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ORIGINAL ARTICLE

Isolation of Pesticide Degrading Microorganisms from soil

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ABSTRACT
In developing nations like India pesticides play an important role in agricultural. Their importance in crop yield improvement is understood and well accepted. But their highly toxic nature posses a fatal environmental threat. Present study is directed towards development of biodegradation method to reduce the environmental burden of the pesticide. During this study six bacterial and four fungal isolates have shown significant ability to carry out the degradation of pesticides, these cultures were identified as Acinetobacter radioresistens, Pseudomonas frederiksbergensis, Bacillus pumilus, Serratia liquefaciens, Serratia marcescens, Burkholderia gladioli among bacteria and Aspergillus niger, Ganoderma austral, Trichosporon , Verticillium dahaliae among fungus.
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INTRODUCTION

According to Third World Network reports, more than one billion pounds (450 million kilograms) of toxins are released globally in air and water. The contaminants causing ecological problems leading to imbalance in nature is of global concern. A vast number of pollutants and waste materials including heavy metals are disposed into the environment per annum. Approximately 6 **x** 10^6 chemical compounds have been synthesized, with 1,000 new chemicals being synthesized annually. Almost 60,000 to 95,000 chemicals are in commercial use.

Pesticides a quit significant among these chemicals, which are defined as any substance or mixture of substances which are used to control destructive pests such as insects, plant disease organisms and weeds, including many other living organisms such as nematodes, arthropods other than insects, and vertebrates that endanger our food supply, health, or comfort. In particular, the term pesticide refers to chemical substances that alter biological processes of living organisms deemed to be pests, whether these are insects, mould or fungi, weeds or noxious plants. Pesticides are widely used in most areas of crop production to minimize infestations by pests and thus protect crops from potential yield losses and reduction of product quality [1]. These pests potentially cause damage or interfere in any other way in the production, elaboration, storage, transport, or commercialization of food, agricultural products and wood products or animal food. Pesticides may be given to animals to prevent insects, arachnids or other plagues in or over their bodies [2].

Certainly, pesticides have improved longevity and quality of life, chiefly in the area of public health. Insect control programs have saved millions of lives by combating diseases such as malaria, yellow fever and typhus. In addition, the use of pesticides constitutes an important aspect of modern agriculture, as they are absolutely necessary for economical pest management [3]. The most promising opportunity for maximizing benefits and minimizing risks is to invest time, money, and effort into developing a diverse toolbox of pest control strategies that include safe products and practices that integrate chemical approaches into an overall and ecologically based framework which will optimize sustainable production, environmental quality, and human health [1].

However, the application of pesticides may cause adverse effects among the different forms of life and among the ecosystems; this will depend on the sensibility grade of the organisms and the pesticide [4]. Approximately 90% of agricultural pesticide application never reaches its target organisms but is, instead, dispersed through the air, soil, and water. As a result, they are routinely detected in air, surface and ground water, sediment, soil, vegetable, and to some extent in foods. In addition, many soil-applied pesticides are also intentionally introduced into the soil environment for the control of soil borne pests and pathogens, which results in the accumulation of their residues and metabolites in soil at unacceptably high levels [5,6]. The inadequate application practice is one of the most important ways of pollution, which has a profound impact not only on the soils of the areas in which they are applied. Pesticides are washed into aquatic ecosystems by water runoff and soil erosion. Pesticides also can drift during application and contaminate aquatic systems. Some soluble pesticides are easily leached into streams and lakes. Wild birds and mammals are damaged and destroyed by pesticides and these animals make excellent "indicator species". Deleterious effects on wildlife include death from the direct exposure to pesticides or secondary poisonings from consuming contaminated food; reduced survival, growth, and reproductive rates from exposure to sub-lethal dosages; and habitat reduction through the elimination of food resources and refuges. Pesticides easily find their way into soils, where they may be toxic to arthropods, earthworms, fungi, bacteria, and protozoa. Small organisms are vital to ecosystems because they dominate both the structure and function of ecosystems. Like pest populations, beneficial natural enemies and biodiversity (predators and parasites) are adversely affected by pesticides.

Bioremediation is described as the use of microorganisms to destroy or immobilize waste materials [7]. This process of detoxification targets the harmful chemicals by mineralization, transformation, or alteration [8]. For centuries, civilizations have used natural bioremediation in wastewater treatment, but intentional use for the reduction of hazardous wastes is a more recent development. Modern bioremediation and the use of microbes to consume pollutants are credited, in part, to George Robinson [9].

Interest in the microbial biodegradation of pollutants has intensified in recent years as humanity strives to find sustainable ways to clean up contaminated environments. These bioremediation and biotransformation methods endeavour to harness the astonishing, naturally occurring ability of microbial xenobiotic metabolism to degrade, transform or accumulate a huge range of compounds including hydrocarbons (e.g. oil), polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), heterocyclic compounds (such as pyridine or quinoline), pharmaceutical substances, radionuclides and metals. Major methodological breakthroughs in recent years have enabled detailed genomic, metagenomic, proteomic, bioinformatic and other high-throughput analyses of environmentally relevant microorganisms providing unprecedented insights into key biodegradative pathways and the ability of organisms to adapt to changing environmental conditions.

The present study focuses on the isolation and characterization of bacterial cultures for biodegradation of pesticides commonly used in the agriculture.

MATERIALS AND METHOD

Collection of soil samples

Soil samples were collected from 2 agricultural areas of Beed District, Maharashtra, with history of continued farming activities for more than 30 years. Surface soil from 0-15 cm were collected, placed in plastic bags, transported on ice to the laboratory and stored at 4°C until analysis. Soil samples were air-dried and sieved through a 10 mm mesh prior to bacterial screening.

Screening and isolation of Pesticide degrading bacteria

Ten grams of soil sample were added to 100 ml MSYM and enriched with an addition of 25 ppm pesticide. Samples were incubated on rotary shaker (150 rpm) at 30°C for 7 days and then transferred to a fresh medium and incubated at the same conditions, after which, the cultures were regularly transferred every 3-4 days or until increased turbidity were evidenced. After 3-4 times of repeated sub-culturing, 0.1 ml culture broth was pipetted and spreaded on MSYM+pesticide agar. Single colonies were selected and streaked on nutrient agar (NA) supplemented with 25, 50 and 100 ppm of the pesticide. Cultures were incubated at 30°C for 3 days. Pesticide degrading isolates were selected from isolates which developed clear zone surrounding their colonies when grown on NA supplemented with 25, 50 and 100 ppm of the pesticide.

Biodegradation of Pesticide by bacterial isolates

Selected bacterial isolates were grown in MSYM + pesticide and incubated on rotary shaker at 150 rpm, 30°C for 10 days. Samples were collected every 24 hrs for the determination of pesticide concentration and cell growth via total protein measurement by Lowry's method (Stoscheck, 1990). To determine

pesticide concentration, 2 ml culture broth was extracted with 4 ml ethyl acetate (repeated 3 times)and evaporated under vacuum condition until dryness. The extract was redissolved with hexane to a volume of 2 ml and analyzed with GC-ECD.

Characterization of bacterial isolates

Morphological studies

Bacterial isolates were grown in MSYM+pesticide and incubated at 30°C until either turbidity or colony was observed. Gram's stain and cell morphology was investigated under microscope (1000x magnification).

16S rDNA analysis

Genomic DNA was extracted by boiling method (Yamada et al., 2002). One ml of cell culture grown in nutrient broth (NB) at 30°C for 18-24 hrs was centrifuged at 10,000 rpm for 10 min then washed 2 times with TE-buffer (pH 7.8). Washed cell was resuspended with 0.3 ml TE-buffer (pH 7.8) then boiled at 100°C for 10 min, followed immediately by cooling at 0°C for 5 min. This boiling/cooling step was repeated 3 times. 16S rDNA from the cell extract obtained with the above procedure was amplified by PCR using 63F (5'- CAGGCCTAACACATGCAAGTC-3') and 1492R (5'- ACGGCTACCTTGTTACGACTT-3') primers. The reaction composition consisted of 0.2 mM each dNTP, 0.2 M of each primer, 5 l 10x PCR buffer, 10 l cell extract, 2.5 units Taq DNA polymerase and sterile deionized water to a final volume of 50 l. Following a hot start (94°C for 3 min), 25 cycles of amplification were used (94°C for 1 min, 50oC for 45 sec, 72°C for 2 min) followed by a final single extension of 72°C for 10 min. The PCR products were separated by electrophoresis on a 1% agarose gel and visualized under UV light after staining with ethidium bromide. The amplified PCR product was purified using QIA quick PCR Purification Kit (QIAGEN, Inc.) according to the manufacturer's instruction. DNA sequencing was performed by Macrogen, Inc. (South Korea) and 16S rDNA sequences were BLAST searched against GenBank database (http:// www.ncbi.nlm.nih.gov/). Phylogenetic tree was constructed using Treeview software (Page, 1996).

Isolation, Identification and Screening of fungus:

The collected soil samples were amended with 25 ppm of each pesticide mixed thoroughly and this mixture is directly incubated at room temperature for about two weeks. The pretreated soil samples were washed with distilled water and allowed to stand still for 30 minutes. After all the soil debris has settled down, the supernatant was decanted into a sterile test tube and serially diluted. Dilutions below 10^{-6} were plated in Potato Dextrose Agar (PDA) +pesticide medium. After 6-7 days of incubation a number of fungal strains were observed on the plate. The most prominent fungus was selected and identified as Aspergillus sp. The isolated fungal colonies were transferred on to the PDA slants and afterwards once again tested to grow in presence of pesticides by culturing them in PDA + pesticide medium, cultures showing highest degree of degradation were selected for further studies.

Bioremediation Assay:

To study the bioremediation of pesticide using Aspergillus sp., two different culture media were prepared in triplicate – medium containing PDA and 0.5 percent (w/w) of pesticide and medium containing PDA, 0.5 percent (w/w) of pesticide inoculated with the spores of Aspergillus sp. The plates were then maintained for about 8 days at 28°C in an incubator.

RESULTS AND DISCUSSION

During this study six (06) bacterial isolates (Table 1) were found to show significant ability to carry out the degradation of selected pesticides, these cultures were identified as *Acinetobacter radioresistens, Pseudomonas frederiksbergensis, Bacillus pumilus, Serratia liquefaciens, Serratia marcescens, Burkholderia gladioli.* The findings for degradation potential of these isolates for selected isolates are tabulated in Table 2.

Acinetobacter radioresistens, has shown considerable degradation of Chlorpyrifos with 38% degradation and marginal degradation of Malathion with 1% degradation, while for rest of the pesticides there was no degradation by this culture.

Pseudomonas frederiksbergensis, was found to be capable of degrading Endosulfan, Chlorpyrifos and Malathion, but this ability was only marginal with 4%, 4% and 9% degradation respectively.

Bacillus pumilus, has shown effective degradation of Malathion and Dimetoate with 45% and 37% degradation respectively and was also able to partially degrade Chlorpyrifos and Igepal-CO 210 with 15% and 8% degradation respectively.

Serratia liquefaciens, was found to degrade Diazinon at 51% and marginal degradation of Malathion with 11% degradation.

Serratia marcescens, has shown partial degradation of Diazinon (34%) and marginal degradation of Chlorpyrifos, Igepal CO-210 and Methyl Parathion with 8%, 2% and 1% degradation.

Burkholderia gladioli. Has only shown marginal degradation of Dimetoate with 3% degradation, while other pesticides were not degraded at al by this culture.

Table 1 : Isolation of Microorganisms from the pesticide contaminated soil samples

S.No.	Isolates	**Identified cultures**
1	*B6*	*Acinetobacter radioresistens*
2	*B11*	*Pseudomonas frederiksbergensis*
3	*B12*	*Bacillus pumilus*
4	*B14*	*Serratia liquefaciens*
5	*B15*	*Serratia marcescens*
6	*B17*	*Burkholderia gladioli*

Table 2 : % Pesticide degradation by the isolated cultures

No.	Microorganism	Methyl parathion	Endosulfan	Isoproturon	Chlorpyrifos	Igepal CO-210	Dimetoate,	Malathion	Diazinon	Lindane
1	*Acinetobacter radioresistens*	--	--	--	**38**	--	--	**01**	--	
2	*Pseudomonas frederiksbergensis*	**10**	**04**	--	--	--	**04**	**09**		
3	*Bacillus pumilus*	--	--	--	**15**	**08**	**37**	**45**		
4	*Serratia liquefaciens*	--	--	--	--	--	--	**11**	**51**	
5	*Serratia marcescens*	**01**	--	--	**08**	**02**	--	--	**34**	
6	*Burkholderia gladioli*	--	--	--	--	--	**03**	--		

During this study four (04) fungal isolates were found to show significant ability to carry out the degradation of selected pesticides, these cultures were identified as *Aspergillus niger, Ganoderma austral, Trichosporon* , *Verticillium dahaliae .*

Aspergillus niger, has shown maximum 59% degradation of Endosulfan, and it has degradaed 29% of Lindane, it was not effective against other pesticides.

Ganoderma austral, has shown highest 61% degradation for Lindane whereas for Chlorpyrifos degradation was only 16%.

Trichosporon sp., has given highest degradation of Chlorpyrifos at 55%, and for Endosulfan and , Lindane it was 10% and 7% respectively.

For *Verticillium dahaliae*, maximum degradation of 64% was seen for Chlorpyrifos, whereas for Endosulfan it was 8%, , for Malathion it was 10% and for Lindane it was 10%.

Table 3 : Isolation of Microorganisms from the pesticide contaminated soil samples

S.No.	Isolates	**Identified cultures**
1	*F24*	*Aspergillus niger*
2	*F43*	*Ganoderma austral*
3	*F79*	*Trichosporon sp.*
4	*F102*	*Verticillium dahaliae*

Table 4 : % Pesticide degradation by the isolated cultures

S.No.	Microorganism	**Methyl parathion**	**Endosulfan**	Isoproturon	**Chlorpyrifos**	**Igepal CO-210**	Dimetoate,	Malathion	**Diazinon**	Lindane
1	*Aspergillus niger*	--	**59**	--	--	--		--		**29**
2	*Ganoderma austral*	--	--	--	**16**	--		--		**65**
3	*Trichosporon*	--	**10**	--	**55**	--		--		**07**
4	*Verticillium dahaliae*	--	**08**	--	**64**	--		**10**		**10**

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