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SCREENING AND CHARACTERIZATION OF CYPERMETHRIN DEGRADING BACTERIA FROM POLLUTED SAMPLES

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Abstract: Extensive use of chemicals around human environment, in soil and natural waters is a constant source of concern for public health authorities. Pesticide use is one of the biggest cause of chemical contamination in our environment. Bacteria have a great potential as a decomposer of biopolymers and synthetic chemicals in organic waste. In this study the role of bacteria in the degradation of widely used pyrethroid pesticide, cypermethrin is being reported. For this purpose the polluted soil and water samples were collected from different pesticide polluted areas of the Punjab. Eight bacterial strains were isolated on cypermethrin-containing M9 agar plates. On the basis of physical and chemical characterization, these isolates belong to genera *Bacillus, Sporosarcina, Pseudomonas, Marinococcus, Arthrobacter, Cupriavidus, Aeromonas* and *Micrococcus*. The optimum growth pH and temperature of the isolates in LB broth was ranged from 6-7 and 30-40°C respectively. In LB broth typical growth patterns of bacterial isolates were observed. These isolates were found sensitive to four metals *i.e.,* Hg^{2+}, Co^{2+}, Cr^{2+} and Cu^{2+}. The antibiotic sensitivity of isolates was also evaluated against nine antibiotics. All the isolates were resistant to ampicillin, furazolidone, and fusidic acid.

Key words: Insecticide degradation, pesticide bioremediation. Pyrethroid, microbial degradation, soil bacteria

INTRODUCTION

Pakistan has an agri-based economy and its major population obtained livelihood through agriculture or agriculture based industry. To meet the demands, agriculture production needs to be increased. One way to increase production is by reducing the crop and stored grains damage by controlling pest population with pesticides. These pesticides however, have been used indiscriminately resulting in severe environmental

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contamination. The pesticides belonging to organochlorine, organophosphates and pyrethroids are freely available in Pakistan. Due to long half life and stability in nature these poisonous chemicals are likely to persist in the environment and gain entry into food chain and cause serious health hazards (Ellis *et al.*, 2001; Chaudhry *et al.*, 2002; Yan *et al*., 2007). To get rid of their toxic effects, these compounds must be degraded and removed from food chains and environement.

Cypermethrin is a synthetic pyrethroid that is persistent in soil and sediment. In addition to target, this compound is extremly toxic to aquatic organisms. Freshwater fish *(Channa punctatus)* exposed to cypermethrin has low levels of red blood cells and proteins (Saxena *et al*., 2002). Amphibian fauna is also declined due to these synthetic pyrethroids because they cause many developmental abnormalities and growth inhibition. Cypermethrin interfere with voltage dependent sodium channel and ATPase in neuronal membranes. It also binds to nuclear-DNA, causing its destabilization and unwinding (Patel *et al.,* 2006).

Bacteria have a potential to degrade the insecticide by utilizing them as energy source for growth and reproduction. These organisms have evolved many mechanisms including catabolic pathways and a system of enzymes like phosphotriesterases, hydrolases and organophosphoric acid anhydrolases. They all work via the hydrolysis of the R3 phosphoester bond (Ellis *et al.*, 2001; Chaudhry *et al.*, 2002; Yan *et al*., 2007; Scott *et al.*, 2008; Gao *et al*., 2010).These organs convert the pesticide into natural products and make them eco-friendly. Now a days, contaminated sites are supplemented with inorganic nutrients and indigenous bacteria that degrade the toxicant (Grant *et al*., 2002; Sogorb and Vilanova, 2002; Murugesan *et al*., 2010; Ortiz-Hernández and Sánchez, 2010).

During oil spill bioremediation in marine environments, bacteria play very important role to degrade hydrocarbons (Cappello *et al*., 2007). Bacteria have potential to live in extremely cold and hot places by degrading various environmental contaminants. Bacteria are also isolated even from the volcano island of Santorini and it was found that that they have ability to degrade crude oil and used it as carbon source (Pollmann *et al*., 2006).

The present study was conducted with an aim to isolate cypermethrin degrading bacteria from local environment and evaluates

their characteristics for the purpose of bioremediation of insecticide contaminated soil and waste water.

MATERIALS AND METHODS

Sample Collection

The soil samples were collected from agricultural lands of Lahore, Faisalabad, Sahiwal, Raheem Yar Khan, Sargodha and Gujranwala, having known history of pesticides use since at least last five years. The samples were collected in sterilized screw capped glass bottles and brought to laboratory for analysis. The physical state, location, temperature and code number of these isolates are given in Table I.

Selection of media and insecticide used

Three types of media *i.e.,* L.B agar, M9 with glucose and M9 with insecticide (instead of glucose) were used. Different concentrations of technical grade cypermethrin (a pyrethroid insecticide) was used for screening of bacterial isolates. The media pH was adjusted 7.2-7.4, sterilized in autoclave at 15lb pressure for 20 minutes. The solutions like metals, sugars, ethidium bromide were sterilized through Bacto-filter (pore size 0.45µm) in already sterilized screw capped bottles.

Isolation and preservation of cypermethrin resistant bacteria

Soil samples (1g each) were suspended in 9 ml of distilled water and kept at room temperature. After 24 hours, 50µl of the supernatant was spread on M9+insecticide medium for the isolation of insecticide resistant bacteria. The plates were incubated at 37°C for 24 hours. The isolates that grew on M9+insecticide medium were considered resistant. To determine the minimum inhibitory concentration (MIC), the isolated colonies were streaked on M9 medium plates with different concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0%) of cypermethrin till the isolates failed to grow even after seven days of incubation. These insecticide degrading bacteria was purified and preserved as glycerol stock for further analysis.

Table I: Sample collection sites, their physical state and other characteristics

Sr. No.	Collection site	Polluted sample type	Sample temperature	Sample pH	Isolates code
1	Manawala, Faisalabad	Soil	30°C	6.9	BCTL-178
2	Bilalpur, Gujranwala	Soil	30°C	7	BCTL-179
3	Phatak VegetableMandi, Lahore	Rotten vegetables	30°C	7	BCTL-180
4	Ravi, Lahore	Soil	27°C	7	BCTL-181
5	Chak 48, Sargodha	Soil	35°C	6.9	BCTL-182
6	Dodhai, Rahimyarkhan	Soil	35°C	7	BCTL-183
7	Chandraey, Lahore	Soil	30°C	7	BCTL-184
8	Farooqa, Sahiwal	Soil	32°C	7	BCTL-185

Physical and biochemical characterization of isolates

Physical and biochemical characterization was performed on the basis of colony morphology, motility, Gram staining, oxidase, catalase, coagulase, urase, sporulation, methyl red, Voges-Proskauer and indole tests. Tyrosine decomposition, starch hydrolysis, citrate utilization and nitrate reduction analysis was also performed to characterize them.

Growth at different temperatures and pH

To study the optimum temperature, bacterial isolates were streaked on LB agar plates and incubated overnight at 5, 10, 45 and 65°C for determination of optimum temperature. For optimum pH determination, isolated colonies of bacteria were also streaked on LB agar plates,

containing media with pH at 4.5, 5.9, 6.1 and 9.6. The isolates were incubated at their respective optimum temperatures.

Acid release from sugars (glucose, lactose, xylose)

This test is used to differentiate organisms that ferment a particular sugar; consequently acid and or gas may be produced. The basal medium was prepared and added to different test tubes, 5ml each. A pair of sterilized test tubes was used for one isolate (for each sugar), one for oxidation and gas production and other for fermentation. 250µl of 10% (w/v) filter-sterilized sugar (glucose, lactose, manitol, arabinose and xylose) was added into basal medium. The bacterial isolate (50µl of log phase culture in peptone water) was inoculated at the bottom of the test tube.

The inoculated medium of one tube was covered with 10mm deep layer of sterile liquid paraffin for excluding oxygen to check the fermenting ability of the isolate for a particular sugar. The gas production is revealed by the formation of a void in the inverted (dipped) vial of the Durham tube. The test tubes were incubated at 37°C overnight and upto one week for the confirmation of test result (Benson, 1994).

Heavy metal resistance

Small sterilized filter paper discs (9mm diameter) were loaded with 1% solution of copper, choromium, mercury and cobalt salts. They were allowed to dry and then placed on the surface of the inoculated nutrient agar. After incubation at 37°C for 24 hours the plates were observed for any growth around the discs.

Antibiotic resistance of the isolates

The isolates were spread on LB-agar plates separately. Antibiotic sensitivity discs of nine antibiotics *i.e*. clarithromycin, ampicillin, spectinomycin, nitroforaution, erythromycin, kanamycin, fusidic acid, furazolidone, and vanacomycin, were placed on these plates and incubated overnight. The plates were checked for any growth around antibiotic disc. The zone of inhibition was also measured for each disc and interpreted according to BBL and Difco chart (Benson, 1994).

RESULTS

Insecticide degrading bacteria

The soil samples were collected from ten different areas of the Punjab province. Eight bacterial isolates were found resistant to cypermethrin. The maximum numbers of colonies in the cypermethrin treated plates were five in the isolates namely BCTL-180, 184, and 185, whereas the minimum colony was observed in isolate BCTL-178 (Table II).

Physical, morphological and biochemical characterization of bacterial isolates

Physical and morphological characterization was performed to identify the isolates. Out of eight isolates BCTL-178,179, 181, 182 and 185 were found to be gram positive while others were gram-negative. Four bacterial isolates were cocci and four (BCTL-178, 180,182, and 183) were bacilli. BCTL-184 was of orange and BCTL-185 was of pink color while all other were of creamy color (Table III). Different biochemical tests were performed to characterized the bacterial isolates (Table IV).

Antibiotic sensitivity test

Antibiotic sensitivity test showed that all isolates were resistant to all antibiotics. In comparison to antibiotic resistance these isolates were more resistant against fusidic acid, spectinomycin, furazolidone and ampicillin than clarithromycin, erythromycin and vanacomycin. In case of clarithromycin, erythromycin and vanacomycin clear zone of inhibition showed that these isolates were least resistant to these antibiotics as shown in Fig.2.

Heavy metal sensitivity test

All isolates showed clear zone of inhibition around the heavy metal strips but large clear areas were present around Hg^{+2} and Cr^{+3}. This showed that all the isolates were sensitive to heavy metals' especially to Hg^{+2} and Cr^{+3} (Fig. 3).

Effect of different temperatures and pH on growth

As from the optimum pH is concerned, all bacterial isolates collected from different polluted samples were mesophiles because they all had a temperature between 30-37°C (Fig. 4). All the collected isolates showed good growth between 7-8 pH and are considered alkaliphilic (Fig. 5).

Table II: Screening of cypermethrin (100mg/l) degrading bacteria from the contaminated soil and vegetable samples.

Sr. #	Code of isolate	No. of colonies in M9+glucose	No. of colonies in M9+cypermethrin
1	BCTL-178	50	1
2	BCTL-179	30	2
3	BCTL-180	20	5
4	BCTL-181	25	2
5	BCTL-182	60	4
6	BCTL-183	69	2
7	BCTL-184	70	5
8	BCTL-185	50	5

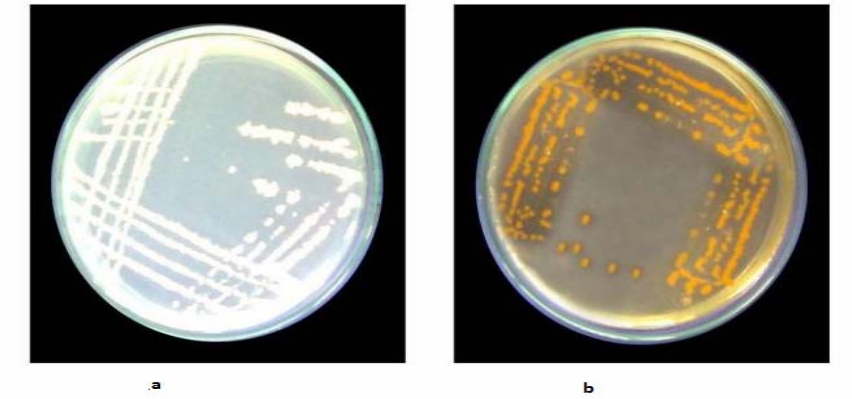

Figure 1: Bacterial strain BCTL-183 (a) and BCTL-184 (b) streaked on LB agar plate.

Table III: Colony morphology of cypermethrin resistant isolates collected from various polluted samples

Sr. No.	Strain Code	Colony Color	Colony Margin	Colony Elevation	Colony configuration	Motality	Gram staining	Isolates
1	BCTL -178	Off white	Regular	Concave	Round	+ve	+ve Bacilli	*Bacillus pumilus*
2	BCTL -179	Off white	Slightly wavy	Flate	Irregular	+ve	+ve Cocci	*Sporosarcina ureae*
3	BCTL -180	Off white	Regular smooth	concave	Round	+ve	-ve Bacilli	*Pseudomonas aeruginose*
4	BCTL -181	Off white	Regular smooth	Concave shiny	Round	+ve	+ve Cocci	*Marinococcus halophilus*
5	BCTL -182	Off white	Irregular wavy	Concave shiny	Irregular	+ve	+ve Bacilli	*Arthrobacter sp.*
6	BCTL -183	Off white	Irregular wavy	Concave	Irregular	+ve	-ve Bacilli	*Cupriavidus necator*
7	BCTL -184	Orange	Regular smooth	Concave from above	Round	+ve	-ve Cocci	*Aeromonas eurenophila*
8	BCTL -185	Pink	Regular Smooth	Flate	Round	+ve	+ve Cocci	*Micrococcus roseus*

Table IV: Biochemical characterization of cypermethrin resistant bacterial isolates.

Sr. No	Biochemical Test	Bacterial isolates							
		178	179	180	181	182	183	184	185
1	Oxidase	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
2	Catalase	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
3	Starch Hydrolysis	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve
4	Ureaset	-ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve
5	Tyrosine Decomposition	-ve	-ve	-ve	-ve	+ve	+ve	-ve	-ve
6	Sporulation	+ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve
7	Voges Boskaner	+ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve
8	Indole	-ve	+ve	-ve	+ve	-ve	-ve	+ve	-ve
9	Methyle Red	+ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve
10	Nitrate	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve
11	Citrate	+ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve

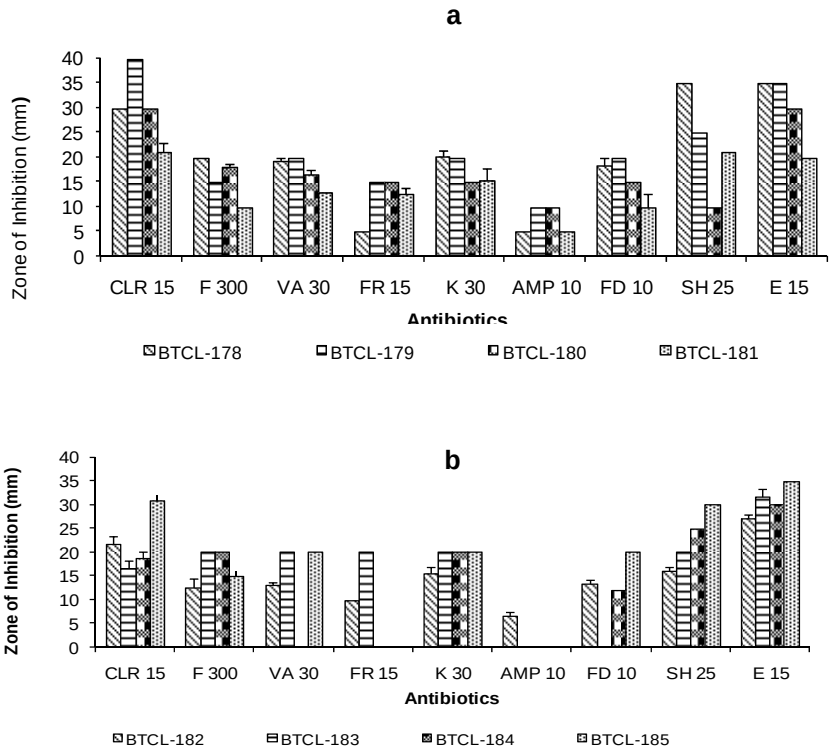

Figure 2: Effect of various antibiotics on the growth of cypermethrin-resistant isolates.

Abbreviations used: CLR, Clarithromycin; F, Nitroforaution; VA, Vanacomycin; FR, Furazolidone; K, Kanamycin; AMP, Ampicillin; FD, Fusidic acid; SH, Spectinomycin; E, Erythromycin.

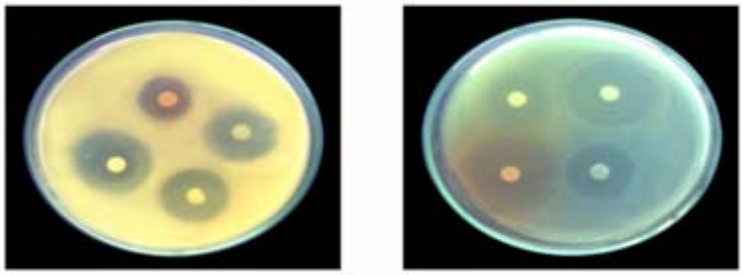

Figure 3: Heavy metal resistance test of cypermethrin resistant bacterial Isolates BCTL-179 (a) and BCTL-181 (b) are showing the zone of inhibition against Hg^{+2}, Co^{+2}, Cr^{+2} and Cu^{+2} salts.

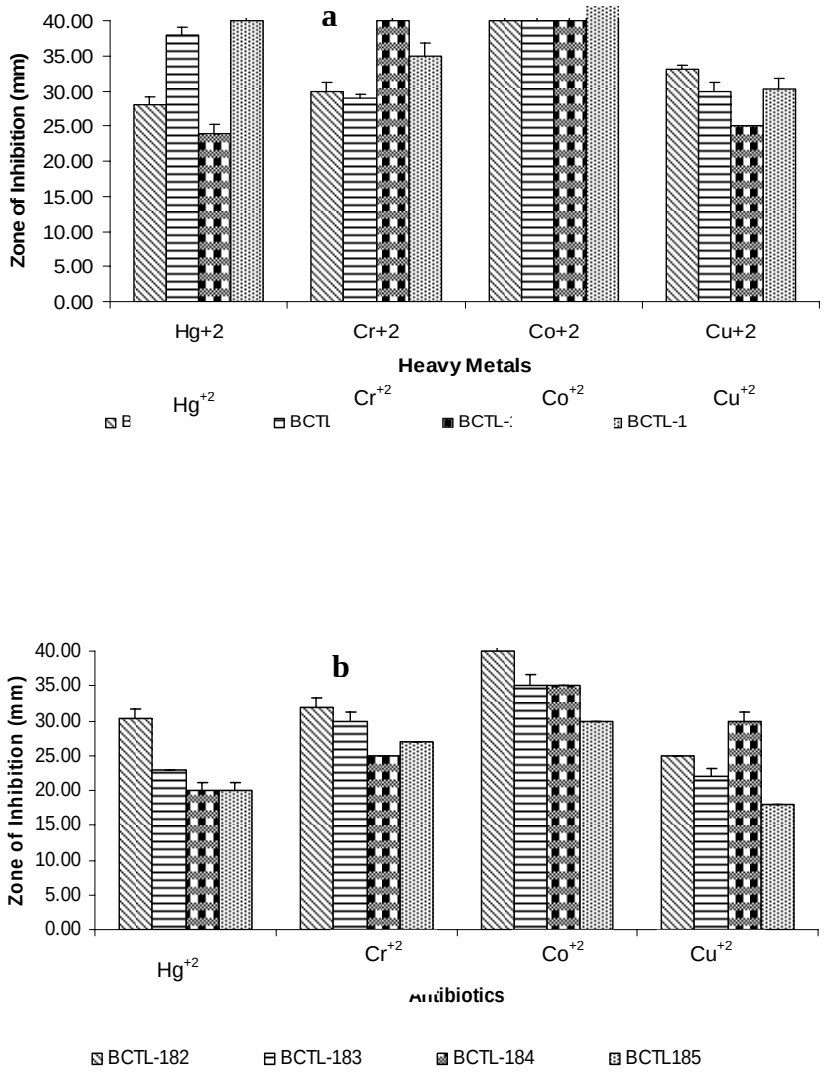

Figure 4: Effects of various heavy metals (Hg^{2+}, CO^{2+}, Cr^{2+}, Cu^{2+}) salts on the growth of cypermethrin resistant isolates

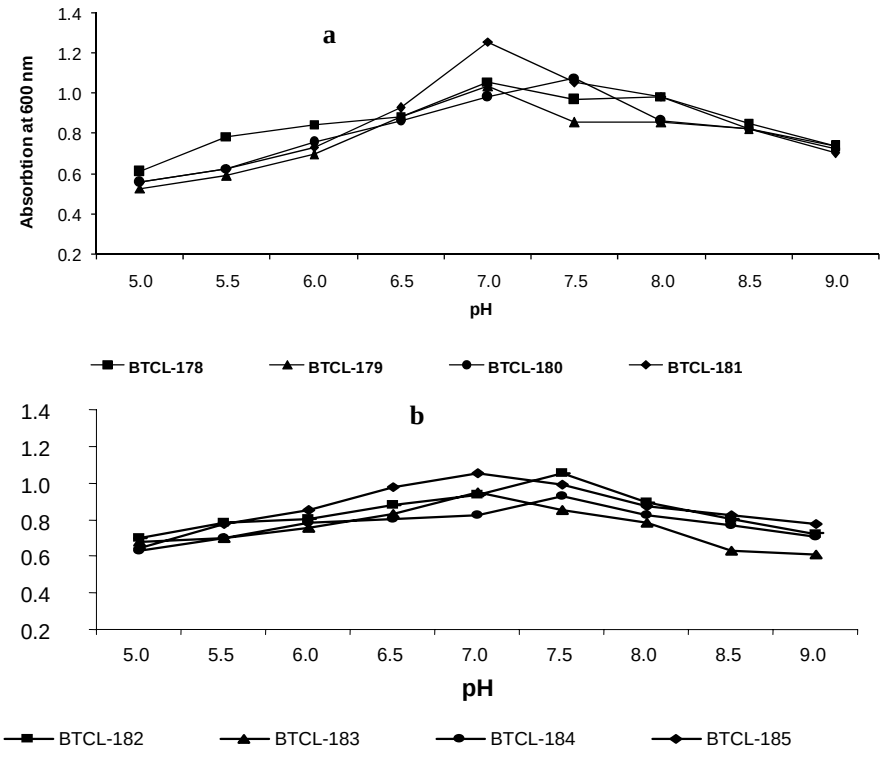

Figure 5: Effect of various pH on the growth of cypermethrin resistance isolates

DISCUSSION

Interest in the microbial biodegradation of pollutants has intensified in recent years as humanity strives to find sustainable ways to clean up contaminated environments. The present study describes the screening, purification, identification and characterization of the bacterial capable of degrading a pyrethroid insecticide cypermethrin. Isolation of bacteria was from polluted soil samples. The degradation of pesticides by soil-borne microorganism was earlier demonstrated by Audus (1949). Abundant reports correlated degradation of a wide range of pesticides with microbial activity and the ability of numerous species of actinomycetes and fungi to

degrade one or more pesticides (Kaufman and Blake, 1973). In many instances the ability of microorganism to use pesticides as their sole energy source were demonstrating using soil enrichment techniques (Audus, 1964). It has been recognized that major pesticide degradation in natural environment is microbial (Felsot, 1989).

In the present study, eight bacterial strains were isolated having the ability to degrade cypermethrin for use as energy source. After morphological and biochemical analysis they were identified as *Bacillus pumilus, Sporosarcina ureae, Pseudomonas aeruginose, Marinococcus halophilus, Arthrobacter sp., Cupriavidus necator, Aeromonas eurenophila* and *Micrococcus roseus.* Several studies from other laboratories have reported isolation of such microorganism capable of utilizing pollutants as source of carbon or energy. Biodegradation of pesticides by using variety of bacteria have been reported from different parts of the world (Hemmingson, 1993; Suett *et al.*, 1994; Dick *et al.,* 1996; Thomas *et al*., 1996; Thompson *et al*., 1998; Poelarends *et al*., 2000; Park *et al*., 2002; Sutherland *et al*., 2002; Coleman and Spaith, 2003; Aklen *et al.*, 2004; Nicholson and Fathepure, 2004). Chaudhry (1988) reported that biodegradation during treatment is greatly affected by low solubility of compounds in an aqueous system. Cypermethrin is co-metabolized by bacteria in soil. Two soil-bacteria *Pseudomonas intragenic* and *Serratia plymuthica* have been reported, which show the ability able to degrade cypermethrin (Grant *et al.,* 2001). A synthetic pyrethroid is often a mixture of different isomers and bacteria successfully degrade some enantiomers over others (Liu *et al*., 2006).

In present work the isolates collected from different contaminated samples were allowed to grow on pesticide containing agar plates for 72 hours. This phenomenon is termed as adaptation which may occur by Induction of specific enzyme, genetic changes resulting in new metabolic capabilities and selective enrichment of organism capable of transforming compound of interest (Ashok and Saxena, 1995). Pyrethroids metabolizing enzymes have been isolated which are responsible for biodegradation of cypermethrin or other pyrethroids, such as permethrinase from *Bacillus cereus* SM3 (Maloney *et al.,* 1993). These enzymes are considered as first steps in catabolic pathways, and the hydrolysis products of these enzymes are further catabolized. In the present study out of eight isolate one was identified as *Bacillus pumilus* which is capable of utilizing the toxic

xenobiotics including chlorinated pesticide (Wallnofer, 1969; Jagnow and Haider, 1972; Martens, 1976). It is concluded from this study that these pesticides degrading bacteria isolated during this study have a strong potential to be used in any future bioremediation procedure in nature.

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