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Biological Treatment of Industrial Wastewater Using Biosimulator

[1]Seema Jilani and [2]M. Altaf Khan

[1]Department of Environmental Studies, Sindh Madressatul Islam University,
Aiwan-e-Tijarat Road, Karachi-74000, Pakistan
[2]Institute of Environmental Studies, University of Karachi, Karachi-75270, Pakistan

Abstract: In Pakistan, besides pesticides contamination from agricultural field, the agricultural industries are also contributing by relatively high quantities of toxic pesticides into the environment. In this study, a microbial isolate, *Pseudomonas*, designated as IES-*Ps*-1, was used to assess its potential for pesticide removal from industrial wastewater using the biosimulator (activated sludge). During experimental studies conducted in the flask as well as in biosimulator, it was observed that IES-*Ps*-1 grows normally at low concentration of added insecticides when compared with the control test (without pesticide). However, at high concentration the microbial count decreased but no death occurred and the culture remained in lag phase. In many cases, the growth of organisms in the presence of the particular substrate serves as an indication about its metabolic potential. However, to confirm these results, chemical oxygen demand (COD) and HPLC analysis was performed. Under aerobic culture conditions using mechanical aerators in biosimulator, almost complete removal of cypermethrin at 20mg/L dose occurred during 48 hours. The results finding indicated that IES-*Ps*-1 strain, can be used for the treatment of the pesticides contaminated environment.

Key words: Toxic Pesticides · Industrial Wastewater · Microbial Isolate · Biosimulator · COD · HPLC Analysis

INTRODUCTION

In Pakistan, insecticides, particularly cypermethrin is mainly used for cotton crop protection, for forestry and public health management. Because of low water solubility and relatively high lipoaffinity, its presence indicates a strong bioconcentration potential in aquatic organisms [1, 2]. It is reported that cypermethrin acts on the nervous system and is toxic to bees, other beneficial insects, earthworms, fish and shrimps [3].

In developing countries farmers are using high amount of fertilizers and pesticides but at the expense of environment and health. These pesticides may be toxic and mutagenic and they may be bioaccumulated or biomagnified by the biota [4, 5]. At present, besides pesticides contamination from agricultural field, the agricultural industries are also contributing by relatively high quantities of toxic pesticides into the environment and most of them have no treatment facilities or have a grossly inadequate arrangement. The Karachi coastal region has become the dumping ground of hazardous waste, receiving huge quantities of untreated industrial and agricultural wastes. Prevention of water pollution and protection of human health and ecosystem in the country needs, an appropriate wastewater treatment system; easy to operate and suitable for environmental conditions.

Among various treatment technologies, the bioremediation technology has been found to be very effective, environmental-friendly and economical for the treatment of hazardous waste [6- 8]. It is well known that specific bacterial culture is capable to degrade the hazardous organic compounds if provided the right environmental conditions for their growth and metabolism. Many researchers have tried to isolate and identify the microorganisms from soil or water and then examine their biodegradation capabilities [9-16].

It has also been observed that these isolated microorganisms perform their activity efficiently in the activated sludge system [17, 18]. Although, most synthetic organic compounds biodegrade easily, making

Corresponding Author: Seema Jilani, Department of Environmental Studies Sindh Madressatul Islam University Aiwan-e-Tijarat Road Karachi, Pakistan. Tel: +92 21 99217501-3, Fax: +92 21 99217504.

the biological treatment a technically feasible alternative for many environmental problems [19- 24]. However, in some cases, specific compounds have either resisted the biodegradation, or their degradation occurs very slowly thus make biological treatment ineffective. Therefore, it is essential for the biological treatment processes, to promote and maintain a microbial population that can metabolize the target wastes. The objective of present research study was to assess the growth and biodegradation potential of a bacterial isolate in the presence of pesticide using biosimulator (activated sludge system).

MATERIALS AND METHODS

Chemicals, Media and Bacterial Culture: Commercial grade organophosphate (Malathion, Methamidophos), carbamate (Cartap) and pyrethroid (Cypermethrin) pesticides were purchased from local markets and used in the present research study. The physical and chemical characteristics of these pesticides are listed in Table 1. Because of low aqueous solubility of pesticides, a stock solution of Malathion (5.7 mg/ml), Methamidophos (10 mg/ml) and Cypermethrin (1 mg/ml) were prepared in GR grade methanol (Merck). However, Cartap solution (9.7 mg/ml) was prepared in sterile distilled water.

The bacterial culture, Pseudomonas (IES-*Ps*-1) was available for this research study and was isolated by Hashmi [11] from agricultural soil using enrichment culture technique. However, cypermethrin adapted bacterial culture were obtained by acclimatization of IES-*Ps*-1 strain in a gradually increased concentration of cypermethrin (20 to 125 mg/L) in a nutrient medium. Adapted IES-*Ps*-1 was stored at 4°C on nutrient agar slopes containing 0.1 mg/L cypermethrin. When a new batch of test was performed with different dose of pesticides, the stock culture was first sub-cultured into 10 ml nutrient broth, aerobically grown and subsequently utilized for characterization, growth and biodegradation studies.

Characterization of IES-*Ps*-1 was performed using morphological, cultural and biochemical tests methods described by Colins and Daugulis [26] up to the stage of genus.

Growth Kinetics and Biodegradation Studies: For growth study, 2.5ml of 24 hours old culture prepared in nutrient broth was inoculated into a 25 ml nutrient broth flask and flasks containing different concentrations (ranged from 35-250 mg/L) of Malathion, Methamidophos, Cartap and Cypermethrin. Non shaken condition was used as it actually represents the environmental conditions where microorganisms are usually exposed. A control experiment without insecticide in nutrient broth was used for comparison. Samples from each flask were drawn at specific time intervals, tested and counted. The growth of IES-*Ps*-1 in biosimulator was determined by viable cell enumeration immediately after inoculation and at 24, 48, 72, 96 hr later. Miles and Misra technique [27] was used for bacterial growth study.

In order to determine the IES-*Ps*-1 capability for cypermethrin degradation, batch experiments were performed using the biosimulator (activated sludge) at different temperatures, dissolved oxygen and cypermethrin concentration. Approximately 350 ml bacterial culture inoculated into wastewater sample (8.5 liters) containing an appropriate quantity of cypermethrin was transferred into the borosilicate glass jar of a compact bench scale stainless steel biosimulator.

Table 1: Characteristic of Pesticides

Properties	Malathion	Methamidophos	Cartap	Cypermethrin
Molecular Formula	$C_{10}H_{19}O_6PS_2$	$C_2H_8PNO_2S$	$C_7H_{16}ClN_3O_2S_2$	$C_{22}H_{19}O_3NCl_2$
Molecular Weight	330.36 g/mol	141.12 g/mol	273.81 g/mol	416.3 g/mol
Appearance	Amber liquid	Crystalline solid with off white color	A white crystalline solid with slight odor	Pure isomers are colorless crystals Mixed isomers are viscous/viscous yellow liquid
Water Solubility	143 mg/L	90 g/L	Soluble in water	0.01 mg/L
Hydrolysis Half Life	In soil (24 hrs to 6 days) In water (15 days to 21 weeks)	3 days at pH 9 27 days at pH 7 309 days at pH 5	In moist soil 21 days - 12 weeks	> 50 days Aerobic 6-20 days Anaerobic <14 days

*Data reproduced from EPA's pesticide fact sheet database [25]

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 was achieved by diffused aeration using pressure pump and mechanical aeration regulated through continuous agitation.

Analytical Method: The water samples were analyzed for temperature, pH, dissolved oxygen and COD as per standard procedures [28]. However, for HPLC analysis, the water samples extracted in n-hexane reagent were allowed to dry at 70°C using a vacuum rotary evaporator. The dried residue was then dissolved in 10 ml methanol (GR grade) and filtered through a 0.2 µm membrane. An aliquot 20 µL, was taken from the organic phase and the quantification of cypermethrin was carried out using HPLC method described earlier by Jilani and Khan [29]. Each sample was analyzed 3 times to obtain mean value.

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). A guard column filled with µBondapak C_{18} analytical waters µBondapak reversed phase column, effluents were monitored by using UV-detector (visible spectrophotometer detector SPD-10A; wave length = 220 nm). The output of the detector was connected to a chromatopack (CR6A). 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 the runs.

Principal Component Analysis: In this study multivariate statistical process control such as Principal Component Analysis (PCA) methodology is applied to predict the real situation likely to control the biosimulator performance. The statistical software package used for statistical analysis was STATISTICA for Windows release 5. The data obtained was subjected to PCA, which is a variance oriented technique where the component score is directly derived by a linear transformation. The use of PCA permit an objective summarization of the variables in the data matrix by extracting a new set of variables called principal components. Generally the first three components account for high proportion of the total variance in the original data set. The data set consist of 36 observations and 10 variables related to biosimulator performance, including COD_{IN}-COD_{OUT}, COD_{OUT}/COD_{IN}, CYP_{IN}-CYP_{OUT}, CYP_{OUT}/CYP_{IN}, dissolved Oxygen, organic load, total cypermethrin concentration, temperature, pH and retention time. The relationships among and within these variables were determined by PCA using the program package developed by Orloci [30].

RESULTS AND DISCUSSION

Characterization and Adaptation of IES-PS-1: Bacterial characterization, based on the morphological, cultural and biochemical tests indicated that the IES-*Ps*-1 strain belongs to the genus *Pseudomonas* according to "Bergey's Manual of Determinative Bacteriology" [31]. Further, the experimental results of the present study, as well as by other researchers, indicate that bacteria belonging to the genus *Pseudomonas* have been able to degrade phenolic compounds [32] and other aromatic substances [11, 14, 33- 37].

During the period of cypermethrin adaptation, it was observed that the bacteria under go stressed as its growth consequently slowed down. Further, the microscopic observation showed that the bacteria change its normal rod-shaped morphology to coccus. However, this change was temporary, as the cells were recovered in the original rod form after a few days.

Bacterial Growth in Flask: The comparative growth response of IES-*Ps*-1 in the presence of different concentrations of Malathion, Methamidophos, Cartap and Cypermethrin as shown in Figure 1 revealed the degree of bacterial sensitivity or resistance and the amount of growth stimulation when exposed to different concentrations and types of pesticide. Comparison of the viable count data with an earlier study conducted on shaking water bath by Hashmi [11], indicated that the growth performance of IES-*Ps*-1 in control experiments with no pesticides was much better. The observed generation time was 45 minutes with specific growth rate of 0.0219 min^{-1}. Whereas, without shaking condition, used in the present study, the generation time increased to 69 minutes with specific growth rate of 0.014 min^{-1}. These results proved that as an IES-*Ps*-1 culture is aerobic, it performed better in a shaking water bath where a greater quantity of oxygen might enhance the oxidation capability of these organisms.

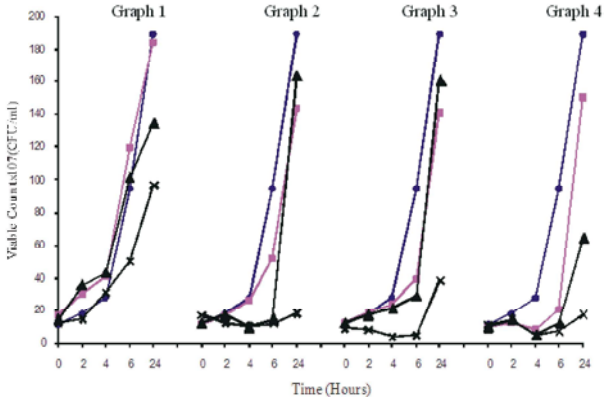

Fig. 1: Comparative growth response of IES-*Ps*-1 in nutrient broth and broth containing different concentration of pesticides

• = IES-*Ps*-1 growth in nutrient broth containing no pesticide (Control)

Graph 1 = IES-*Ps*-1 growth in the presence of Malathion (■ 35, ▲ 50 & ✖ 120 mg/L)

Graph 2 = IES-*Ps*-1 growth in the presence of Methamidophos (■ 60, ▲160 & ✖ 250 mg/L)

Graph 3 = IES-*Ps*-1 growth in the presence of Cartap (■ 60, ▲ 80 & r 120 mg/L)

Graph 4 = IES-*Ps*-1 growth in the presence of Cypermethrin (■ 40, ▲ 60 & ✖ 80 mg/L)

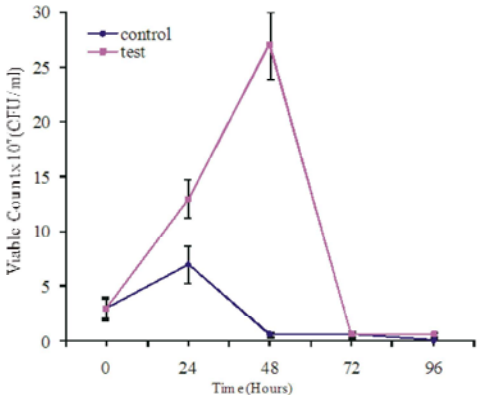

Fig. 2: Total cell count of bacteria in biosimulator containing 40 mg/L Cypermethrin

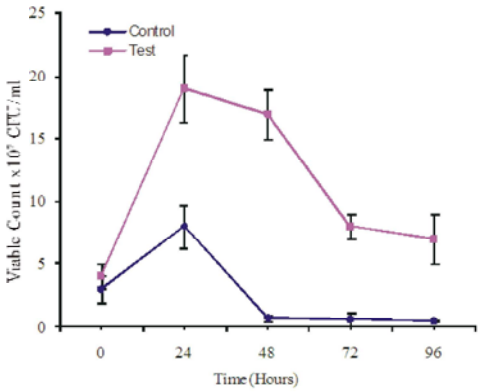

Fig. 3: Total cell count of bacteria in biosimulator containing 80 mg/L Cypermethrin

Since the solubility of pesticides in aqueous solution varies and is generally less, therefore a corresponding increase in turbidity and decrease in growth was observed with the increase pesticide concentration in a nutrient medium. This increased turbidity might cause a decreased solubility of dissolved oxygen in the medium which is essentially required for the growth of IES-*Ps*-1, suggesting that the growth was directly proportional to pesticide concentration. Similar growth studies have been conducted and reported by other researchers as they provide information about the biodegradation potential of microorganisms [10, 11, 34, 36- 38].

The overall findings of this research study suggested that IES-*Ps*-1 can grow in the presence of added pesticides. However, the toxicity pattern observed was as follows: cartap > cypermethrin > methamidophos ~ malathion. From these results it appears that cartap and cypermethrin pesticides are more toxic than methamidophos and malathion. It is worth mentioning here that since the culture was malathion adapted, therefore IES-*Ps*-1 growth in the presence of organophosphorus pesticides increased its tolerance and hence more growth was observed even at high concentration of methamidophos and malathion.

During growth kinetic studies as explained above using different concentrations of pesticides, the death phase was not observed even at high concentrations; however the viable count gradually decreased. The possible explanation of these may be the non adaptation of IES-*Ps*-1 in the presence of added pesticide as an acclimation period is necessary for microorganisms in order to activate the production of degradative enzymes. This may account for the prolonged lag phase which was observed at high concentration of all the added pesticides. Another reason may be the less availability of dissolved oxygen (DO), as it is reported that the increased organic load might decrease the DO concentration [39]. Based on the present study findings as well as by Hashmi [11], it can be concluded that the IES-*Ps*-1 strain could be used for remediation of pesticide contamination.

Bacterial Growth in Biosimulator (Activated Sludge System): The growth of IES-*Ps*-1 in the presence of cypermethrin (Fig. 2 and 3) indicated that bacteria can grow fast in biosimulator as a higher number of cells were

Table 2: Evaluation of performance of IES-*Ps*-1 in cypermethrin degradation after 48 hours

		COD removal		Cypermethrin degradation	
Parameters	pH	Conc.(mg/l)	% removal	Conc.(mg/l)	% degradation
	Effect of Cypermethrin Concentration (mg/l)				
20 mg/L	8.60	80	97	-	No Peak
40 mg/L	8.30	1080	82	8.2	81
80 mg/L	7.81	4500	54	45	51
125 mg/L	7.83	13767	24	118	18
	Effect of Temperature (°C) at 80 mg/L dose				
18-25°C	7.80	4500	54	42	51
28-30°C	7.33	867	89	9.0	88
38-40°C	7.50	4000	52	42	48
	Effect of Dissolved Oxygen (mg/L) at 80 mg/L dose				
5-6 mg/L	7.87	5000	31	50	32
8-9 mg/L	7.81	4500	54	42	51
8-9 mg/L (30°C)	7.33	867	89	9.0	88
11-12 mg/L	8.20	1300	83	17	78

*Data indicate average values of three experiments

observed compared to the shaken flask condition. The maximum count at 24 hours with 40mg/L cypermethrin was $13\pm1.73x10^7$ CFU/ml and with 80mg/L, it was $19\pm2.65x10^7$ CFU/ml. However, the generation time at these concentrations (40 and 80mg/L) were 57 and 53 minutes respectively. On the other hand in control experiments, the cell count at 24 hours was relatively low (7 ± 1.73 x10^7 CFU/ml) with a marked increase in generation time (98 minutes).

It was further noted that the growth at 40mg/L dose was significantly increased after 48 hours incubation. But at 80mg/L dose, the growth was slightly less but continued till 96 hours incubation and a count of $7x10^7$ CFU/ml was recorded. The observed growth may be due to the availability of nutrients and favorable environmental conditions in biosimulator which allow the cells to survive till 96 hours. In contrast, the population density in the control experiment (no cypermethrin) was comparatively less ($0.1x10^7$ CFU/ml). Since 78-88% cypermethrin degradation was observed after 48 hours, it appears that biodegradation actually occurred by the acclimated IES-*Ps*-1 culture bioaugmented in wastewater samples. Further the presence of bacterial cells in log phase during biodegradation indicated that substrate conversion would be at its maximum as also described by Gray [40] and similarly observed in this study.

Influence of Physicochemical Conditions on Biodegradation Rates pH: The data as reported in Table 2, indicated that IES-*Ps*-1 can retain their degradation ability in a wide range of pH with optimum degradation at 30°C temperature where the pH of the water sample was found near neutral range (pH 7.33). This finding is supported by Ashok and Seth [40], who reported that isolated *Pseudomonas* strain can grow between pH 5.5 to 9.5 with optimum growth at pH around 7.0. Similarly, Mandelbaum *et al*. [9], found that atrazine degradation in the pH range of 5.5 to 8.5 by *Pseudomonas* strain was not affected. Moreover it is reported that the tolerable limits for pH in activated sludge ranged between pH 6.0 to 9.0 [41].

Temperature: Temperature is among the important environmental parameters that can influence the microbial growth as well as treatment efficiency [43]. Therefore the influence of temperature on biodegradation of Cypermethrin was investigated by performing experiments at temperatures of 18-25°C (ambient temperature), 28-30°C and 38-40°C. The results indicated the direct correlation between temperature and microbial activity. Significant removal of cypermethrin by IES-*Ps*-1 strain was observed when biosimulator operated at 28-30°C temperature using 8-9mg/L DO, whereas moderate degradation occurred at ambient and 38 temperatures (Table 2, Figure 4). Similar optimal temperature (28-30°C) for *Pseudomonas* growth in activated sludge was also reported by Schlegel [44].

Dissolved Oxygen: Comparative performance evaluation at different dissolved oxygen concentration for cypermethrin degradation is presented in Table 2 and shown in Figure 5. It was noted that the initial dissolved oxygen concentration in the wastewater sample ranged

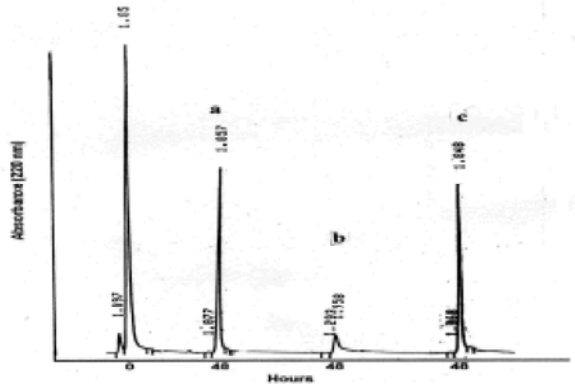

Fig. 4: HPLC chromatograms showing comparative effect of temperature on cypermethrin (80 mg/L) degradation at 9 mg/L DO. a: ambient temp.; b: 30°C; c: 38°C

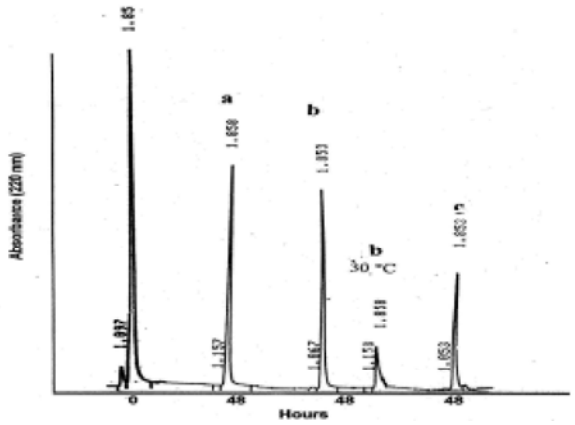

Fig. 5: HPLC chromatograms showing comparative effect of DO on cypermethrin (80 mg/L) degradation. a: 6 mg/LDO; b: 9 mg/L DO; c: 12 mg/L

between 1.5-2.6 mg/L. In order to maintain aerobic conditions in biosimulator, the mechanical aeration was provided, which not only maintain the sufficient dissolved oxygen in the reactor but also kept the cypermethrin in suspension, such that the bacterial growth and COD removal were greatly enhanced. During experiment it was observed that the biodegradation performance of IES-*Ps*-1 significantly improved at 8-9 mg/L dissolved oxygen using mechanical aeration (250 rpm) in the temperature ranged of 28°C-30°C.

Chemical Oxygen Demand: Results as summarized in Table 2 explain a good agreement between COD removal and cypermethrin degradation rates especially at low concentration. These results are in accordance with previous findings reported by Berchtold *et al*. [45], who noticed the same correlation between the COD removal

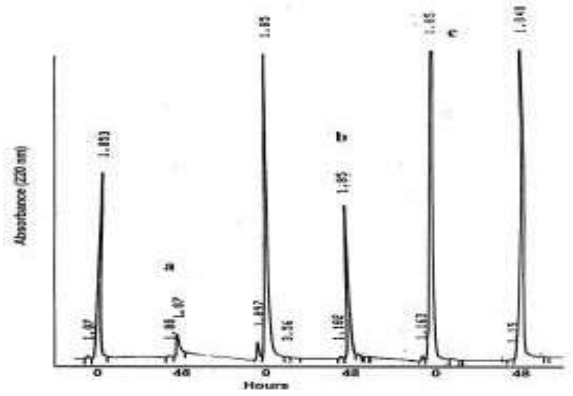

Fig. 6: HPLC chromatograms showing comparative biodegradation rates at different cypermethrin Conc. a: 45 mg/L; b: 80 mg/L; c: 120 mg/L

and biodegradation of 2,4-DAT and 2,4 and 2,6 diamino toluene degradation by acclimated bacteria [46]. Moreover, similar correlations were also observed by Ramanathan and Lalithakumari [35], during biodegradation of hazardous chemicals. During treatment of cypermethrin, it was also observed that even at high organic load, the biosimulator function satisfactorily when operated under controlled temperature and dissolved oxygen with retention time of 48 hours. These findings are in agreement with Toprak [47] who found that COD removal during treatment mainly depends on temperature and influent COD concentration.

Biodegradation of Cypermethrin: The data reported in Table 2 and HPLC chromatogram shown in Figure 6 clearly indicated that the increased cypermethrin concentration (40 mg/L to 125 mg/L) in biosimulator decreased the degradation rates. However, the complete removal occurred at 20mg/L dose [29]. Similar results of lower degradation at high concentration of organic pollutants were also reported by other researchers [10, 26, 34, 41, 46, 48].

During the experimental work it was observed that the optimum temperature for growth and degradation of cypermethrin by IES-*Ps*-1 culture remains between 28-30°C. Using a similar concentration of cypermethrin (80 mg/L), when the temperature of the reactor was decreased or increased (38°C), the degradation rates were significantly reduced. At ambient temperature, 51 % cypermethrin degradation occurred whereas at 38ºC, it was 48 %. In contrast, when biosimulator temperature was maintained between 28 to 30°C using mechanical aeration (8-9 mg/L), keeping cypermethrin concentration constant (80 mg/L), > 88 % removal was achieved after 48 hours of

aerobic treatment (Figure 4). Similar optimal temperature (28-30°C) for growth of *Pseudomonas* in activated sludge was reported by Schlegel [44] and Chaterjee *et al*. [49]. Moreover, Karpouzas and Walker [38] reported that unlike high temperature, low growth temperatures for *Pseudomonas putida* strain are usually not lethal.

Overall the study findings described that high concentration of cypermethrin degradation is achieved in short retention time of 48 hours. Although the transformation of permethrin (50 mg/L) by pure culture of *Pseudomonas fluorescence* under aerobic conditions with a half-life of less than 5 days was reported by Maloney *et al*. [50] and the removal of technical grade cypermethrin from 60 to 6mg/L by *Pseudomonas* specie in 20 days was reported by Grant and Betts [51].

It can be concluded that biodegradation performance mainly depends on cypermethrin concentration. But optimizing the treatment conditions like temperature and dissolved oxygen in activated sludge, IES-*Ps*-1 could effectively remove higher concentrations of toxic organics.

Principal Component Analysis: The multivariate statistical method, principal component analyses exposed the groups of correlated variables and their importance in the data structure. Based on the principal components I, II and III, which explains 86% of the total variance, the highest proportion of the total variance, PC1 explains 59.3% of the variance and is primarily a function of COD_{OUT}/COD_{IN}, CYP_{OUT}/CYP_{IN}, COD_{IN}-COD_{OUT}, CYP_{IN}-CYP_{OUT}, total cypermethrin concentration, organic load and retention time. The second PC (PC2) explained 15.4% of the variance and seemed to be governed by pH, dissolved oxygen and temperature. The third PC (PC3) accounts for 11.6% of the variance and is mainly based on temperature and retention time. Overall, PCA results indicate that the main factors governing the biosimulator performance are cypermethrin concentration, organic load (COD) and retention time. Other factors are operational parameters such as pH, temperature and DO. These findings explain that high values of effluent COD are directly related to cypermethrin concentration. It is likely that complete removal of cypermethrin at high organic load with a short retention time would only be possible, if the system operates at optimum pH, temperature and with extended aeration. Therefore from this wide range study, principal component data exhibited the trend of controlling parameters that is likely to affect the overall treatment system.

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