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Malathion Degradation by *Pseudomonas* Using Activated Sludge Treatment System (Biosimulator)

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Abstract: This study was aimed at determining the growth kinetics and degradation potential of malathion by inoculation of the bacterial culture. Growth kinetics studies of *Pseudomonas* were determined by SPC technique using 4.0 mg L^{-1} dissolved oxygen (DO) in sterile undiluted nutrient broth and sterile diluted (1:10) nutrient broth, whereas the degradation studies were determined using high pressure liquid chromatographic (HPLC) techniques, which were conducted using continuous cultivation technique with similar DO level as that of growth kinetic studies using biosimulator, with two different set of conditions (with-culture; using pure culture of *Pseudomonas* and without-culture; indigenous microorganisms). A critical comparison of data revealed that at each sampling hour the viable population density was greater in the presence of undiluted nutrient broth than in the presence of diluted nutrient broth. Similarly the mean of degradation studies revealed that malathion degradation was more pronounced in studies with inoculation (with-culture) as compared with indigenous bacteria (without-culture). The study demonstrated that inoculation of bacterial culture studied under laboratory conditions can be used in bioremediation of environmental pollution caused by pesticides.

Key words: Activated sludge, degradation, wastewater treatment, malathion

INTRODUCTION

Pesticides have played an important part in dramatic increases in agricultural productivity, which have been achieved in the developed world over the last few decades. Groundwater and surface water pollution by pesticides has been reported by many authors[1,2]. Recently, the significance of the mode of growth of microorganisms has received considerable attention due to its potential biotechnological application in bioremediation environmental pollution through bioreactors[3]. Today, environmental engineers are designing and operating treatment facilities that utilize living organisms to bring about the destruction or transformation of organic and inorganic waste material. Bioremediation technology to treat hazardous waste has gained considerable attention as it is ecologically sound and economical relative to other technologies and it has been used successfully in many countries of the world[4,5]. Microorganisms have a number of vital functions in pollution control. It is the microbial component of aquatic ecosystems that provides the self-purification capacity of natural waters in which microorganisms respond to organic pollution by increased growth and metabolism. Species belonging to genera such as *Achromobacter, Alcaligenes, Arthobacter, Bordetella, Flavobacterium, Pseudomonas and Xanthobacter* have been isolated from soils and shown to degrade pesticides in liquid nutrient media[6-8]. Application of mixed substrates also allows us to understand the mechanisms of microorganism's adaptation to real substrates. Along with the many benefits that these pesticides have rendered, their use has also caused many serious problems in terms of harmful effects on non-target organisms. Even if present in minute quantities, their variety, toxicity and persistence have an adverse effect on ecological systems[9-11].

In the present study, an organophosphate pesticide named malathion was selected and studied. It is a non-systemic, one of the world's most widespread general-purpose broad spectrum insecticide with high selective toxicity that is mostly used for the control of sucking and chewing insects including mosquitoes, aphids, turf insects and many floral and vegetable crops and fruits. It also finds use as a control for animal parasites (ectoparasites) and head and body lice[12].The extent to which pesticide residues could be removed from treated produce by washing may be influenced by a variety of factors such as the chemical properties and formulation

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type of the pesticide, the nature of the commodity, the length of time that the residue has been on the commodity's surface and the rinsing time and rinsing agents used[13].The study determined that inoculation of bacterial culture studied under laboratory conditions can be used in bioremediation of environmental pollution caused by pesticides.

MATERIALS AND METHODS

Pesticide used: The pesticide used in the present study is commercially available as malathion, chemical name S–1, 2–bis (ethoxycarbonyl) ethyl O, O–dimethyl phosphorodithioate. Table 1 represents the different physical and chemical properties of malathion, whereas the chemical structure is presented in Fig. 1.

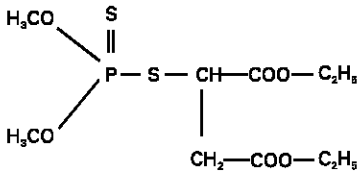

Fig. 1: Chemical structure of malathion

Isolation and maintenance of malathion degrading bacterial culture: The bacterial culture capable of degrading malathion was isolated from the evenly mixed soil, obtained within the campus premises 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 malathion and stored at 4°C. The bacterial culture was subcultured after every 3 months period. The optimum concentration of malathion for bacterial growth was determined by the inoculation of the bacterial culture on nutrient agar plates containing 2.85, 5.7 and 8.55 mg ml^{-1} concentration of malathion. The plates were incubated at 37°C for 24 h.

The identification and characterization of the bacterial culture was made using morphological, cultural and biochemical tests. Morphological characteristics 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 min and pH was adjusted at 7.2. Similarly, for determining fermentation of sugars, peptone water with 1% sugar containing Andrade's indicator was used[15].These sugar solutions were filter sterilized using pre sterilized millipore membrane filter of 0.45 μm pore size and dispensed in 5 ml 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 the 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 h grown agar slopes and also in broth cultures and tubes which 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

Preparation of the medium and inoculum: For the growth kinetic studies of the malathion degrading bacterial culture, nutrient broth and agar (Acumedia; pH=6.8±0.2) were used. The medium was dispensed in 10 ml quantity in screw cap tubes and in 500 ml quantity in screw cap flasks. These were autoclaved at 121°C, 15 psi for 30 min. Sterility was checked for 48 h 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 h; this seeded culture was used to inoculate 500 ml of nutrient broth and again incubated at 37°C for 24 h. The turbidity of the culture was finally matched with mcfarland's turbidometric index (3×10^{9} bacteria ml^{-1}). This was then used to inoculate 8 L broth in the biosimulator.

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 saline 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 h and CFU were determined[15]. The samples were plated within 30 min after collection. When samples could not be examined promptly, they were kept in a refrigerator and processed within 2 h. The samples stored for more than 2 h were discarded.

Growth kinetic and malathion degradation studies in biosimulator: The viable count and performance efficiency of *Pseudomonas* for malathion degradation was evaluated in the biosimulator (model MF-114; NBS-New Brunswick Scientific Company). The general layout of the biosimulator is illustrated in Fig. 2.

Table 1: Physical and chemical properties of malathion[15]

Physical		Chemical	
Properties	Value	Properties	Value
Nature	Amber liquid	Molecular formula	$C_{10}H_{19}O_6PS_2$
Melting point	2.85°C	Molecular weight	330.36 gm mol^{-1}
Boiling point	156–157°C (10.7 mm Hg)	Half life in soil	24 h to 6 days
Vapor pressure	130°C (4x10-5 mm Hg)	Half life in water	1.5 days to 21 weeks
Specific gravity	1.23 (25°C)	Log octanol/water	
Refractive index	1.4985	partition coefficient	2.36
Organic solvent	Miscible	Solubility in water	145 ppm (20°C)
Auatic toxicity	High		

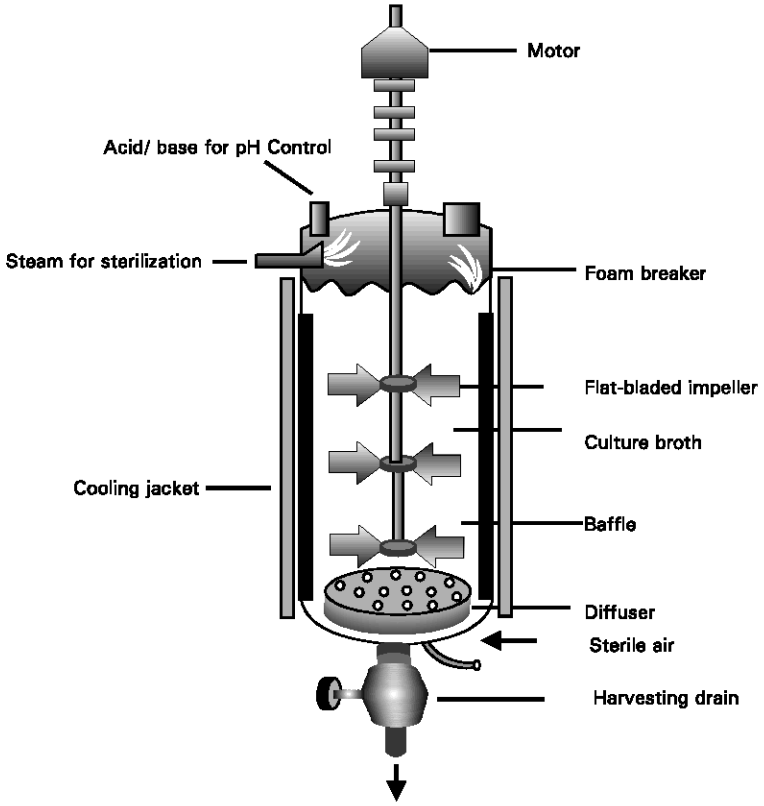

Fig. 2: General layout of a biosimulator (activated sludge treatment system)

In order to determine growth rates of *Pseudomonas,* the nutrient broth was adjusted to two different levels (a) sterile undiluted nutrient broth (high COD value) and (b) sterile diluted (1:10) nutrient broth (low COD value). For growth kinetic studies the biosimulator was operated at ambient temperature (25-28°C) for 29 h at 4.0 mg L^{-1} DO concentration. In undiluted nutrient broth the total malathion concentration added was 126.35 mg L^{-1} whereas for diluted nutrient broth (1:10) it was 12.635 mg L^{-1} (10 times diluted). Finally 500 ml of inoculum prepared earlier was added into 7.5 L of diluted and undiluted nutrient broth.

Similarly, for continuous cultivation the biosimulator was operated for 51 h at 4.0 mg L^{-1} DO concentration at ambient temperature. 126.35 mg L^{-1} of malathion was added twice times (0 and 25 h) during the experimental conditions, followed by addition of 500 ml culture inoculum.

Approximately 8.0 L of the sample (raw wastewater; culture inoculum) was transferred carefully into the heavy wall, borosilicate glass jar of a compact bench scale stainless steel biosimulator. The sample was strongly agitated by impeller with flat stirring paddles and by the four vertical baffles. Agitation was continuously monitored on a calibrated electrical tachometer, which provides accurate speed indication. Air was metered through a pressure regulator, needle valve flow meter and a stainless steel filter. pH and DO was measured by using pH and DO controller of NBS, whereas chemical oxygen demand (COD) was determined by the

potassium dichromate reflux method using HACH-COD analyzer[16].

Sample processing: Samples were collected from the biosimulator vessel as per schedule. Thin layer chromatographic procedures were employed to identify malathion[17]. A 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 (20X20 cm) immediately. The plates were air-dried and activated at 105-110°C for 2 h. 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 min, permitted detection of malathion to visualize the spots[18] whereas for 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 was used and effluents were monitored using a UV-detector (visible spectrophotometer detector SPD-10A; ë= 220 nm). 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[19]. 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 1.5 ml min^{-1} with total elution time of 12 min 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

Isolation and study of bacterial culture for malathion degradation: On the basis of morphological, cultural and biochemical characteristics, the culture was found to belong to the genus *Pseudomonas*, according to the Bergeys Manual of Determinative Bacteriology[20]. Bacteria belonging to the genus *Pseudomonas* are highly oxidative, aerobic and metabolically versatile and have been reported to degrade aromatic hydrocarbons, oil, petroleum products and pesticides and are being used for in situ biorestoration. Thus they play a significant role in controlling pollution due to liquid effluents[21].

Growth kinetics of *Pseudomonas* in undiluted nutrient broth (high COD value) using biosimulator: Growth kinetic results of *Pseudomonas* in undiluted nutrient broth in a biosimulator are shown in Fig. 3.

It is seen from the figure that the phase of acclimatization of *Pseudomonas* continued up to almost 4 h after initial inoculation indicating that the organism remained in the lag phase for a relatively longer period of time and the mean maximum viable population density was $30.13x0^7$ CFU ml^{-1}. After remaining in the lag phase for up to 4 h, the cells in the undiluted nutrient broth seem to have entered into the phase of positive acceleration. At 8 and 10 h, the total viable counts were 168 $X10^7$ CFU ml^{-1} and 216.66 x 10^7 CFU ml^{-1}, respectively, indicating that the culture after remaining in the lag phase (phase of adjustment) entered into the phase of positive acceleration. After 24 h of inoculation, the cells were in the typical log phase and the viable count was 284.66 $x10^7$ CFU ml^{-1} and at 29 h it was 1786.66 x 10^7 CFU ml^{-1}.

Growth kinetics of *Pseudomonas* in sterile diluted (1:10) nutrient broth (low COD value) using biosimulator: The results of this study are shown in Fig. 3. The general growth kinetic pattern of *Pseudomonas* at low nutrient level (low COD value) revealed that the lag phase was much longer up to 9 h as compared to the previous study where it was up to 4 h. In the previous study, cells entered the phase of positive acceleration after 4 h whereas in the diluted nutrient broth (1:10) the cells entered the phase of positive acceleration after 9 h and the mean maximum viable population density was 44 $x10^7$ CFU ml^{-1}. A comparison of data Fig. 3 would indicate that at each sampling hour the viable population density was greater in the presence of undiluted nutrient broth (higher COD values) than in the presence of diluted nutrient broth (lower COD values). At 4, 10, 24 and 25 h the maximum viable population density was 30.13, 216.66, 284.66 and $498.66x10^7$ CFU ml^{-1}, respectively in undiluted nutrient broth whereas the corresponding counts were 10.5, 89, 173 and $268.33x10^7$ CFU ml^{-1} in sterile diluted (1:10) nutrient broth. After 29 h the maximum population viable density in undiluted nutrient broth was $1786.66x10^7$ CFU ml^{-1} whereas in diluted nutrient broth it was $1423.66x10^7$ CFU ml^{-1} indicating an increase by almost 25% in the former case. The low bacterial density in diluted nutrient broth is considered to be due to its low nutrients content (low COD level). The total quantity of nutrients would also affect the total viable population density. In all these studies, decline phase was not achieved even after 29 h

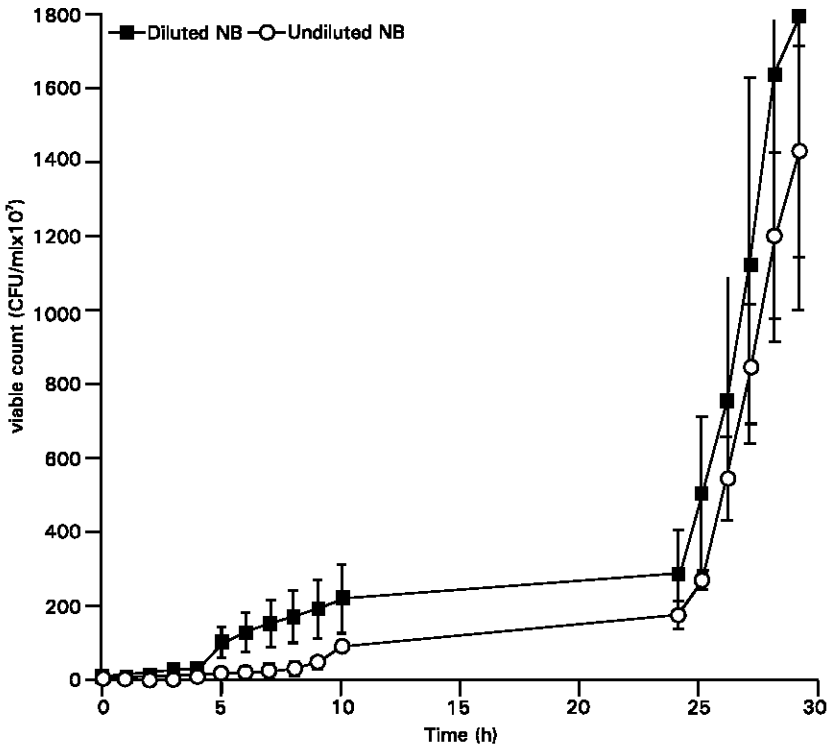

Fig. 3: Growth kinetics of *Pseudomonas* at 4.0 mg L^{-1} DO in sterile undiluted nutrient broth and diluted (1:10) nutrient broth using biosimulator

of incubation indicating that the nutrients were still available for the cells to grow and that other environmental condition were also favorable. The bacterial cells in the log phase would mean that substrate conversion (and hence COD reduction) would be at its maximum[22,23].

Malathion degradation studies in the biosimulator (continuous cultivation): Data taken during this study to assess the performance of *Pseudomonas* for malathion degradation are presented and discussed. The mean results of both these sets of conditions (with-culture and without-culture) are shown in Table 2. This study was performed to compare the efficiency of *Pseudomonas* with the normal bacterial flora of the raw wastewater. Malathion when present in the 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.

Very slight variation in dissolved oxygen concentration was observed and it was more or less the same as adjusted prior to the treatment. However, the trend of pH was towards alkaline side during the treatment (tables not shown). The change in pH during the present study may also be helpful in removing malathion from the system[24].

The degradation of malathion under two sets of conditions is reported in Table 2 and illustrated in Fig. 4. Although the same concentration of malathion (126.35 mg L^{-1}) was used in both the conditions but the degradation efficiency was different.

Malathion degradation by *Pseudomonas* in with-culture studies was more pronounced. More than 50% of malathion degradation was achieved within 6 h but in the other case it was only 28%. A previous study conducted showed that numerous bacteria isolated from salt marsh environment capable of degrading malathion up to 90% when supplied with additional nutrients as energy and carbon sources[25]. After 10 h the malathion degradation in both the conditions was 90.01 and 38.29%, respectively. However, it is interesting to note here that at 24 h the mean total percent of malathion degradation was almost similar and the respective values were 97.47 and 96.39%, respectively. It is also reported that 97% degradation of malathion occurs in both sterile and unsterile conditions[26]. The COD removal rate looks quite consistent with time. The COD removal seems proportional to the microbial cell density in the log phase.

Table 2: Degradation of malathion (%) at 4.0 mg L^{-1} DO by *Pseudomonas* under continuous cultivation using biosimulator (with-culture and without culture)

Time (h)	Parameters: Concentration (mg l^{-1})* With culture	Concentration (mg l^{-1})* Without culture	Retention time (min) With culture	Retention time (min) Without culture	Peak area With culture	Peak area Without culture	% degradation With culture	% degradation Without culture
0	126.35(1160)	126.35(1180)	2.10	2.09	1748725	1657792	---	---
2	104.70(1000)	121.58(1140)	2.10	2.09	1449035	1595334	17.13	3.76
4	77.30(920)	105.67(1060)	2.10	2.09	1069771	1386459	38.82	16.36
6	39.63(880)	90.55(1000)	2.09	2.09	548538	1188195	68.63	28.32
8	20.89(760)	82.17(980)	2.09	2.09	289094	1078167	83.46	34.96
10	12.62(680)	77.95(940)	2.09	2.08	174569	1022858	90.01	38.29
24	3.18(280)	4.56(420)	2.18	2.16	44096	59842	97.47	96.39
25	35.93(1020)	38.66(1220)	2.08	2.09	497318	507267	71.56	69.40
27	6.73(860)	35.86(1140)	2.04	2.09	93147	470560	94.67	71.61
29	6.20(780)	14.69(1040)	2.07	2.08	85819	192789	95.09	88.37
31	5.08(620)	6.93(960)	2.04	2.16	70330	90945	95.97	94.51
33	4.44(600)	6.51(880)	2.17	2.12	61577	85480	96.47	94.84
34	4.22(560)	5.93(820)	2.17	2.17	58428	77840	96.65	95.30
49	2.70(400)	4.73(300)	2.17	2.17	37455	62155	97.85	96.25
51	1.21(320)	3.98(220)	2.16	2.16	16854	52250	99.03	96.84

Table 3: Growth kinetics of *Pseudomonas* at 4.0 mg L^{-1} Do in sterile undiluted nutrient broth and diluted (1 : 10) nutrient broth using biosimulator

Time (h)	Sterile undiluted nutrient broth eperiment No. (Viable count CFU ml^{-1} x 10^7) I	II	III	Mean	Sterile diluted (1:10) nutrient broth experiment No. (Viable count CFU ml^{-1} x 10^7) I	II	III	Mean
0	8.5	8.81	7.68	8.33	6.93	7.0	7.1	7.04
1	6.3	7.90	5.40	6.53	2.20	2.2	2.3	2.24
2	11.2	13.10	11.70	12.00	4.90	5.4	4.8	5.03
3	21.9	26.00	22.40	23.43	6.90	7.8	8.7	7.80
4	29.6	31.00	29.80	30.13	9.80	11.5	10.2	10.50
5	101.0	110.00	91.00	100.66	13.00	14.7	11.5	13.06
6	122.0	129.00	130.00	127.00	19.10	17.6	15.5	17.40
7	140.0	158.00	154.00	150.66	22.30	21.0	20.7	21.33
8	163.0	170.00	171.00	168.00	29.10	29.3	29.6	29.33
9	189.0	186.00	193.00	189.33	38.00	45.0	49.0	44.00
10	214.0	217.00	219.00	216.66	94.00	91.0	82.0	89.00
24	284.0	291.00	279.00	284.66	203.00	186.0	130.0	173.00
25	510.0	496.00	490.00	498.66	269.00	271.0	265.0	268.33
26	790.0	745.00	730.00	755.00	460.00	540.0	630.0	543.33
27	1140.0	1135.00	1110.00	1128.33	890.00	836.0	820.0	848.66
28	1680.0	1650.00	1560.00	1630.00	1290.00	1196.0	1100.0	1195.33
29	1860.0	1790.00	1710.00	1786.66	1440.00	1421.0	1410.0	1423.66

Better COD removal efficiency was observed when the cells were in the log phase of growth. As it is quite evident that with the increase in organic loading COD removal efficiency also increased.

The unsterile condition represents a heterogeneous population of microorganisms, some of which may utilize malathion as a carbon source. These organisms are of major interest. Some workers reported the degradation 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 degrading malathion[27,28]. Infact the present bacterial isolate (*Pseudomonas*) is a soil isolate. After 25 h when the fresh inoculum was added, the degradation was 71.56 and 69.40%, respectively. After 27 h the degradation reached 94.67% whereas in another condition it was 71.61%. This was the situation that was attained within the initial 2 h of the experiment up to 49 h not much difference in % degradation with in the two sets of conditions were observed. At the end of experiment (51 h), the degradation of malathion was almost 99.03% in the experiment with-culture studies whereas in the other condition the same was 96.84%. It is difficult to account for inconsistent results obtained during the two different sets of conditions (with-culture and without-culture). However, it becomes clear that addition of *Pseudomonas* into raw wastewater did enhance malathion degradation. Based on the uninoculated system it appears that wastewater borne microorganisms were also contributing towards malathion degradation. Carefully controlled experiments are required to prove or disprove this concept. Results of the present study are in accordance with the findings of previous work reported in the literature by many scientists[29,30].

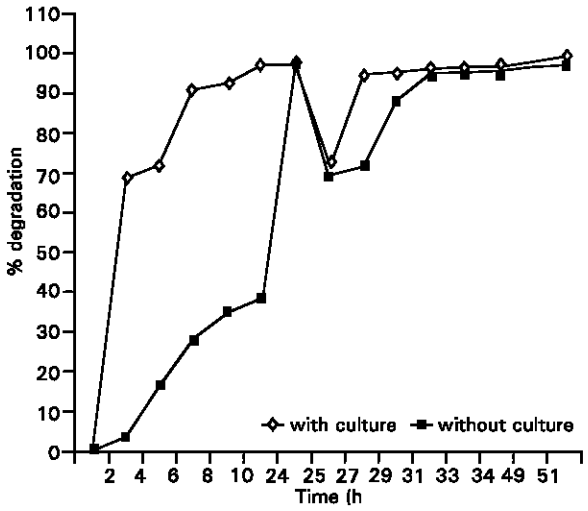

Fig. 4: Degradation of malathion (%) at 4.0 mg L^{-1} DO by *Pseudomonas* under continuous cultivation using biosimulator (with-culture and without-culture; "0" h means start of experiment whereas "25" h means fresh batch of wastewater was introduced into the biosimulator)

The total mean percentage of malathion degradation was almost the same in condition with with-culture and without-culture studies which indicates that the malathion degrading bacteria were present in the system but they first adapted themselves to the malathion containing environment. Therefore, not much significant percentage of malathion degradation was observed within the first few hours. However, once the culture established in the new environment rapid degradation of malathion was observed as reported in Table 3.

DISCUSSION

A comparison of the growth kinetics studies of *Pseudomonas* under sterile undiluted nutrient broth and sterile diluted (1:10) nutrient broth using biosimulator indicates that bacterial viable population density correlates to some extent, with the degradation rate in the first 10 h. When the cells entered the log phase and multiplied geometrically, a corresponding increase in degradability was lacking. As expected, the degradation of malathion was much better with *Pseudomonas* within the first 10 h of activated sludge treatment system. The rate of degradation in the activated sludge system in with-culture studies for the first 10 h was less than half of that of without-culture. After 24 h of activated sludge treatment, the magnitude of degradation was almost equal in both the conditions. It can be concluded that the activated sludge treatment system for malathion degradation using *Pseudomonas* becomes more than 2.5 times as effective within the first 10 h. In other words, 2.5 times less time is required to achieve the same level of degradation; hence making the system more economical. Slowing down of degradation rate after the initial 10 h up to 24 h does not correlate well with the growth kinetics model of the log phase cells (Fig. 3 and 4). This would mean that after the initial rapid degradation for the first 10 h the substrate concentration (malathion) reduced slowing down the rate of forward reaction but when more malathion (substrate) was made available (continuous cultivation) a rapid degradation rate reestablished indicating that degradation rate was a function of substrate concentration and bacterial cell count. As mentioned earlier bacterial culture continue to remain in the log phase even up to 29 h (Fig. 3). High malathion degradation rate in the initial stages (first 10 h) of bacterial growth is difficult to explain. Perhaps molecular oxygen is playing some role in oxidizing malathion thereby adding to the biological breakdown of malathion. As the culture (*Pseudomonas*) is highly aerobic in nature it performed better in biosimulator at a dissolved oxygen concentration of 4.0 mg $L^{-1}$where greater quantity of oxygen is more readily available. Further the study also revealed that malathion did not interfere with the growth of *Pseudomonas* in the concentration used. The present study also suggests that pesticide degrading bacterial culture should preferably be used for the management of pesticide containing wastewater. Pesticide manufacturing industries are expected to generate such a wastewater.

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