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Growth Response of a Selected Bacterial Population (*Pseudomonas*) Exposed to Malathion

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Abstract: Growth kinetics of *Pseudomonas* in sterile nutrient broth was studied using nutrient broth alone and broth supplemented with malathion (8.55 mg/ml malathion). The percent biodegradation of malathion by *Pseudomonas* was determined under sterile and unsterile conditions. The mean percentage biodegradation of malathion under unsterile conditions varied from 88.57 per cent for 0.00285 µg/ml, 70.49 percent for 0.0057 µg/ml and 78.52 for 0.00855 µg/ml whereas in sterile conditions the fate of microorganisms capable of biodegrading malathion showed the following percentage mean values which varied from 53.26 percent for 0.00285 µg/ml, 53.95 percent for 0.0057 µg/ml and 80.61 percent for 0.00855 µg/ml. The study determined that inoculation of bacterial culture under laboratory scale conditions can be used in bioremediation of environmental pollution caused by xenobiotics.

Key words: activated sludge, biodegradation, wastewater treatment, malathion

Introduction

Pakistan is an agro based country and most of the land is generally meant for cultivation. Due to rapid increase of population in the country (growth rate of 3% per year), the need for food is also rising. The farmers who constitute approximately 70-75% of the population produce a large proportion of dietary requirements of the country. To produce this much amount of agricultural commodities, higher input of fertilizers and pesticides are being used, but at the expense of environment and human health. The dilemma of using pesticide is that, they protect crops from insects, weeds, plant diseases but their use is also associated with hazards ranging from acute toxicity to cumulative health effects such as cancer, central nervous system disorders, birth defects and genetic mutation (Morgan, 1992; McClintock *et al*., 1994; Ahmad *et al*., 1995; Hussain, 1998).

Residues of applied pesticides stay in the environment. They can stay in soil, in free or bound form and can also enter the ground and surface water compartments. At high temperature, pesticides rapidly volatilize into atmosphere and ultimately sink into aquatic ecosystem. Most of the developing countries are located in the tropical belt, where high temperature and rainfall are rather common. The tropical agro-ecosystem characterized by the above climatic feature may facilitate the rapid dissipation of toxic contaminants through air and water. In fact a typical case was observed in Nigeria where approximately 98% of DDT applied to the cow pea crop was volatilized during a period of 4 years (Perfect, 1980). The investigation on the fate of the insecticides in the tropical paddy ecosystem in South India provides much more evidence (Ramesh *et al*., 1991).

Pesticides contamination as a result of agricultural and industrial activities poses serious threats to the environment and indeed to human life. Today Environmental Engineers are designing and operating the treatment facilities that utilize living organisms to bring about the destruction or transformation of organic and inorganic waste materials (Schnoor, 1992; Ritmann *et al*., 1988). The use of bio-remediation technology to degrade organic contaminants including hazardous waste has received a great deal of attention in recent years (Thomas *et al*., 1987; Sterrit and Lester, 1988). Numerous reports are available indicating the use of microorganisms for pollution control and for the production of chemicals of economic importance. Additionally, techniques are being used to select microorganisms that can degrade extremely toxic compounds (Anonymous, 1991; Bitton, 1998). Reports are available regarding efficiency and use of biological sludge system and anaerobic digestion for biotransformation of pesticides (Kobayashi and Rittman, 1982; Buisson *et al*., 1988). The removal of several organic toxicants by the activated sludge and trickling filter processes has been investigated (Ramalho, 1977; Robert, 1998). In general, the activated sludge process is quite efficient in decreasing the concentration of many priority pollutants and other xenobiotics to concentrations below detection and permissible limits. Tucker *et al*. (1975) showed that commercial polychlorinated biphenyl (PCB) mixture underwent primary bio-degradation by the activated sludge treatment system. The suitability of pesticides for a particular region must be based on its climate, ecological, agronomic, social economic and environmental conditions. One must check the suitability, efficacy, safety or fate of pesticides under the conditions where it may ultimately be used. Moreover, multinational companies are engaged in the lucrative business of those pesticides that have been banned in their own countries since long because of their ill effects on human health and environment, thus exposing the developing world to severe health hazards. For the future sustainable development, it is the time that countries should move quickly to implement control over waste disposal if they are to avoid high cleanup cost in future and intensify our research and development work towards environment to preserve essential natural resources and to protect human health.

Malathion is a non-systemic, one of the world's most widespread general-purpose organophosphate insecticide with high selective toxicity that is mostly used for the control of sucking and chewing insects on fruits and vegetables and for controlling mosquitoes and flies (Wester and Cashman, 1989). This study was aimed at determining the viable count and performance efficiency of *Pseudomonas* capable of biodegrading malathion using two sets of conditions (I) nutrient broth alone and broth supplemented with malathion (8.55 mg/ml) and (ii) sterile and unsterile conditions in a shaking water bath.

Materials and Methods

The present research investigation was carried out in the Institute of Environmental Studies, University of Karachi, Pakistan. Wastewater was collected from pilot plant within the university.

Pesticide used: The pesticide used in the present study belongs to the class organophosphate. It is commercially available as malathion. It is selected on the basis of its wide application and present market trends.

Isolation and maintenance of malathion degrading bacterial culture: The bacterial culture capable of biodegrading malathion was isolated from the soil using enrichment technique, with varying concentration of malathion in the medium. The bacterial culture isolate showing highest tolerance to malathion was purified,

identified and preserved. Isolated culture was maintained on nutrient agar (Acumedia) slants containing 1 % malathion concentration and stored at 4°C. The bacterial culture was subcultured after every 3-months.
The optimum concentration of malathion for bacterial growth was determined by the inoculation of bacterial culture on nutrient agar plates containing 2.85, 5.7 and 8.55 mg/ml concentration of malathion. The plates were incubated at 37°C for 24 hours.

Identification and characterization of bacterial culture: The identification and characterization of the bacterial culture was made using morphological, cultural and biochemical tests.
Morphological character of the culture was studied by using Gram's staining method whereas for cultural characterization the dehydrated medium (Acumedia) was used to prepare nutrient agar and broth in sterile distilled water which were autoclaved at 15 psi for 15 minutes and pH was adjusted at 7.2. Similarly for determining fermentation of sugars, peptone water with 1 % sugar containing Andrade's indicator was used (Collins and Lyne, 1985). These sugar solutions were filter sterilized using pre sterilized millipore membrane filter of 0.45µm pore size and dispensed in 5ml quantity in sterile screw cap tubes. The reactions were noted up to 15 days at 37°C. Glucose phosphate broth was used for determining methyl red test and for the study of production of acetyl methyl carbinol. Indole production was determined by using peptone tryptone. Similarly catalase activity was also determined by adding a few drops of 3 % hydrogen peroxide on 24 hours grown agar slopes and also in broth cultures and tubes were examined for the evolution of oxygen. Appearance of bubbles in the tubes was taken as catalase positive.

Growth kinetic studies of malathion degrading bacterial culture: Washing and sterilization of glassware: All glassware that was used in the present study was cleaned first with a suitable detergent. The glassware were kept in chromic acid solution for overnight, then washed thoroughly with running tap water and finally rinsed with distilled water and dried in an oven. The dried glassware were wrapped in paper and sterilized in an autoclave at 121°C, 15 psi for 30 minutes. The sterilized glassware was dried in an oven.

Preparation of the medium and inoculum: For the growth kinetic studies of malathion biodegrading bacterial culture nutrient broth and agar (Acumedia) were used. Nutrient broth was prepared as mentioned earlier. The medium was dispensed in 10 ml quantity in screw cap tubes, and in 250 ml quantity in screw cap flasks. These were autoclaved at 121°C, 15 psi for 30 minutes. Sterility was checked for 48 hours at 37 °C.
Inoculum was prepared by taking a loopful of the bacterial culture from the nutrient agar plate, inoculated first into 10 ml nutrient broth and incubated at 37° C for 24 hours; this seeded culture was used to inoculate 250 ml of nutrient broth in shaking water bath. The turbidity of the culture was finally matched with Mcfarland's Index (3 X 10^9 bacteria/ml).

Determination of the viable count: The viable count was measured by the SPC technique using nutrient broth as the growth medium and reported as CFU/ml. Samples of the culture were drawn at regular time intervals. Serial dilutions of the samples were made in 9-ml sterile blanks (0.85 % NaCl; pH 7.0) the appropriate dilutions were transferred into sterile petri plates and molten nutrient agar was poured. The plates were incubated at 37°C for 24-48 hours and CFU (colony forming unit) were determined by the method described by Collins and Lyne (1985). The samples were analyzed within thirty minutes after collection. When samples could not be examined promptly, they were kept in a refrigerator and processed within two hours. The samples stored for more than two hours were discarded.

Growth kinetic studies in shaking water bath: The viable counts of culture were determined using nutrient broth and the broth supplemented with 8.55 mg/ml malathion in shaking water bath. The working details of the shaking water bath are shown in Table 1.

Table 1: Technical data of shaking water bath

General layout	
Shaking water bath	Model Grant Type SS30
Speed	240-260 r.p.m.
Temperature	Ambient
Retention time	29 – hours
Working details	
Total Hydraulic load *	
(I)Unsterile conditions {RWW (KUC), TMC, Manure (10g) }	250 ml
(ii)Sterile conditions (Nutrient broth, TMC, culture inoculum)	250 ml
Total malathion concentration (TMC)	
(I)Standard I	0.00285 µg / ml
(ii)Standard II	0.0057 µg / ml
(iii)Standard III	0.00855 µg / ml
Size of inoculum **	3.0 X 10^9 bacteria/ml

* RWW (KUC) = Raw wastewater, Karachi University Campus, Pakistan.
TMC = Total malathion concentration i.e. Standard I, II, III.
** Size of inoculum = 24-hours old grown culture.
(Culture was streaked on nutrient-agar slants and incubated for 24-48 hours. Washes were taken with nutrient broth and matched with Mcfarland's Index)

Malathion degradation studies in shaking water bath: The ability of the *Pseudomonas* for malathion biodegradation was determined in shaking water bath under sterile and unsterile conditions to monitor the fate whether the bacteria can utilize malathion as a carbon substrate. TLC and HPLC techniques were used for determining the degradation.

Extraction of metabolites for chromatographic identification: Samples were collected as per schedule and subjected to n–hexane extraction. The hexane layer was separated and evaporated on a water bath at 70°C, the residue was finally dried. After complete drying, the residue was recovered in 10 ml of HPLC-grade methanol. The resulting solution was used for TLC and HPLC analysis.

Thin-layer chromatography (TLC): Thin layer chromatographic procedures described by Kadoum (1970) were employed to identify the malathion. The suspension of silica Gel-G absorbent was prepared in distilled water with a ratio of 1:3. The slurry was poured onto the clean grease free glass plates (20 X 20cm^2) immediately. The plates were air dried and activated at 105-110 °C for 2-hours. About 5-10 μl of the sample was applied with the help of a calibrated micropipette. Standards were also applied to identify the components of the sample. The following solvent systems were used for chromatographic identification: benzene-hexane-acetic acid (40:40:20), hexane-acetic acid-ether (75:15:10), benzene-acetic acid (4:1). The spots of separated components were visualized by using freshly prepared DCO (2,6-dibromo-N-chloro-p-quinoneimine diluted to 0.5 % in acetone) which was sprayed on plates and heated at 110°C for 20 minutes, permitted detection of malathion to visualize the spots as has also been described by Menn *et al.* (1957) and Jaglan and Gunther (1970). The plates were also visualized under UV-light.

High pressure liquid chromatography (HPLC): The 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 uBondapak C_{18} analytical waters uBondapak reversed-phase column, effluents was monitored by using UV-detector (visible spectrophotometer detector SPD-10A). The output of the detector was connected to a chromatopack (CR6A). Mobile phase consisted of methanol (Merck analytical

grade) since malathion is miscible with alcohols (Montgomery, 1993). The methanol was first distilled twice and further purified by filtration through Millipore filtration unit (0.2 and 0.4 μm Millipore filter; Micropore, Nylon). The filtered methanol was degassed prior to use by sonication. The flow rate was adjusted at 1.5ml/min with total elution time of 12 minutes for each run. The column was flushed with deionized distilled water and methanol whenever required removing impurities and was allowed to equilibrate between runs.

Results and Discussion

Isolation and study of bacterial culture for malathion degradation: The soil bacterial isolates in present study were identified up to the level of genus. No attempt was made for speciation. On the basis of morphological, cultural and biochemical characteristics the culture was found to belong to genus *Pseudomonas* (Table 2). Bacteria belonging to genus *Pseudomonas* are highly oxidative, aerobic and metabolically versatile and have been reported to degrade the aromatic hydrocarbons, oil, petroleum products and pesticides (Choudhary *et al.*, 1988; Kimbara *et al.*, 1989). Because of being highly oxidative, aerobic and metabolically versatile, members of the genus *Pseudomonas* are being used for the degradation of hydrocarbons (phenols, cresols etc.), oil and petroleum products and pesticides. Thus they play a significant role in controlling pollution due to liquid effluents.

Growth kinetics of *Pseudomonas* in sterile nutrient broth using shaking water bath: From Table 3 and Fig. 1 it is seen that the phase of acclimatization of *Pseudomonas* continued upto almost 6 hours after initial inoculation. Initially the count at 0 hour (2.22 X 10^7 CFU/ml) dropped to 1.03 X 10^7 CFU/ml and then started increasing slowly. At 6 hours the total viable count was 37 X 10^7 CFU/ml with the generation time of 45 minutes and specific growth rate of 0.0219 (generation time and specific growth rate not shown in the table). At 8 and 10 hours the total viable counts were 86.42 and 166.14 X 10^7 CFU/ml respectively, indicating that the culture after remaining in the lag phase (phase of adjustment) for 6 hours entered into phase of positive acceleration. The total viable count became 682.85 X 10^7 CFU/ml after 25 hours of incubation showing that the culture was in the log phase.

Growth kinetics of *Pseudomonas* in malathion containing nutrient broth (8.55 mg/ml malathion) using shaking water bath: *Pseudomonas* was also grown in malathion containing nutrient broth. The results are shown in Table 3 and Fig. 1. Comparison of data in Table 3 and Fig. 1 indicates that the general growth pattern of *Pseudomonas* in the absence and presence of malathion is very much similar. In two different environments (absence and presence of malathion), the viable counts at 6 hours were 25.53 X 10^7 CFU/ml, at 8 hours 84.71 X 10^7 CFU/ml and at 10 hours 179.43 X 10^7 CFU/ml respectively. After 25 hours of incubation the counts were 738.57 X 10^7 CFU/ml respectively. The generation time of *Pseudomonas* in malathion was calculated to be 47 minutes at 6 hours after inoculation (results not shown in the table).

Further it indicates that malathion did not interfere with the growth of *Pseudomonas* in the concentration used.

Table 2: Characteristics of *Pseudomonas*

Tests	*Pseudomonas*
Shape	Thin short rods
Gram stain	Gram negative
Motility	Motile
Growth on nutrient agar	Colonies are round, smooth and convex
Starch hydrolysis	Positive
Nitrate reduction	Positive
Indole production	Negative
Oxidase and catalase	Positive
Methyl Red	Negative
Voges-Proskauer	Negative
Citrate utilization	Positive
Acid without gas from glucose, fructose, sucrose, mannose, ribose, xylose, mannitol and lactose	Positive

According to "Bergey's Manual of Determinative Bacteriology" 8th Ed., (1974) the culture was classified in the genus *Pseudomonas*.

Malathion degradation studies in shaking water bath: In this study the performance efficiency of *Pseudomonas* was evaluated in shaking water bath using sterile and unsterile conditions. Malathion present in wastewater, which is subjected to activated sludge treatment process, encountered many physical and biological forces. Among biological reactions, microbial activities are regarded as the most important ones in the elimination of malathion from the wastewater. In order to confirm the capability of *Pseudomonas* to degrade malathion, a complete and thorough analysis was performed using TLC and HPLC. Table 4 represents the mean values of malathion biodegradation under two different conditions.

Unsterile conditions (indigenous micro-organisms): HPLC was performed to confirm the bacterial biodegradation of malathion.

Table 3: Growth kinetics of *Pseudomonas* in sterile nutrient broth and broth supplemented (8.55 mg/ml malathion) in shaking water bath

Time	Nutrient broth (Viable count CFU/ml x 10^7)								NB supplemented (Viable count CFU/ml x 107)							
	I	II	III	IV	V	VI	VII	Mean	I	II	III	IV	V	VI	VII	Mean
0	1.02	0.76	2.54	1.94	1.32	3.86	4.12	2.22	3.42	2.54	1.94	1.32	3.86	4.12	3.6	2.97
2	1.19	1.16	0.64	0.97	0.73	1.35	1.18	1.03	1.57	0.73	0.90	0.79	1.71	1.55	1.49	1.25
4	8.40	9.90	3.60	5.40	3.30	7.10	4.10	5.97	2.33	2.60	4.90	5.50	9.50	4.10	2.28	4.46
6	36.00	38.00	48.00	41.00	28.00	34.00	34.00	37.00	29.40	20.00	28.00	26.00	22.00	24.00	29.3	25.53
8	97.00	89.00	94.00	80.00	101.00	64.00	64.00	86.42	96.00	81.00	82.00	87.00	84.00	76.00	87.0	84.71
10	164.00	189.00	160.00	127.00	176.00	166.00	166.00	166.14	176.00	182.00	199.00	175.00	184.00	176.00	164.0	179.40
25	620.00	730.00	640.00	670.00	700.00	780.00	780.00	682.85	780.00	790.00	690.00	790.00	730.00	710.00	680.0	738.60

Note = Based on mean of seven replicates CFU = Colony forming unit (viable count is a mean of three replicates)

Table 4: Concentration and percentage degradation of malathion in working standards

Standard Concentration (0-hour) (µg/ml)	Unsterile conditions (% degradation)				Sterile conditions (% degradation)			
	Min. (%)	Max. (%)	Mean (%)	Conc. (29 hours((µg/ml)	Min. (%)	Max. (%)	Mean (%)	Conc. (29 hours((µg/ml)
0.00285	88.05	89.68	88.57	0.00032	48.22	59.57	53.26	0.00132
0.0057	70.73	89.66	70.49	0.00119	48.91	61.94	53.95	0.00262
0.00855	69.79	84.69	78.52	0.00183	77.25	84.11	80.61	0.00165

Based on mean of 8-replicates

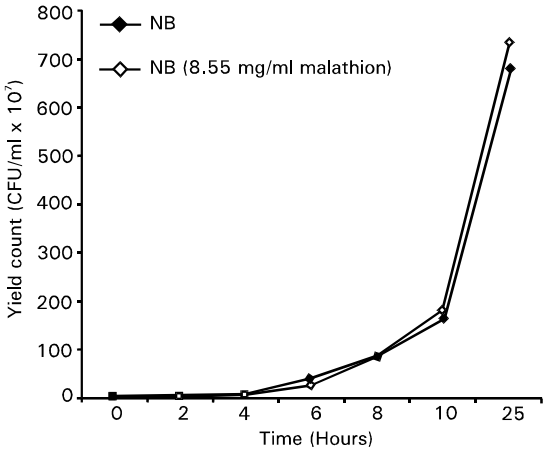

Fig. 1: Growth kinetics of *Pseudomonas* in sterile nutrient broth and broth supplemented (8.55mg/ml malathion) using shaking water bath.

The mean percentage biodegradation of malathion using unsterile conditions (RWW) was 88.57 % for 2.85 mg/ml, 70.49 % for 5.7 mg/ml and 78.52 % for 8.55 mg/ml after 29 hours. The unsterile condition represents heterogeneous population of microorganisms, some of which may utilize malathion as a carbon source. These organisms are of major interest. Paris *et al.* (1975) reported the biodegradation of low concentration of malathion by heterogeneous bacterial population. It was also reported that several bacterial and fungal isolates obtained from soil and wastewater are also capable of biodegrading malathion (Chakrabarty, 1982). In fact the present bacterial isolate (*Pseudomonas*) is a soil isolate.

Sterile conditions (using pure culture of *Pseudomonas*): The results of biodegradation of malathion for different concentrations by *Pseudomonas* in sterile nutrient broth using shaking water bath are summarized in Table 4. It is difficult to account for inconsistent results obtained in the presence of different concentrations of malathion under two different conditions (raw wastewater and sterile nutrient broth). However, it becomes clear that addition of *Pseudomonas* into raw wastewater did enhance malathion biodegradation. It may be that wastewater borne microorganisms were also contributing towards malathion biodegradation. The results of malathion biodegradation at 2.85 and 5.7 mg/ml were almost the same. The mean percentage biodegradation was 53.26 and 53.95 % respectively. The minimum and maximum biodegradation ranged between 48.22-59.57 and 48.91-61.94 % respectively after 29 hours. This was the condition when the *Pseudomonas* was used. It is important to note that when the concentration increased thrice, the percentage biodegradation was at its maximum (80.61 %) with minimum and maximum values ranged between 77.25-84.11 %. The maximum biodegradation in this condition may be due to the fact that *Pseudomonas* had adapted itself to the highest concentration of malathion. Results of the present study are in accordance with the findings of Matsumura and Boush (1966) who found that malathion was rapidly metabolized by the *Pseudomonas* species due to the action of carboxyesterase. Rosenberg and Alexander (1979) also reported the enzymatic hydrolysis of malathion by two *Pseudomonas* species grown on malathion. Walker (1976) reported 97 % biodegradation of malathion in both sterile and unsterile estuarine water when incubated in the dark for 18 days.

From the studies it is evident that a pure and mixed bacterial culture (sterile and unsterile conditions) studied under laboratory conditions can be used in bioremediation of environmental pollution caused by xenobiotics.

Decline phase was not achieved even after 29 hours of incubation indicating that the nutrients were still available for the cells to grow and that other environmental conditions are also favorable as has also been reported by Pirt (1975). The bacterial cells in the log phase would mean that the substrate conversion would be at its maximum as has also been described by Gray (1989). Not much difference in the viable count of *Pseudomonas* was observed under two sets of conditions (nutrient broth alone and broth supplemented with malathion; 8.55 mg/ml malathion) using shaking water bath.

The percentage degradation in sterile and unsterile conditions indicates that the malathion degrading bacteria were present in the system but they first adapted themselves to the malathion containing environment. However, once the culture established in the new environment rapid degradation of malathion was observed. Malathion degradation by *Pseudomonas* was more pronounced as expected. Bourquin (1977) isolated numerous bacteria from salt marsh environment capable of degrading malathion upto 90 % when supplied with additional nutrients.

A comparison of the growth kinetics of *Pseudomonas* with that of biodegradation under shaker conditions reveals that bacterial density correlates to some extent with the degradation rate. The shaking water bath (activated sludge treatment system) has proved to be a rapid and efficient method for treating wastewater containing pesticides, within a short period of time, thus it was necessary to investigate the method of analysis very carefully so that their estimation at such low levels was possible, with sufficient precision.

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