Biodegradation of chlorpyrifos using Actinomycetes, isolated from coffee plantation soil of Chikmagalur, Karnataka, India.

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ABSTRACT: Environmental pollution caused by pesticides and their degradation products is a major ecological problem. Pesticides are the synthetic compounds introduced into agricultural systems to protect crops against weeds, insects, fungi and other pests.. Organophosphate pesticides are commonly used in agriculture for pest control. Organophosphate pesticides constitute the largest group of pesticides account for about 38% of total pesticides used worldwide. Chlorpyrifos is broad spectrum moderately toxic organophosphate pesticide. Pesticide biodegradation capacity developed by soil microorganisms is a major issue which maintains the fertility of soil. Actinomycetes are group of bacteria known for their metabolic capacities and have considered potential for the biotransformation and biodegradation of pesticides. The present study focused on biodegradation of chlorpyrifos pesticides by actinomycetes isolated from coffee plantation soil's of Chikmagalur and their residual analysis. Soil samples were collected and the isolation of actinomycetes was carried out. Total 29 isolates were recovered. The isolates were further subjected for morphological and biochemical characterization studies. Based on morphological and Biochemical characterization, the isolates belongs to *Streptomyces* species. The isolates which were capable of utilizing chlorpyrifos as the sole source of carbon were subjected to degrade chlorpyrifos in bulk. The residual analysis of fermentation extract was done by AOAC method.

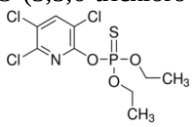
Keywords: Actinomycetes, *Streptomyces*, Chlorpyrifos, Biodegradation, Residual analysis.

I. INTRODUCTION

Agriculture is the important need of the increasing population to meet their food demand. Pesticides are the major reason for large amount of crop yield. The high ability to control the harmful pest is the main reason for the increasing demand of the pesticide all over the world (Rajalakshmi and Raju, 2017) Pesticides are synthetic organic compounds, million tons of pesticides are applied annually in modern agriculture to increase the production through controlling harmful effects caused by the target organisms including insects, fungi, bacteria, viruses as well as grasses grown in between the economical crops (Nawaz *et al., 2011*; Aly *et al*., 2017). However the majority of the applied pesticides, even if sprayed on foliage of crop plants and weeds, well eventually reach the soil, which may affects the growth and activity of soil microbial communities.(Xiaoqiang *et al.,2008).*

Organophosphorus pesticides (OPs) are the most widely used group of pesticides, accounting for more than 36% of the total world market (Briceno. G *et al., 2012*). OPs insecticides are ester or thiol derivatives of phosphoric acid, whose mode of action is through the inhibition of enzyme Acetylcholinesterase, which is responsible for nerve transmission. Chlorpyrifos [O,O-diethyl O-(3,5,6-trichloro-2-pyridyl phosphorothiote)]is an important OPs insecticide, widely used against a broad spectrum of agricultural crops throughout the world at concentration of 3 to 15 kg/ha. Chlorpyrifos, first introduced into the market in 1965, has been widely used globally as an insecticide to control crop pests in agriculture. The extensive usage of chlorpyrifos having a half life from 10 to 120 days in soil has resulted in widespread environmental contamination affecting beneficial non-target soil microorganisms. Chlorpyrifos shows a wide spectrum of biological activity and is used to control wide range insects pests as well as soil dwelling grubs, rootworms, borers and subterranean termites. (George *et al*., 2014; Kumar, 2011; Supreeth.M *et al*., 2016).

Chlorpyrifos [O,O-diethyl O-(3,5,6-trichloro-2-pyridyl phosphorothiote)



Coffee forms an important commercial crop and its cultivation is extensively carried out in South India. The coffee plantations with the best suit of climatic conditions have been located in the Western Ghats belt of Karnataka *viz.*, Chikmagalur. Coffee being a woody perennial evergreen dicotyledonous plant and plantations being carried out intermingled with shade trees, leaf litter accumulation is prominently seen with rich humus formation. Humus being rich in organic content harbours a rich and diverse microbial life.

Soil borne bacteria and fungi by their degradative capacity play an important role in the formation and enrichment of humus. Soil actinomycetes are prokaryotes with extremely various metabolic possibilities (Moncheva *et al*., 2002). Actinomycetes are gram positive filamentous bacteria, characterized by the formation of aerial mycelium and spores on solid media with DNA high in G+C content of 60-70 mol%. They are important class of bacteria since they produce numerous natural products such as antibiotics and enzymes (De Schrijver and De Mot, 1999). Actinomycetes have been potentially known for their secondary

metabolism, degradative capacities and significant source of commercially important compounds such as antibiotics, enzymes, pesticide degradative agents and immunosuppressant's etc.

The present study focused on the morphological diversity of actinomycetes isolated from coffee plantations. The use of microorganisms for the degradation and detoxification of numerous toxic pesticides, proved to be an efficient tool to decontaminate the polluted sites in the prevailing environment to maintain soil fertility through their roles in nutrient cycling and organic matter decomposition.

II. MATERIALS AND METHODS

2.1 Soil sampling and Isolation of actinomycetes

The soil samples were collected from different regions of Coffee Plantation of Chikmagalur, Karnataka. Sterilized polythene bags were used for soil collection (Sahin and Ugur, 2003; Supreeth. M *et al*., 2016). The samples were air dried at 50°c for 6 to 7 days to remove moisture (Kumari *et al*., 2006). The standard serial dilution plate culture method was employed to isolate the pure culture of actinomycetes. The dilutions were plated on different culture media like starch casein Nitrate (SCN) agar, Actinomycetes isolation agar (Himedia), International Streptomyces Project medium (ISP) Yeast extract malt extract agar(ISP-02), Oat meal agar (ISP-03). The plates were incubated at 30±2°c for 10 to 14 days. (Shirling and Gottlieb, 1966; Gautham *et al., 2012)*

2.2 Characterization of actinomycetes

Isolated actinomycetes were subjected for morphological, microscopic and biochemical characterization. A morphological study including substrate mycelium, aerial mycelium, sporulation and pigmentation status of the actinomycetes (Lin *et al.,* 2011; Williams *et al.,* 1923), after their adequate growth on starch casein agar was carried out as per the procedure prescribed in Goodfellow (1989), Bergey's manual of determinative Bacteriology (Holt *et al., 2000*). The isolates were then identified up to genus level based on their spore chain arrangement by covers slip method (Shirling and Gottlieb, 1966)

2.2.1 Staining

All the isolates were subjected for gram's and acid fast staining (Aneja, 1996; Cappucino, 1999).

2.2.2 Biochemical characterization

Biochemical characterization includes Starch hydrolysis, Catalase test, Carbohydrate fermentation (glucose, sucrose, lactose, maltose and starch**),** Indole productin, MethyRed – VogesProskauer, Citrate utilization, Gelatin hydrolysis, Nitrate reduction, Hydrogen sulfide production test (Aneja, 1996; Cappucino, 1999).

2.3 Screening of pesticide degrading Actinomycetes

All isolates were subjected for screening of pesticide like Chlorpyrifos, in the concentration of 1000 ppm was incorporated in starch casein nitrate agar and isolates were inoculated by streak method and incubated at 30^0C for 14days. Actinomycetes which grow on these pesticides were further screened by using pesticide as a sole source of carbon ranging from $2x10^5$, $4 x10^5$, $6 x10^5$, $8 x10^5$ and $1 x10^6$ ppm was incorporated in starch casein nitrate agar and isolates were inoculated by streak method and incubated at 30^0C for 14days (Jayabarath *et al., 2010;* Lin *et al., 2011*).

2.4 Solvent extraction and Residual analysis of pesticides

The screened actinomycetes were subjected for degradation of pesticide in bulk and incubated at 30^0C for 14days. Culture filtrate of broth culture was solvent extracted with Ethyl acetate in the ratio 1:1. Ethyl acetate extract of pesticides were concentrated and residual analysis of extract was done (Pankaj *et al*., 2016; Chandra *et al.*, 2010).

III. RESULTS

3.1 Soil sampling and Isolation of actinomycetes

Different soil samples were collected in a distance of 2 kilometres from coffee plantation soil of Chikmagalur, Karnataka, India. About 29 actinomycetes species were isolated Starch casein nitrate medium was the best medium for isolation yielding high number of actinomycetes isolates, followed by ISP-03, ISP-04, ISP-02 and Actinomycetes Isolation agar. First and second dilution yielded highest number of colonies followed by third to nine.

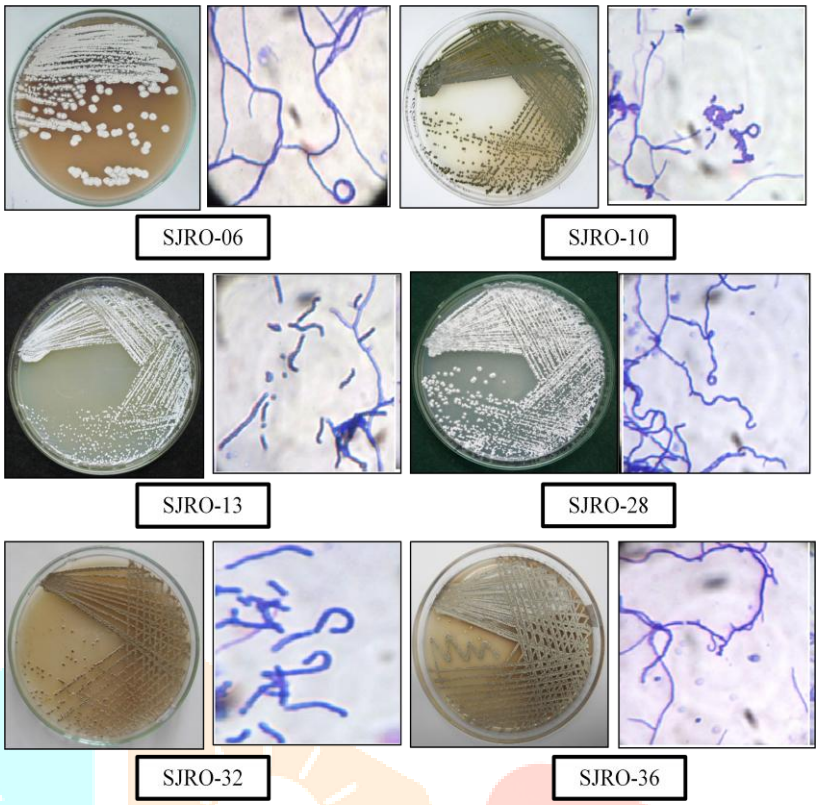
3.2 Characterization of actinomycetes

3.2.1 Morphological and Staining Characteristics:

All the isolates (29) were subjected for further studies. The results revealed a diverse morphological characteristics with varied spore colours, colony morphology, aerial and substrate mycelium colourations. The spore morphology showed different arrays of spore arrangement varying from rectus, flexibilis, retinaculum aperatum – open loops, hooks and spira– simple spirals, short and compact spirals (Table 1; Figure 1). Based on the spore chain arrangements the isolates were assigned to the genus Streptomyces. All the isolates were found to be gram positive and non-acid fast

Table -1 Morphological characterization of Actinomycetes isolates:

Isolates	Colony morphology	Aerial mycelium colour	Substrate mycelium colour	Background	Spore arrangement
SJRO-01	Powdery	Creamy	Creamy	-	Rectus Straight
SJRO -03	Powdery	White	White	-	Rectus Straight
SJRO -06	Powdery	White	Brown	Brown	Simple spira
SJRO -07	Powdery	Grayish white	Gray	-	RA- Hooks
SJRO -09	Powdery	Gray	Gray	-	RA- Open loops
SJRO -10	Powdery	White	White	-	Compact Spira
SJRO -11	Powdery	Green	White	-	Simple spira
SJRO -13	Powdery	White	Gray	-	Rectus Straight
SJRO -14	Spotted	Black	Black	-	RA- Open loops
SJRO -15	Powdery	Blue	Cream	-	Rectus Straight
SJRO -16	Powdery	Gray	White	-	Rectus Straight
SJRO -17	Powdery	Gray	White	-	Rectus Straight
SJRO -18	Velvety	Orange	White	Orange	Rectus Straight
SJRO -19	Velvety	Orange	White	Orange	Compact Spira
SJRO -21	Powdery	Gray	White	-	RA- Open loops
SJRO -22	Powdery	Gray		-	Rectus Straight
SJRO -23	Raised Powdery	Orange	White	Orange	Simple spira
SJRO -24	Powdery	Gray	White	-	RA- Hooks
SJRO -25	Powdery	Cream	White	-	Compact Spira
SJRO -26	Velvety	Yellow	Cream	Yellow	RA- Open loops
SJRO -28	Powdery	Cream White	White	-	Simple spira
SJRO -31	Powdery	Gray	White	-	Rectus Straight
SJRO -32	Powdery	Gray	White	-	RA- Hooks
SJRO -34	Powdery	Gray	White	-	RA- Open loops
SJRO -35	Powdery	Gray	White	-	Rectus Straight
SJRO -36	Powdery	Cream White	White	-	Compact Spira
SJRO -37	Raised Powdery	Brown	Red	Red	Simple spira
SJRO -39	Raised Powdery	Brown	Red	Red	Simple spira
SJRO -40	Raised Powdery	Brown	Red	Red	Simple spira



Figre-1: Isolated Actinomycetes and their Microscopic view

3.2.2 Biochemical Characteristics

The isolates showed varied physiological characteristics. All the isolates were catalase positive, 27 isolates were positive for starch hydrolysis, 07 isolates were positive for gelatin hydrolysis, 10 isolates were positive for nitrate reduction test, 03 isolates were positive for H_2S production, 5 isolates were positive for citrate utilization test. 2 isolates were positive for methyl red test and all the isolates were negative for voges proskauer test (Table 2). The carbohydrate utilization test showed diverse results. The results showed acid, gas and alkali production. The acid and gas production was noted by the colour change from red to yellow and accumulation of gas in the Durham's tube. The alkali production was observed by the change of colour from red to dark pink. Most of the isolates showed positive results for alkali production in all the sugars tested, alkali production was considerably high in disaccharides and polysaccharides (Table 3).

Table 2: Biochemical characterization of Actinomycetes isolates:

Isolates	Gelatin hydrolysis	Starch hydrolysis	Nitrate reduction	MR	VP	H_2S production	Citrate utilization	Catalase test
SJRO-01	-	+	+	-	-	-	-	+
SJRO -03	-	+	-	-	-	-	+	+
SJRO -06	-	+	+	+	-	-	-	+
SJRO -07	-	+	-	-	-	-	-	+
SJRO -09	+	+	-	-	-	-	-	+
SJRO -10	-	-	-	+	-	+	-	+
SJRO -11	-	+	-	-	-	-	-	+
SJRO -13	-	+	+	-	-	-	-	+
SJRO -14	-	-	-	-	-	-	-	+
SJRO -15	+	+	-	-	-	+	-	+
SJRO -16	+	+	-	-	-	-	+	+
SJRO -17	-	+	-	-	-	-	+	+
SJRO -18	-	+	+	-	-	-	-	+
SJRO -19	-	+	-	-	-	-	-	+
SJRO -21	+	+	+	-	-	-	-	+
SJRO -22	-	+	-	-	-	-	-	+
SJRO -23	-	+	+	-	-	+	-	+
SJRO -24	-	+	-	-	-	-	+	+
SJRO -25	+	+	-	-	-	-	-	+
SJRO -26	-	+	-	-	-	-	-	+
SJRO -28	-	+	-	-	-	-	-	+
SJRO -31	-	+	+	-	-	-	-	+
SJRO -32	-	+	-	-	-	-	-	+
SJRO -34	-	+	-	-	-	-	+	+
SJRO -35	+	+	-	-	-	-	-	+
SJRO -36	+	+	+	-	-	-	-	+
SJRO -37	-	+	+	-	-	-	-	+
SJRO -39	-	+	+	-	-	-	-	+
SJRO -40	-	+	-	-	-	-	-	+

('-' Negative, '+' Positive)

Table 3: Biochemical characterization of Actinomycetes isolates:

Isolates	Carbohydrate Fermentation test														
	GLUCOSE			SUCROSE			MALTOSE			LACTOSE			STARCH		
	Ac	G	Alk	Ac	G	Alk	Ac	G	Alk	Ac	G	Alk	Ac	G	Alk
SJRO-01	-	-	-	-	-	+	-	-	-	-	-	+	-	-	+
SJRO -03	-	-	-	-	-	+	-	-	-	-	-	+	+	-	-
SJRO -06	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+
SJRO -07	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+
SJRO -09	-	-	+	-	-	+	-	-	+	-	-	+	+	-	-
SJRO -10	+	-	-	-	-	+	+	-	-	-	-	+	+	-	-
SJRO -11	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+
SJRO -13	-	-	+	-	-	+	-	-	-	-	-	+	-	-	-
SJRO -14	-	-	+	+	-	-	-	-	-	-	-	+	+	-	-
SJRO -15	-	-	+	-	-	+	-	-	+	+	-	-	-	-	+
SJRO -16	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+

SJRO -17	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+
SJRO -18	-	-	+	+	-	-	-	-	+	-	-	+	-	-	+
SJRO -19	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+
SJRO -21	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+
SJRO -22	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+
SJRO -23	-	-	+	-	-	+	-	-	-	-	-	+	-	-	+
SJRO -24	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+
SJRO -25	-	-	-	-	-	+	-	-	+	-	-	+	-	-	+
SJRO -26	+	-	-	+	-	-	+	-	-	-	-	-	-	-	+
SJRO -28	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+
SJRO -31	+	-	-	-	-	+	-	-	+	-	-	+	+	-	-
SJRO -32	-	-	-	-	-	+	-	-	+	-	-	+	-	-	+
SJRO -34	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+
SJRO -35	-	-	+	-	-	+	-	-	+	-	-	+	+	-	-
SJRO -36	-	-	-	-	-	+	-	-	-	-	-	+	-	-	+
SJRO -37	-	-	-	-	-	+	-	-	-	-	-	+	-	-	+
SJRO -39	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-
SJRO -40	-	-	-	-	-	+	-	-	+	-	-	+	-	-	+

(Ac-Acid, G- Gas, Alk- Alkali, '-' Negative, '+' Positive)

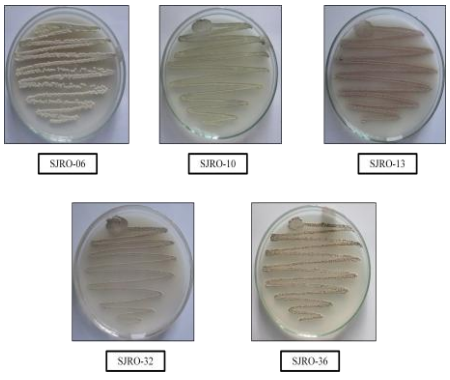
Based on Morphological and biochemical characteristics the Actinomycetes belong to the family *Streptomycetes.*

3.3 Screening of pesticide degrading Actinomycetes

Out of 29 isolates only 3 isolates SJRO-06, SJRO-10 and SJRO-36 grown on medium incorporated with chlorpyrifos as sole source of carbon in concentration range $2x10^5$, $4 x10^5$, $6 x10^5$, $8 x10^5$ and $1 x10^6$ ppm (Table 4: Figure 2).

Table 4: Screening of Chlorpyrifos pesticide by Actinomycetes:

ORGANISMS	CONCENTRATION OF CHLORPYRIFOS (ppm)				
	$2 x10^5$	$4 x10^5$	$6 x10^5$	$8 x10^5$	$1 x10^6$
SJRO-03	++++	+++	+	+	-
SJRO-06	++++	+++	+++	++	++
SJRO-10	++++	+++	+++	++	++
SJRO-13	+++	+++	++	+	+
SJRO-16	++++	+++	++	-	-
SJRO-21	+++	++	+	-	-
SJRO-24	+++	++	+	-	-
SJRO-28	++	+	++	++	-
SJRO-32	++++	++++	+++	++	+
SJRO-36	++++	+++	++	++	++



Figre-2: Chlorpyrifos Degrading Isolates

3.4 Solvent extraction and Residual analysis of pesticides

Actinomycetes SJRO-06, SJRO-10 and SJRO-36 were bulk cultured in broth medium. Culture filtrate was extracted with the solvent ethyl acetate. Pesticide suspended with ethyl acetate extract was residual analysed by AOAC method. Residual content of pesticide chlorpyrifos after degradation by actinomycetes listed in table 5.

Table 5: Residual analysis of pesticides Chlorpyrifos

S. No	Particulars	Results	References
01	Description	Colorless liquid	-
	Chlorpyrifos	19ppm	AOAC
02	SJRO-06	06ppm	AOAC
03	SJRO-10	03ppm	AOAC
04	SJRO-36	18ppm	AOAC

IV. DISCUSSION

Pesticides are the synthetic compounds used to protect agricultural crops from disease causing pests. The applied pesticide will reach target pests by only 1 % and the remaining will come into contact with soil, where they undergo a variety of transformations that provide a complex pattern of metabolites. Fertility of soil is dependent on the soil microbial richness and diversity (Supreeth.M *et al*., 2016). Organophosphorus pesticides are the most widely used group of pesticides. In this present study chlorpyrifos pesticide is selected which commonly used in coffee plantation to prevent berry borer.

Actinomycetes are Gram positive bacteria distributed widely in soil and constitute a significant part of soil microflora. These are well known for their secondary metabolite production besides that actinomycetes are also known for the degrading complex organic materials in soil and sediments. The soil samples were collected from coffee plantation of Chikmagalur, Karnataka. Serial dilution procedure yielded 29 isolates. Kumari *et al.*, (2006) and Xu *et al.*, (1996) were isolated actinomycetes across the world in different landscapes and geographical locations.

The 29 isolates based on the morphological and biochemical characterization studies such as Catalase, Starch hydrolysis, Carbohydrate fermentation, Citrate Utilization, MRVP, Geltain hydrolysis, Nitrate reduction test and H_2S production test and the colony characteristics the organism was identified as *Streptomycetes* spp. The results of the morphological and biochemical studies are also similar with earlier results of Moncheva *et al*. (2000-2002)**.**

Streptomyces species which are able to use cypermethrin and chlorpyrifos pesticides as a sole source of carbon were screened and among 29 isolates SJRO-06, SJRO-10 and SJRO-36 isolates degrade chlorpyrifos at 1000mg/lt. Similar work was carried out by Lin *et al.*, (2011), Jayabarath *et al*., (2010) and Eissa *et al*., (2014) reported the isolated actinomycetes which are able to degrade cypermethrin, carbofuran and chlorpyrifos respectively. Similar review was given by Javaid *et al* (2016). Briceno *et al.*, (2012) used chlorpyrifos at the concentration of 25mg/lt and 50mg/lt and degraded by Streptomyces sp.

The residual analysis of pesticide chlorpyrifos was carried out by extracting culture filtrate with ethyl acetate solvent. The solvent extract of pesticide residues analysed by AOAC method, the similar work was done by Pankaj *et al*., (2016) studied contaminated agriculture field with cypermethrin and their novel pathway of degradation by Bacillus sp. Strain SG2. Chandra *et al.*, (2010) also carried pesticide residual analysis in vegetables.

V. CONCLUSION

Actinomycetes being a prominent microbial community which are known for their diverse degradative metabolic capacities are isolated from coffee plantations which are located in the Western Ghats regions, Karnataka.

The investigations have been successful in isolating and characterising *Actinomycetes* which are known for their secondary metabolite production. The isolates are screened for chlorpyrifos degradation and the potent isolates SJRO-06, SJRO-10 and SJRO-36 degrades chlorpyrifos and used as sole carbon source and residual analysis of solvent extract successfully done by AOAC method. These isolates belong to the family *Streptomycetes* and genus *Streptomyces spp.*

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