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Isolation, Biochemical Characterization and Growth Kinetics Studies of Chlorpyrifos and Malathion Degrading Bacterial

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Abstract: ***Among the different pesticides used worldwide, organophosphorus (OP) pesticides form the major and most extensively used class of agricultural pesticides. The toxic effects of OP were well known on humans, animals and soil microbiota. Some bacterium in Soil capable of utilizing organophosphate based pesticides as sole carbon source was isolated by selective enrichment on mineral medium containing chlorpyrifos and malathion individually. Total fifteen bacterial isolates which can capable of degrading chlorpyrifos and malathion were isolates were isolated from five different agriculture soils. KSCM-08 was proved to be high potential isolate against chlorpyrifos and malation degradation. Biochemical characteristics revealed that KSCM-08 was gram positive bacilli. Growth studies showed that KSCM-08 utilized chlorpyrifos to grow in Luria-Bertani broth containing different concentrations of chlorpyrifos at 50 -500 ppm. However, the optimum concentration that supported bacterial growth over 24 h was found to be 50-200 ppm. Further concentrations were proved to be decreasing their bacterial growth and become completely lethal at 500 ppm for both chlorpyrifos and malathion.***

Keywords: organophosphorus, microbial degradation, soil microflora and growth kinetics

1. Introduction

The application of pesticides for rice-based cropping systems is common practice in India. Insecticides are the dominant group of pesticides used in most rice-growing countries like India. Since pesticides are very toxic by design, they have the potential to adversely impact on ecosystem health. Organophosphorous (OP) insecticides such as parathion, methamidophos, malathion and chlorpyrifos are a group of highly toxic agricultural chemicals widely used in plant protection. It is commonly used as a general purpose insecticide for the control of agricultural soil-dwelling insects, household insects, flies and animal parasites (Singh et al., 2012). OPs are neurotoxic due to their ability to inhibit the acetyl cholinesterase (AChE), a key enzyme for normal nerve signal transmission (Cherian et al., 2005; Singh and Walker 2006; Theriotand Grunden 2011).

As these pesticides cause extensive damage to non-target organisms, studies regarding their degradation have received considerable attention from soil microbiologists. Several reports suggest that contamination of soil by these pesticides as a result of their bulk handling at the farm yard or following application in the field or accidental release may lead to occasional contamination of a wide range of water and terrestrial ecosystems (Singh et al., 2004). Extensive use of chlorpyrifos contaminates air, ground water, rivers and lakes. The effects on mammals, especially human beings, include leukemia, kidney damage, brain damage, lung damage, etc., while its carcinogenic effects include chromosomal aberrations in human blood cells and gene loss from human DNA (Kanade et al., 2012).

If pesticides are not degraded or detoxified rapidly enough, the risk of their off-site migration may pose a health risk to humans. Increasing awareness of the potential adverse effects of pesticides has resulted in greater public pressure to assess, monitor and minimize off-site impacts. Soil bacteria that utilize several pesticides have been isolated from the soil. Microbial degradation is generally regarded as the safest, least disruptive and most cost-effective technology. Microorganisms are considered to be the major factors that determine the fate of xenobiotics including OP insecticides in the environment (Singh and Walker, 2006). Numerous bacterial strains belonging to different taxonomic groups have a great degradation potential for organophosphorus insecticides, including malathion (Mohamed et al., 2010; Kanade et al., 2012; Hamouda et al., 2013).

The objectives of the present study were to isolate CP and MT degrading bacteria from different agriculture soils and biochemical characterization of those bacterial isolates.

2. Materials and Methods

2.1 Sample

Twelve soil samples were collected from malathion and chlorpyrifos using agriculture soils located at Bheemali, Rambilli, Tagarapuvalasa, Kottavalasa and Adavivaram areas in Visakhapatnam.

2.2 Pesticide used

Commercial grade insecticide chlorpyrifos (50% E.C.) and malathion (30% E.C.) were used in this study. They were used throughout the experimental studies, because it may more closely resemble the active compound that microorganisms are likely to be exposed to in the soil environment.

2.3 Media

Mineral salts medium (MSM) enriched with chlorpyrifos and malathion were used for isolation and characterization of chlorpyrifos and malathion degrading bacteria. The

carbon source in MSM was replaced with chlorpyrifos and malathion in different concentrations. The MSM has the following composition in (g/L): KH2PO4, 4.8; K2HPO4, 1.2; NH4NO3, 1.0; MgSO4.7H2O. 0.2; Ca (NO3)2.4H2O, 0.04; and Fe(SO4)3, 0.001 with pH 7.0 (Rasul et al., 1988)

Culture enrichment and isolation of chlorpyrifos and malathion degrading bacteria from soil samples

Fivesoil samples were serially diluted and third dilution was inoculated into MS media which contains 0.25% of chlorpyrifos/malathion and 0.75% of glucose as carbon source and incubated in orbital shaker for three days at 28^0C and 240 rpm. Then 100 µl of samples for the above treatments were transferred into fresh tube containing MS media with 0.5% of chlorpyrifos/ malathion and 0.5% of glucose and incubated in orbital shaker for three days at 28^0C and 240 rpm. Then from the above treatments 100 µl of sample was transferred into fresh tube containing MS media with 0.25% of chlorpyrifos/malathion and 0.75% of glucose and incubated in orbital shaker for three days at 28^0C and 240 rpm. Finally 100 µl of sample was inoculated fresh tube containing MS media with 1% of chlorpyrifos/malathion and incubated in orbital shaker for three days at 28^0C and 240 rpm. After incubation 100 µl from each treatment were spread speparately onto mineral agar supplemented with chlorpyrifos (50 mg/L) and mineral agar supplemented with malathion (50 mg/L) and plates were incubated at 37^0C for 24 h.

Biochemical characterization of isolated bacteria

The individual bacterial colonies that grew on the medium were subcultured onto mineral agar containing chlorpyrifos of the same concentration until pure cultures were obtained. Bacterial isolates were subjected to morphological, cultural and biochemical studies.

Viable cell count determinations

Aliquots (2.5 ml) of 24-h old bacterial cultures grown in mineral salts medium were inoculated into 100-ml Erlenmeyer flasks containing25 ml of Luria-Bertani broth supplemented with various concentrations (50- 700 mg/L) of chlorpyrifos and malathion to test their ability to degrade the supplemental substrate (pesticide). A control was maintained with equal volume of broth containing bacterial culture, but without pesticide. Bacterial growth was followed by viable cell counts immediately after inoculation at 0 h and 2, 4, 6 and 24 h of incubation. Abacterial inoculum (1 ml) was drawn at regular intervals from the test and control cultures and serial dilutions were performed using 9ml of sterile saline (0.85% NaCl; pH 7). Appropriate dilutions were plated in triplicate on nutrient agar and the plates incubated at 37^0Cfor 24 h. The bacterial colonies were counted (CFU/ml) with a colony counter.

3. Results and Discussion

Fifteen different chlorpyrifos and malathion degrading bacterial isolates were isolated based on the morphological colony characters from the collected five soil samples using the culture enrichment technique (Figure-1). All bacterial isolates were common to the both pesticides because these two pesticides were belongs to organophosphates family.

All these fifteen isolates were showed distinguish difference at biochemical level characters. In the eleven isolates three isolates were cocci shaped and remaining eight were rod shaped bacteria. On the other hand five bacteria were gram negative bacteria and six bacteria were gram positive bacteria. Among the all bacteria KSCM-04, 05, 06, 09, 10, 11 and 14 isolates utilize citrate and the remaining isolates were not able to utilize citrate. Among the bacteria KSCM-02, 03, 04, 08, 09, 10, 14 and 15 were able to utilize and hydrolyze starch and remaining bacteria were not able to utilize starch as carbon source. KSCM- 01, 04, 07, 09, 13 and 15 isolates were able to reduced nitrate and the remaining bacteria were not able reduce nitrate. KSCM- 01, 04, 07, 08, 12, 14 and 15 isolates were showing positive results for methyl red test and the remaining bacteria were showed negative results for methyl red test. On the other hand KSCM- 04, 06, 08, 12, 14 and 15 isolates were showing positive results for Voges-Proskauer test and the remaining isolates were showing negative results for VP test. Only KSCM-06 and 13 isolates were able to produce H_2S and remaining isolates did not able to produce H_2S gas. Only KSCM 01, 08, 10 and 11 isolates were able to hydrolysis casein and remaining isolates were not able to utilize casein protein. On the other hand isolates KSCM -06, 09, 10 and 15 isolates were able to hydrolysis Gelatin and the remaining isolates were not able to hydrolysis Gelatin. KSCM – 1, 2, 3, 6, 8, 10, 12, 13 and 15 isolates showed positive results for catalase and remaining isolates were showed negative results. Only KSCM-04, 05, 06 11, 12, 14 and 15 isolates were able to produce in dole and the remaining isolates were showed negative results for indole production. And KSCM- 01, 02, 03, 07, 10, 11, 12 and 15 isolates showed positive results for urea test and the remaining isolates showed negative results for urea test. (Table-1)

Growth kinetics studies of KSCM-08

Growth curve experiments were performed to confirm the high pesticide tolerance isolate and to determine the optimum concentration of chlorpyrifos and malathion that stimulates the growth KSCM-08. A Similar strategy has been reported previously by (Karpouzas and Walker, 2000; Haugland et al., 1990) while performing pesticide degradation using isolated strains of microorganisms.

At 200 ppm all 15 isolates show visible growth both in chlorpyrifos and malathion treatments. After 6 hours the growth was gradually increasing, and the growth was variable for each isolate. Between 6 hours and 24 hours the growth was dynamically increased. At 24 hours KSCM-02 maximum viable count in both chlorpyrifos and malathion amended conditions, the viable counts were 109.14 x 10^6 and 85.72 x 10^6 consequently. These viable counts were very high when compared to the rest of the all isolates. So further pesticide tolerance growth kinetics studies were carried with KSCM-08

By observing the growth kinetics studies we observe that the phase adoption was continued up to 6 h and the total viable count at the initial period was 0.48 x 10^6CFU/ml in chlorpyrifos and 3.99x 10^6 in malathion at 50 ppm concentration (Table 3 and 5). After incubation for 12 h, the total viable counts were 9.21 x 10^6 and 6.94 x 10^6 CFU/ml

for chlorpyrifos and malathion consequently. The bacterium had a generation time of 77.2 min and specific growth rate (μ) of 0.017/h. (Mansi and Bryce, 2002). With increasing incubation time the bacterial growth also improved. For instance, after incubation for 24 h the total viable count increased significantly decrease from 50 ppm to 500 ppm. This indicated that the bacterial culture, after remaining in lag phase (phase of adjustment to initiate accelerated growth) for 6 h, entered into the log phase. And because of the effect of the negative effect of the pesticide bacteria took another 12 to 18 hours to reach its maximum growth. The total viable count of 0.48 x 10^6 CFU/ml at 6 h had increased to 9.21 x 10^6 CFU/ml at 12 h, and to 321.94 x 10^6 CFU/ml at 24 h in chlorpyrifos, on the other hand the total viable count of 3.99 x 10^6 CFU/ml at 6 h had increased to 6.94 x 10^6 CFU/ml at 12 h, and to 184.31 x 10^6 CFU/ml at 24 h in malathion. These results revealed that KSCM-08 growth was higher in chlorpyrifos than malathion. The generation time was calculated to be 137 min at 50 mg/L and 165 min at 200 mg/L of chlorpyrifos, respectively (Mansi and Bryce, 2002). The results also indicated that growth of the bacterial isolate in the presence of chlorpyrifos was stimulated in the concentration range of 50-200 mg/L during 24 h of incubation (Figure 3). The total viable count at 24 h was 321.94 x 10^6, 287.31 x 10^6, 109.14 x 10^6, 33.03 x 10^6, 4.04 x 10^6 and 0.35 x 10^6 with 50 ppm, 100 ppm, 200 ppm, 300 ppm, 400 ppm and 500 ppm respectively for chlorpyrifos and 184.31 x 10^6, 123.90 x 10^6, 85.72x 10^6, 21.98 x 10^6, 1.36 x 106 and0.09 x 10^6 with 50 ppm, 100 ppm, 200 ppm, 300 ppm, 400 ppm and 500 ppm respectively for malathion. These observations indicated that the rate of substrate utilization decreased, which prolonged the lag phase in the presence of higher concentrations of chlorpyrifos. As growth kinetic studies providing an evidence of mineralization potential of organism, therefore such studies were reported by several other researchers (Maria et al., 2002; Karpouzas and Walker, 2000; Lee et al., 1998; Smith and Adkins, 1996) while performing pesticide degradation using isolated strains of microorganisms.

The results obtained in this study were in agreement with earlier reports that indicated the involvement of different species of *Enterobacteriaecae* in the degradation of organophosphorous insecticides like chlorpyrifos (Singh et al., 2004), phosphonate (Lee et al., 1992) and glyphosate (Dick and Quinn, 1995). Singh et al. (2004) reported that *Enterobacter* strain B-14 used chlorpyrifos as a source of carbon and phosphorous. Sethunathan and Yoshida (1973) isolated a *Flavobacterium* sp. that could use parathion as source of phosphorous but not diazinon as carbon source. Although utilization of chlorpyrifos and malathion by several soil bacteria has been reported (Singh, 2004; Racke et al., 1990).

A possible explanation may be that microorganisms need an adaptation period to produce the necessary degradative enzymes. This may account for the prolonged lag phase at high concentrations of added chlorpyrifos (Jilani and Khan, 2004). Degradation of different pesticides at high concentrations by microorganisms has been reported previously (Karpouzas and Walker, 2000; Struthers et al., 1998). *Enterobacter* sp. was able to degrade chlorpyrifos at concentrations as high as 250 mg/L in less than two days (Singh et al., 2004). In the presence of high concentrations of insecticides, the bacterium was greatly stressed and its growth was slowed as a consequence. During the adaptation period, the bacteria changed from a rod shape to a coccus form with an increase in insecticide concentration (Jilani and Khan, 2004). However, this change was temporary and after seven days the strain recovered to its original rod shape. The soil used for the present study had been exposed to chlorpyrifos for about ten years. Hence, the tolerance of the organism might be due to previous exposure or due to its ability to hydrolyze the supplemental substrate. Successful removal of pesticides by the addition of bacteria had been reported earlier for many compounds, including coumaphos (Mulbry et al., 1996), ethoprop (Karpouzas and Walker, 2000) and atrazine (Struthers et al., 1998).

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Table 1: Biochemical characters of eleven bacterial isolates

Isolates	Shape	Grams Stain	Citrate	Starch hydrolysis	Nitrate reductase	MR	VP	H_2S production	Casein hydrolysis	Gelatin hydrolysis	Catalase test	Indole production	Urea test
KSCM-01	**Rod**	**Positive**	-	-	+	+	-	-	+	-	+	-	+
KSCM-02	**Rod**	**Positive**	-	+	-	-	-	-	-	-	+	-	+
KSCM-03	**Rod**	**Negative**	-	+	-	-	-	-	-	-	+	-	+
KSCM-04	**Rod**	**Negative**	+	+	+	+	+	-	-	-	-	+	-
KSCM-05	**Cocci**	**Positive**	+	-	-	-	-	-	-	-	-	+	-
KSCM-06	**Cocci**	**Positive**	+	-	-	-	+	+	-	+	+	+	-
KSCM-07	**Cocci**	**Positive**	-	-	+	+	-	-	-	-	-	-	+
KSCM-08	**Rod**	**Positive**	-	+	-	+	+	-	+	-	+	-	-
KSCM-09	**Rod**	**Negative**	+	+	+	-	-	-	-	+	-	-	-
KSCM-10	**Cocci**	**Positive**	+	+	-	-	-	-	+	+	+	-	+
KSCM-11	**Cocci**	**Positive**	+	-	-	-	-	-	+	-	-	+	+
KSCM-12	**Rod**	**Negative**	-	-	-	+	+	-	-	-	+	+	+
KSCM-13	**Cocci**	**Positive**	-	-	+	-	-	+	-	-	+	-	-
KSCM-14	**Cocci**	**Positive**	+	+	-	+	+	-	-	-	-	+	-
KSCM-15	**Rod**	**Positive**	-	+	+	+	+	-	-	+	+	+	+

Table 2: Effect of Chlorpyrifos at 200 ppm against eleven bacterial isolates growth kinetics

S.No	Bcaterial Isolates	Viable bacteria x 10^6			
		6hr	12hr	18hr	24 hr
1	KSCM-01	0.10	0.87	3.19	7.09
2	KSCM-02	0.09	0.38	6.00	15.92
3	KSCM-03	0.03	0.29	37.09	87.23
4	KSCM-04	0.11	1.72	9.08	32.09
5	KSCM-05	0.12	0.17	5.98	12.05
6	KSCM-06	0.21	0.98	8.73	19.03
7	KSCM-07	0.10	0.36	17.17	48.69
8	**KSCM-08**	**0.31**	**7.96**	**39.13**	**109.14**
9	KSCM-09	0.08	0.38	4.98	23.73
10	KSCM-10	0.09	3.82	31.43	93.96
11	KSCM-11	0.10	1.04	9.93	19.01
12	KSCM-12	0.10	4.92	12.45	34.82
13	KSCM-13	0.23	12.31	48.75	105.80
14	KSCM-14	0.29	11.94	50.42	81.22
15	KSCM-15	0.13	0.95	8.21	18.91

Table 3: Effect of chlorpyrifos at different concentrations against KSCM-08 bacterial growth kinetics

S.No		Viable bacteria x 10^6			
		6hr	12hr	18hr	24 hr
1	50 ppm	0.48	9.21	49.81	321.94
2	100 ppm	0.36	9.19	42.87	287.31
3	200 ppm	**0.31**	**7.96**	**39.13**	**109.14**
4	300 ppm	0.26	3.21	17.04	33.03
5	400 ppm	0.11	0.38	1.89	4.04
6	500 ppm	0.02	0.09	0.12	0.35

Table 4: Effect of malathion at 200 ppm against eleven bacterial isolates growth kinetics

S.No	Bacterial Isolates	Viable bacteria x 10^6			
		6hr	12hr	18hr	24 hr
1	KSCM-01	0.19	2.31	8.04	28.11
2	KSCM-02	0.11	0.98	4.29	19.21
3	KSCM-03	0.28	3.01	6.04	34.29
4	KSCM-04	0.10	3.38	11.68	35.19
5	KSCM-05	0.09	1.03	8.21	63.01
6	KSCM-06	0.11	2.79	12.16	49.61
7	KSCM-07	0.09	2.72	09.47	22.82
8	**KSCM-08**	**0.31**	**4.28**	**18.53**	**85.72**
9	KSCM-09	0.14	6.14	14.51	83.62
10	KSCM-10	0.22	2.89	7.24	19.45
11	KSCM-11	0.15	1.24	5.82	19.72
12	KSCM-12	0.16	4.29	15.90	81.32
13	KSCM-13	0.23	6.28	23.19	63.01
14	KSCM-14	0.18	2.76	8.31	21.90
15	KSCM-15	0.31	4.09	18.03	53.41

Table 5: Effect of malathion at different concentrations against MGPD-02 bacterial growth kinetics

S.No		Viable bacteria x 10^6			
		6hr	12hr	18hr	24 hr
1	50 ppm	3.99	6.94	145.14	184.31
2	100 ppm	2.10	6.53	94.29	123.90
3	200 ppm	**0.31**	**4.28**	**18.53**	**85.72**
4	300 ppm	0.23	0.35	8.24	21.98
5	400 ppm	0.21	0.18	0.97	1.36
6	500 ppm	0.03	0.06	0.08	0.09

Figure 1: Chlorpyrifos and malathion added MSM agar plates showing bacterial colonies after 7 days of incubation