BIOREMEDIATION OF PESTICIDE-CONTAMINATED AGRICULTURAL SOILS AND EFFLUENTS

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PREFACE

In the present study an effort was made to identify efficient microbial strains capable of degrading two of the most widely used pesticides in Indian agriculture and also in many parts of the world, namely, the s-triazine herbicide, **atrazine** and cyclodiene insecticide, **endosulfan**. Several bacterial and fungal strains isolated using enrichment technique, along with a few other strains, were individually screened to test their ability to bring about the transformation of these pesticides. In addition to this, many experiments related to biodegradation of atrazine and endosulfan were conducted and also resting cell experiments; chemical degradation of atrazine and endosulfan; pH profile of all the fungal strains; synthesis and purification of three metabolites of endosulfan such as endosulfan sulfate, endosulfan diol and endosulfan ether; an experiment on use of α- or β- isomers of endosulfan as sole source of carbon; percent disappearance studies and other related experiments.

The results from these studies revealed that among the many microbial strains, the fungus, *Mucor thermo-hyalospora* was found to be most efficient in bring about the transformation of endosulfan molecules. The identification of endosulfan metabolites by Gas Liquid Chromatography using Electron Capture Detector (GLC-ECD), ^{1}H Nuclear Magnetic Resonance (NMR), Mass Spectrometry (MS) and Infrared spectra (IR) revealed the formation of non-toxic endosulfan diol as a major metabolite. A significant amount of endosulfan sulfate was also produced during the later part of incubation. This indicates that the fungus is involved in both oxidative and hydrolytic pathways of degradation of this compound.

Introduction

During the last fifty years, new technologies and scientific methods of farming have caused a shift in mainstream agriculture, creating a system that depends on agrochemicals, new varieties of crops and modern labour saving farm machineries. The practice of scientific agriculture has enhanced world food production to a remarkably high level from 700 million tonnes to 2000 million tonnes per year. In India, where 70% of the population depends on agriculture, similar trends have been experienced. In 1950, the country's food production was 52 million tonnes and by 1995, it had increased to 195 million tonnes . It is estimated that by 2000 A.D., population may increase to over 1000 million and even at moderate levels of food grain consumption, India need to produce an additional food grains of 45 to 50 million tonnes per annum.

It is estimated that worldwide over one lakh species of agricultural pests destroy the crops worth of Rs. 60,000 crores per annum. The crop loss in our country is estimated to be nearly Rs 6,000 to 7,000 crores per year. Out of the total loss, the crop weeds accounted for nearly 33% followed by plant diseases 26%, insects and rodents 26%, storage pests 7% and miscellaneous pests 8%. In modern agriculture, pesticides have become an inevitable tool and these chemicals are extensively used to bring down the pest population. During the last five decades, the annual pesticide consumption in our country has increased by fifty four times. India is one of the top ten producers of pesticides in the world and it is the second largest manufacturer of basic pesticide chemicals among the South-Asian and African countries. Out of the total pesticides used in the country, insecticides, herbicides and fungicides contribute 77%, 12% and 8% respectively (Dutta and Raghavan, 1995) and it is being used at a rate of 570 grams per hectare in India (Chandran 1996).

Use of pesticides has no doubt benefited the modern society by enhancing the agricultural production. However, indiscriminate and improper use has caused serious threat to human health and environment. One of the major environmental concerns world over is the remediation of pesticide contaminated ecosystems. Various physico-chemical

processes can be used for pollution reduction. However, bioremediation is a safe, inexpensive and effective technology, where biological agents are stimulated to break down both anthropogenic and naturally occurring pollutants in the soil and aquatic environment. Among the various groups of pesticides, the s-triazine herbicide, **atrazine** and the cyclodiene insecticide, **endosulfan** are extensively used in India and also in many parts of the world. The bioremediation studies of these chemicals are of importance because of their persistence in nature, potential carcinogenecity and also their effect on non-target organisms including human beings. Isolation or identification of efficient microorganisms capable of degrading these chemicals will help in detoxifying the contaminated environment.

In view of this, the **National Institute of Advanced Studies** had initiated a collaborative research programme on "**Bioremediation of pesticide contaminated soils and effluents**" in association with **Indian Institute of Science**, Bangalore and **Bhabha Atomic Research Centre**, Mumbai. This project was sponsored by the Board of Research in Nuclear Sciences (BRNS), Department of Atomic Energy, Government of India. The main objective of this programme was to identify an efficient microbial strains capable of degrading the atrazine and endosulfan molecules and the possibility of use of such organisms for bioremediation of contaminated environment.

Chapter I

Atrazine is a s-triazine herbicide, used to control the broad leafed weeds of maize, sorghum, sugarcane and also in irrigated cotton crops. Because of their broad-spectrum activity, this compound occupies the sixth position among the herbicides used in India with an annual consumption of 210 tonnes/annum (Mathur and Bahl, 1994). As a result of its widespread use for over thirty years (as a selective and non-selective chemical), atrazine residues have been detected in ground and surface waters in several countries (Koshinen and Harper, 1990). Atrazine is also found to be relatively persistent in the soil and acquifer sediments with varying half lives of a few days to several months (Jones, *et. al.*, 1982; Rodosevich, *et. al.*, 1993). However, in certain cases residues of atrazine have been detected years after its application (Schaivon, 1988). Using ^{14}C techniques, 18% of the parent material was found to be mineralized in 550 days in soil (Wolf and Martin, 1975). The absorption and desorption characteristics, soil pH and dissolved organic carbon are known to influence the persistence of atrazine in soil (Calvet, 1980 ; Koshinen and Harper, 1990). Half life of atrazine has been shown to exceed 170 days in aquifer sediments (Radosevich, *et. al.*, 1993). The frequent detection of atrazine has been reported in surface water, rain, tile drain and ground waters by exceeding the limit of the contaminating levels of 3ppb (Muir and Baker, 1976; Belluck, *et. al.*, 1991; Thurman, *et. al.*, 1992). Atrazine contamination levels of 15.9 ppb was also detected in river waters of Australia (Peerce and Whally, 1993). Atrazine has been classified as a class 'C' carcinogen by the United States Environment Protection Agency(USEPA,1990)with a preventive action level of 0.3 ppb. The widespread use of atrazine and its subsequent detection in water systems have caused great concern due to its toxicity. Atrazine is known to exhibit the phytotoxicity and also inhibit the growth of cyanobacteria (Stratton, 1984).

Bacterial degradation of atrazine has been reported by several workers (Wolf and Martin 1975 ; Cook and Hutter, 1984 ; Mandelbaum, *et. al.*, 1993). Atrazine was found utilize as a sole carbon source by *Pseudomonas* sp. (Khan and Behki, 1990), *Nocardia* sp. (Giardini, *et. al.*, 1980), *Rhodococcus* sp. (Behki, *et. al.*, 1993; Behki and Khan, 1994 and Zwieten and Kennedy, 1995) and Strains of *Klebsiella* (Hapemann, *et. al.*, 1993).

The transformation of the s-triazine herbicide by soil fungi has been reviewed by many workers (Kaufman, *et. al.*, 1965; Kearney, *et. al.*, 1965 and Kaufman and Blake, 1970). The Dealkylation was found to be the major mechanism of fungal transformation of atrazine however, dehalogenation or formation of hydroxy-atrazine has also been reported (Cough, *et. al.*, 1965; Armstrong and Harris, 1965 ; Harris, 1967 and Skipper, *et. al.*, 1967). Two marine fungal species were found to utilize the atrazine as a source of carbon / nitrogen (Schocken, *et. al.*, 1982). The experiments carried out by Schocken and Speedie (1984) showed that atrazine was degraded by a higher marine fungi *Periconia prolifica* to deisopropyl products. Similarly, biotransformation of atrazine has been reported by a white rot fungi (Masaphy, *et. al.*, 1993 and Mougin, *et. al.*, 1994).

The traditional methods of isolation of microorganisms of interest involve the enrichment of soil followed by isolation of pure cultures. Earlier, several workers selected soil and sewage as inoculum for enrichment (Cook, *et. al.*, 1978 ; Cook and Hutter 1981). Further this was treated with the s-triazine herbicide. Enrichment and isolation of microbes under laboratory conditions had been described by Harder (1981), and Behki and Khan (1986). They followed soil perfusion and batch culture technique for isolation of microorganisms.

Endosulfan is a chlorinated cyclodiene insecticide used to control aphids, shoot and fruit borers of egg plant and also pests of cotton, tea and coffee plantations. It occupies the second position among the insecticides used in India. Endosulfan is a broad-spectrum insecticide extensively used in agriculture and in public health programmes since the ban in use of other organochlorine insecticides such as DDT, aldrin and dieldrin. It is classified as moderately hazardous chemical by the World Health Organization (1984). Even at the recommended doses of application, endosulfan is found to be extremely toxic to fish (Murthy & Devi, 1982; Verma, *et. al.*, 1983). Endosulfan is called as a pseudo cyclodiene molecule because of presence of the cyclic sulfite ester. Thus it varies markedly from the other cyclodiene insecticides. Technical endosulfan is a crystalline substance consisting of alpha- and beta - isomers in the ration of 7:3. Both the endosulfan isomers are fairly resistant to photodegradation. They are strongly adsorbed to the soil particles whereas alpha-isomer is rapidly dissipated when compared to beta-isomer (Beyers, *et. al.*, 1965). The alpha-isomer has a shorter half life when compared to the beta-isomer (Steward and Cairns, 1974). The half-life of endosulfan in run-off water is approximately 4 days (Eichelberg and Lichtenberg, 1971) and 7 days in normal waters. The half life is greatly affected by pH and dissolved oxygen content. Endosulfan contamination of water bodies is only through run-off where as none of the two isomers are prone to leaching in the soil (Steward and Carins, 1974).

The concern regarding contamination of endosulfan in the environment is due to its frequent detection in food, feed, fruits and vegetables in appreciable quantities. The study of Corneliussen (1969) has established that endosulfan residues in food and feed were predominantly due to the residues deposited on the fruits and vegetables. Out of 240 samples of leafy vegetables, seven samples showed the residues of 0.004-0.002mg/kg. Similarly the fruits also showed traces upto 0.006mg/kg of endosulfan residue. According to Food and Agricultural Organization, the acceptable daily intake (ADI) levels of endosulfan by the human beings is 0.008mg/kg body weight i.e based on NOAEL (No Observed Adverse Effect Level) doses. Endosulfan is highly toxic to fish (Gopal, *et. al.*, 1981; Singh and Srivastava, 1981; Murthy and Devi, 1982 and Verma, *et.*

al., 1983). It is also moderately toxic to honeybees (Stevenson, *et. al.*, 1978) with a reported contact LD_{50} of 7.1 ug/bee and oral LD_{50} of 6.9 ug/bee. Although toxicity of endosulfan to warm blooded vertebrates in the form of teratogenecity, carcinogenecity and mutagenecity has not been determined.

The technical endosulfan is sensitive to moisture, acid and alkali where it undergoes slow hydrolysis by producing SO_2 and endosulfan alcohol via the intermediate compound i.e endosulfan sulfate (Martens, 1977). In soil, endosulfan sulfate is the primary degradation product of endosulfan along with lesser amounts of endosulfan diol and lactone (Martens, 1977). In aquatic environment endosulfan diol is produced with smaller amounts of sulfate (Eichelberger and Lichtenberg, 1971).

The role of microorganisms in endosulfan degradation has been explained by various workers (Martens, 1976 and Katayama and Matsumura, 1993). Nearly 15 strains of bacteria tested by Martens (1976) have shown to degrade endosulfan to endosulfan sulfate and endosulfan diol. Further it was reported that majority of soil fungi degraded endosulfan to endosulfan sulfate. In soil suspension, Perscheid and coworkers (1973) have identified endosulfan diol to be the major degradation product of endosulfan. El Zorgani and Omer(1974) reported the degradation of endosulfan by *Aspergillus niger*. Similarly Katayama and Matsumura (1993) observed the transformation of endosulfan to endosulfan sulfate and endosulfan diol by *Trichoderma harzianum*. In studies conducted by El Beit and Coworkers (1981) showed that endosulfan was degraded by both bacteria and fungi.

The strains of bacteria which were capable of degrading endosulfan have been isolated from soil suspension (Perscheid, *et. al.*, 1973). Martens (1976) reported the isolation of the fungal strains from soil enrichment technique. Wherein different concentrations (100, 200, 500 and 1000 ug/ml) of commercial formulations of endosulfan were added to soil suspension (lg/100ml) prepared in a Czapek-Dox broth. The fungal strains growing on the surface of the broth were selected and purified on Czapek-Dox agar plates. These strains were used to study the transformation of endosulfan. The methods of isolation of

fungi and bacteria differ greatly since the compounds are degraded extracellularly by fungi and need not be internalized for metabolism. The bacterial metabolism on the other hand, involves catabolic degradation and enzymatic conversion. Hence, the fungal metabolism is advantageous over bacterial conversion for the sheer non specificity of the mechanism of degradation (Barr and Aust, 1986).

Chapter II

Chemical : Atrazine (2 chloro, 4 ethylamino, 6-isopropylamino, 1,3,5-triazine) was obtained from Rallis India Ltd, Bangalore and technical grade endosulfan (6,7,8,9,10,10-hexachloro - 1,5,5a,6,9,9a-hexalydro-6,9 methane-2,4,3-benzodioxathiepine - 3 oxide) was provided by Recon India Ltd, Bangalore. The technical grade atrazine and endosulfan were further purified by silica gel column chromatography. All the other chemicals used in the experiment were of analytical grade. The commercial grade atrazine (50% WP) and endosulfan (35% EC) used in enrichment studies were obtained from pesticide distribution units in Bangalore.

Synthesis of metabolites of endosulfan : The following metabolites or by-products of endosulfan were chemically synthesized in the laboratory and these metabolites were purified and confirmed by using the analytical techniques.

Endosulfan sulphate : Technical endosulfan (102g) dissolved in chloroform (250ml) and acetic acid (500ml) was stirred and aqueous solution of barium permanganate (125ml, 0.15 mole) was added dropwise over one hour while the reaction temperature was held at 5°C. After one hour at 5°C, a solution of sodium bisulphite (50g, in 300ml) was added slowly while the temperature was permitted to rise to 25°C. The hydrated barium sulphate was filtered and aqueous layer was extracted with chloroform (100ml). It was dried and distilled. The residue contained 68gm of sulphate. It was recrystallised from cyclo-hexane (m.p. 235-238°C).

Endosulfan ether : Technical endosulfan (5g) was placed in a stoppered test tube and put in an oil bath preheated to a constant temperature of 235°C. The solid melted and rapidly discoloured. After one hour no liquid remained in the test-tube and an off white solid coated the walls. The solid was recovered to give 3.5gm of product. Recrystallised from absolute ethanol and norit treatment gave a white solid, m.p.196-203°C.

Endosulfan diol : Technical endosulfan (10g) in methanolic KOH (100ml, 5%) was heated under reflex for one hour. The dilution, acidification followed by extraction with ether yielded endosulfan diol as colourless needles from methanol, m.p 203-205°C, recrystallised from methanol.

Soil collection : Soil samples were collected from the agricultural fields in Hessaraghatta, Bangalore; organic wastes and sewage samples from discharge areas of Binny Mills; banks of Vrishabhavathi river; waste discharge sites of pesticide formulation units in Bangalore(Karnataka) and also black cotton soils from Guntur(Andhra Pradesh). Apart from this, the soil samples with previous history of exposure to pesticides of interest and also soils (laterite, red and black) of different agro-climatic regions of Karnataka have been selected and used for enrichment studies. The soil samples were air dried and sieved through a 2mm sieve. The soil samples were divided into two parts, the first part was used for soil enrichment with atrazine and endosulfan and the second part was used for isolation of atrazine or endosulfan degrading microorganisms by following soil dilution technique.

Soil enrichment method : Five hundred grams of the composite soil samples were taken in the plastic pots. The soil was maintained in an open environment protected from rain, direct sunlight and other physical and biological disturbances. The pots were maintained at field capacity. The soil was enriched by spraying commercial grade of atrazine or endosulfan at a rate of 2% separately. The enrichment was carried out on weekly intervals for a period of 10 weeks. The control pots were maintained without addition of pesticides.

After ten weeks of enrichment, the soil types were serially diluted in a sterile saline (85% Nacl) from 10^{1} to 10^{-4}. The dilutions were plated on BMMA/E agar (basal mineral media containg: K_2HPO_4 , 1g; KH_2PO_4 ,1g; NH_4NO_3 ,1g; $MgSO_4.7H_20$, 0.2g; $Fe(SO_4)_3$, 5mg; $Na_2MoO_4.2H_2O$, 5mg; $MnSO_4.4H_2O$, 5mg; Distilled water-1000ml; pH - 7.00; supplemented with 100 ppm of technical grade atrazine or endosulfan) plates for isolation of microorganisms and also on Martins rose bengal agar plates (Glucose, 10g; Peptone,

5g; Potassium dihydrogen phosphate, 1g; Magnesium sulphate, 0.5g; Rose bengal, 0.03 g; Agar 20g; distilled water 1000ml; streptomycin and pH 6.00) for fungal colonies. Fungal isolates were purified by streaking on Czapek-Dox agar plates for 3-4 times. The purified isolates were maintained at 4°C on BMMA/E and modified Czapek- Dox slants for bacteria and fungi respectively. The strains were sub-cultured at fortnightly intervals.

Soil dilution method : Five grams of air dried and sieved soil from each soil types were suspended in 50ml of buffer salt solution and kept in a mechanical shaker at 220rpm for one hour and it was allowed to settle for 30 minutes. The supernatant was filtered through a Whatman filter paper No.1 and the filtrate was used as inoculum. The Erlenmayer flasks containing 100 ml of basal mineral media (BMM) supplemented with 100 ppm of technical grade atrazine or endosulfan (BMMA/E) and 5ml of inoculum were kept in a mechanical shaker at 220rpm at 28°C for 2 weeks. After the incubation period, 1ml of the inoculum was transferred to 50ml sterile BMMA/E media and maintained under similar conditions. This was repeated for 8 times with a gap of 2 weeks. Finally, 0.2ml of inoculum was added to 20ml of sterile BMMA/E media and maintained in a shaker at 28°C for 10 days. After this, the dilutions were made in a sterile saline and plated on BMMA/E plates. These plates were incubated at 30°C for 5 days. The resultant colonies were picked up at different intervals and purified on BMMA/E plates. The isolated colonies were further grown on BMMA/E agar plates/broth. This was repeated for 3 times to obtain a pure strain.

Isolation of microorganisms from industrial effluents :

The industrial effluents were collected from different atrazine/endosulfan manufacturing units and these were further screened for the presence of suitable microorganisms which can degrade the atrazine or endosulfan molecules. Four fungal strains were (AF- 6, AF-7, AF-8, AF-9) isolated from the effluents and purified. These strains were further subjected for degradation studies.

pH profile of fungal strains : For degradation studies, those fungal strains which maintained the pH of the broth between 6.0 and 8.0 were selected. In order to study the

pH profile, known number of spores were added to the experimental flasks containing 100ml of Czapek-Dox broth (ranging from 10^5 spores/ml of suspension - the haemocytometer was used for counting fungal spores in liquid suspension). The control flasks without inoculum were maintained. The pH variations were monitored for 5 days at every 24 hours interval.

Organisms :10 bacterial strains and 40 fungal strains were isolated from enrichment technique(soil enrichment or soil dilution technique) and a few laboratory identified strains such as *Trichoderma harzianum* (BARC, Mumbai), *Mucor thermo-hyaiospora* (MTCC 1384), *Mucor mucedo* (MTCC 353), *Mucor hiemalis* (MTCC 157), *Aspergillus* sp. and *Trichoderma* sp. were tested for their ability to bring about transformation of endosulfan and atrazine molecules. The fungi were grown and maintained in Czapek - Dox agar medium containing-Sucrose, 30grams, Sodium nitrate, 3grams; Dipotassium phosphate, 1gram; Magnesium Sulphate, 0.5gram; Potassium chloride, 0.5gram; Ferrous Sulphate, 0.01gram; Distilled water 1000ml ; the pH was 7.0.

Inoculum: The fungal strains were grown in 250ml Erlenmeyer flask containing 50ml of Czapek - Dox agar and incubated for 3 days at 28 ± 2°C at 100 rpm in a Newtronic orbital shaking incubator. After the incubation, the Mycelia were collected from the agar medium in sterile water, homogenized and filtered through sterile muslin cloth. Enumeration of mycelial suspension was determined by dilution plate technique and $2x10^5$/ml of mycelial inoculum were added to experimental flasks.

Degradation studies: Cultures were grown in carbon - deficient (0.1% sucrose) Czapek - Dox broth in a Newtronic orbital shaking incubator at 100rpm at 28 ± 2°C for 48 hours for three days. After preliminary incubation each flask received 28ml of a solution of endosulfan in acetone to give a final concentration of 5 ppm. The cultures were incubated further for different growth periods upto 20 days after addition of endosulfan. A set of heat killed control samples were prepared by autoclaving the cultures which had been pre-grown for 48 hours and with 5 ppm of endosulfan were incubated for the same period of time. Appropriate control experiments were carried out with uninoculated medium. At

intervals cultures were removed and acidified to pH 2 with in Hcl and extracted with equal volume of Hexane-acetone mixture following the methods of Kullman and Matsumara (1996). For large scale degradation studies, the organisms were grown in 100 ml carbon deficient Czapek - Dox broth in 500ml Erlenmeyer flasks, the flasks were incubated for 8 days with appropriate controls and were extracted by following the above mentioned method. The organic extract was dried over anhydrous sodium sulphate and evaporated to near dryness in a rota-vapor. It was redissolved in a suitable organic solvent.

Purification and identification of Metabolites: The metabolites were separated by column chromatography on silica gel using graded concentrations of ethyl acetate (1-20%) in hexane as eluents. Different fractions were examined by thin-layer chromatography (TLC) using 0.25mm silica gel G plates and hexane-chloroform-acetone (9:3:1 v/v/v) as a mobile phase (Martens, 1976) for parent compounds and metabolites. The TLC plates were sprayed with Ortho toluidine (3% solution in acetone) and exposed to sunlight for 15 minutes for the detection of endosulfan and its metabolites.

Spectral studies: Quantitative analysis of endosulfan and its metabolites was done by Gas Liquid Chromatography - Chemito Model 3685, Gas Liquid Chromatogray - equipped with a electron capture detector (GLC - ECD), the operating conditions were as follows : separation were carried out by using a glass column of 10% DC 200+ 15% RF1 on Gaschom QHP. The column temperature was 200° C. Injector and detector temperatures were 220° C and 270° C respectively. Nitrogen was used as a carrier gas at a flow rate of 24ml/min. Infrared (IR) spectra were recorded in neat or nujol mull on a Perkin Elmer model 397 spectrometer. ^{1}H - Nuclear Magnetic Resonance (NMR) spectra were recorded in a JEOL -FX - 90. Mass Spectra (MS) were recorded on a JEOL - JMS - DX 303 instrument with JMA - DA 5000 data system.

Resting cell experiments:

The fungal isolates were grown on Czapek-Dox broth for 24-36 hrs. 0.5 to 1.0mg of endosulfan was added to induce the degradation. The mycelium was harvested by

filtration and washed with 1% saline. The mycelium was suspended in phosphate buffer (50M) and the pH was 7.0. It was divided into 2 parts, to one part endosulfan (1.0mg) was added and the second part served as control (substrate). The flasks were incubated in a shaker for 12-18 hours and endosulfan and its metabolites were extracted and separated by column chromatography. Further the samples were subjected to analytical studies.

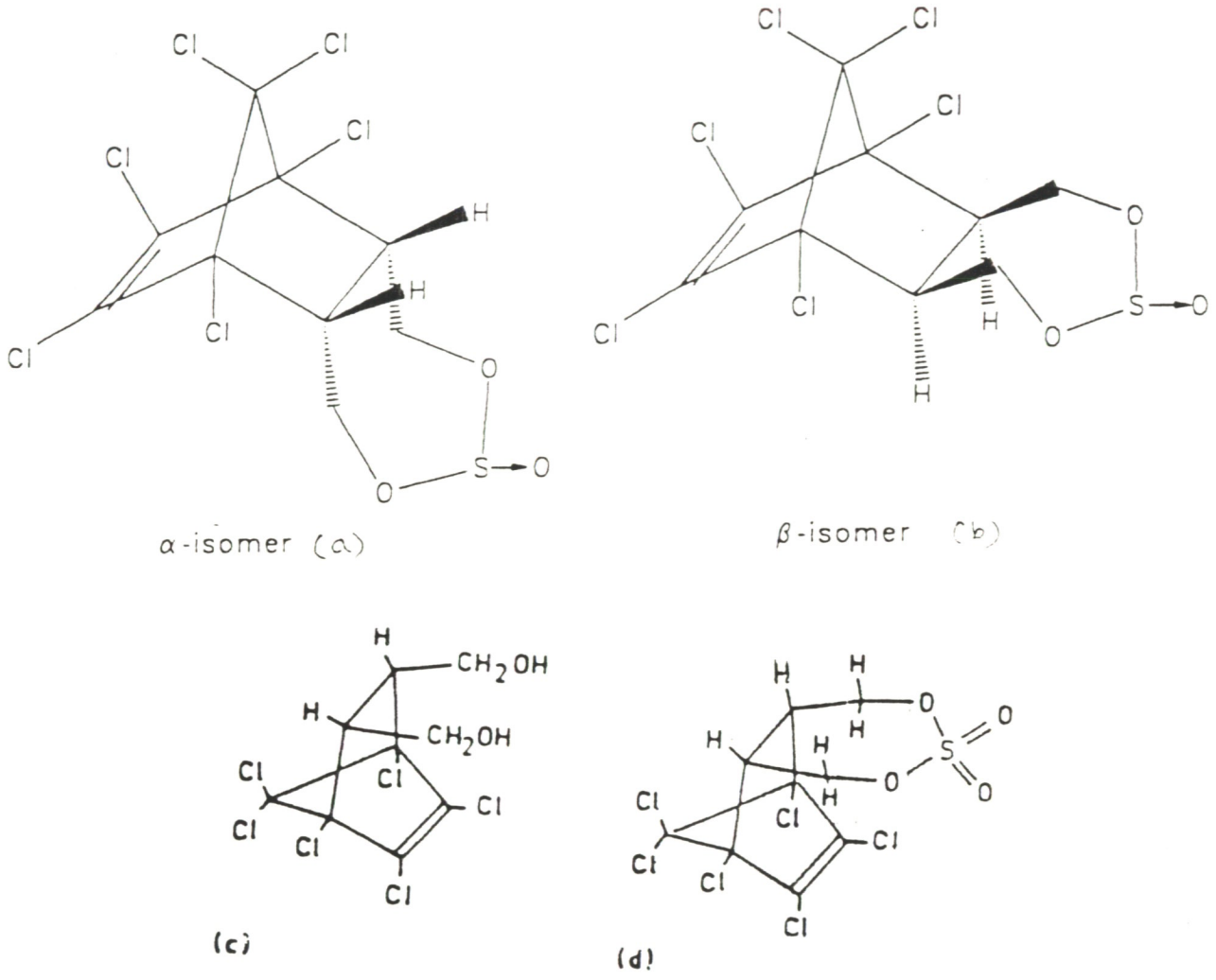


Figure 1. Endosulfan isomers and its metabolites (a) alpha- Endosulfan, (b beta-Endosulfan, (c) Endodiol and (d) Endosulfate.

Chapter III

In the present study out of 40 fungal strains, only thirteen strains were found to maintained the pH between 6.00 and 8.00, whereas other strains were observed to alter the pH to alkali or acidic range. Those fungal isolates which did not maintain the pH of the medium between 6 and 8 were not selected for degradation studies. Out of 10 bacterial strains only three strains were finally used for mixed bacterial fermentation studies.

Degradation studies:

The fungal isolates were individually screened to test their ability to bring about the transformation of endosulfan and atrazine molecules. None of the 40 fungal strains isolated from enrichment technique degraded the atrazine or endosulfan molecules at the concentrations of 30 to 50 ppm levels, whereas a few identified strains namely, *Mucor thermo-hyalospora*, *Mucor mucedo*, *Mucor hiemalis* and *Aspergillus* sp. were degraded these pestecides at this concentrations (Table1).

Among, different fungal strains, *Mucor thermo-hyalospora* found to be efficient in degrading endosulfan molecules. This organism showed the presence of five transformation products of endosulfan which were designated as M I, M II, M III and M IV and M V. The Rf values of these products are shown in Table. 2, along with the Rf values of reference standards. This organism was also found to be more efficient in bringing the transformation of endosulfan in the carbon limiting medium. For large scale preparation, the cells were harvested in batches using Czapek- Dox medium with 0.1 % sucrose as carbon source.

Table 1 : pH profile and transformation of atrazine/endosulfan by fungal isolates

Fungal isolates	pH profile	Transformation		Source
		Atz	End	
1. *Trichoderma harzianum*	+ve	-ve	-ve	BARC
2. *Trichoderma* sp.	+ve	-ve	-ve	BU
3. *Aspergillus* sp.	+ve	-ve	+ve	BU
4. *Mucor thermo-hyalospora*	+ve	+ve	+ve	MTCC 1384
5. *Mucor mucedo*	+ve	-ve	+ve	MTCC 17
6. *Mucor hiemalis*	+ve	+ve	+ve	MTCC 353
7. AF 1	+ve	-ve	+ve	Enrichment
8. AF 2	+ve	-ve	-ve	Enrichment
9. AF 3	+ve	-ve	-ve	Enrichment
10. AF 4	-ve	-ve	-ve	Enrichment
11. AF 5	-ve	-ve	-ve	Enrichment
12. AF 6	+ve	-ve	-ve	Effluent
13. AF 7	-ve	-ve	-ve	Effluent
14. AF 8	-ve	-ve	-ve	Effluent
15. AF 9	+ve	-ve	-ve	Effluent
16. AF 10	+ve	-ve	-ve	Enrichment
17. AF 11	-ve	-ve	-ve	Enrichment
18. AF 13	-ve	-ve	-ve	Enrichment
19. AF14	-ve	-ve	-ve	Enrichment
20. AF 15	-ve	-ve	-ve	Enrichment
21. AF 16	+ve	-ve	-ve	Enrichment
22. AF17	+ve	-ve	-ve	Enrichment
23. AF 19	-ve	-ve	-ve	IISc
24. AF 20	+ve	-ve	-ve	NIAS
25. AF 22	-ve	-ve	-ve	IISc
26. AF 24	+ve	-ve	-ve	BU
27. AF 25	-ve	-ve	-ve	Enrichment
28. AF 26	+ ve	-ve	-ve	Enrichment
29. AF 27	-ve	-ve	-ve	Enrichment
30. AF 28	-ve	-ve	-ve	Enrichment
31. AF 29	-ve	-ve	-ve	Enrichment
32. AF 31	-ve	-ve	-ve	Enrichment
33. AF 32	-ve	-ve	-ve	Enrichment
34. AF 35	-ve	-ve	-ve	Effluent
35. AF 36	+ve	-ve	-ve	BU

Table 2: Rf value (hexane-chloroform-acetone 9:3:1 v/v/v) and retention time (GLC-ECD) of endosulfan and its metabolites in the solvent system.

Endosulfan and metabolites	Rf value	retention time
α-endosulfan	0.78	7.97
β-endosulfan	0.60	14.34
Endosulfan sulfate	0.50	22.91
Endosulfan ether	0.69	3.57
Endosulfan diol	0.24	5.52
Metabolite I	**0.50**	**22.91**
Metabolite II	0.31	------
Metabolite III	0.18	------
Metabolite IV	0.06	------
Metabolite V	**0.24**	5.52

GLC - ECD analysis of hexane - acetone extract and TLC studies revealed the formation of two metabolites (M V and M I) with retention time of 5.52, 22.91 and the Rf value of 0.24, 0.50 respectively. This matched with the retention time and Rf value (the reference standards)of endosulfan diol and endosulfan sulfate respectively. The spectral analysis of the metabolite V (Rf 0.24) had the following characteristics: The IR spectrum (nujol) showed absorption at 3200 cm^{-1} (hydroxyl group) and 1590 (double bond). The ^1H NMR ($CDCl_3$) δ: 3.9 (2H, d, CH_2 -OH), 3.6(2H, t C<u>H</u>, - CH_2) 3.2 (2H, d, CH_2 - OH) 2.35 (2H,bs,OH). The MS data (m/z) for this compound is : 360(M^+), 342(M^+-H_2O), 324(M^+-H_2O -H_2O), 277(M^+ -$CHCl_2$), 229(M^+-$C_2H_5O_2Cl_2$). For the HRMS, $C_9H_8Cl_6O_2$ requires 360.9108 and 360.8802 found. The metabolite I (Rf 0.50) had the following spectral characteristics: the IR spectrum (neat)showed absorption at 1700 cm^{-1}(sulfate group) 1600 cm^{-1}(double bond). The ^{1}HNMR ($CDCl_3$) δ : 4 55 (4H,q,2C<u>H</u>$_2$O), 3.5(2H,bs,2C-H). The MS data (m/z) : 422(M^+), 387(M^+ - Cl), 358(M^+ - SO_2), 272(M^+ -$CH_4Cl_2SO_2$). For the

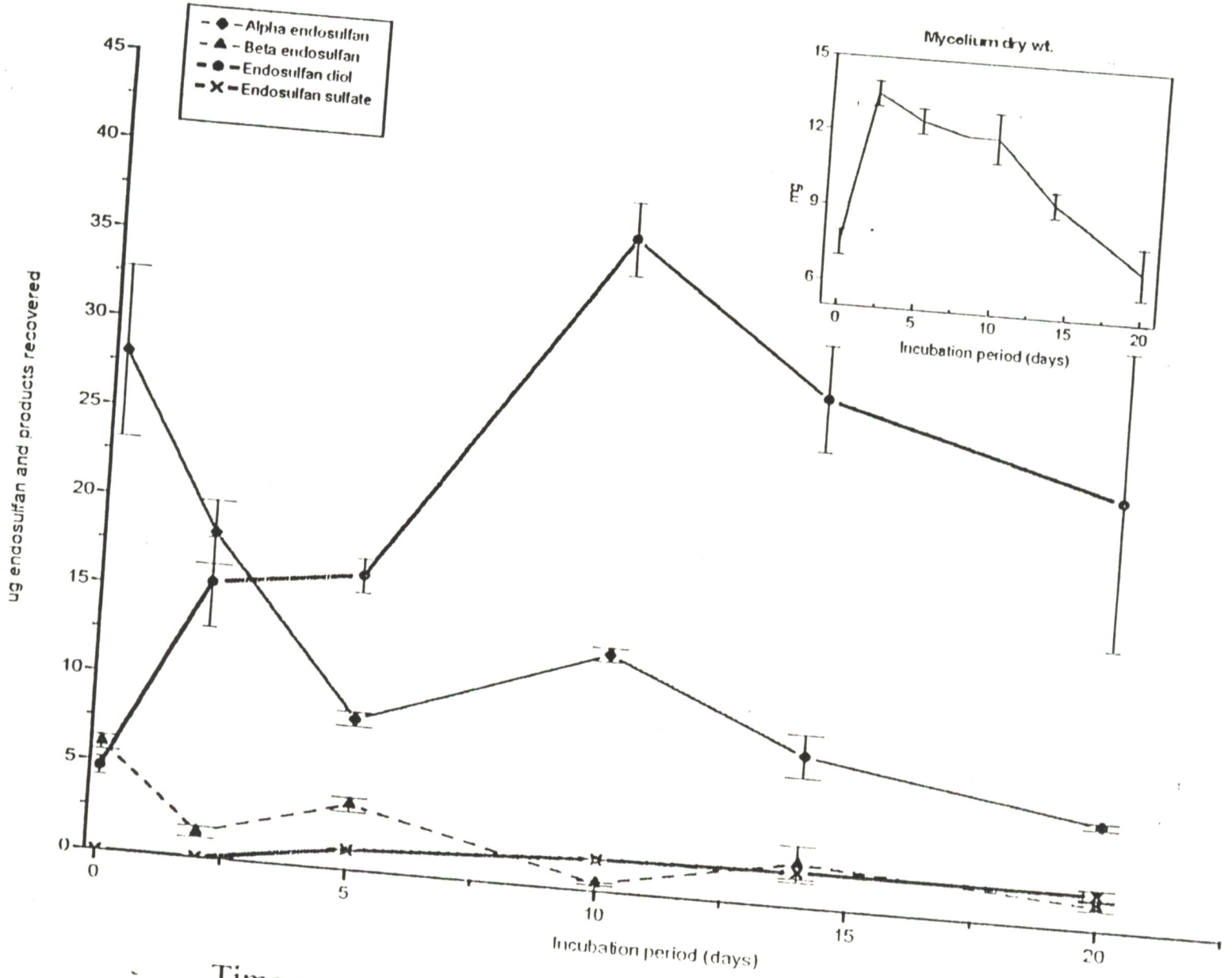
(HRMS), high resolution mass spectrum $C_9H_6Cl_6O_4S$ requires 422.9269 and 422.304 was found.

Apart from this, many preliminary experiments related to biodegradation of atrazine/endosulfan were carried out in the laboratory. This includes, resting cell experiments; chemical degradation of atrazine and endosulfan (checked the stability of the pesticide molecules at different pH ranges and also in different nutrient media); pH profile of all the isolated soil fungi; standardization of TLC and GLC conditions for atrazine and endosulfan, different solvent systems for developing thin layer plates and detection methods; and synthesis and purification of three metabolites of endosulfan such as endosulfan sulfate, endosulfan diol and endosulfan ether.

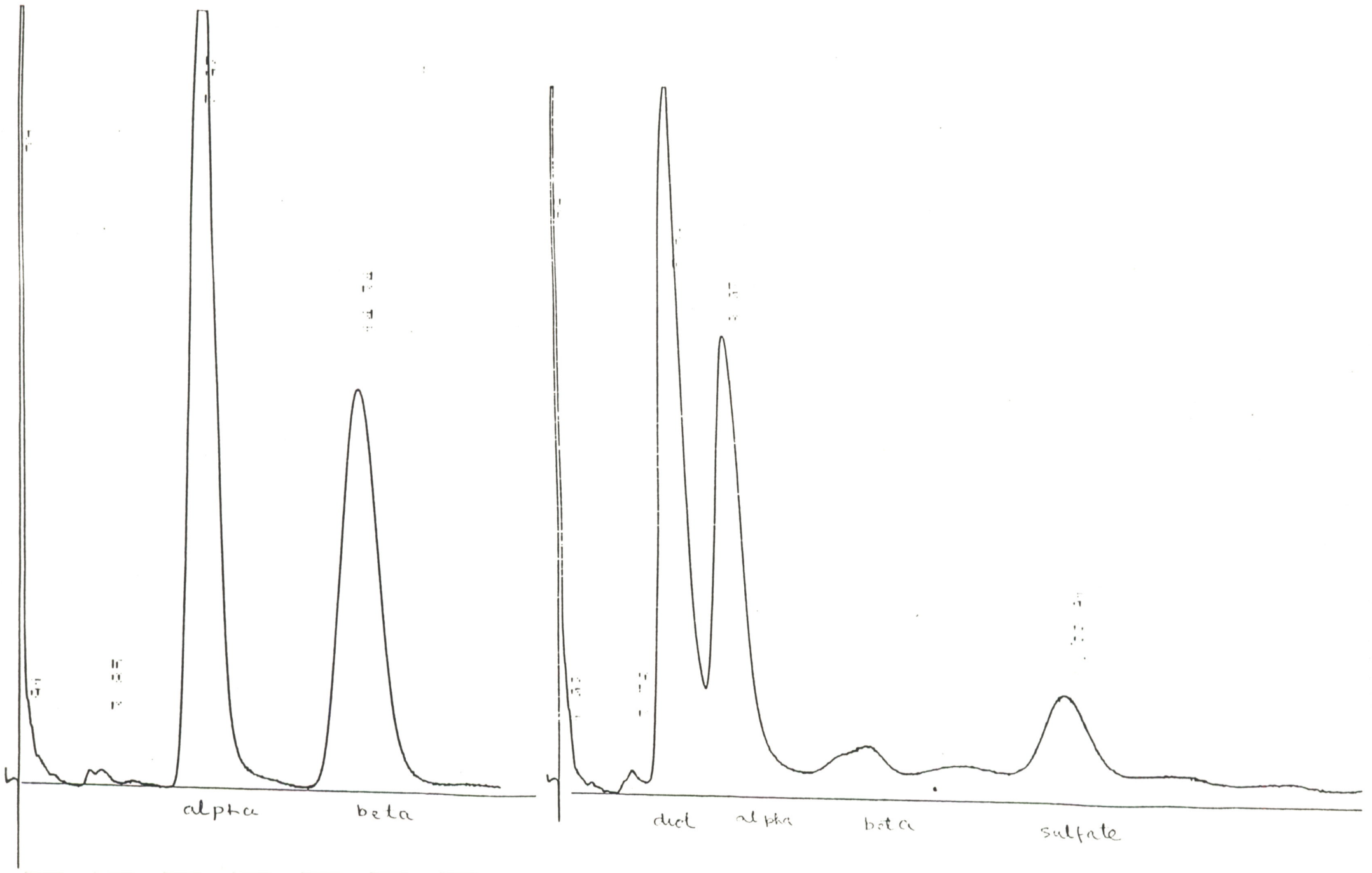
An experiment was conducted by using α- or β- isomers of endosulfan separately as sole source of carbon for *Mucor thermo-hyalospora*. The preliminary analysis revealed that the β- isomer of endosulfan was readily degraded to endosulfan sulfate (Rf 0.50) and to other metabolites.

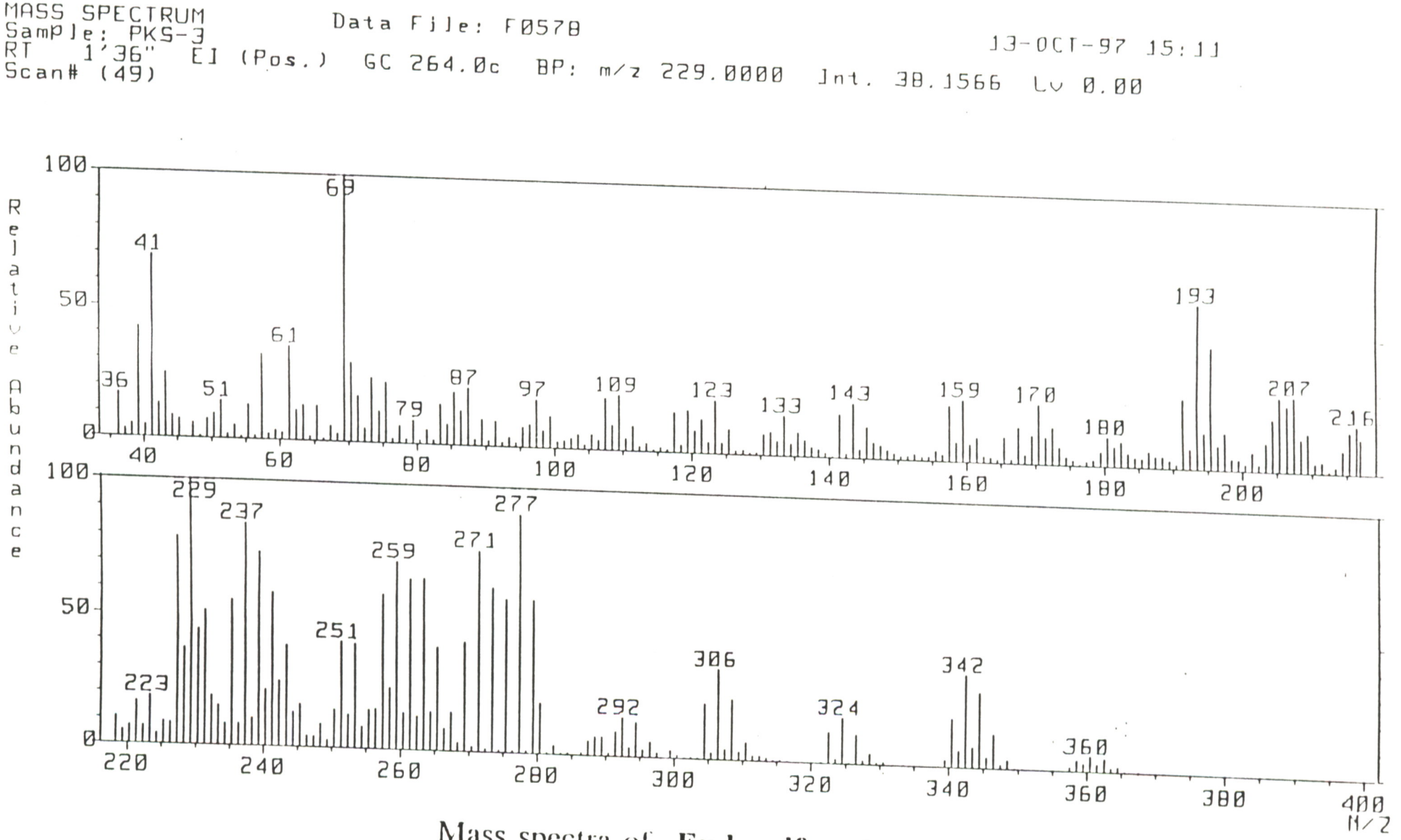
Mucor piriformis isolated from soil has been shown to bring about transformation of atrazine. Fermentation carried out in neutral media for a period of 8-9 days resulted in the formation of atleast three metabolites which were not seen in the control experiments

The bacterial isolates were grown in ten times diluted nutrient broth containing a known concentration of endosulfan. The preliminary analysis of the 10th and 15th day culture extracts on silica gel-G plates revealed the presence of an extra spot (Rf 0.18), with the decrease in the concentration of β-isomer of endosulfan. However, this was not observed in the control experiment and also in the culture extracts of BMM supplemented with different carbon sources. Similar experiments were carried out using atrazine, whereas preparative chromatography did not confirm the degradation of this molecule.



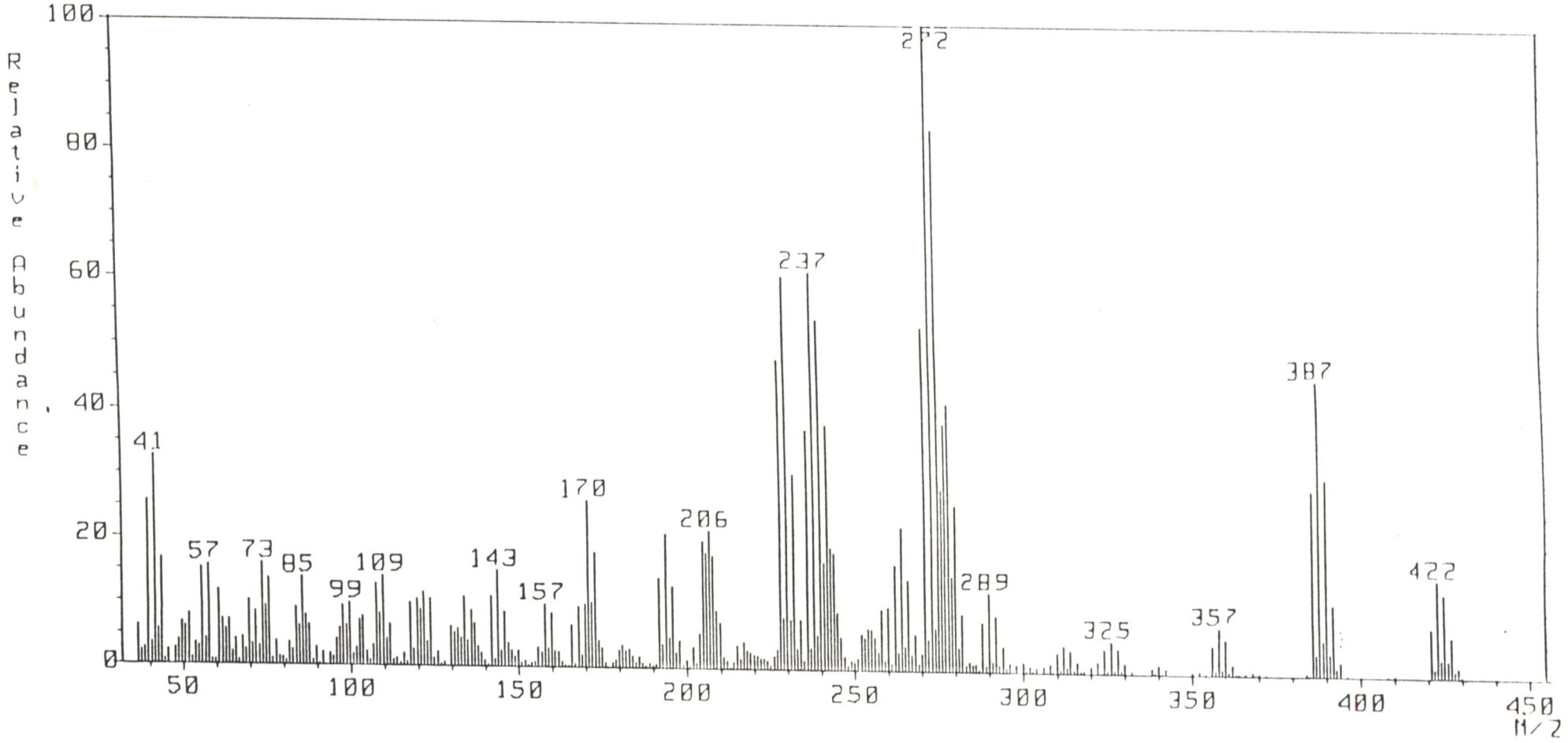
Time course of mycelial mass (*Mucor thermo-hyalospora*)


alpha
beta
dual
alpha
beta
sulfate



Mass spectra of **Endosulfan diol**

MASS SPECTRUM Data File: F0589 14-OCT-97 14:45
Sample: PKS-1
RT 1'16" EI (Pos.) GC 264.0c BP: m/z 272.0000 Int. 49.2357 Lv 0.00
Scan# (39)



Mass spectra of **Endosulfan Sulphate**

Chapter IV

The biodegradation of pesticides in the environment are influenced by various physical and chemical factors such as temperature, pH, sunlight and also competition among microbial populations. In general, microorganisms degrade synthetic pesticides to generate energy and nutrients for their own growth, in the process these organisms detoxify or convert the toxic molecules in to simpler inorganic forms. The microorganisms which are capable of degrading specific pesticide may be isolated from soil by enrichment technique by incubating a source of microorganisms with the substance of interest. The efficient microorganisms or their enzymes or engineered organisms may be successfully utilized in decontamination of pesticide contaminated soil and aquatic environment.

In the present study an effort was made to isolate efficient strains of microorganisms capable of degrading two of the most widely used pesticides in Indian agriculture namely the s-triazine herbicide, atrazine and cyclodiene insecticide, endosulfan. In order to obtain an efficient organism, several bacterial and fungal strains isolated from enrichment technique and also a few identified laboratory strains were screened for their ability to degrade atrazine and endosulfan molecules. The results from this studies revealed that none of the fungal strains isolated from the enrichment technique degraded the atrazine or endosulfan molecules at the concentrations of 30 to 50ppm levels. However, a few identified strains particularly *Mucor thermo - hyalospora* were found to be capable of bring about transformation of endosulfan molecule. The preliminary analysis of the culture extract revealed the presence of two transformation products (M V and M I) with Rf value of 0.24 and 0.50 respectively. These metabolites had the same Rf value as that of reference standards of endosulfan diol (Rf = 0.24) and endosulfan sulfate (Rf = 0.50). This was further confirmed by GLC -ECD studies. The substrate disappearance followed by increase in product formation indicated the degradation of endosulfan by the organism. Further, spectral and analytical studies confirmed the formation of endosulfan diol as the major metabolites followed by the production of endosulfan sulfate. Martens

(1976) reported that the majority of soil fungi found to produce endosulfan sulfate which indicated the oxidative mechanism of metabolism. According to an earlier reports endosulfan sulfate is more persistent and as toxic as the parent isomers, but the formation of endosulfan diol and endosulfan hydroxy ether constitutes a detoxification (Maier-Bode 1968; Guerin and Kennedy 1992). In the present investigation the fungus, *Mucor thermo - hylospora* MTCC 1384 found to be efficient in bringing transformation in carbon deficient medium with formation of a major non- toxic metabolite, endosulfan diol. El Zorghani and Omer (1974) earlier reported the transformation of endosulfan to endosulfan diol by *Aspergillus niger*. Katayama and Matsumura (1993) reported that the oxidative enzyme system in *Trichoderma harzianum* responsible for degradation of endosulfan to endosulfan sulfate, further a hydrolytic enzyme, such as sulfatase, was found to be responsible for the indirect formation of endosulfan diol by hydrolysis of endosulfan sulfate. More recently, Kullman and Matsumura (1996) found the transformation of endosulfan by a white rot fungus, *Phanerochaete chrysosporium* through oxidative and hydrolytic pathways producing endosulfan sulfate and endosulfan diol. In our experiments the preparative chromatography showed three other transformation products, which did not produce in significant quantities, so they were not identified.

Prior to degradation studies, pH profiles of the fungal isolates were monitored in anticipation that at pH extremes, endosulfan or atrazine may undergo chemical degradation. Martens (1976) has reported that above pH 8, chemical degradation of endosulfan is of importance, according to him the conversion is more than 90%, with the formation of endosulfan diol by the hydrolysis of endosulfan. Similarly, several workers have reported the chemical hydrolysis of atrazine in strongly acidic or basic solutions (Armstrong et al., 1967, Giardi et al., 1985). In the present studies, a few fungal isolates tested were found to maintain the pH in the range of 6.00-8.00 and these strains were subjected for a detailed degradation studies along other identified strains. The present study revealed that the atrazine or endosulfan do not undergo the chemical transformation at neutral pH range.

Earlier report showed that α-isomer of endosulfan was readily degraded in soil when compared to β-isomer (Steward and Cairns, 1974). Whereas, Rao and coworkers (1980) have reported that β-endosulfan was more persistent than that of α-endosulfan. The ability of α- and β- isomers to undergo transformation individually was tested using *Mucor thermo-hyalospora*. The organism was found to degrade β-isomer more readily to endosulfan sulfate than that of α-isomer. In an experiment carried out by Mukherjee and Gopal (1994) using *Aspergillus niger* showed that the organism was readily converted β-isomer to endosulfan diol.

The efficiency of *Mucor thermo-hyalospora* to transform endosulfan in carbon rich(3% sucrose) and carbon limiting(0.3% sucrose) conditions were also studied. The fungus was found to transform endosulfan more efficiently in carbon limiting conditions, indicating the utilization of endosulfan as a source of carbon. The influence of media composition on the degradative ability of a white rot fungus *Phanerochaete chrysosporium* has been shown by Kullman and Matsumura (1996). They observed the highest degradation rates in the carbon deficient medium. The present study also revealed that the fungus, *Mucor thermo-hyalospora* was efficiently degraded endosulfan molecules upto the concentration of 56 μl/ml.

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