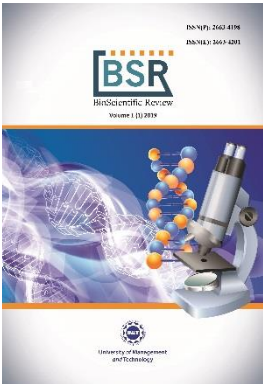

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Isolation and Characterization of Chlorpyrifos Degrading Bacteria from Contaminated Agricultural Lands

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Abstract

Previous literature has confirmed that pesticides are posing serious threats to the lives of human beings and animals alike because of their use in agricultural crops. Pesticides have also increased pollution in aquatic and terrestrial environment. In this study, chlorpyriphos degrading bacterial strains were isolated from pesticide contaminated agricultural soils by the selective enrichment method. Bacterial isolates were biochemically characterized for the strain identification. The effect of different environmental factors on optimum growth was also checked. These factors included pH, temperature, and concentration of pesticide. Isolated strains were also ribotyped for identification. Metal resistance profiling was performed for different heavy metals, that is, $ZnSO_4.7H_2O$ (Zinc sulphate), $CdCl_2.H_2O$ (Cadmium chloride), $CuSO_4$ (Copper sulphate), and K_2CrO_4 (Potassium chromate). It was found that strains $NW3_O$ and $NW3_T$ were sensitive to cadmium except NR2 and NR4, which were resistant at the concentration of 100 and 200 µg ml^{-1}. NR4, NW4 and $NW3_G$ were resistant to chromium upto the concentration of 100 µg ml^{-1}, while NR2, $NW3_O$ and $NW3_T$ tolerated 200 µgml^{-1} of the metal. All the strains were resistant to zinc at different concentrations. NR2 showed maximum resistance to copper by showing growth on all concentrations of the metal. Thin layer chromatography was performed for the detection of different intermediates formed during the degradation of chlorpyrifos. Minimum inhibitory concentration of pesticide was estimated in M9 medium containing chlorpyriphos. The current study resulted in the isolation of efficient chlorpyrifos degrading strains with a wide range of pH and temperature tolerance that can utilize chlorpyrifos upto 700mg/L during lab scale degradation tests (growth on chlorpyrifos supplemented minimal agar and broth).

1. Introduction

Since before 2500 BC, pesticides are used by humans to protect the crops. Synthetic pesticides are produced in large quantity since 1940's. Pesticides target species, including non-target species, bottom sediments, water, food and air. However just 95% herbicides and 98% sprayed insecticides reach a destination (1). The usage of pesticides for protection of the crops, firstly, recorded about 4500 years ago. Paul Muler discovered DDT as a very effective insecticide in 1993. Pesticides in large amount are used in agricultural activities all over the world that are toxic for humans and also for animals. Aquatic and terrestrial ecosystems are contaminated due to inappropriate use of pesticides. Many pesticides are used instantly on different agricultural crops, that increased pollution (2).

In 1989, pesticides consumption was fivefold increase in one year, because public sector transferred the sale of pesticides to private sector. About 80% pesticides are used on cotton plants, and others are applied on fruits, vegetables and paddy tobacco (3). It has been assessed that throughout the world about 2.5 million tons pesticides are used every year and it is also increasing day by day (4). Pakistan shows the same trend. In Pakistan and other Asian countries, nitrates and pesticides causes the contamination in the shallow groundwater (5-7).

Conventional methods using for degradation of Chlorpyrifos results in accumulation of unmanageable residual and many toxic products. So, biodegradation for its removal from environment by using native microorganisms is quite attractive. The most common Chlorpyrifos biodegradation products such as 3, 5, 6-trichloro-2-pyridinol (TCP) has antimicrobial activity (8), and by the US environmental protection agency (EPA) it has been persistent, mobile and toxic with a half-life about 65 to 360 days in soil (9). It causes large scale contamination of aquatic and soil environments, because, due to its greater water solubility it is more portable than the parent molecule. So, study of TCP biodegradation is also important (10). Sphingomonas sp. Dsp-2, Enterobacter strain B-14 and Stenotrophomonas sp. YC-1 have been described as Chlorpyrifos degrading bacteria (11-13). Chlorpyrifos degrading fungi such as *Vertocollium*, *Phanerochaete chrysosporium*, and *Aspergillus terreus* sp. have also been reported (14-16). Unfortunately, TCP accrued in the medium without further metabolism and degradation of bacteria was only partial. Only *Pseudomonas* sp. (TCP mineralizing bacterium) has been describe earlier (17), less data is available on the microbial metabolism of TCP (18). Aim of the presented study is to isolate Chlorpyriphos degrading bacterial strains from pesticide contaminated agricultural soils as well as to biochemically characterize the isolated bacterial strains. The effect of different environmental factors on optimum growth have also been checked these factors include pH, temperature, concentration of pesticide etc. Isolated strains were also ribotyped for identification.

2.Methodology

Agricultural soil samples with pesticide usage history were collected from different cities of Punjab as shown in table 1. By using sterile scalpel the sampling of soil attained from the depth of 5cm (Figure 1) and moved into polythene bag to store at 4ºC until used.

Table 1: Pesticide Contaminated Samples

S. No.	Sample Collection Site
1.	Kala shah kaku Rice fields
2.	Faisalabad Rice fields
3.	Effluent from Ali Akbar group of pesticides
4.	Sheikhu pura Rice fields
5.	Faisalabad Cotton fileds

Then for the removal of any hard particle the samples were sieved. Bacteria that have the capability of degrading Chlorpyriphos were isolated from the soil samples, by selective enrichment techniques. The soil samples were air dried, sieved. Then samples were suspended in the flask containing 50 ml of the minimal salt medium supplemented with Chlorpyrifos (50 mg L^{-1}). Minimum inhibitory concentration of pesticide was estimated in M9 medium having 500µg/ml 300µg/ml, 200µg/ml and 100µg/ml of Chlorpyriphos. After 48 hrs incubation growth was observed that showed the strains were resistant to the pesticides.

Figure 1. Sample collection

For colony morphology 24 hrs incubated fresh bacterial cultures at 37ºC were grown on N-agar plates. After this, under the size, color, shape, margins and evaluation of the bacterial colonies was done under microscope. To check the multiple resistivity of the isolates to other heavy metals, following heavy metals were used: Zn^{2+} ($ZnCl_2$), Cu^{2+} ($CuSO_4$), Cr^{2+} (k_2CrO_4), and Cd^{2+} ($CdCl_2$). For this purpose, in autoclaved distilled water the 10% stock solutions were prepared for all heavy metals mentioned above was prepared as it was very toxic. The results were noted as negative or positive. Antibiotic resistance of the isolates was also estimated, five different antibiotics were used: Ampicillin, Erythromycin, Tetracycline, Chloremphenicol and Gentamycin.

3.Results

3.1. Characterization and isolation of Chlorpyriphos degrading Bacteria

The bacterial strains were isolated from different agricultural land fields that had earlier history of Chlorpyriphos contamination (Table 1) with the help of careful enrichment techniques. Strains

NR2, NR4, NW4, $NW3_T$, $NW3_G$ and $NW3_o$ were selected which had the capability to use the pesticide as the sole source of energy and carbon. These strains were finally purified and maintained under stress condition.

3.2. MIC of the pesticide

The isolated strains were examined for degradation of pesticide at dissimilar concentrations such as; 100, 200, 300 and 500 µg/L. All the strains showed growth in the presence of 100 and 200 µg/L of the pesticide. Only NR2 showed the growth on 300 and 500 µg L^{-1} of the pesticide and NR4 also showed the growth on 300 µg/L^1 of the pesticide (Table 2 & Figure 2).

Table 2. Minimum Inhibitory Concentration of Chlorpyriphos Degrading Bacteria

S. No.	Strains	Concentrations of Pesticide µg ml^{-1}			
		100	**200**	**300**	**500**
1.	NR2	+	+	+	+
2.	NR4	+	+	+	-
3.	NW4	+	+	-	-
4.	$NW3_O$	+	+	-	-
5.	$NW3_G$	+	+	-	-
6.	$NW3_T$	+	+	-	-

Figure 2. Minimum Inhibitory Concentrations (MIC) of the pesticide (a) Growth on plate with the concentration of pesticide 100 µg ml-1 (b) 200 µg ml-1 (c) 300 µg ml-1(d) 500 µg ml-1

3.3. Colony morphology

Colony morphology of the bacteria strains were examined on N-agar. The strain NR2 showed off-white colonies and is thus characterized as *Bacillus spp*. Greenish, flat and opaque colonies were observed in the case of strains NR4 and $NW3_G$, which shows that it belongs to *Pseudomonas spp.* While all other showed off-white colonies, as shown in the table 3 and figure 3.

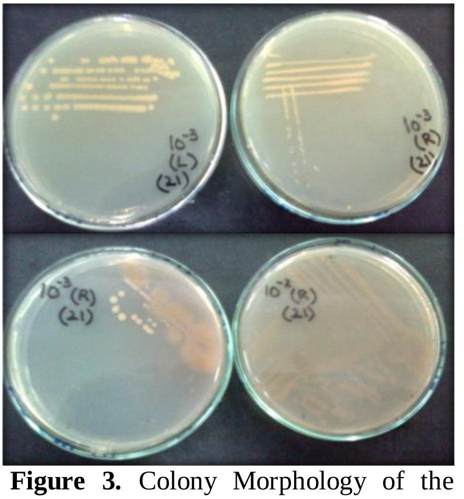

Figure 3. Colony Morphology of the isolated strains (a) NR2 (b) $NW3_O$, (c) NR4 and (d) $NW3_G$

Table 3. Colony Morphology of Pesticide Degrading Bacteria.

S. No.	Strains	Shape	Size	Color	Margin	Elevation	Transparency
1.	NR2	Circular	Small	Off-white	Entire	Flat	Opaque
2.	NR4	Circular	Medium	Greenish	Entire	Flat	Opaque
3.	NW4	Circular	Small	Off-white	Entire	Raised	Opaque
4.	$NW3_O$	Circular	Pinpoint	Off-white	Entire	Flat	Opaque
5.	$NW3_G$	Circular	Pinpoint	Greenish	Entire	Flat	Opaque
6.	$NW3_T$	Circular	Medium	Off-white	Entire	Flat	Opaque

Gram staining was done after growing the strains on LB agar and incubating them for 24 hours. According to the staining results, all the strains except NR2 were gram negative (Table 4 & Figure 4).

3.4. Triple Sugar Iron test

To examine the fermentation pattern of the isolated pesticide degrading bacterial strain the triple sugar iron test was done. The slants color changed so, results of triple sugar iron tests are understood. The results of triple sugar iron test are took by the change in color of the slants. Strain NR2, NW4, $NW3_O$, $NW3_G$ and $NW3_T$ did not ferment glucose except NR4 which gave yellow butt indicated that it had the ability to ferment glucose. All the strains showed red slants which indicated that none of them can ferment lactose and sucrose (Table 5 & Figure 5).

Table 4. Cell Morphology of Pesticide Degrading Bacteria

S. No.	Strains	Shape of cells	Gram staining	Spore staining	Motility
1.	NR2	Rods	+	+	+
2.	NR4	Rods	-	-	+
3.	NW4	Rods	-	-	+
4.	$NW3_O$	Cocci	-	-	-
5.	$NW3_G$	Rods	-	-	+
6.	$NW3_T$	Cocci	-	-	W^+

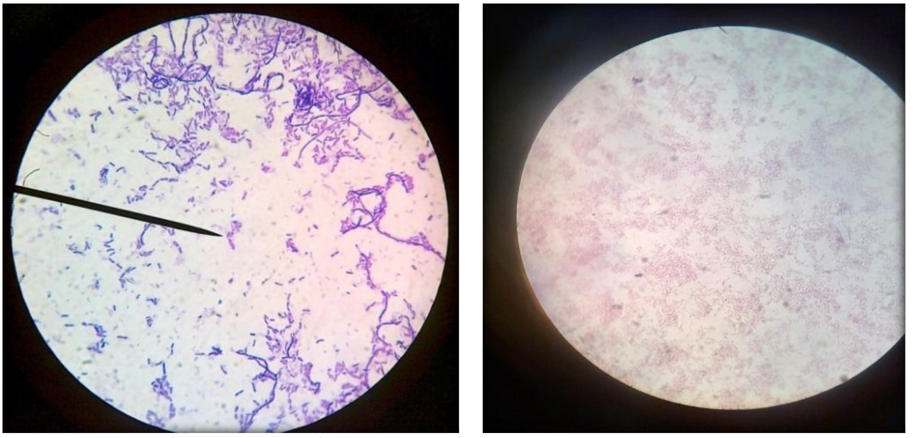

Figure 4. Cell morphology of pesticide degrading bacteria (a) Gram positive rods, (b) Gram negative cocci

Table 5. Triple Sugar Iron Test of the Pesticide Degrading Bacteria

S. No.	Bacterial strains	Carbohydrate Fermentation			H_2S Production		Gas production
		Butt color	Slant color	Carbo-hydrate Fermented	Blackening	H_2S	
1.	NR2	Red	Red	None	No	-	No
2.	NR4	Yellow	Red	Glucose	No	-	No
3.	NW4	Red	Red	None	No	-	No
4.	$NW3_O$	Red	Red	None	No	-	No
5.	$NW3_G$	Red	Red	None	No	-	No
6.	$NW3_T$	Yellow	Red	None	No	-	No

Figure 5. Triple sugar iron test of the isolated, pesticide degrading strain

3.5. Metal resistance profiling

Ten percent stock solutions of $ZnSO_4.7H_2O$ (Zinc sulphate), $CdCl_2.H_2O$ (Cadmium chloride), $CuSO_4$ (Copper sulphate), and K_2CrO_4 (Potassium chromate) were prepared to monitor multiple metal tolerance profile of pesticide degrading bacterial strains. Metal solutions were added into autoclaved nutrient agar media to make working concentrations of 100 µg ml^{-1}, 200 µg ml^{-1}, and 300 µg $ml^{-1}$and 500 µg ml^{-1}. Strains were marked on metal supplemented plates and growth was checked after 24 hours. NR2 showed considerable growth on almost all the metals. NW3$_O$ and NW3$_T$ were sensitive to cadmium except NR2 and NR4, which were found resistant at the concentration of 100 and 200 µg ml^{-1}. NR4, NW4 and NW3$_G$ were resistant to chromium upto the concentration of 100 µg ml^{-1}, while NR2, NW3$_O$ and NW3$_T$ tolerated 200 µgml^{-1} of the metal. All the strains were resistant to zinc at different concentrations. NR2 showed maximum resistance to copper by showing growth on all concentrations of the metal. NR4 and NW3$_G$ grew on 300 µg ml^{-1}, whereas NW4, NW3$_O$ and NW3$_T$ showed growth on the concentration 100 µg ml^{-1} (Table 6 and figure 6).

3.6. Antibiotic Resistance Profiling

NR2 was sensitive to Ampicillin and Tetracycline. However, it was weak positive to Erythromycin, Gentamycin and Chloremphenicol at 50 µg ml^{-1}. NW4 was sensitive towards Erythromycin and 100 µg ml^{-1} concentration of Gentamycin, Chloremphenicol and Tetracycline. However, it showed strong positive and weak positive growth on 50 and 100 µg ml^{-1} of ampicillin. NW3$_T$ and NR4 were sensitive to all. NW3$_O$ was strongly positive against 50 µg ml^{-1} concentration of Ampicillin, while it was weak positive against 50 µg ml^{-1} of Erythromycin, Chloremphenicol and Tetracycline. NW3$_G$ was sensitive to all; however, it showed resistance towards results at 50 µg ml^{-1} of Chloremphenicol (Table 7).

3.7. Thin layer chromatography

Degradation experiment of Chlorpyrifos was preceded for 7 days. After 7 days extraction, running and development of TLC for the detection of different intermediates formed during degradation of Chlorpyrifos was done (Figure 6).

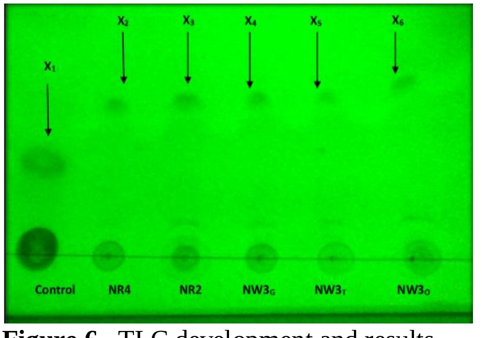

Figure 6. TLC development and results under UV illuminator for the Degradation of chlorpyrifos

Table 6. Metal Resistance Profiling of the Isolated Strains

Metals	μg ml^{-1}	NR2	NR4	NW4	NW3$_O$	NW3$_G$	NW3$_T$
Copper (Cu)	100	+	+	+	+	+	+
	200	+	+	-	-	+	-
	300	+	+	-	-	-	-
	500	+	-	-	-	-	-
Cadmium (Cd)	100	+	+	+	-	+	-
	200	+	-	+	-	-	-
	300	-	-	-	-	-	-
	500	-	-	-	-	-	-
Chromium (Cr)	100	+	+	+	+	+	+
	200	+	-	-	+	-	+
	300	-	-	-	-	-	-
	500	-	-	-	-	-	-
Zinc (Zn)	100	+	+	+	+	+	+
	200	+	+	-	+	-	-
	300	+	-	-	-	-	-
	500	-	-	-	-	-	-

.

Table 7. Antibiotic Resistance Profile of Pesticides Resistant Isolates.

S. No.	Bacterial strains	Antibiotics Used (μg ml^{-1})									
		Erythromycin		Gentamycin		Chloremphenicol		Tetracycline		Ampicillin	
		50	100	50	100	50	100	50	100	50	100
1.	NR2	W$^+$	-	W$^+$	-	W$^+$	-	-	-	-	-
2.	NR4	-	-	-	-	-	-	-	-	-	-
3.	NW4	-	-	W$^+$	-	W$^+$	-	W$^+$	-	++	W$^+$
4.	NW3$_O$	W$^+$	-	-	-	W$^+$	-	W$^+$	-	+++	W$^+$
5.	NW3$_G$	-	-	+	+	W$^+$	-	-	-	-	-
6.	NW3$_T$	-	-	-	-	-	-	-	-	-	-

Keywords: +; Positive results, W+; Weak positive, ++/+++; strong positive, - ; negative

Different intermediates have different mobility rates during the development of TLC plate. So different R_f values were obtained for different intermediates as mentioned in table 8.

Table 8. R_f Values of Different Components (chlorpyrifos) in TLC

S. No.	Components	R_f values
1.	X_1	0.55
2.	X_2	0.64
3.	X_3	0.64
4.	X_4	0.64
5.	X_5	0.64
6.	X_6	0.67

4. Discussion

It has been predicted that between 1995 and 2020 the food grain demand is likely to be doubled; for vegetables more than 2.5 times and for fruits 5 times. Consequently, the increase in the consumption of pesticides is likely to be at least 2 to 3 times more in the years to come. Extensive use of pesticides is inevitable since they provide a sure cover to the farmer to protect his investment in seeds, fertilizers, irrigation and his own hard labor from the insects and pests. Environmental pollution due to pesticide residues, therefore, will continue and strategies like biodegradation and bioremediation will have to be followed (19).A large number of microorganisms which can degrade organophosphorus compounds by mineralization have been characterized and isolated. Most of these microbes have the ability to work in the natural environment but some modifications can be brought about to encourage the organisms to degrade the pesticide at a faster rate in a limited time span. This capability of microbe is sometimes utilized as the technology for removal of contaminant from actual site. Breaking down of toxic pesticides into nontoxic compounds and in som case, breakdown into the original elements from which they derived is described as pesticide degradation. Three types of pesticide degradation are microbial, chemical, and photo degradation. Degradation in soil is commonly carried out by microorganisms, mainly bacteria and fungi. Although, a lot of work has been reported on biodegradation, particularly of parathion and methyl parathion, meager data is available on the application of microbial cultures in bioremediation of soil contaminated with organophosphorous.

In this work, bacterial strains were isolated from different pesticides contaminated agricultural soils which have the ability to degrade pesticides. A well-known technique 'selective enrichment' was used to isolate the pesticide degrading bacteria (20). Six strains NR4, NR2, NW4, $NW3_O$, $NW3_G$ and $NW3_T$ were isolated from the pesticide contaminated sites and were found to be highly efficient in degrading the pesticide used in this study, that is, chlorpyriphos. These strains had the ability to utilize chlorpyriphos as their sole source of carbon and energy. These isolated pesticide degrading bacteria were maintained on minimal agar containing pesticide, so that their ability of pesticide degradation was not lost. A bacterial strain Bacillus sp., capable of degrading chlorpyrifos at concentration as high as 1g/l, was also reported in several studies**.** chlorpyrifos was also reported for the phenomena of accelerated biodegradation (21, 22). *Pseudomonas fluorescens* and *Serratia plymuthica* was reported for the degradation of chlorpyrifos at very high concentrations (23). In another report, Zhu et al (2010) isolated *Bacillus licheniformis* from soil that could degrade chlorpyrifos upto 100mg/kg in 14 days. Cycon et al., (2009) *Pseudomonas sp*. isolated from soil that could degrade diazinon (24). *Pseudomonas diminuta* was isolated as methyl parathion degrading species by Chaudhry et al., (1987). Similarly, Flavobacterium ATCC 27551 (25) and Sphingomonas Dsp-2 (26) can utilize chlorpyrifos and TCP as the sole source of energy and carbon. These organisms can also perform in mixed cultures for the degradation of a variety of organophosphates. As (27) reported that some organophosphorous insecticides serve as carbons sources for growth, such as chlorpyrifos, parathion, malathion, ethion, gusathion and diazinon are susceptible to microbial hydrolysis. In an earlier report, *Flavobacterium* as a source of carbon or phosphorus, was not used as organophosphorus pesticide (28). However, in this study, *Flavobacterium odoratum* was isolated as a bacterium that can use chlorpyrifos as a carbon source.

Strains were checked for multiple metal tolerance. Resistance against Cu^{2+} ($CuSO_4$), Cd^{2+} ($CdCl_2$), Cr^{6+} (K_2CrO_4) and Zn^{2+} ($ZnSO_4.7\ H_2O$) was checked. Strain NR2 and NR4 showed maximum resistance to copper at maximum metal concentration. NW3O and NW3T were sensitive to cadmium, whereas NR2 and NW4 were resistant upto 200µg/ml. Chromium resistance at a concentration of 200µg/ml was shown by NR2, $NW3_O$ and $NW3_T$. NR2, at the concentration of

300µg/ml, showed maximum tolerance against zinc.

Sensitivity and resistance frequency was calculated for bacterial strains at different concentrations of Erythromycin, Gentamycin, Chloremphenicol, Tetracycline and Ampicillin. NR2 showed weak positive growth on 50 µg/ml concentration of Erythromycin, Gentamycin and Chloremphenicol. Strain $NW3_T$ and NR4 were sensitive to all the antibiotics used. $NW3_O$ showed weak positive growth at 100µg/ml and maximum tolerance against 50µg/ml of Ampicillin. Biodegradation of chlorpyrifos was confirmed through TLC. Different intermediates with different R_f values were obtained after developing TLC plate. Similar R_f values were also reported for yeast strains which had the ability to degrade chlorpyrifos. The R_f values suggest that the degraded compound may be TCP, which is the most common compound formed when chlorpyrifos was degraded. Non-polar CP was determined as the spot that appeared on the TLC plate which showed the R_f value to 0.55; whereas, the other spot showed R_f value of approximately 0.66. Same results have been stated by Yun et al 2009 for lactic acid bacteria (LAB) that are capable of degrading chlorpyriphos during kimichi fermentation (29). Spots with R_f value approximately 0.66 indicate the presence of TCP, which is the most common degraded product of chlorpyriphos. As TCP is more polar than CP, so its R_f value is higher as compared to CP. On the whole, this study resulted in the isolation of efficient chlorpyrifos degrading strains with a wide range of pH and temperature tolerance that can utilize chlorpyrifos upto 700mg/L.

5. Conclusion

The curent study resulted in isolation of efficient chlorpyrifos degrading strains with a wide range of pH and temperature tolerance that can utilize chlorpyrifos upto 700mg/L during lab scale degradation tests (growth on chlorpyrifos supplemented minimal agar and broth).

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