Nature Environment and Pollution Technology © Technoscience Publications	Vol. 6	No.3	pp. 399-406	2007

A COMPARATIVE STUDY ON BIODEGRADATION OF CHLOROBENZENE BY *PSEUDOMONAS AERUGINOSA*, *NOCARDIA HYDROCARBOXYDANSE* AND *MYCOPLANA DIMORPHA*

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ABSTRACT

Microorganisms have the ability to conform to a variety of environmental conditions. Their versatility makes them useful for various biotechnological applications. One such application is the use of microorganisms for removal of pollutants from the environment, called bioremediation. The first goal of the present work was to identify a microorganism which could grow on the model pollutant monochlorobenzene. The microbes used for degradation are *Pseudomonas aeruginosa, Nocardia hydrocarboxydanse,* and *Mycoplana dimorpha*. The batch-wise degradation is carried out on a laboratory scale using monochlorobenzene (MCB) as a substrate. The pH and temperature were maintained at 7.0 and 30°C throughout the experiment. The degradation was studied at various substrate concentrations (100, 200, 500 ppm.). Following a 2-5 hr post-treatment lag phase, degradation of the substrate occurred within 48 h. Various factors such as inocula, concentration of chlorinated aromatic hydrocarbons (CAHs), pH, temperature, presence of co-substrates and the type of carbon source used influenced the degradation of CAHs in shake flasks. Results show that the degradation was highest at an initial concentration of 200 ppm of MCB by *Pseudomonas aeruginosa*. Results also show that when the substrate concentration was increased to 500 ppm, the degradation became slower.

INTRODUCTION

Chlorobenzenes are important class of aromatic substituted hydrocarbons. They are used in the manufacture of numerous pesticides and insecticides to control the attack of microbes on plants. They are precursors for the manufacture of paints, dyes and industrial solvents. They are introduced into the environment as pesticides applied on farmlands, and enter the food chain through leaching of soil by rainwater. Since, even traces of these chemicals are carcinogenic to human and pollute the environment, especially their potential for bioaccumulation in many food chains, their degradation is essential to prevent the adverse effects on the environment.

Possible fates for chlorobenzenes, released into the environment include volatilization, photooxidation, chemical oxidation, bioaccumulation and adsorption on soil particles. Biodegradation of these compounds is favoured than chemical treatment as the products of degradation of latter are themselves sources of pollution. Growth of microorganisms on chlorobenzenes involves the conversion of the substrates into chlorocatechols via deoxygenase and diol dehydrogenase reactions.

There have been number of studies on the ability of soil and aquatic microorganisms to dissimilate chlorinated aromatic hydrocarbons such as chlorotoluene, chlorobenzenes, chlorobenzoates, chlorophenols, chloroacetamide, 4- chlorophenylacetate and chlorophenoxyacetates (Rasul & Chapalamadugu 1991).

Microbial degradation of chloro-substituted aromatics such as chlorobenzoates, chlorophenols, chlorobenzenes or chlorophenoxyacetates has been described via chlorocatechols as central intermediates, and a catechol 1, 2- dioxygenase with relaxed substrate specificity and high activity against chlorocatechols as a key activity in a variety of those organisms (Andrea et al. 1990).

The two species of *Achromobacter* were used to degrade mono and dichlorobiphenyls, and subsequently a number of investigators have shown that axenic cultures of microorganisms are capable of degrading congeners of polychlorobiphenyls (Ahmed & Focht 1973, Adriaens et al. 1989).

Pseudomonad sp. WR912 was isolated by continuous enrichment in three steps with 3-chloro-, 4-chloro-, and 3, 5-dichlorobenzoate as sole source of carbon and energy and studied for the metabolism of chlorobenzoate (Hartmann et al. 1979). A similar study was carried out on *Acinetobacter* sp. strain 4CB1 from a polychlorobiphenyl polluted soil sample by using 4-chlorobenzoate (Adriaens et al. 1989).

An experiment was conducted to study the microbial degradation of chloroaromatics. In this method toluene and chlorobenzene were used as carbon source for the growth of *Pseudomonas putida* GJ31 and identified the meta-cleavage pathway for mineralization of these compounds (Mars et al. 1997). The bacterium was isolated from groundwater and soils contaminated with chlorobenzene to study the mineralization of toxic compounds and found that approximately 54% of the chlorobenzene was mineralized within 7 days and reported that the strain followed ortho metabolic cleavage pathway (Nishino et al. 1992).

Microbial growth was studied by using environmental contaminants as substrates and confirmed that the biomass increased on subsequent biodegradation of the contaminants (Okpokwasili & Nweke 2005). The microbial reductive dehalogenation was reported on various substances such as alkyl solvents, aryl halides, polychlorinated biphenyls and other xenobiotic compounds (Mohn & Tiedje 1992).

Microbial degradation of a number of recalcitrant, aromatic pollutants, including trichloroguaiacol and di, tri, and pentachlorophenol was conducted using the ^{14}C-labeled compounds and found that dichlorophenol was the most rapidly degraded pollutant (Larsson et al. 1988).

The growth of *Pseudomonas* sp. strain JS150 on phenol, ethyl benzene, toluene, benzene, naphthalene, benzoate, p-hydroxybenzoate, salicylate, chlorobenzene and several 1, 4-dihalogenated benzenes was confirmed and specific experiment was designed based on strain containing the genes for the degradative pathways to study the degradation of multiple substrates simultaneously (Haigler et al. 1992).

In this paper, an investigation has been made on the biodegradation of MCB, using three microorganisms *Pseudomonas aeruginosa, Mycoplana dimorpha* and *Nocardia hydrocarboxydanse* in a batch culture. As in many environmental applications, the goal was to minimize chlorobenzene concentration at the end of the degradation process with the highest biodegradation rate. The degradation was carried out in batches at different initial concentrations of MCB. Comparisons were made to measure the effects of changes in the substrate concentration and microbes degrading them.

MATERIALS AND METHODS

Chemicals: Monochlorobenzene of 99+ % analytical standards and bovine serum albumin were purchased from Ranbaxy Labs**.** The chemicals for the preparation of mineral inoculum medium (ammonium sulphate, ammonium nitrate, calcium chloride, glucose, magnesium sulfate, potassium hydrogen phosphate potassium dihydrogen phosphate and sodium chloride) were of Himedia Chemicals, India which were of analytical reagent grade. Stock solutions of chlorobenzene dissolved in n-butanol were initially established at concentrations of 1000 ppm and then diluted to required concentrations such as 100 ppm, 200 ppm and 500 ppm before being used in the experiments.

Microorganisms: The species selected for the work were *Pseudomonas aeruginosa* (NCIM-2074), *Mycoplana dimorpha* (NCIM-2383) and *Nocardia hydrocarboxydanse* (NCIM-2386). The strains were obtained from NCIM, Pune, India. The strains were cultured on nutrient agar slants and used for further experimentation. Different concentration of chlorobenzene was mixed with the mineral medium (Table 1) to carry out batch kinetic degradation study. The components used were in sterile condition and dissolved in 1000 mL of sterile distilled water. The pH of the solution was adjusted to 7.0 by using 2N H_2SO_4 or 2N NaOH solution. 50 mL of the medium was taken in each of 250mL Erlenmeyer flasks and were sterilized at 1.5 kg/cm^2 (gauge) for 20 minutes. After cooling to room temperature, the medium was inoculated with the strains in a laminar flow chamber. The flasks were then incubated on a rotary shaker for 48 hours at 30°C and 135 rpm for full growth of the strain. The growths of the strains were tested by the optical density values, the subcultured strains were stored at 5°C.

Preparation of degradation medium: 50 mL of the medium was taken in each of the 250 mL Erlenmeyer flasks. The flasks were numbered and were sterilized before use. The initial pH was adjusted to 7.0 and the flasks were sterilized at 1.5 kg/cm^2 (gauge) for 20 minutes. After cooling to room temperature, 1mL of the inoculum was transferred to the growth medium aseptically in a laminar flow chamber. All the flasks were kept on a rotary shaker at 135 rpm speed for growth of the strain.

Preparation of pollutant standards: 100 µL of monochlorobenzene dissolved in 10 mL of n-butanol gives 10000 ppm of MCB. This solution was further diluted using n-butanol to give stock solutions of 100 ppm, 200 ppm and 500 ppm. This is used in calibration charts for measuring concentration of MCB by spectrophotometry. The broth sample (after degradation) containing MCB was centrifuged at 5000 rpm for 20 minutes. The supernatant liquid was separated and 10 mL of butanol was added. The mixture was thoroughly stirred for 10 minutes and allowed to separate as aqueous and organic layers. n-butanol was separated by using a separating funnel.

Experimental design: Batches of experiments were carried out with initial substrate concentration of 100ppm. Different time intervals like 0, 4, 8, 12, 24, 36 and 48 hours were selected to monitor the time course degradation of chlorobenzene. Required number of sterile 250 mL Erlenmeyer flasks was taken. 50 mL of the growth medium was taken in each of the flasks. A known quantity of chlorobenzene was added as a pollutant into the growth medium. It was ensured that there was no other carbon source in the growth medium. To start the degradation, 1 mL of inoculum was added to each flask and the time was noted. All the flasks were kept on the rotary shaker rotating at 135 rpm maintained at an ambient temperature. The flasks were removed at scheduled time and were stored at 5°C to arrest further degradation till the analysis was carried out. The fermented broth was analysed for concentration of residual pollutant which is a measure of degradation, and amount of soluble protein which is measure of growth of microbes due to assimilation of the chlorobenzene.

The procedure was duplicated for different concentrations of chlorobenzene. Monochlorobenzene was used as carbon source for experimentation with microbes namely *Pseudomonas aeruginosa*, *Mycoplana dimorpha* and *Nocardia hydrocarboxydanse.*

Table 1: Composition of mineral medium.

S. No	Ingredients	Concentration
1	NH_4NO_3	1.0 g/L
2	$(NH_4)_2SO_4$	0.5 g/L
3	NaCl	0.5 g/L
4	K_2HPO_4	1.5 g/L
5	KH_2PO_4	0.5 g/L
6	$MgSO_4.7H_2O$	0.5 g/L
7	$CaCl_2$	0.01 g/L
8	Double distilled water	1 L

Analysis of residual concentration of MCB: The residual chlorobenzene in the broth was analysed by spectrophotometry using UV-Vis spectrophotometer. The broth sample containing MCB was centrifuged at 5000 rpm for 20 minutes. The supernatant liquid was separated and 10 mL of n-butanol was added. The mixture was thoroughly stirred for 10 minutes and allowed to separate into organic and aqueous layers. The n-butanol was separated using a separating funnel and the light absorbance was read at 264.4 nm.

Analysis of soluble protein: The soluble protein is measure of growth of the microorganism in the pollutant medium. Its estimation is done by Lowry's method by using crystalline bovine serum albumin.

RESULTS AND DISCUSSION

Degradation of MCB by *Pseudomonas aeruginosa*

Fig. 1 shows the time course of degradation of MCB at different concentrations by *Pseudomonas aeruginosa*. It was found that with an initial concentration of 100 ppm of MCB, 40% of MCB is degraded within 12 hr. Further degradation of MCB is slow. At the end of 48 hours of observation, only 50% is removed. Comparatively, with an initial concentration of 200 ppm, 85% is degraded fast up to 4 hrs and there is decrease in the rate of degradation thereafter. The total removal of MCB was 86% at the end of 48 hrs. With the initial concentration of 500 ppm of MCB 62% was degraded fast within 8 hrs. Further degradation was slow. The overall removal of MCB was 64% in 48 hrs.

From these observations it is evident that *Pseudomonas aeruginosa* has shown better growth on MCB as a sole source of carbon. It is also noticed that the growth of strain is very fast in the beginning of the degradation. The consumption of MCB in 48 hrs is appreciable for these concentrations. The comparison of degradation ability of *P. aeruginosa* with different initial MCB concentrations shows that around 50-86% of MCB is degraded within 48 hrs.

Growth of *Pseudomonas aeruginosa* on MCB

Fig. 2 shows the growth kinetics of the strain on different concentrations of MCB. The growth of

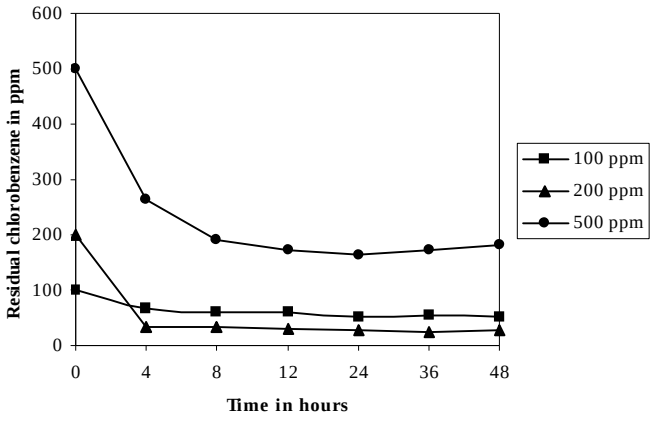

Fig. 1 Degradation of chlorobenzene at 100, 200, 500 ppm by *Pseudomonas aeruginosa*

organism is indirectly determined by estimation of soluble protein. After an initial lag period of 2 hours, there is a continuous increase of 30 % in biomass at the end of 48 hours. Similar observations were made with 200 and 500 ppm of MCB. On comparison of growth at three-concentration level, it was found that in all cases the growth of *Pseudomonas aeruginosa* was similar.

Degradation of MCB by *Mycoplana dimorpha*

Fig. 3 shows the time course of degradation of MCB at different concentrations by *Mycoplana dimorpha*. It was found that 8% of MCB at an initial concentration of 100 ppm is degraded very fast within 4 hours. The degradation of MCB later was slow and from 12th hour, no further appreciable degradation was found up to 48 hrs. At the end of 48 hours, 50% of initial MCB was removed.

Similarly with 200 ppm of initial concentration of MCB, 25 % was degraded fast within the first four hours. The total removal of MCB was 45% for a 48 hour period by *Mycoplana dimorpha*. When the initial concentration of MCB was 500 ppm, only 33 % degraded fast within 4 hrs. The overall removal of MCB was 77% for 48 hours.

From these observations, it is apparent that *Mycoplana dimorpha* has shown degradation capability. On comparison of degradation ability of *M. dimorpha* at different MCB concentrations, it shows that around 50-77 % of MCB is degraded at the ranges of initial concentration of 100 to 500 ppm.

Growth of *Mycoplana dimorpha* on MCB

Fig. 4 shows the growth kinetics of the strain of MCB. After a lag period of 4 hours, a maximum of 17 % increase in biomass at the end of 48 hour was obtained. Similar results were obtained with 200 and 500 ppm of MCB with biomass formation of 64.51 and 62.23 µg/mL. *M. dimorpha* has shown similar growth with three concentrations.

Degradation of MCB by *Nocardia hydrocarboxydanse*

Fig. 5 gives the time course degradation of MCB at different concentration by *Nocardia*

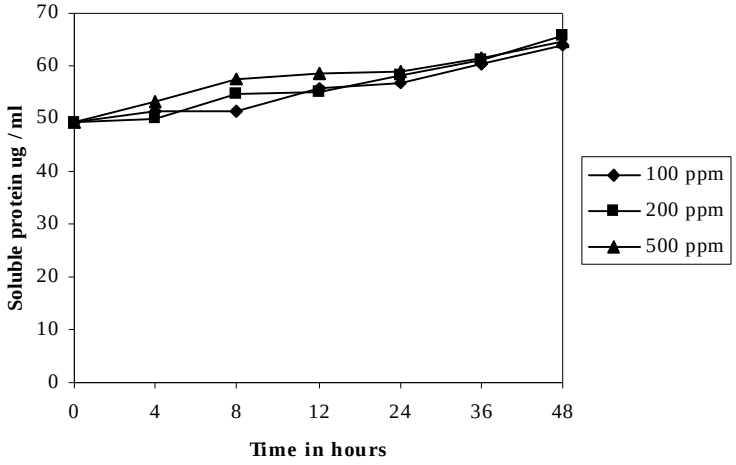

Fig. 2 Growth Kinetics of *Pseudomonas aeruginosa* at different concentration of chlorobenzene

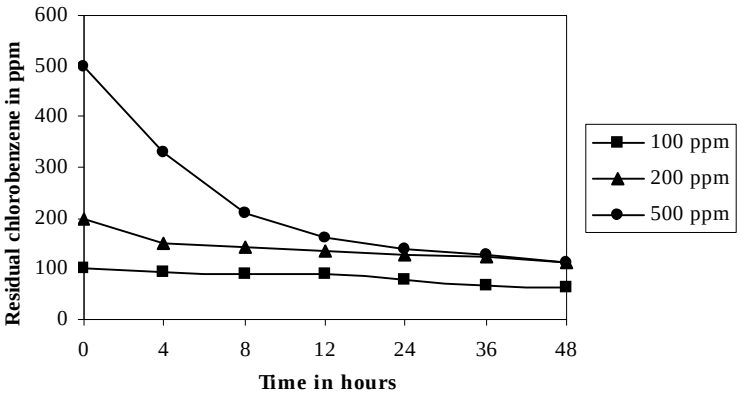

Fig. 3 Degradation of chlorobenzene at 100, 200, 500 ppm by *Mycoplana dimorpha*

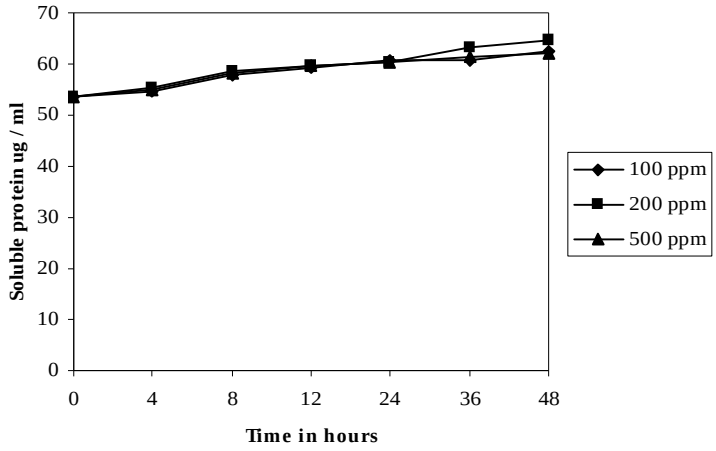

Fig. 4 Growth Kinetics of *Mycoplana dimorpha* at different concentration of chlorobenzene

hydrocarboxydanse. It was found that 32% of initial concentration of 100 ppm of MCB is degraded within 4 hours. Further degradation of MCB is slow with 40% and levels off up to 48 hours. The overall degradation is 42% in 48 hours.

With 200 ppm of MCB initial concentration, 57% was degraded fast within 6 hour and later there was slow degradation up to 48 hr. The total removal of MCB was 63% for 48 hr by *N. hydrocarboxydanse*.

When the initial concentration of MCB is 500 ppm, 50% is degraded fast within 4 hrs. The overall removal of MCB was 79 % for 48 hour period experimentation.

From the above observations, it is experienced that *N. hydrocarboxydanse* has shown less degradation in the beginning. The strain has shown appreciable consumption of MCB in 48 hrs for three concentrations. On comparison of degradation ability of *N. hydrocarboxydanse* at different MCB concentrations it shows that around 48 to 79% of MCB is degraded in the range of 100 to 500 ppm.

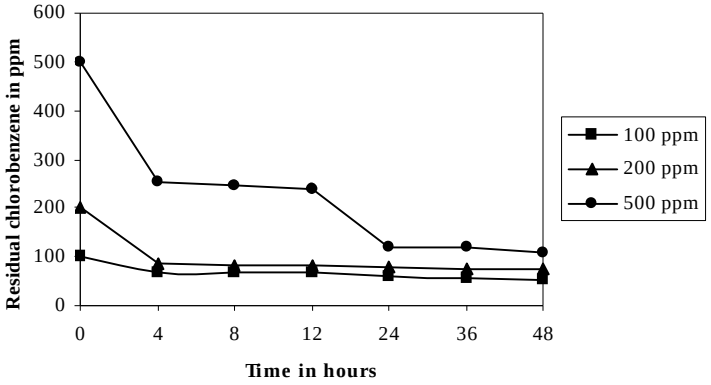

Fig. 5 Degradation of chlorobenzene at 100, 200, 500 ppm by *Nocardia hydrocarboxydanse*

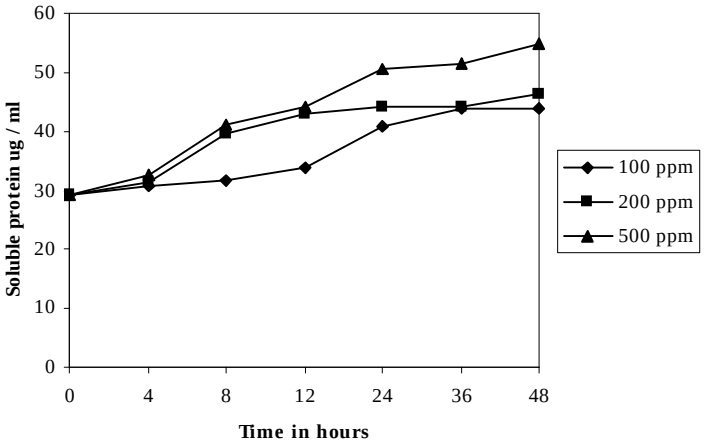

Fig. 6 Growth Kinetics of *Nocardia hydrocarboxydanse* at different concentration of chlorobenzene

Growth of *Nocardia hydrocarboxydanse* on MCB

Fig. 6 presents the growth kinetics of the strain *Nocardia hydrocarboxydanse* with different concentrations of MCB. The growth of organism was determined by formation of soluble protein. A lag period of 2 hrs was observed, later increased to give a maximum of 43.82 µg/mL at the end of 48 hours. Similar observations were made with an initial concentration of 200 and 500 ppm of MCB with maximum of 46.17 µg/mL and 54.78 µg/mL of biomass at the end of 48 hours respectively.

CONCLUSION

Three bacterial strains have been selected to study the degradation of MCB and p-DCB. The following conclusions were drawn:

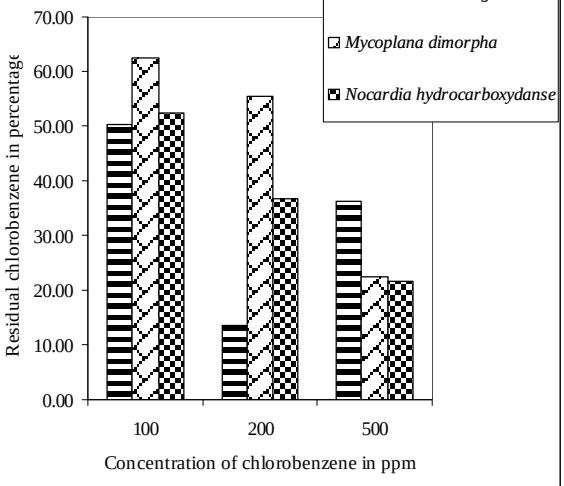

Fig. 7 Comparison of degradation ability of the microbes to degrade chlorobenzene at different concentration

Pseudomonas aeruginosa, *Mycoplana dimorpha* and *Nocardia hydrocarboxydanse* can be used to degrade monochlorobenzene. The degradation was faster in the initial stages of 4 hours and slowed down thereafter. No further degradation was noticed beyond 48 hrs.

The percentage of degradation in 48 hours by the three microorganisms is shown in Fig. 7. Growth of the three microorganisms exhibited the general characteristics *viz.*, lag, exponential growth and stationary phases. The degrading ability of *Pseudomonas aeruginosa* is better than the other strains for all the concentrations of MCB but the performance of *Mycoplana dimorpha* is better at 500 ppm level of substrate. Thus, the selected strains have the capacity to grow on chlorobenzene as a sole source of carbon and energy.

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