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Isolation, Growth and Identification of chlorpyrifos degrading microorganisms from Agricultural soil

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Abstract : *Pesticides are a large and varied group of substances that are specifically designed to kill organisms including of weeds, insects, and the indiscriminate use of pesticides in agricultural field resulted into contamination of soil environment leading to toxicity. The current method for removing such contaminants from the environment through biodegradation has been shown to be more effective than any other method that is biodegradation using native microorganisms for pesticides removal from the environment is quite attractive .Chlorpyrifos is a broad-spectrum, moderately toxic pesticide that has been widely used in the prevention of agricultural pests. In the present study, we collected soil sample having history of chlorpyrifos from four different soil sample (Rice,wheat,maize and vegetable). Nine chlorpyrifos pesticide utilizing bacteria were isolated and identified through cultural and biochemical tests as strains of Bacillus sp, Staphylococcus sp. Coccus sps. Their growth in minimal salt medium supplemented with 200µg/ml and 250µg/ml of Chlorpyrifos was monitored at optical density 600nm. The result showed that Staphylococcus sp., Streptococcus sp. and Bacillus sp. had maximum growth at twelve days, while Coccus sp. Gram-ive Bacillus sp. shows highest growth upto four days of incubation at200µg/ml and upto eight days of incubation at 250µg/ml of Chlorpyrifos. The results of this research indicated that the isolated bacteria can be used for bioremediation of Chlorpyrifos contaminated soil.*

Keywords *Isolation, Bacteria, Chlorpyrifos, chlorpyrifos containinated Agricultural Soil.*

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Introduction :

India is an agriculture based country and maximum portion of it's economy is dependent on agriculture. The promotion of high yielding varieties of crops has led to large scale use of chemicals as pesticides. The increased demand of agro-products in changing regional climate has resulted in an increase in consumption and application of pesticides (Shetty et al.,2008).

Application of pesticides on agricultural soil is now a common practice in all over the world and is an important factor of integrated pest management (IPM) strategies. Some of these pesticides persist in the soil to form pollutants which may occasionally lead to surface and groundwater contamination.

Improper handling and unsafe spraying of the agrochemicals cause high risk of health hazards.

Chlorpyrifos, is one of the most commonly and widely used commercial orgnophosphate pesticide which considers as one of the most frequently used chlorinated organophosphorus pesticides (Maya et al., 2011). It is a broad-spectrum, moderately toxic pesticide that has been widely used in the prevention of both agricultural pests and urban public health pests. It was introduced in 1965 by Dow Chemical Company India. It has large blights on public health and environment resulting from its long residual period in soil and water.

The microbial action in the environment causes the natural degradation of the pesticides which might convert parent compounds to intermediates or comparatively less toxic compounds. The adaptability of microorganisms during bioremediation releases certain enzymes, which metabolizes wide spectrum of anthropogenic chemicals.

Many bacteria that are able to degrade organophosphate pesticides have been isolated from soil around the world, which could utilize Chlorpyrifos as the only source of carbon and phosphorous Yang et al.(2006).

The first step in dealing with pollution caused by Chlorpyrifos is the isolation and screening of microbial species that can degrade the chlorpyrifos effectively.

The main objective of this study is to isolate and identify Chlorpyrifos utilizing microbes from agricultural soil using an enrichment culture technique and determining the growth response of bacteria in minimal salt medium supplemented with chlorpyrifos.

Materials and Methods :

The present research work was conducted in the Department of Industrial Microbiology, Patna Women's College, Patna during the period July to october, 2016.

Commercial grade pesticide Chlorpyrifos with mg/ml concentration was obtained.

Nutrient Agar medium used for isolation of bacteria contain the following (g/l): (pH7.2- 7.4, peptone 5g ,beef extract 3g , sodium chloride 5g , agar 15g , D.W1000ml) and potato Dextrose Agar media used for isolation of fungi contain the following (g/l): (pH 5.4, potato 200g ,dextrose 20g , agar 20 g , D.W 1000ml)

Urea agar, Nutrient agar, Simon Citrate agar, MacConkey agar, and Nutrient broth were also used during the isolation and identification of Chlorpyrifos degrading bacteria.

Agricultural soil were taken from different area of Bihar. The rice field in Belwar, Madhubani, Chitkohra, patna, and Mohammadpur, Khagaul,Patna ,as well as maize and wheat field in Laie, Bihta and vegetable field in Mohammadpur, Khagaul having past of history of chlorpyrifos used in pest control were selected for this study.

Samples were collected randomly from three rice fields and three other crops fields from 12 - 15cm depth of the field and stored aseptically for further analysis.

For isolation of microbes from different soil samples sereial dilution were carried out by dissolving 0.1g of each soil samples in 9.9ml of normal saline solution.

For isolation of bacteria 1ml of soil suspension of different samples were spread over the pre sterilized petri plates containing nutrient agar media at dilution 10^{-5},10^{-6} and 10^{-7} and culture plates were incubated at 37^0C for 24h.

For isolation of fungi from different soil samples were done by spreading 1ml of soil suspension of different samples over the pre sterilized petri plates containing potato dextrose agar media at dilution 10^{-3},10^{-4} and 10^{-5} and culture plates were incubated at 26^0 C for 48h. And the isolated colonies were selected and pured on PDA plates for further studies.

Chlorpyrifos utilizing microbes were isolated from soil samples by the enrichment culture technique nutrient culture medium, using chlorpyrifos as the sole source of carbon. This was done by sub-culturing those pure culture of bacteria and fungi of pesticides containing NA media and PDA media plates and incubated at 37^0C for bacterial plates and 26^0C for fungal plates for 24-48h.

The Fungi isolates were identified based on the appearance, mycelia, spores and colour accordingly with lactophenol cotton blue test.

Fungal cultures from PDA plates were taken and mounts of the fungal isolates were made each with lactophenol cotton blue and examined individually microscopically using 10X and 40X objective lens and identified.

The bacterial isolates grown on Chlorpyrifos agar were subjected to physiological and biochemical tests. The tests carried out include: Gram staining, catalase test, citrate utilization, oxidase test, indole production, motility, sugar fermentation, methyl-red test, nitrate reduction, starch hydrolysis, Voges-Proskeur test and hydrogen sulphide production.

Growth curve experiments were performed with different doses of chlorpyrifos in order to determine the optimum concentration of chlorpyrifos that stimulates the growth of isolates in liquid medium at different concentration(i.e 200µg,250µg) of chlorpyrifos at interval of 2,4,8,12,and14 days using spectrophotometer at 600nm by taking O.D(optical density).

And then, the optimization of those bacterial isolates which shows better growth response in different concentration of chlorpyrifos have been done to determine the degrading ability of isolates to degrade chlorpyrifos pesticide at different pH, (2,4,7,9,11) and temperature (4,6,25,37,40 and 60^0C).

Results and Discussion :

From different rice soil samples (R_1, R_2, R_3,) the number of fungi isolated are in the following order:

From R_1 *Rhizopus* sp., from R_2 *Aspergillus flavus ,* from R_3 *Mucor* and *Aspergillus fumigates*, from wheat *Penicillium* sp. were obtained and from maize and of vegetable soil *Rhizopus* sp. were isolated.

The result of fungal characterisation are shown in Table1.

Table 1. Culture Characteristics of isolated fungi

Soil Samples	Types of colonies	Colony characteristics			Organisms
		Colour	Margin	Texture	
R_1	(a)	white	white	Cottony	*Rhizopus sp.*
R_2	(b)	Green		Powdery	*Aspergillus flavus*
R_3	(a)	White grey		cottony	*Mucor sp.*
	(b)	white	white	cottony	*Aspergillus fumigatus*
Maize	(a)	White		cottony	*Rhizopus sp.*
Wheat	(a)	Olive green	White	powdery	*Penicillium sp.*
Vegetable	(a)	white	white	cottony	*Rhizopus sp.*

Bacterial Isolates The bacterial isolates grown on Chlorpyrifos agar were subjected to physiological and biochemical tests. The results of morphological, cultural and biochemical testscarried out are shown in Table 2.

Table 2. Morphological, cultural and biochemical characteristics of the Isolates

Isolates	Colony Morphology	Gram's Reaction	Methyl Red	Voges-Proskauer	Indole	Catalase test	Citrate	Starch	urease	H_2S	Glucose Fermen-tation	Identity
R_1(a)	Off whitish, slimy and elevated, looking colony	+ve/rod in chain	-	+	-	-	-	+	-	-	A/G	***Bacillus sp.***
R_1(b)	Slimy texture, white in colour, elevated colony	+ve /coccus in chain	-	-	-	-	-	-	-	-	A/G	***Strepto-coccus sp.***
R_2	Orangish, smooth,slimy, elevated colony	+ve /coccus in chain	+	-	-	+	+	-	-	-	A	***Staphylo-coccus sp.***
R_3	Slimy texture, white in colour, flat colony	-ve / rods	-	+	-	-	+	-	+	-	A/G	***Klebsiella sp.***
Maize(a)	Offwhitish,slimy and elevated, looking colony	+ve /coccus	-	-	-	-	+	-	-	-	A/G	***Coccus sp.***
Maize(b)	Whitish, smooth, slimy, elevated colony	+ve /rod in chain	-	-	-	-	-	+	-	-	A	***Bacillus sp.***
Wheat(a)	Slimy texture, yellow in colour, elevated colony	+ve /coccus in clusture	-	-	-	+	-	-	-	-	A	***Staphylo-coccus sp.***
Wheat(b)	off Whitish, slimy and elevated, looking colony	+ve /coccus	-	-	-	-	+	-	-	-	A/G	***Coccus sp.***
Vegetable (a)	Off whitish, slimy, elevated colony	+ve /coccus in clusture	+	-	-	-	-	-	-	-	A	***Staphylo-coccus Sp.***

- a and b are type of isolate from same sample.

The result of the growth response of the isolates in the presence of Chlorpyrifos showed that all the isolates utilized the pesticide as the only carbon source.

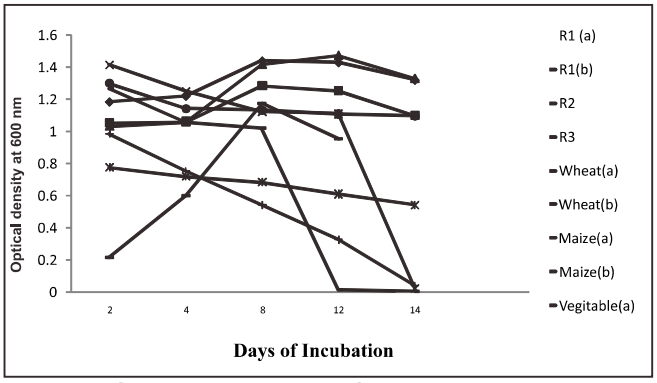

Fig.1. Growth response of all the isolates in the presence of chlorpyrifos

At 250µg/ml concentration of chlorpyrifos, maximum growth were shown by isolate R_2(a) during incubation period of 12 days.

And isolate, R_1(a) & (b), maize(b) and vegetable(a) have shown maximum growth at 8 days of incubation period whereas, isolate R_3(a) and wheat(a) & (b) has maximum growth at 4 days of incubation periods.

Out of eleven bacterial isolates only nine isolates were capable of utilizing Chlorpyrifos as the source of carbon. All of the isolates were Gram +ive bacteria,except one isolate i.e R_3(a) which was Gram -ive rods i.e *Klebsiella* sp Munazza et al., (2005) and Furham et al., (2013) have also reported *Klebsiella* sp. as a chlorpyrifos utilizer.

After, observing the growth kinetics of all bacterial isolates in different concentration of chlorpyrifos it was observed that *Staphylococcus* sp. from R_2 and vegetable soil sample, *positive rods* and *streptococcus* sp. from R_1 soil sample showed maximum growth at 200µg/ml concentration of chlorpyrifos in twelve days of incubation while at concentration 250µg/ml of chlorpyrifos only *Staphylococcus* sp. from R_2 showed maximum growth than other isolates and It was confirmed that these isolates were able to utilize and degrade chlorpyrifos.

It has been suggested that cultures of bacteria with the ability to degrade specific compounds can be used for bioremediation process of pesticide polluted sites (Kavi Karunya and Saranraj, 2014). The result of optimization of above four isolates at different pH and temperature were also reported in this way that the maximum growth rate of bacteria was recorded at pH 7, 9 and10.5. according to the method described by Zajic and Supplisson (1972) to reach the optimum pH values for microbial growth. The least growth rate of was recorded at pH 4 of *Staphylococcus* sp. of sample R_2 and vegetable. The maximum growth rate of bacteria was recorded at temperature 25°C, 37°C,and 40^{0}C by all four isolates and it was reported that the most rapid degradation of chlorpyrifos was observed at 30 ºC (Singh et al., 2004 and Racke et al., 1990) and least growth rate at 6^{0}C was shown by *Staphylococcus* sp. of sample R_2 and vegetable. And no growth was recorded at 60^{0}C and 4^{0}C. Hence, those isolates which showed luxurious growth at higher temperature i.e at 40^{0}C, may be thermophillic bacteria.

Conclusion :

The present study reports the identification of a bacterium, as *Staphylococcus* sp., *Streptococcus* sp. *and Rods* which is capable of utilizing chlorpyrifos as a source of carbon. Utilization of xenobiotic compounds by soil microorganisms is a crucial phenomenon by which these compounds are removed from the environment, thus preventing environmental pollution. Results from the present study suggest that the isolated bacteria *Staphylococcus* sp., *Streptococcus* sp. *and Rods* are able to grow in medium in the

presence of added pesticide (100µg/ml - 250µg/ml)and also shows better growth response at higher temperature and pH and may therefore be used for bioremediation of pesticide-contaminated soil.

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