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CHLORPYRIFOS DEGRADATION BY ACTINOMYCETES ISOLATED FROM COFFEE PLANTATION SOIL AND RESIDUAL ANALYSIS BY GC-MS

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ABSTRACT

Microorganisms are an important biological component of the soil ecosystem and play an vital roles in soil fertility through their roles in nutrient cycling and organic matter decomposition. 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. 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%. Actinomycetes have considerable potential for the biotransformation and biodegradation of pesticides. Hundreds of pesticides in different chemical moieties are widely used for agricultural purpose. Chlorpyrifos is a broad spectrum organophosphate pesticide widely used to control pest. Exposure to this moderately hazardous pesticide creates health problems due to choline esterase inhibition, immunological effects, psychological and neurotoxicity. Intensive use of chlorpyrifos has resulted in its ubiquitous presence as a contaminant in soil and surface water streams. Thus, it is critically important to develop bioremediation methods to degrade and break down the pollutant from environment. The present investigation focused on isolation of actinomycetes from coffee plantation soil, degradation of chlorpyrifos and their residual analysis by GC-MS. Total 29 isolates were recovered and subjected for morphological and biochemical characterization studies, isolates are belonging to Streptomyces species. Biodegradation of pesticides were detected by using chlorpyrifos as sole source of carbon in different concentrations and further bulk cultured with potent isolates to detect residues by GC-MS.

KEY WORDS

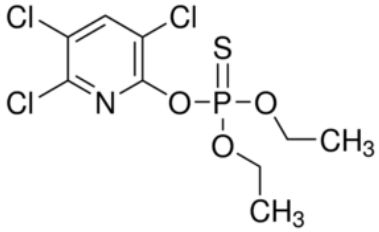
Biodegradation, Soil microorganisms, Streptomyces, pesticide, Chlorpyrifos, GC-MS.

I. INTRODUCTION

Hundreds of pesticides in different chemical moieties are widely used for agricultural purpose, terrestrial ecosystem, water and soil receive large amount of it even from handling, direct application or else which lead to occasional contamination besides accumulation lead to many health hazards associated with it [1]. Environmental pollution caused by pesticides and their degradation products is a major ecological problem. It has been documented that organophosphorus pesticides (OP) constitute the largest group of highly toxic agricultural chemicals widely used for plant protection [2, 3]. Chlorpyrifos [O, O diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate], a phosphorothioate insecticide, has been commercially used since the 1960s. Globally, chlorpyrifos ranks first among the conventional pesticide active ingredients in the agricultural sector with the production of 3.64-4.99 million kg during 2007. In India, chlorpyrifos was the second most used agricultural insecticide in 2013 to 2014 with the production of 9540 tons. Used particularly for the control of broad-spectrum insect

pests of economically important crops. The extensive usage of chlorpyrifos having a half-life from 1day to more than 240 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 [4, 5, 6, 7].

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



Chlorpyrifos was the fourth largest consumed organophosphate pesticide next to monocrotophos, acephate and endosulfan. According to WHO classification, chlorpyrifos belongs to class II pesticides with moderate toxicity. The excessive and injudicious application of pesticides led to environmental deterioration and human health concerns. The Chlorpyrifos enter in to the human body via inhalation, ingestion and through skin contact and inhibits acetylcholine esterase enzyme in the central nervous system. The exposure of this OP compound during pregnancy led to children with birth defects. The ingestion of this compound can also cause liver toxicity, immunological disorders and its long-term accumulation in body tissues, proteins, fats and bones create other health problems. The chlorpyrifos residues in the soil and water environment resulted in biodiversity loss, alter its quality and productivity. The microbial bioremediation play an important role in chlorpyrifos degradation in the environment [8].

Microorganisms are an important biological component of the soil ecosystem and play vital roles in soil fertility through their roles in nutrient cycling and organic matter decomposition. Biological decomposition of pesticides is the most important and effective way to remove hazardous compounds from the environment. Microorganisms have the ability to interact both chemically and physically, with substances leading to structural changes or complete degradation of the target molecule. Among the microbial communities, bacteria, fungi and actinomycetes are the main transformers and pesticide degraders [5, 9].

Actinomycetes population has been identified as one of the major group of soil population, which may vary with the soil type. 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%. They are important class of bacteria since they produce numerous natural products such as antibiotics and enzymes [10, 11]. Actinomycetes being a prominent microbial community are group of organisms which have been renowned for their diverse degradative metabolic capacities. These are well known for their antibiotic production besides that actinomycetes are also known to produce several enzymes capable of degrading complex organic materials in soil and sediments. Actinomycetes have considerable potential for the biotransformation and biodegradation of pesticides [12, 13].

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 [14].

The objective of this study was to isolation, characterization and identification of actinomycetes were capable of degrading chlorpyrifos from coffee plantation soil and residual analysis of chlorpyrifos. Hence isolation of indigenous actinomycetes capable of metabolizing pesticides has received considerable attention thus providing an environmental friendly detoxification. Preliminary screening and bulk culture of actinomycetes and detection of chlorpyrifos residues by GC-MS method.

II. MATERIALS AND METHODS

Soil sampling, Isolation and identification of actinomycetes

The soil samples were collected from different regions of Coffee Plantation of Chikmagalur, Karnataka. The standard serial dilution plate culture method was employed to isolate the pure culture of actinomycetes. The dilutions were Plated on culture media like starch

casein Nitrate (SCN) agar (Starch 10g; Casein 0.3g; Potassium nitrate 2.0g; Sodium chloride 2.0g; Magnesium sulphate 0.05g; Calcium carbonate 0.02g; Ferrous sulphate 0.01g; Agar 20g; Distilled water 1000ml; pH 7.1). The plates were incubated at 30±2°c for 10 to 14 days [15, 16]. Isolated actinomycetes were subjected for morphological, microscopic and biochemical characterization [17, 18], after their adequate growth on starch Casein agar were carried out as per the procedure prescribed in [19], Bergey's manual of determinative Bacteriology [20, 21]. The isolates were then identified up to genus level based on their spore chain arrangement by coverslip method [22, 23, 24, 25].

Screening of pesticide degrading Actinomycetes

All isolates were subjected for screening of pesticide like Chlorpyrifos (Commercial formulation of chlorpyrifos 20% EC, HYBAN)), 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 this pesticide 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 [26, 17].

Solvent extraction and Residual analysis of pesticide by AOAC

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 pesticide was concentrated, and residual analysis of extract was done [27, 17].

Detection of chlorpyrifos residues by GC-MS

Culture filtrate of medium containing chlorpyrifos was extracted with ethyl acetate. Ethyl acetate was evaporated, and residues dissolved in small amount of ethyl acetate. The extracts were analyzed by GC-MS. The GC-MS analysis was performed in electron ionization (EI) mode with a Thermo Scientific GC Trace 1310 Equipped with Thermo Scientific MS TSQ 8000 detector. An Agilent DB 5MS (30-meter X 0.25 mm) capillary column was used with a temperature program of Initial 40^0C hold for 2 min, Ramp at 5^0C to 240^0C, Ramp at 20^0C to 300^0C hold for 2 min. Helium was used as the carrier gas. MS transfer line temperature of 300^0C and Ion source temperature of 230^0C [17, 1].

III. RESULTS

Soil sampling, Isolation and identification 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 on Starch casein nitrate medium. First and second dilution yielded highest number of colonies followed by third to nine. All Isolates were subjected for further studies. The results revealed a diverse morphological characteristic 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. 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. Based on Morphological and biochemical characteristics the Actinomycetes belong to the family *Streptomycetes.*

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-1.

Table 1: Screening of Chlorpyrifos pesticide by Actinomycetes:

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

Solvent extraction and Residual analysis of pesticides

Culture filtrates of SJRO-06, SJRO-10 and SJRO-36 were extracted with the solvent ethyl acetate. Pesticide suspended with ethyl acetate extract was residual analyzed by AOAC method. Residual content of pesticide chlorpyrifos in control 19ppm, SJRO-06 06ppm, SJRO-10 03ppm and SJRO-36 18ppm after degradation by actinomycetes Table 2.

Table 2: Residual analysis of pesticides Chlorpyrifos by AOAC

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

Detection of chlorpyrifos residues by GC-MS

The degradation products of chlorpyrifos by actinomycetes were extracted and identified by GC-MS. The metabolite peaks were identified using documented date from National Institute of Standards and Technology (NIST) library database. The residual content of chlorpyrifos in Control, SJRO-06 and SJRO-10 are 100%, 79.23% and 71.54% respectively (Table 3) and Retention Time (RT) of chlorpyrifos in Control, SJRO-06 and SJRO-10 are 21.58min, 21.61min and 21.59min respectively. The GC-MS chromatogram are shown in Figure 1, 2 and 3. NIST library of degraded samples are listed in Table 4 and 5.

Table 3: Summary of Pesticide residue in samples

Sample	R. T	Area	Dilution Factor	Residual pesticide content
Chlorpyriphos Control	21.58	1132648491.74	20	100.00
SJRO-06	21.61	5982834464.87	3	79.23
SJRO-10	21.59	1620687932.39	10	71.54

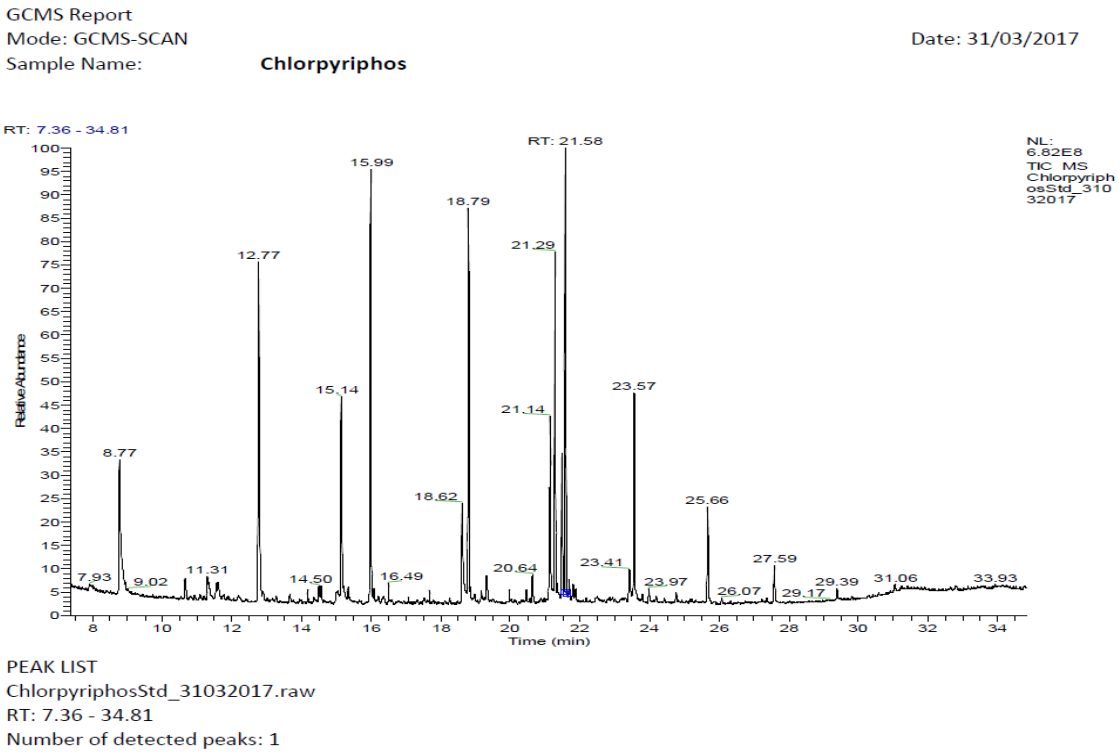


Figure 1: GC-MS total Ion Chromatogram of chlorpyrifos Control

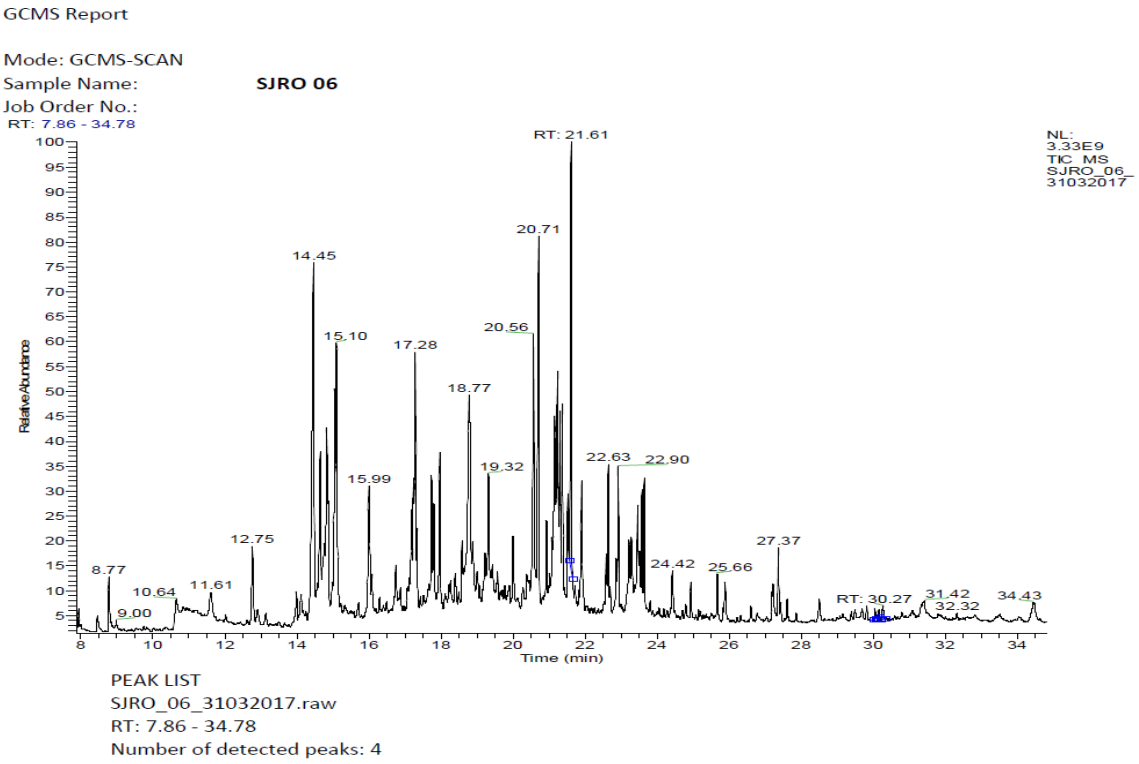


Figure 2: GCMS Total Ion Chromatogram of SJRO-6 sample

Table 4: NIST library of SJRO-6 sample

Sl.No.	Peak RT	NIST Library Hits
1	14.45	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-
2	15.10	(3R,3aR,7R,8aS)-3,8,8-Trimethyl-6-methyleneoctahydro-1H-3a,7-methanoazulene
3	15.99	1-Nonadecene
4	17.28	aR-Turmerone
5	18.77	Tetradecanoic acid
6	19.32	Spiro [4.5] decan-7-one, 1,8-dimethyl-8,9-epoxy-4-isopropyl-
7	20.56	Hexadecanoic acid, methyl ester
8	20.71	7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione
9	21.61	Chlorpyrifos
10	22.90	Methyl stearate
11	22.63	10-Octadecenoic acid, methyl ester
12	27.37	Diisooctyl phthalate

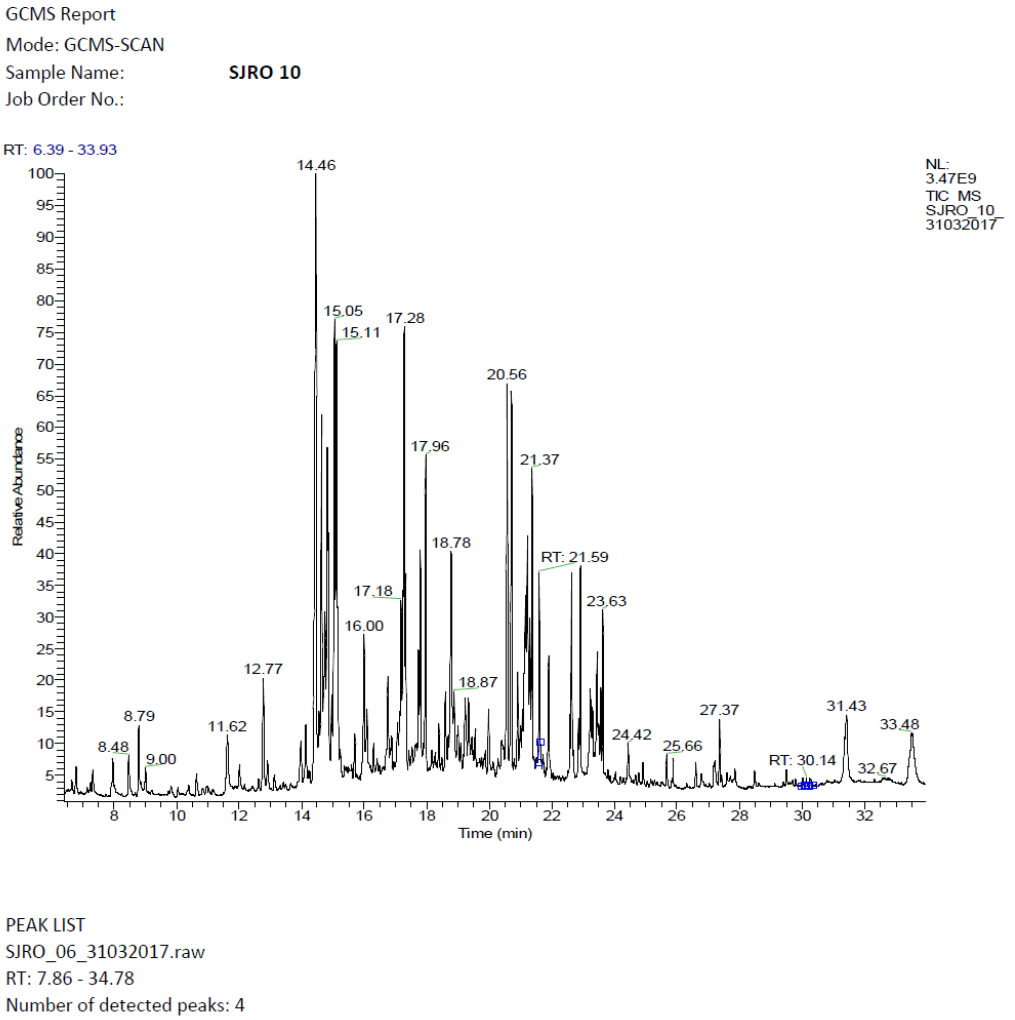


Figure 3: GCMS Total Ion Chromatogram of SJRO-10 sample

Table 5: NIST library of SJRO-10 sample

Sl.No.	Peak RT	NIST Library Hits
1	14.46	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-
2	15.05	Dodecanoic acid, methyl ester
3	15.11	Cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methylene-, [S-(R*, S*)]-
4	17.28	aR-Turmerone
5	17.96	Methyl tetradecanoate
6	18.78	Oleic Acid
7	20.56	Hexadecanoic acid, methyl ester
8	21.37	Hexadecanoic acid, ethyl ester
9	23.63	Octadecanoic acid, ethyl ester
10	27.37	Diisooctyl phthalate
11	31.43	2-Phenanthrenecarboxylic acid, 1-(1,3-dithian-2-ylmethyl)-7-hydroxy 2,4b-dimethyl-1,2,3,4,4a,4b,5,6,7,8,10,10a-dodecahydro-, methyl ester
12	33.48	Eicosanoic acid, 2-[(1-oxohexadecyl) oxy]-1-[[(1-oxohexadecyl) oxy] methyl] ethyl ester

IV. DISCUSSION

Synthetic pesticides are purposely introduced into agricultural systems to protect crops against weeds, insects, fungi and other pests. However, the majority of the applied pesticides, even if sprayed on foliage of crop plants and weeds, will eventually reach the soil. Organophosphorus insecticides have been extensively used in agricultural practice for more than 40 years and it is one of the major group of pesticides accounting 38% of total global pesticide consumption that replaced organochlorines to a greater extent against crop loss by pest attack and improving yield. Chlorpyrifos is one of the broad spectrum organophosphate pesticide used to control against various agriculture and household pests. It is used for crop protection in cotton, rice, sugarcane, peanuts, tobacco, vegetables, fruits and ornamental plants against sucking, chewing and boring insects [4, 5, 8].

Actinomycetes are widely distributed in soil and constitute a significant part of soil. Soil actinomycetes are prokaryotes with extremely various metabolic possibilities [28]. 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 %. 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 [10, 29, 30].

The 29 isolates based on the morphological, microscopic and biochemical characterization the Organisms were identified as *Streptomycetes* spp. Taxonomical criteria's the classification outline recommended in the International *Streptomyces* Project Guidelines and Bergey's Manual of Determinative Bacteriology [20] have been extensively adapted in *Streptomyces* characterization studies and similar results have been observed by [28, 29, 31, 32].

Streptomyces species which are able to use chlorpyrifos pesticide 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 [2, 16, 17, 26] reported the isolated actinomycetes which are able to degrade chlorpyrifos, cypermethrin, and carbofuran respectively. Similar review was given by [33]. [34] 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 [27]. The degradation products of chlorpyrifos were extracted and identified by GC-MS. The metabolite peaks were identified using documented date from National Institute of Standards and Technology (NIST) library database. Retention Time (RT) of chlorpyrifos in Control, SJRO-06 and SJRO-10 are 21.58min, 21.61min and 21.59min respectively, similar GC-MS studies was carried out by [1, 17] for malathion and cypermethrin respectively, RT of malathion is 15.60min and cypermethrin is 14.793min. [2] studied biodegradation of chlorpyrifos by microbial strains and a residual study was done by GC-FPD.

V. CONCLUSION

Bioaccumulation of pesticides has been a significant problem leading to health hazards. Microbial degradation of pesticides has been recognized as the most important process controlling their environmental fate. Therefore, biodegradation using native microorganisms for pesticide removal from the environment is quite attractive. The degradative capacity of soil borne actinomycetes can be a beneficial aspect if these bio resources are tapped.

The present study has been successful in isolating and characterising Actinomycetes which have been known for their diverse metabolic activities and metabolites. The isolates belong to the family *Streptomycetes* and genus *Streptomyces spp.* Isolates are screened for chlorpyrifos degradation and the potent isolates are SJRO-06 and SJRO-10 degrades chlorpyrifos and used as sole carbon source. The residual content of chlorpyrifos analysed by GC-MS.

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