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# BIOREMEDIATION OF AN INDUSTRIAL EFFLUENT CONTAINING CYPERMETHRIN

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## ABSTRACT

Cypermethrin is a pyrethroid insecticide mainly used in Pakistan to increase cotton crop production. Being highly insoluble and toxic to aquatic organisms, its treatment need especial attention. In this study malathion degrading bacterial isolate designated as IES-*Ps*-1 was used to assess its biodegradation potential for Cypermethrin. The data indicate that bioaugmented conventional activated sludge system able to treat Cypermethrin contaminated wastewater and a complete removal at 20 mg/L dose was observed after 48 hours. However, further loading of organic compound (40, 80 & 120 mg/L) correspondingly decreased the removal efficiency. But under mechanical agitation using 8-9 mg/L dissolved oxygen in a wide range of pH (6.8-8.8) at 28-30 °C temperature, >85% removal efficiency was achieved even at high organic load (80 mg/L). Experimental findings proved the potential of this bacterium to be used in bioremediation of pesticide contaminated effluent. Such study would be valuable to scientist and engineers who are trying to develop methods for the clean up of contaminated soils and water.

**Keywords**: Cypermethrin, biodegradation, activated sludge, wastewater.

## INTRODUCTION

In Pakistan, Cypermethrin is mainly used for cotton crop protection and for forestry and public health management. The very low water solubility of Cypermethrin and relatively high lipoaffinity indicate a strong bioconcentration potential in aquatic organism when available as solute (Sapiets *et al*., 1984; Kollman and Segawa, 1995). Cypermethrin actually acts on the nervous system and is toxic to bees, other beneficial insects, earthworms, fish and shrimps (Stepheson, 1982).

At present, besides pesticide contamination from agricultural field, the agricultural industries are also contributing relatively high quantities of toxic pesticides into the environment, since most of them have either no treatment facilities or have grossly inadequate arrangement. The Karachi coastal region has become the dumping ground of hazardous waste, receiving huge quantity of untreated domestic, industrial and agricultural wastes. An effective pesticide waste treatment technology is therefore needed to prevent water pollution and to comply with increasing regulatory pressures.

Recently, the bioremediation has been proven to be a suitable method for the treatment of polluted aquifers containing hazardous waste that could be implemented either in situ or off-site in specially designed reactors or wastewater treatment plants (Bharati *et al.,* 2002; Kao *et al*., 2004; Quan *et al.,* 2004; Chen *et al*., 2005). Moreover, in most cases, it has been found the most cost-effective and environmentally friendly treatment method. The purpose of present study is to identify potential microorganism able to biodegrade pesticide in an aquatic environment using biological treatment system. Such studies would improve design and operation of biomechanical treatment system used for the degradation of toxic compounds like pesticides which are resistant otherwise to conventional treatment.

## MATERIALS AND METHOD

### Pesticide, medium and culture used

The pesticide used in this study belong to the class pyrethroid and is commercially available as Cypermethrin. Due to low water solubility, stock aqueous solution of Cypermethrin (1mg/ml) was prepared in sterile HPLC grade methanol (Merck).

Nutrient broth media having composition of Beef extract 3.0gm and Pancreatic digest of gelatin 5gm, whereas nutrient agar media containing additional 20.0gm Agar, were prepared according to the manufacturer's instruction (8 gm in 1000 ml purified water, pH 7.2 and autoclaved at 121°C, 15 psi for 30 minutes). The medium was used for growth and biodegradation studies.

The bacterial culture (IES-*Ps*-1) capable of degrading malathion was isolated by Hashmi (2001) from agricultural soil using enrichment technique and was used in present study. Cypermethrin degrading culture was obtained through acclimating IES-*Ps*-1 strain with gradually increased concentration of Cypermethrin from 10 to

120 mg/L in nutrient medium. Adapted IES-*Ps*-1 was stored at 4°C on slopes of nutrient agar containing 0.1 mg/L Cypermethrin and subcultured after every three months.
When a new batch of test was performed at different temperature using varying dose of Cypermethrin, the stock culture was first subcultured into 10 ml nutrient broth, aerobically grown and subsequently utilized for characterization, growth and biodegradation studies.

**Characterization and growth of IES-*Ps*-1**

Characterizations of IES-*Ps*-1 was performed using morphological, cultural and biochemical tests using methods described by Colins and Lyne (1985) up to the stage of genus. Growth of IES-*Ps*-1 in biosimulator was determined by viable cell enumeration immediately after inocculation and at 24, 48, 72, 96 h later. Miles and Misra technique (938) was used for bacterial growth study.

**Cypermethrin degradation studies using biosimulator (activated sludge)**

The compact bench scale biosimulator (Model MF-114) consists of a stainless steel reactor with a heavy wall glass jar of borosilicate glass equipped for monitoring and controlling rate of agitation and aeration was used.

The effect of temperature (ambient temperature, 30ºC and 38ºC), on the performance of IES-*Ps*-1 for Cypermethrin (80 mg/L) degradation was evaluated. Approximately 8.5 liters wastewater sample, inoculated with 350 ml culture and an appropriate quantity of Cypermethrin was transferred into the biosimulator. The sample was strongly agitated by impeller with flat stirring paddles and by four vertical baffles. The required temperature was maintained by the built in thermostat and the DO concentration of 8-9 mg/L was achieved by mechanical aeration regulated through continuous agitation.

**Analytical procedure**

The sample from biosimulator was withdrawn at timed intervals of 8, 24, 32, 48 hours and analyzed for pH, temperature, dissolved oxygen and COD as per standard procedure laid down in APHA (1998).

**Extraction of Cypermethrin for HPLC analysis**

Samples were collected from biosimulator as per schedule and were extracted two times with n-hexane (75 ml and 50 ml) by vigorous shaking for 15-20 minutes in a separatory funnel. The hexane layer was separated and evaporated to dryness at 70 °C using vacuum rotary evaporator (BUCHI Rotavapor R- 200/205). The dried residue was then dissolved in 10 ml HPLC grade methanol. After gently vortexing and filtering through a 0.2 μm membrane filter, an aliquot of 20 μL, was used for HPLC analysis. Each sample was injected 3 times and the mean was calculated.

**High Pressure Liquid Chromatography (HPLC)**

HPLC (Shimadzu, Japan) chromatographic system consisted of a solvent delivery pump LC-10 AS, connected with an autoinjector model SIL-6A and a rheodyne injection valve fitted with a sample loop (20 μl). The chromatographic separation was achieved on a reverse phase $C_{18}$ column with a guard column and monitored by UV-detector (visible spectrophotometer detector SPD-10A) set at 220 nm. The output of the detector was connected to a chromatopack (CR6A). The mobile phase consisted of methanol (Merck HPLC grade) since Cypermethrin is miscible in alcohol. The filtered methanol was degassed prior to use by sonication. The flow rate was adjusted at 2 ml/minute with total elution time of 10 minutes for each run. The column was flushed with deionized distilled water and methanol whenever required for removing impurities and was allowed to equilibrate between runs.

## RESULTS AND DISCUSSION

**Characterization and adaptation of bacterial isolate**

On the basis of morphological, cultural and biochemical characteristics, the bacterial isolate was identified as a member of the genus *Pseudomonas* according to "Bergey's Manual of Systematic Bacteriology" (Palleroni, 1986). Characterization studies of the isolate from experimental results, as well as of those by other researchers, indicate that bacteria belonging to the genus *Pseudomonas* are gram-negative, rod-shaped, highly oxidative and metabolically versatile, able to degrade aromatic hydrocarbons, oil, petroleum products and pesticides (Maloney *et al*., 1988; Ramos *et al*., 1995; Lee *et al*., 1998; Ramanathan and Lailithakumari 1999; Martin *et al*., 2000).

During adaptation, it was observed that in the presence of high concentration of Cypermethrin, the bacterial growth slowed down due to stress. Further, the bacteria changed its normal rod-shaped morphology to that of a coccus. However, this change was temporary, because the cells recovered the original rod form after a few days.

**Bacterial growth in biosimulator**

The results as shown in Figure 1 and 2, clearly indicate that Cypermethrin had pronounced effect in promoting better growth of IES-*Ps*-1. As in presence of Cypermethrin, the bacteria grow fast and a higher number of cells were observed when compared with the control (without Cypermethrin). The maximum count at 24 hours with 40mg/L Cypermethrin was 13±1.73x$10^7$ CFU/ml and with 80mg/L, it was 19±2.65x$10^7$ CFU/ml respectively. However, the generation time at these concentrations (40 and 80mg/L) were noted to be 57 and 53 minutes. On the other hand in the control experiments, the cell count at 24 hours was relatively low (7±1.73 x$10^7$ CFU/ml) with marked increase in generation time (98 minutes).

It was further noted that the growth at 40mg/L Cypermethrin dose significantly increased after 48 hours incubation. But the growth at 80mg/L dose was slightly less but continued to grow till 96 hours incubation and a count of 7x$10^7$ CFU/ml was observed. This may be due to availability of nutrients and favourable environmental conditions in biosimulator which allow the cells to survive till 96 hours. In contrast, the population density in control experiment (no pesticide) was comparatively less (0.1x$10^7$ CFU/ml). This may be because of the presence of limited concentration of nutrient in wastewater sample (no Cypermethrin), which does not allow the cells to grow to higher numbers.

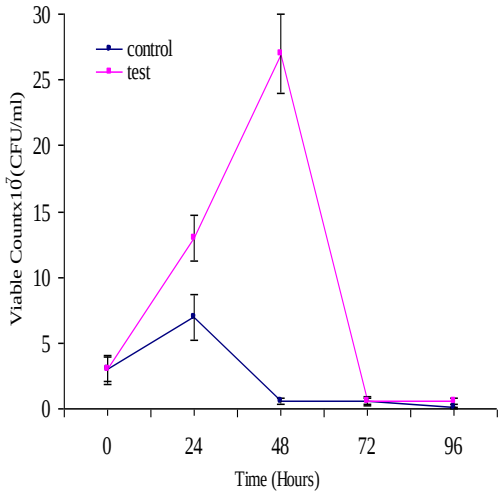


**Fig. 1 Total cell count of bacteria in biosimulator containing 40 mg/L Cypermethrin**

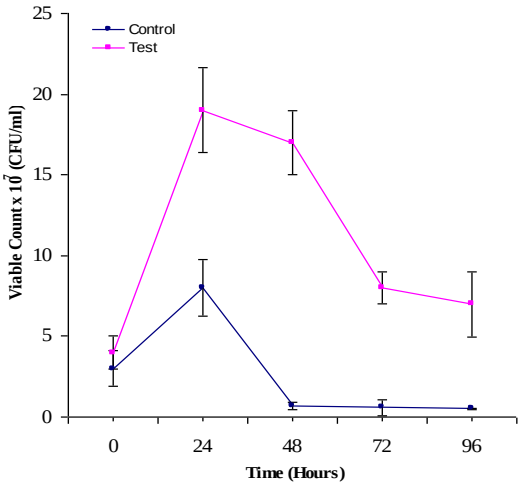


**Fig. 2 Total cell count of bacteria in biosimulator containing 80 mg/L Cypermethrin**

Since 78-88% degradation of Cypermethrin observed after 48 hours of aerobic treatment in biosimulator, it appears that biodegradation actually occurred by the acclimated culture of IES-*Ps*-1 in wastewater samples. The bacterial cells in log phase during the period of biodegradation indicate that substrate conversion would be at its maximum as also described by Gray (1989a,b) and similarly observed in this study.

**Influence of physicochemical conditions on biodegradation performance at different temperature**

Temperature is one of the important environmental parameters, that can influence the microbial growth as well as the treatment efficiency. The present study was conducted to observe the effect of temperature on COD removal and Cypermethrin degradation rate.

**pH:** The pH fluctuations during biodegradation of Cypermethrin at different temperature indicate that IES-*Ps*-1 can retain their degradation ability in a wide range of pH (7.27- 8.00) with optimum degradation observed at 30°C temperature where the pH of water sample was found within the neutral range after 48 hours treatment. Similar data were obtained by Mendelbaum *et al*., (1995) and Karpouz and Walker (2000), who found pesticide degradation by *Pseudomonas* strain in the pH range of 5.5 to 8.5 with low degradation below optimum pH.

The present work has shown that although bacterial growth and degradation occurred at pH 8 for the temperature investigated (ambient temperature ranged from 18 to 25°C and 38°C), but the degradation rates were low which might be due to decreased metabolic activity of microorganisms at these temperatures. In contrast, the results presented in Table 1 showing greater degradation efficiency at 30°C temperature with pH around 7.33. These

data suggested that optimum temperature and pH for the growth of IES-*Ps*-1 may be near 30°C with pH around neutral. Similar findings were also reported by Ashok and Singh (1988), who found that isolated *Pseudomonas* strain able to grow between pH 5.5 to 9.5 with optimum growth at pH around 7.0. Moreover, the optimum pH for most biological treatment of wastes in the aeration tank is reported around 7 to 7.5 (Metcalf and Eddy, 1991). The same was also observed in this study.

**Dissolved oxygen:** The dissolved Oxygen concentration during biodegradation of Cypermethrin at different temperature was maintained at 8-9mg/L using mechanical aeration in biosimulator (activated sludge process). During experiment, it was noted that the initial dissolved oxygen concentration in the wastewater sample ranged between 1.5-2.3mg/L. However, using mechanical aeration in the reactor not only sufficient aerobic conditions was maintained but it also dispersed (mixed) Cypermethrin in wastewater, such that bacterial growth and COD removal rates were greatly enhanced. It was also noticed that biodegradation performance significantly improved in the temperature ranged of 28°C-30°C at 8-9 mg/L using mechanical aeration (250 rpm).

**Chemical oxygen demand:** To determine the dependence of temperature on biodegradation rates, COD analysis were carried out keeping Cypermethrin concentration (80 mg/L) constant, the maximum concentration that supported IES-*Ps*-1 growth in biosimulator. At ambient temperature the COD after 48 hours of treatment was 4500mg/L (54% removal), whereas, at 30°C, COD decreased from 8167mg/L to 867mg/l (89% removal) and at 38°C COD diminished from 8333mg/L to 4000mg/L (52% removal) after 48 hours respectively. During the experiment, it was also observed that COD gradually decreased with time and the removal efficiency at ambient temperature and 38°C was almost same after 48 hours of aerobic treatment. In contrast at 30°C the removal efficiency significantly improved (89%). The overall data presented in Table 1 indicate a good correlation between COD removal and Cypermethrin degradation rates analyzed by HPLC.

From these results it can be concluded that IES-*Ps*-1 can degrade relatively high concentration of toxic organic pollutants provided the biosimulator operating temperature maintained at optimum level. Previous work also demonstrated removal of several organic toxicants by activated and trickling filter processes (Hannah *et al*., 1988). But activated sludge process was found to be quite efficient in decreasing the concentration of many priority pollutants and other xenobiotics to concentration below detection limits (Grady, 1986 & 1990).

**Table 1. Comparative performance evaluation of Cypermethrin (80mg/L) degradation at different temperature after 48 hours using biosimulator.**

| Temperature (°C) | pH | COD Values | | HPLC data | |
|---|---|---|---|---|---|
| | | Conc. (mg/L) | % removal | Conc. (mg/L) | % degradation |
| Ambient Temp. (18-25) | 7.80 | 4500 | 54 | 44 | 51 |
| 28-30 | 7.33 | 867 | 89 | 69 | 88 |
| 38-40 | 7.50 | 4000 | 52 | 39 | 48 |

*Results based on mean of three replicates

**Effect of temperature on Cypermethrin degradation using biosimulator**

Experimental findings as shown in Table 1 indicate that optimum temperature for the growth and degradation of Cypermethrin by IES-*Ps*-1 culture remain between 28°C-30°C. Using similar concentration of Cypermethrin, it was noticed that as temperature of the reactor decreased or increased (38°C), degradation rates were significantly reduced. At ambient temperature Cypermethrin degradation was only 51% whereas at 38ºC, it was 48%. In contrast the IES-*Ps*-1 can grow well at temperature ranging between 28°C to 30°C and a marked increase in bacterial growth and Cypermethrin degradation rates were observed after 48 hours of aerobic treatment. Similar optimal temperature (28°C-30°C) for the growth of *Pseudomonas* in activated sludge process was reported by Schlegel (1969). These results are in accordance to our expectation, as most *Pseudomonas* species and all *Pseudomonas putida* strains are unable to grow at 42 °C and would unlikely to persist at this temperature (Palleroni, 1986).

Previous research work on biodegradation also demonstrated that microbial communities growing at high temperature bioreactors are less proficient in simultaneously utilizing multiple substrates compared to analogous mesophilic systems (LaPara *et al*., 2000) and have difficulty in maintaining membrane integrity under substrate depleted conditions (Konopka *et al*., 1999). Further reported that below optimum temperature, the metabolic activity of bacteria decreased because of reduced enzymes activities and a loss of fluidity of the cell membrane. These changes actually restricted the transport mechanism of substrate molecule and therefore the degradation rates (Irvine and Wilderer, 1988). Present findings have demonstrated good agreement with previous work on pesticide degradation by *Pseudomonas* strain in liquid culture systems and bioremediation studies in soil or natural water matrices. Karpouzas and Walker (2000), reported that Ethoprophos degradation by *Pseudomonas putida* strain was most rapid at temperature of 25 and 37°C. However, at 5°C, the strain was capable to degrade pesticide but at a slower rate. In contrast, the dissipation was markedly reduced when culture were incubated at 42°C. Similarly, Chaterjee *et al*., (1982) found the optimum temperature for degradation of 2,4,5-T in soil by *Pseudomonas cepacia* was 30°C, which was also the optimum temperature for its growth in liquid media.

Many studies have shown that unlike high temperature, low growth temperatures are usually not lethal. Instead, many cells have the ability to become dormant. In order to observe this effect, the treatment time of the experiments at ambient temperature (18-25°C) and 38°C was increased from 48 hours to 72 hours. Degradation was somewhat improved but low rate observed at ambient temperature and only 62% of Cypermethrin degradation was observed compared to 51% removal at 48 hours. In contrast at 38°C, no further Cypermethrin degradation by IES-*Ps*-1 strain was observed even after 72 hours of treatment (data not shown). These data explain that IES-*Ps*-1 is able to degrade Cypermethrin at ambient temperature and 38°C, but relatively at a slower rate with optimum degradation rates at 30ºC which is also the optimum temperature for the growth of *Pseudomonas* species.

It is worth mentioning here that previous work on Cypermethrin degradation, reported that increased concentration gradually decreased the biodegradation efficiency of IES-*Ps*-1 and a complete removal observed at 20mg/L dose (Jilani & Altaf, 2006). However, in the present study, it was noticed that using mechanical aeration and maintaining suitable environmental conditions like temperature, DO, rate of agitation/aeration in the reactor, IES-*Ps*-1 can effectively reduce higher concentration of Cypermethrin (80 mg/L) in short retention time of 48 hours.

## CONCLUSIONS

Following conclusions may be drawn from this study:

- Malathion degrading bacterial isolate, IES-*Ps*-1, can be used for biodegradation of pesticides wastes, as IES-*Ps*-1 showed potential to grow in the presence of Cypermethrin.
- In biosimulator, temperature was found to be the principal governing factor in removing Cypermethrin.
- Because of the low aqueous solubility of Cypermethrin, mechanical aeration in biosimulator proved to be very effective in reducing the concentration of Cypermethrin. As mechanical aeration not only provided the maximum dispersion of Cypermethrin in wastewater but also maintained the sufficient dissolved oxygen required for the growth of IES-*Ps*-1.
- Complete removal of Cypermethrin (20 mg/L) would only be possible if appropriate organism (IES-*Ps*-1) and optimum conditions (temperature, dissolved oxygen, mechanical aeration) be maintained in biosimulator.

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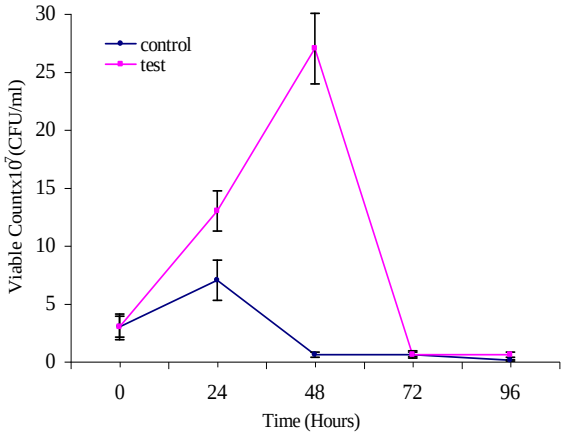


**Fig. 13 Total cell count of bacteria in biosimulator containing 40 mg/L Cypermethrin**

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