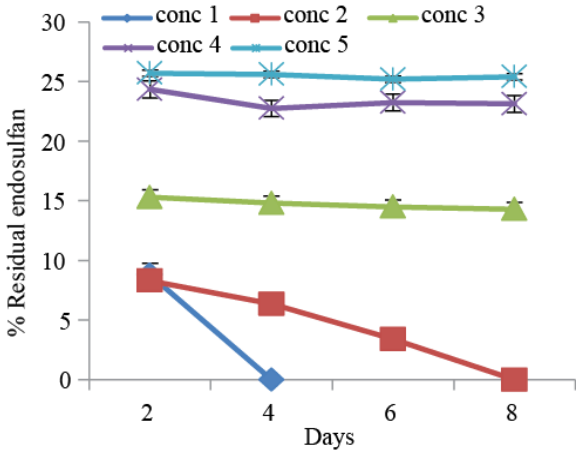

Fig. 3.Effect of different concentrations of endosulfan on the biodegradation of endosulfan by strain AE. The standard deviation (SD≤±0.05) between the three replicates is represented by Y- error bars

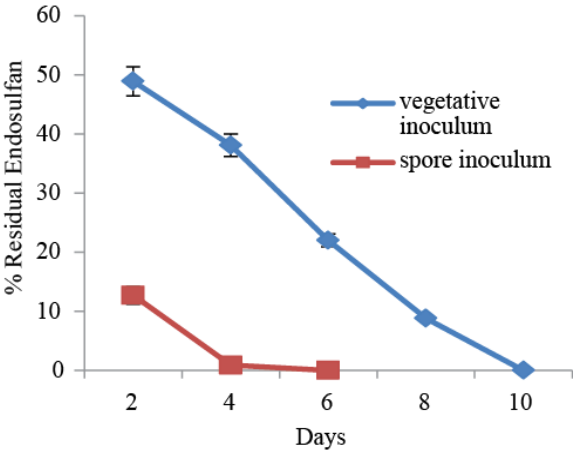

Fig. 4. Effect of type of inoculum on the biodegradation of endosulfan by strain AE. The Standard deviation (SD≤±0.05) between the three replicates is represented by Y- error bars

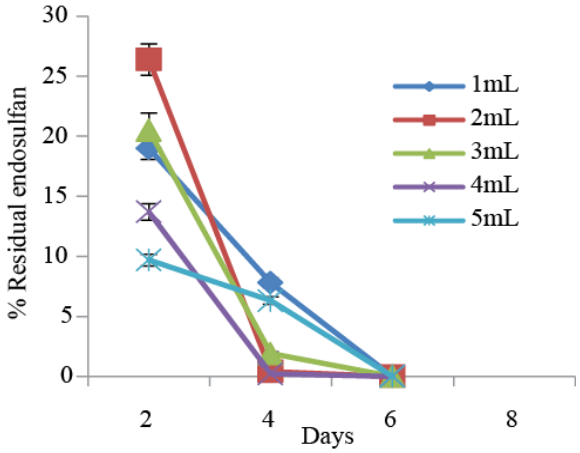

Fig. 5. Effect of volume of inoculum on the biodegradation of endosulfan by strain AE. The Standard deviation (SD≤±0.05) between the three replicates is represented by Y- error bars

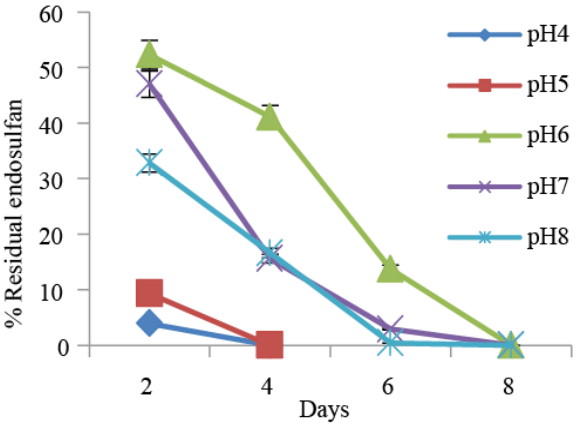

Fig. 6. Effect of medium pH on the biodegradation of endosulfan by the strain AE.Y The Standard deviation (SD≤±0.05) between the three replicates is represented by Y- error bar

The spore inoculum was found to be very efficient in degrading endosulfan as compared to vegetative inoculum. Vegetative inoculum degraded 61.9% endosulfan while 99.6% endosulfan was degraded after four days of incubation when spore inoculum was used (Fig. 4). The complete degradation of endosulfan was observed in eight days of incubation by vegetative inoculum while spore inoculum completely degraded endosulfan in less than five days. The degradation rate by vegetative inoculum was slow as compared to the inoculum of spore suspension. This may be due to the possibility that spores required endosulfan as a substrate for their propagation and rapidly degrade endosulfan under controlled conditions. Young and active spores must have some active enzymes that catalyze the breakdown of endosulfan. Fungal mycelia might be less adaptive to the environment containing endosulfan and there is a possibility of the formation of certain metabolic products due to mycelial growth that reduces the utilization of endosulfan.

Spore inoculum was observed to be efficient in the degradation of endosulfan so it was further used to analyze the effect of different amount of inoculum on the rate of degradation of endosulfan (Fig. 5). Strain AE completely degraded the endosulfan in 5 days with all sizes of inoculum ranging from 1 to 5mL (2-10%). This showed that the inoculum size did not significantly affect the degradation of endosulfan but when comparing the degradation rate of endosulfan after 4 days of incubation, it was observed that the inoculum size of 2mL (4%) degraded endosulfan more efficiently (99.6%) as compared to other inoculum sizes. Minimum degradation (91.6%) was observed when inoculum size of 1ml was used.

The effect of medium pH on the degradation of endosulfan by the fungal strain AE was observed. 96% degradation of endosulfan was observed at pH 4 and 90% degradation at pH 5 in just two days of incubation at 30^{o}C as indicated in Fig. 6. It was observed that in previous parameters fungal strain AE degrade the endosulfan in 4 to 5 days of incubation but adjusting the pH between 4 and 5, the degradation rate was increased. This means that this pH condition must be required by the fungal strain AE for its proper growth which enhanced the rate of degradation. 47.7, 53 and 67.2% degradation was observed at pH 6, 7 and 8 after two days of incubation. The complete degradation of endosulfan at pH 6, 7 and 8 was occurred in eight days while complete degradation of endosulfan was occurred in three days when pH was adjusted at 4.

Conclusion

Strain AE isolated from soil had the potential to degrade endosulfan effectively within a short period of time. It can tolerate high concentrations of endosulfan but up to a certain limit and can completely degrade 0.1% endosulfan in four days. The inoculum size had no significant effect while the pH of the media had a profound effect on the degradation of endosulfan.

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