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RESEARCH ARTICLE

ISOLATION AND IDENTIFICATION OF CHLORPYRIFOS DEGRADING BACTERIA FROM AGRICULTURAL SOIL.

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Abstract

The extensive use of pesticides in agricultural fields for pest control pose a serious problem in contaminating soil and water ecosystems. Chlorpyrifos is the major broad spectrum organophosphorus insecticide used in paddy fields against sucking, chewing, boring insects. Due to its toxicity and persistence in the environment, there is an immediate need to eliminate them from contaminated sites by biodegradation. Two pesticide degrading bacteria were screened and isolated from chlorpyrifos contaminated soil by enrichment culture technique and were identified as *Kocuria kristinae* and *Staphylococcus aureus*. The growth response and degradation of chlorpyrifos by the isolates in MSM broth supplemented with 0.5% chlorpyrifos was monitored every 48-72 hrs in spectrophotometer at 600nm. *Kocuria sp* showed maximum growth in 7 days than *Staphylococcus aureus*. The degradation efficiency of the strains were determined and estimated by the removal percentage of chlorpyrifos from the liquid culture. Both the isolate showed the degrading capability of chlorpyrifos in MSM. The isolate, *S. aureus* was more potent in degrading the 80% of the total compound from the media in 2 weeks of incubation than *K. kristinae* which shows 35 % of degradation. These results were further confirmed by GCMS, in which *S. aureus* has degraded **82.06% and** *K. kristinae* has degraded **30.78%** of chlorpyrifos in the medium. This study indicates that the isolate, *Staphylococcus aureus* is more potent in degrading chlorpyrifos in liquid culture and can also be used in bioremediation of chlorpyrifos contaminated soils.

Introduction:-

Pesticides are BIOCIDES, designed to kill, reduce or repel insects, weeds, rodents, fungi or other organisms that can threaten public health and the economy. Pesticides are designed to kill specific pests and the most widely used are insecticides, rodenticides, herbicides, insect repellents and fungicides. Organophosphorus compounds are the most

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widely used insecticides, accounting for an estimated 34% of worldwide insecticide sales (1).

Chlorpyrifos (O, O-diethyl O-3, 5, 6-trichloro-2-pyridyl phosphorothioate) are the esters derived from phosphoric acid is one of the broad spectrum organophosphate pesticide used against sucking, chewing and boring insects (Fig 1). It is used for crop protection and also has acaricidal and termiticidal properties. In man, it acts on the central nervous system by inhibiting acetylcholinesterase, an enzyme that modulates the amount and levels of the neurotransmitter acetylcholine and thus disrupting the nerve impulse. Chlorpyrifos causes hazardous effects to the environment and also toxic to human beings resulting in headache, nausea, muscle twitching, convulsions, birth defects and even death (2).

Due to these effects, the need to remove these residues from the environmental sources should be focused. Degradation of chlorpyrifos by microorganisms has become extensively studied as other methods are impractical or costly or environmentally hazardous (3).

To date, several chlorpyrifos-degrading bacterial strains were isolated *including Enterobacter sp* (4,5,6), *Pseudomonas putida* (7,8), *Ps. aeruginosa* (9,10), *Ps. desmolyticum* (11), *Ps. resinovorans* (12), *Ps. fluorescens* (13), *Bacillus subtilis* (13,14,15,16), *Bacillus sp and Micrococcus sp* (17,18), *B. firmis* (19), *B. cereus* (20), *Stenotrophomonas* (21), *Serratia marcescens, Klebsiella oxytoca (22), Achromobacter sp, Ochrobactrum sp* (23), *Alcaligenes faecalis* (24), *Mesorhizobium* (25), *cellulomonas fimi* (26), *Gordonia* (27), *B. polymyxa* (28), *Kocuria sp* (29), *Staphylococcus sp, Streptococcus sp, azomonas sp, Flavobacterium sp* (30) were extensively studied in degradation of chlorpyrifos.

The objective of this study focuses on isolation and identification of chlorpyrifos utilizing bacteria from agricultural soil especially from paddy field by enrichment culture technique and its ability to degrade chlorpyrifos in liquid culture medium. This study aims at the biotechnological approach in cleaning chlorpyrifos contaminated environment by microbial degradation.

Materials and Methods:-

Pesticide and Chemicals:-

Commercial grade of chlorpyrifos (dursban, EC 20%), a type of organophosphorus pesticide was obtained used for this study. It was purchased from a local agrochemicals shop in Thiruvallur district, Tamil Nadu. The analytical grade chemicals were purchased from HIMEDIA, Chennai.

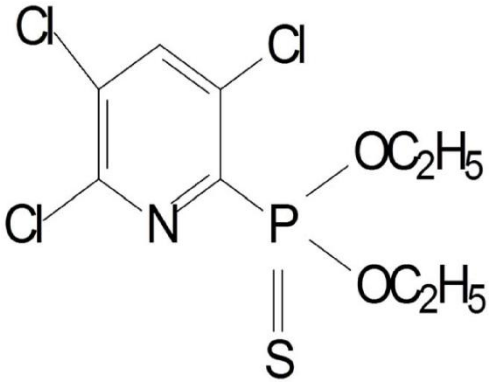

Figure 1:- Structure of chlorpyrifos.

Collection of Soil Samples:-

The soil samples were collected from ten different paddy fields from places in and around Thiruvallur district. The soil samples were collected from topsoil layer (0-15cm) and air dried at room temperature and mixed thoroughly and were stored in sterile bottles in refrigerator at 4°C.

Enrichment Culture Technique:-

The Enrichment culture technique was used for the isolation of bacterial strains capable of utilizing chlorpyrifos as a sole source of carbon and energy. Soil enrichment was carried out in minimal salt medium (MSM, pH 7.0) containing (g/L) Dextrose, 1.0; K_2HPO_4, 7.0; KH_2PO_4, 2.0; Sodium citrate, 0.5; $MgSO_4.7H_2O$, 0.1; $NH_3(SO_4)_2$, 1.0; Agar, 15.0 with pH 7.0 ± 0.2 (4).

Isolation and Screening of Chlorpyrifos Degrading Bacteria:-
One gram of each soil sample was dispersed in 100 ml of sterile distilled water and were serially diluted. The last three dilutions 10^{-5}, 10^{-6} and 10^{-7} were inoculated in MSM broth, supplemented with 0.5 ml/L of chlorpyrifos and incubated at 28°C for 7 days.
From the enrichment culture, one ml of aliquots were transferred to the fresh MSM broth and it was incubated at 28°C for 7 days. This procedure was repeated thrice for selective enrichment and isolation of chlorpyrifos degrading bacteria.
Finally, 1 ml from the above medium were inoculated on MSM agar plates and incubated at 28° for 5 days. Single colony from MSM agar plates were picked up aseptically and were sub-cultured on MSM agar slants and stored at 4°C for further use.

Identification of Bacteria:-
Microscopic and Biochemical tests were performed for determining the phenotypic characterization of the chlorpyrifos degrading isolates. They were examined for the colony morphology, pigmentation, and routine preliminary tests such as Gram's reaction, motility and biochemical tests like IMViC test, Oxidase test, Catalase Test, urease test, Carbohydrate fermentation test, NO_2 reduction test, casein and starch hydrolysis, H_2S production and confirmed by Bergey's manual of determinative Bacteriology (31, 32).

The identification of bacteria was done by using The BD Phoenix™ Automated Microbiology System for the in vitro rapid identification (ID) and quantitative determination of antimicrobial susceptibility by minimal inhibitory concentration (MIC)

Degradation of Chlorpyrifos:-
The bacterial isolates were examined for their potential to degrade chlorpyrifos in liquid medium. 0.5% of chlorpyrifos was added to 100 ml of MSM medium and the isolates were inoculated with a uniform cell density of 0.8 OD. It was incubated at 30°C for 14 days in a rotary shaker at 150 rpm. Un-inoculated flasks were also prepared to check for the abiotic degradation under similar conditions. The experiment was conducted with three replicates.

Growth response of the isolates and degradation were observed at every 48-72 hrs interval in spectrophotometer at 600 nm (23). The content of the flasks were checked by taking 5 ml of culture drawn from mineral based medium and centrifuged at 5000 rpm for 10 minutes. The pellet was discarded and the supernatant was analyzed by spectrophotometer.
Percent degradation of compound was determined by using the formula

Percent degradation= Ab-Aa ÷ Ab × 100
Where,
Ab is absorbance of compound before degradation.
Aa is absorbance of compound after degradation (11).

Extraction of Chlorpyrifos from Samples:-
The media was centrifuged at 5000 rpm for 15 minutes. The supernatant was extracted and it was used for GC-MS analysis.

10 ml of sample which was obtained in the previous step was taken in centrifuge tube with 2 ml of ethyl acetate and thoroughly mixed in vortex mixture for 10 minutes. Then it was centrifuged at 5000 rpm for 5 minutes and the ethyl acetate layer was separated and injected in GC-MS. Control was set up with the same additions except the degraded sample.

Gas Chromatography Mass Spectrometry (GC-MS) Analysis:-
Gas chromatography mass spectrometry (GC-MS) analysis were performed with an Agilent triple Q GCMSMS 7890A gas chromatograph, equipped with a HB 5 MS (30 m × 0.25 mm × 0.25 µm film thickness), an auto-injector and an Agilent network mass selective detector. Nitrogen was used as the carrier gas with a constant flow rate of 0.8 ml/min. The injector and transfer lines were 280°C and 300°C, respectively. The chromatography program was as follows: total runtime 11 min, initial temperature of column 60°C, a temperature increase of 25°C/min and final heating to 310°C. The retention time of chlorpyrifos were detected and percent of degradation were determined by qualitative analysis report.

Results and Discussion:-

Ten samples were processed by the enrichment culture technique for the isolation of bacterial strains. Soil enrichment was carried out in minimal salt medium with the chlorpyrifos (0. 5%), which are capable of utilizing it as a sole source of carbon and energy. The soil samples S7, S6, S10 and S9 showed maximum absorbance of 0.49, 0.40, 0.35 and 0.33 respectively, in enrichment culture technique after 7 days of incubation (Table 1). Among all the isolates, the organisms with maximum OD values (S7 and S6) were processed for identification. Both the organisms were identified by cultural and biochemical tests and confirmed by BD Phoenix Automated Microbiology system. Isolate S6 was identified as *Kocuria kristinae* and S7 as *Staphylococcus aureus* (Tables 2 and 3 and Figs 2 and 3) (17, 18).

The growth response of the isolates in MSM supplemented with chlorpyrifos (0.5%) showed that both the isolates utilized the insecticide as the only carbon source (34). Similar results are obtained by Nagavardhanan et al. (33), who studied on the growth of *Kocuria sp.* in chlorpyrifos incorporated MSM as the only carbon source. The OD values were observed at 600 nm at every 48-72 hours indicating that *K. kristinae* showed maximum growth in 7 days of incubation when compared with .*S. aureus* (Table 4 and Figs 4, 5, and 6).

The degradation efficiency of the strains were determined and estimated by the removal percentage of Chlorpyrifos from the liquid culture. Both the isolate showed the degrading capability of Chlorpyrifos in MSM. The isolate, *S. aureus* was more potent in degrading the 80% of the total compound from the media in 2 weeks of incubation followed by *K. kristinae* which shows 35 % of degradation. These results were confirmed by GCMS (35). From the qualitative analysis report, the RT value of chlorpyrifos was found to be 9.63 min, which was accordance with the study conducted by Rokade et al. (11), who recorded the retention time of chlorpyrifos as 9.55min in GC MS.

The residual concentration of chlorpyrifos in sample 2 (*Staphylococcus aureus*) and sample 1 (*Kocuria kristinae)* were estimated as 17.94% and 69.22% respectively. From the results, it was concluded that the *S. aureus* has degraded **82.06% (100 - 17.94)** and *K. kristinae* has degraded **30.78% (100 - 69.22)** of chlorpyrifos in MSM (Figs. 7 to 11, Tables 5 to 7).

The difference is the degradation percentage at the same concentration (0.5% v/v) of chlorpyrifos is due to the ability of the microbe to utilize available chlorpyrifos. The substrate availability is a key factor determining the rate of degradation of the pesticide by the bacterial agents. According to Sharma et al. (17), the chlorpyrifos degrading organisms *Bacillus sp* and *Micrococcus sp* showed 71. 6% at 0.1% v/v and 46% at 0.05 %v/v of chlorpyrifos after 10 days of incubation where as in the case of *Bacillus sp* 40% and 44% at the same concentrations.

Conclusion:-

The present study focusses on isolation of two bacteria *Kocuria kristinae* and *Staphylococcus aureus* from chlorpyrifos contaminated soil and their degrading ability in medium containing pesticide. Both the isolates were able to utilize and grow in the presence of chlorpyrifos indicates that they may be used for bioremediation of pesticide contaminated soil.

Table 1: Absorbance of samples at 600 nm

Absorbance at 600 nm	Samples	S. No
0.03	S1	1
0.15	S2	2
0.03	S3	3
0.29	S4	4
0.06	S5	5
0.40	S6	6
0.49	S7	7
0.21	S8	8
0.33	S9	9
0.35	S10	10
0.00	Control	11

Figure 2:- Isolated colonies of *Kocuria kristinae* (Sample 6**)**. Pale yellow with round, translucent, mucoid colonies with measure 2-5mm in diameter.

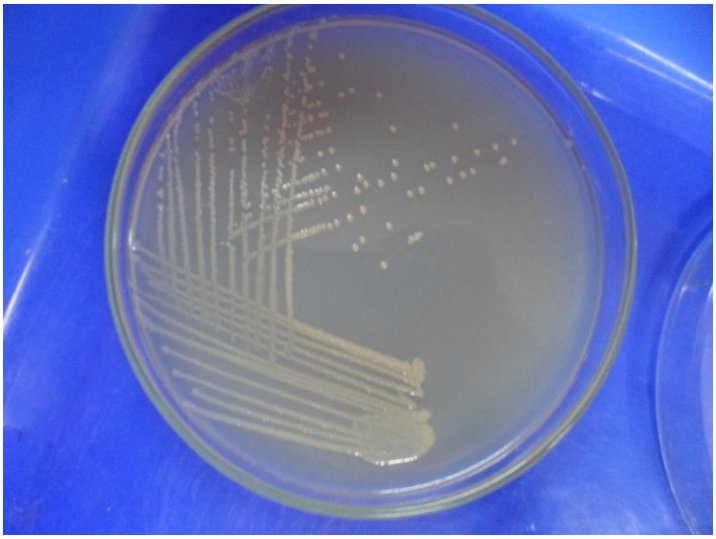

Figure 3:- Isolated Colonies of *Staphylococcus aureus* (Sample 7). Round, mucoid, with yellow pigment with measures 2-4 mm in diameter

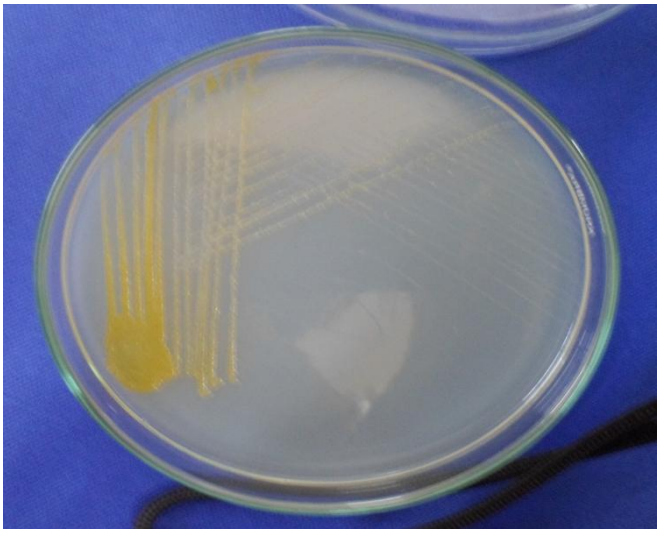

Table -2:- Identification test results for *kocuria kristinae*

Laboratory Report

Printed Dec 11, 2016 15:00 IST
Printed by:

Patient Name:
Isolate Group: WHITE-8

Patient ID:

Bionumber: 010030302011211
Selected Organism: Kocuria kristinae

Comments:	

Identification Information	Card:	GP	Lot Number:	2420022103	Expires:	Dec 18, 2017 12:00 IST
	Completed:	Dec 9, 2016 20:45 IST	Status:	Final	Analysis Time:	5.00 hours
Selected Organism	93% Probability		Kocuria kristinae			
	Bionumber:	010030302011211			Confidence:	Very good identification
SRF Organism						
Analysis Organisms and Tests to Separate:						
Analysis Messages:						
Contraindicating Typical Biopattern(s)						
Kocuria kristinae	NOVO(1),O129R(7),					

Biochemical Details

2	AMY		4	PIPLC		5	dXYL		8	ADH1	+	9	BGAL	-	11	ACLU	-
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	-
20	LeuA	+	23	ProA	+	24	BGURr	-	25	AGAL	-	26	PyrA	-	27	BGUR	-
28	AlaA	+	29	TyrA	+	30	dSOR	-	31	URE	-	32	POLYB	-	37	dGAL	-
38	dRIB	-	39	ILATk	+	42	LAC	-	44	NAG	-	45	dMAL	-	46	BACI	-
47	NOVO	+	50	NC6.5	-	52	dMAN	-	53	dMNE	+	54	MBdG	-	56	PUL	-
57	dRAF	-	58	O129R	+	59	SAL	-	60	SAC	+	62	dTRE	-	63	ADH2s	-
64	OPTO	+															

Table 3:- Identification test results for *Staphylococcus aureus*

Microbiology Chart Report

Printed Dec 11, 2016 14:59 IST

Patient Name:
Location:
Lab ID: yellow

Patient ID:
Physician:
Isolate Number: 9

Selected Organism : Staphylococcus aureus

Source:

Collected:

Comments:	

Identification Information	Analysis Time:	5.00 hours	Status:	Final
Selected Organism	95% Probability Bionumber:	Staphylococcus aureus 070402076763271		
Organism Quantity:				
ID Analysis Messages				

Biochemical Details

2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	+	11	AGLU	+		
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	+		
20	LeuA	-	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-		
28	AlaA	-	29	TyrA	-	30	dSOR	-	31	URE	+	32	POLYB	+	37	dGAL	+		
38	dRIB	-	39	ILATk	+	42	LAC	+	44	NAG	+	45	dMAL	+	46	BACI	+		
47	NOVO	-	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-		
57	dRAF	-	58	O129R	+	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	+		
64	OPTO	+																	

Table 4:- Growth response of the isolates in MSM with chlorpyrifos

Absorbance of 600 nm			
Staphylococcus aureus	***Kocuria kristinae***	**Control**	**Days**
1	1	1	1
1.02	1.12	1	4
1.25	1.44	1	7
0.86	1.09	1	10
0.20	0.65	1	14

Figure 4:- Growth response of *K. kristinae* (X axis = days: Y axis = absorbance at 600 nm).

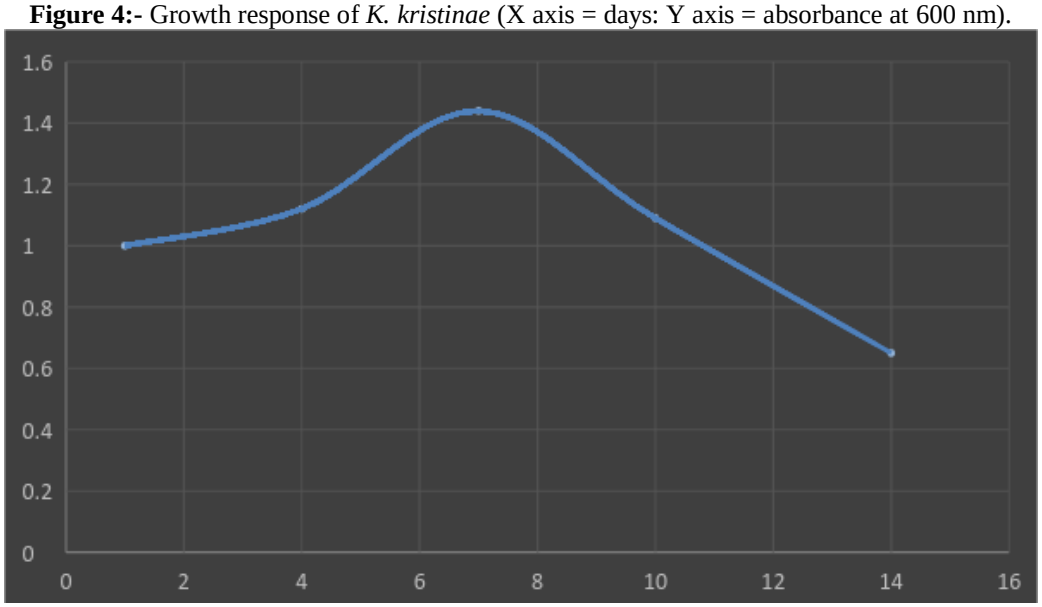

Figure 5:- Growth response of *S. aureus* (X axis = days: Y axis = absorbance at 600 nm).

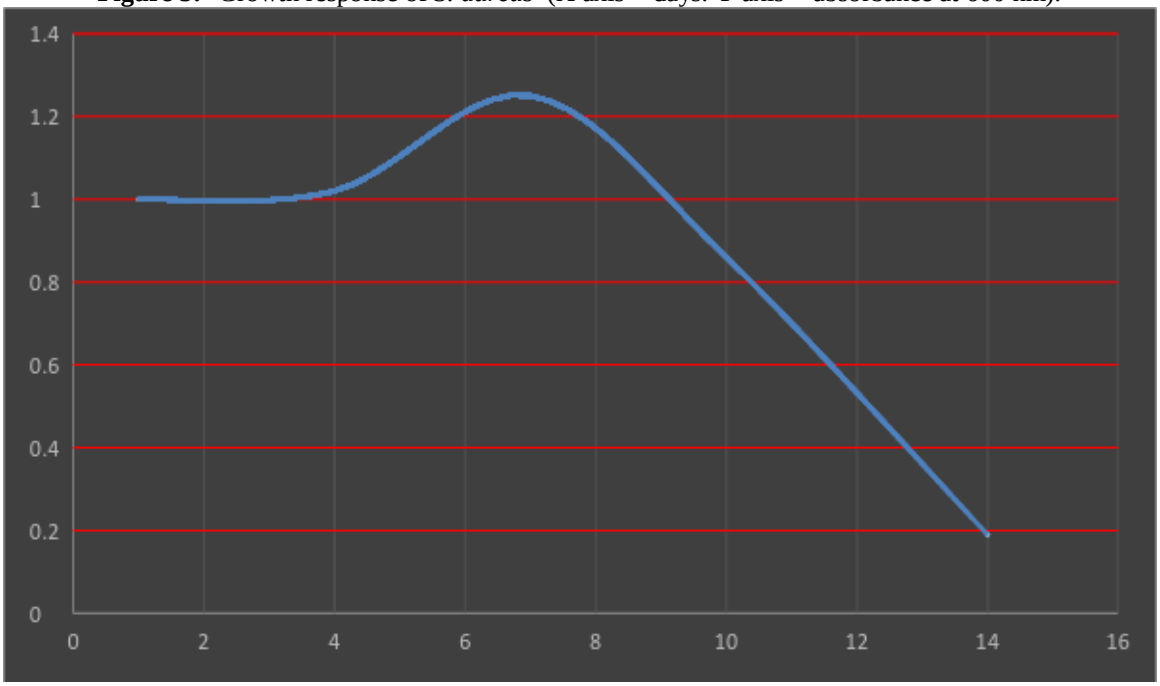

Figure 6:- Degradation of chlorpyrifos by spectrophotometric analysis.

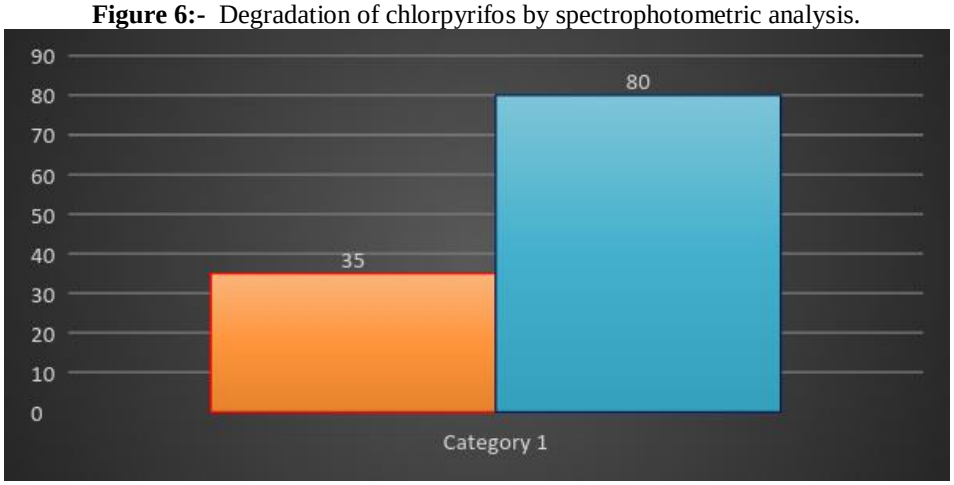

Figure 7:- GC scan showing the peaks for degrading chlorpyrifos by *Staphylococcus aureus*.

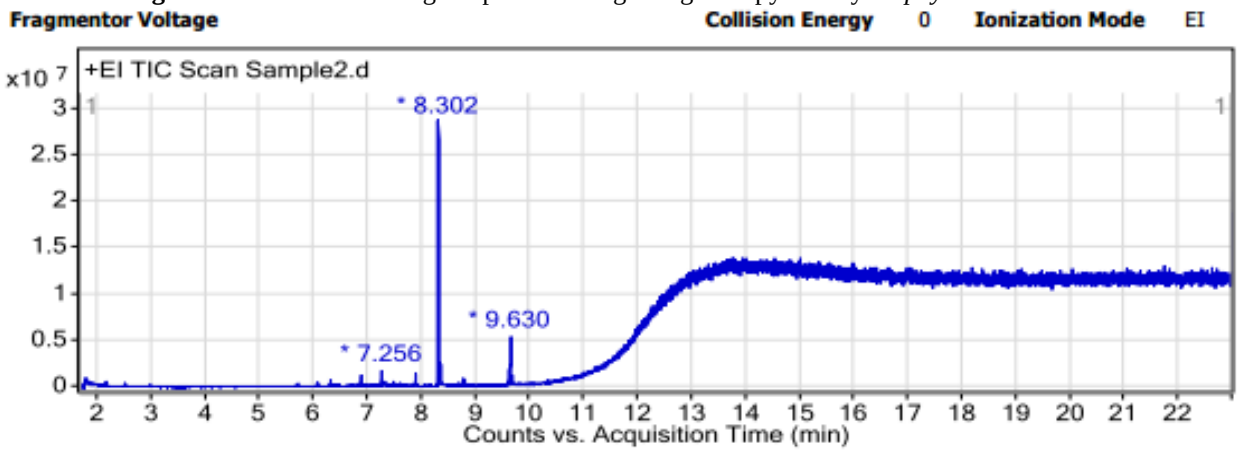

Figure 8:- Peaks of degraded compounds.

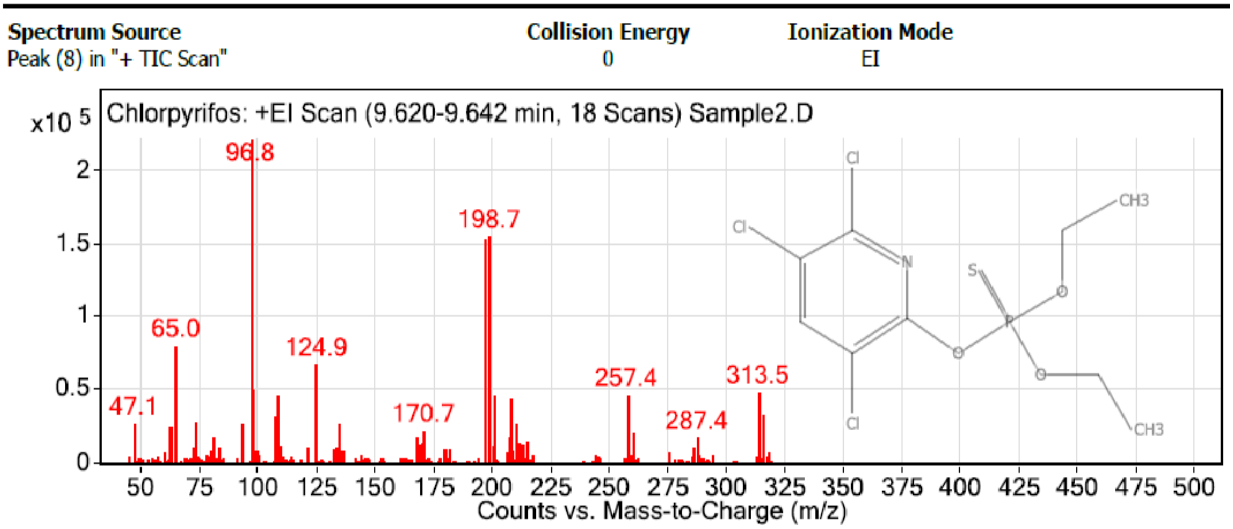

Table 5:- GC scan showing the results for area percentage for each peak

Qualitative Analysis Report

Integration Peak List

Peak	Start	RT	End	Height	Area	Area %	AreaSumPercent
1	2.147	2.162	2.177	480474.99	338065.53	1.59	1.08
2	6.275	6.31	6.331	721453.27	558329.09	2.63	1.78
3	6.86	6.868	6.933	1072515.65	853714.3	4.02	2.72
4	7.229	7.256	7.28	1561522.84	1423655.21	6.7	4.54
5	7.849	7.874	7.9	1198120.83	840287.66	3.96	2.68
6	8.278	8.302	8.318	28515911.45	21242970.58	100	67.7
7	8.32	8.334	8.351	2449293.57	2311159.77	10.88	7.37
8	9.618	9.63	9.642	5172075.27	3810633.1	17.94	12.14

Figure 9:- GC scan showing the peaks for degrading chlorpyrifos by *Kocuria kristinae*.

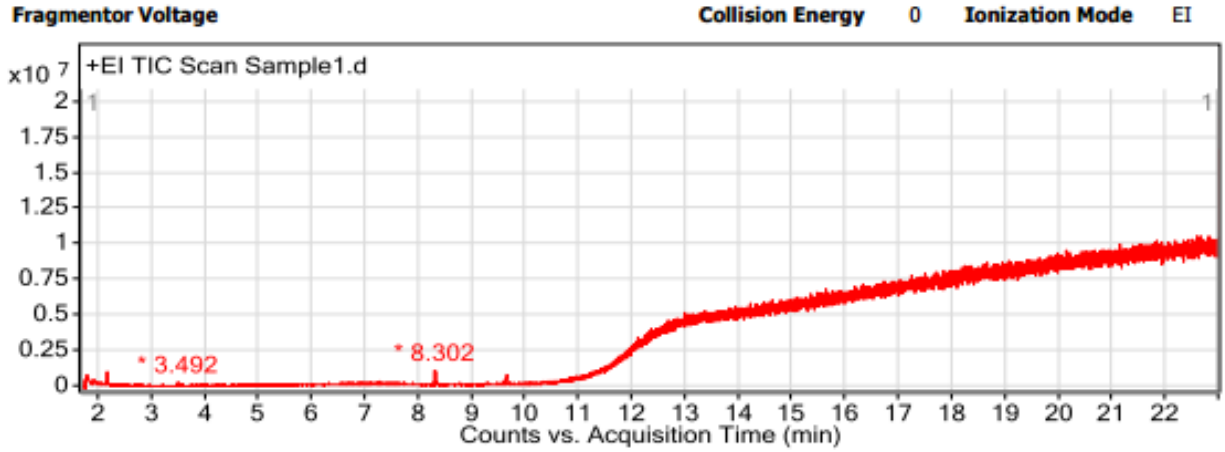

Figure 10:- Peaks of degraded compounds.

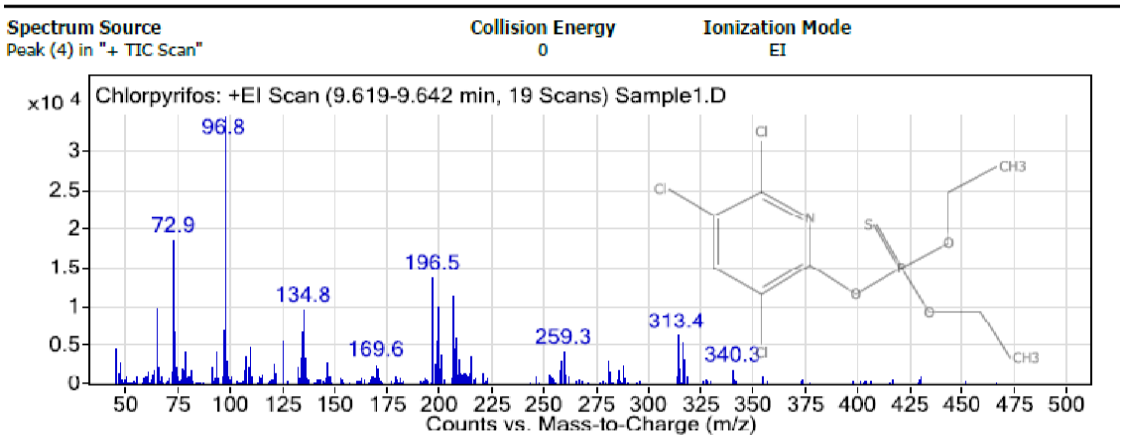

Table 6: GC scan showing the results for area percentage for each peak in Sample -1 (*K. kristinae*)

Qualitative Analysis Report

Integration Peak List

Peak	Start	RT	End	Height	Area	Area %	AreaSumPercent
1	2.141	2.16	2.186	919084	636540.61	71.69	26.53
2	3.477	3.492	3.528	255566.95	260366.89	29.32	10.85
3	8.275	8.302	8.315	1026427.1	887954.84	100	37.01
4	9.616	9.632	9.652	693557.89	614686.68	69.22	25.62

Control:

Figure 11:- GC scan showing the peaks for chlorpyrifos.

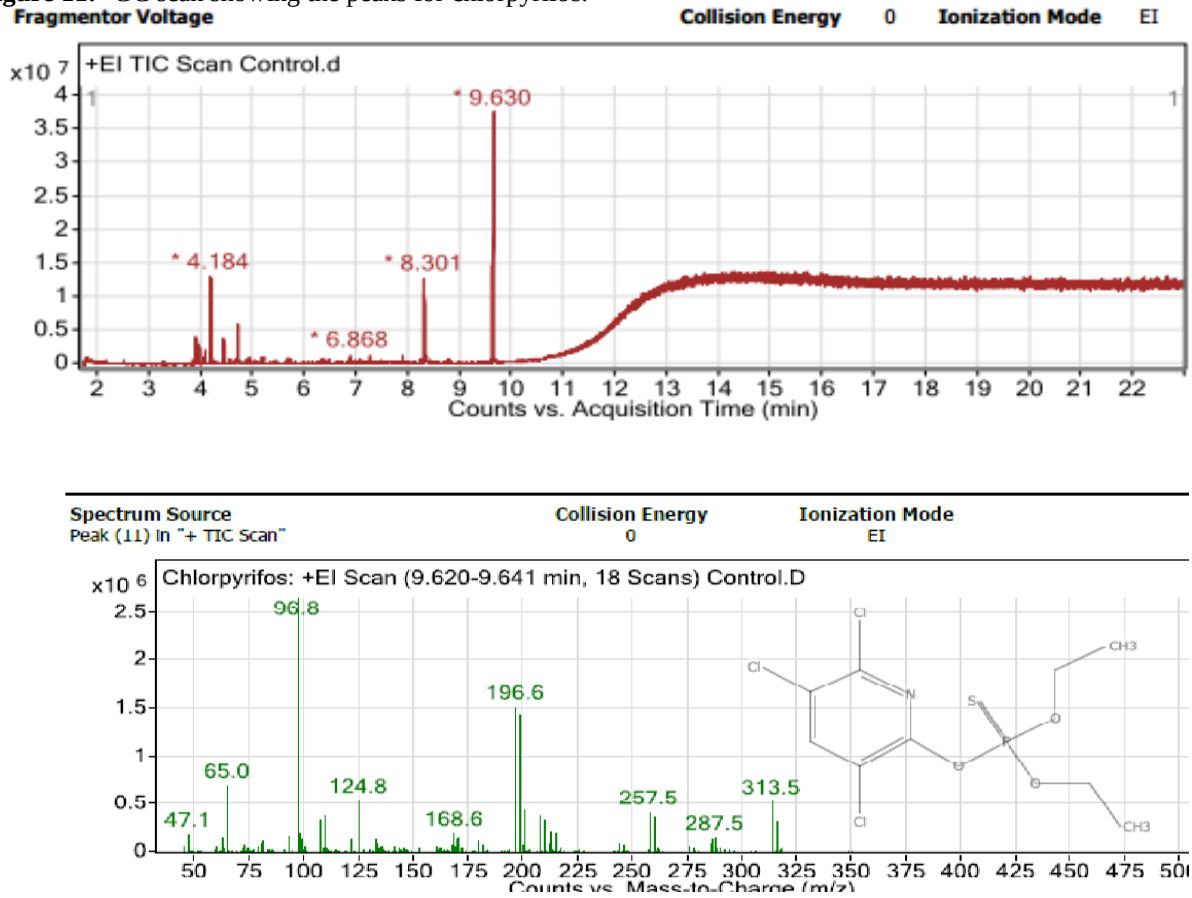

Table 7:- GC scan showing the results for area percentage for each peak

Qualitative Analysis Report

Integration Peak List

Peak	Start	RT	End	Height	Area	Area %	AreaSumPercent
1	3.874	3.901	3.943	3964175.68	4714731.06	16.36	6.87
2	3.953	3.964	3.981	2801125.95	2370339.3	8.22	3.45
3	4.027	4.068	4.088	1955152.06	1581598.46	5.49	2.3
4	4.153	4.184	4.199	12832037.81	10592335.47	36.75	15.43
5	4.399	4.435	4.459	3798412.02	3324263.69	11.53	4.84
6	4.673	4.7	4.72	5802795.04	5222630.69	18.12	7.61
7	6.848	6.868	6.89	1041823.78	722147.62	2.51	1.05
8	7.243	7.253	7.281	974690.71	991680.47	3.44	1.44
9	8.28	8.301	8.316	12525470.2	9545538.44	33.12	13.91
10	9.6	9.63	9.65	37254024.23	28819076.33	100	41.98

Reference:-

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