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STUDIES ON DEGRADATION OF CHLOROBENZENE BY IMMOBILIZED CRUDE EXTRACT OF *NOCARDIA HYDROCARBONOXYDANS*

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ABSTRACT

In this work *Nocardia hydrocarbonoxydans* has been identified as potential organism to decompose chlorobenzene by its crude extract through immobilization technique. *Nocardia hydrocarbonoxydans* was grown on chlorobenzene as a sole source of carbon and energy. Chlorobenzene was used as an inducer to develop specific intracellular enzymes which will decompose chlorobenzene to nontoxic substance. Crude extracts of the cell contain intracellular enzyme, which were immobilized on sodium alginate beads. The beads were mixed with different concentrations (200, 250, 300 ppm) of chlorobenzene to study the kinetics of degradation of chlorobenzene. The rate of decomposition of chlorobenzene by immobilized crude extracts was measured at different time intervals and it was found that 86 to 96 percent of chlorobenzene can be decomposed in less than ten minutes. The immobilized crude extracts were reused for all other experiments and found that immobilization technique can be used for higher capacity conversion for scale up process.

INTRODUCTION

Of the numerous chemical substances that enter the environment with wastewater and exhaust, great numbers are benzene derivatives and other nonpolar aromatics (Walter et at. 1984). The synthetic chemicals which carry chlorinated aromatic nuclei are released into ecosystems as solvents, lubricants, insulation and hydraulic fluids, biocides, herbicides, plastics, degreasers, synthetic waste products, and others (Erich et al. 1978).

The deleterious effects of aromatic, chlorinated hydrocarbons on natural environments created major concern on surroundings. Although acute toxicity is uncommon, these substances cause sublethal damage, e.g., reduced reproduction and physiological disturbances, to a wide range of organisms, thereby reducing the competitive abilities of organisms. Another distinctive feature of the synthetic chlorinated hydrocarbons is their environmental persistence (Larsson et al. 1988). The capability to utilize aromatic compounds with even complex structures as the sole source of carbon and energy is not an unusual property of microorganisms (Erich et al. 1978).

There have been number of studies cited in journals 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 Chaudhry & 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 was identified 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).

Pseudomonas 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 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). Another study was carried out by using *Pseudomonas* sp. WR912 with 3-chloro, 4-chloro-, and 3, 5-dichlorobenzoate as sole source of carbon and energy (Adriaens et al. 1989). 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).

Although number of workers described about the microbial degradation, limited literature is available on immobilized enzymatic degradation. In the present investigation, crude cell extracts from the enriched strain *Nocardia hydrocarbonoxydans* was immobilized on sodium alginate beads and chlorobenzene was used as substrate to study the degradation.

MATERIALS AND METHODS

Chemicals: Chlorobenzene (CB) of 99+ % analytical standards was purchased from S.D. Fine-Chem. Ltd. The chemicals for the preparation of mineral inoculum medium (ammonium sulphate, ammonium nitrate, calcium chloride, glucose, magnesium sulphate, potassium hydrogen phosphate potassium dihydrogen phosphate and sodium chloride) were purchased from Himedia Chemicals, India which are of analytical reagent grade. The bovine serum albumin used for assay and nutrient agar was obtained from Ranbaxy Labs. Sulphuric acid and sodium hydroxide used for calibration of pH were also purchased from Himedia Chemicals.

Maintenance and cultivation of microorganism: The strain *Nocardia hydrocarbonoxydans* was obtained from NCIM, Pune, India. The strain was subcultured in nutrient broth (Table 1). The broth was incubated in the shaker with 135 rpm and at 37°C overnight. Sterile plates containing nutrient agar (Table 2) of specified composition were streak plated with the overnight cultures. The culture on the plates was used as the source for the entire experiment. The mineral medium with specified composition (Table 3) of chemical substances was prepared to conduct the experiment. The pH of the mineral medium 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 250 mL Erlenmeyer flasks and sterilized at 1.5 kg/cm^2 (gauge) for 20 minutes. After cooling to room temperature, the medium was inoculated in a laminar flow chamber. The flasks were then incubated on a rotary shaker for 48 hours at 30ºC and 135 rpm for full growth of the strain. The growth of the strains was tested by the optical density values; the subcultured strains were stored at 5 ºC.

Inoculum preparation and fermentation conditions: The inoculum was prepared by transferring a loopful of cells from a freshly grown culture into 50 mL of a mineral medium with known concentration of chlorobenzene in 250 mL Erlenmeyer flasks. The flasks were incubated on a rotary shaker at 135 rpm for 24h at 37°C. An aliquot (1 mL) of the culture broth was added aseptically to the 250 mL Erlenmeyer flasks containing 49 mL of mineral medium with known concentration of chlorobenzene identical to that of the inoculum preparation. The bacterial growth was estimated by collecting the samples at regular intervals (30 min) through optical density (OD) measurements using UV visible spectrophotometer (Model Hitachi UV 2800). The growth of *Nocardia hydrocarbonoxydans* was plotted as OD at 380 nm against time.

Table 1: Composition of nutrient broth.

S. No	Components	Composition
1	Beef extract	10 g
2	NaCl	5g
3	Peptone	10 g
4	Double distilled water	1 L

Table 2: Composition of Nutrient Agar medium.

S. No	Components	Composition
1	Beef extract	10 g
2	NaCl	5g
3	Peptone	10 g
4	Double distilled water	1 L
5	Agar	20 g

Table 3: Composition of mineral medium

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

Suspension of washed cells and cell extracts: Cells grown on chlorobenzene as the sole carbon source, were harvested in the mid-exponential growth phase by centrifugation (10,000 rpm for 10 min at 4°C), washed with sodium phosphate buffer (pH 7.0, 50 mM), and suspended in the same buffer. The cell extracts were prepared by disrupting the cells by ultrasonic disintegration (Labsonic-P of Labsonic-Germany). The resulting cell lysate was centrifuged at 10,000 rpm for 15 min at 4°C, and the supernatant, containing approximately 10 to 20 mg of protein per mL, was the crude cell extract (containing chlorobenzene degrading enzyme). The concentrations of protein content in the crude extracts were measured using UV visible spectrophotometer with the aid of standard plot of BSA at 280 nm.

Immobilization of crude cell extract in sodium alginate beads and batch kinetic studies on biodegradation: The immobilization of crude cell extract was carried out in sodium alginate solution. Aliquots (4 mL) of crude cell extracts were

mixed with 100 mL of 3 %(w/v) sodium alginate solution. The crude cell extract suspensions in the alginates were immobilized by extruding drop wise into 2% (w/v) $CaCl_2$ solutions to form immobilized beads (3 mm diameter). After maintaining in the 2% (w/v) $CaCl_2$ solution for 24 h at 5°C, the beads were washed with distilled water.

Samples (20 g) of beads were transferred to 250 mL Erlenmeyer flasks containing 50 mL of 200 ppm chlorobenzene solution. The flasks were incubated in batches at 35°C with agitation at 135 rpm. Aliquots were collected after 1, 2, 3, 4, 5, 6, and 7 min. The analysis of residual amount of chlorobenzene was carried out using UV visible spectrophotometer. The entire enzymatic studies were carried out at an ambient temperature. The samples were collected after every minute and analysed for residual substrate chlorobenzene concentration. The same procedure was repeated to conduct experiments for 250 and 300 ppm of chlorobenzene.

RESULTS AND DISCUSSION

Growth of *Nocardia hydrocarbonoxydans* on chlorobenzene: Fig. 1 shows the growth kinetics of the strain on CB with initial concentration of 50 ppm. After an initial lag period of 4 hours, there was exponential growth up to 12 hours. The growth of *Nocardia hydrocarbonoxydans* was measured up to 24 hours.

Degradation of CB by crude cell extracts of *Nocardia hydrocarbonoxydans*: Figs. 2 to 4 show the time course of degradation of CB at different concentrations by immobilized *Nocardia hydrocarbonoxydans* cell extracts. It is observed from the Fig. 2 that the enzymatic decomposition of CB with an initial concentration of 200 ppm was rapid up to 53% within 1 minute. After this period, the decomposition is very slow reaching 86% for the maximum period of 7 minutes, whereas the microbial degradation time is 4 hours for 85% as reported (Manikandan et al. 2005). This observation indicates that immobilized crude extract degradation is much faster than microbial degradation. The reason for slow decomposition after 4 minutes may be due to formation of products which inhibit the enzymatic decomposition. The similar trend of decomposition (Fig. 3 and 4) was observed for the concentration of 250 and 300 ppm with the percentage decomposition at the end of 7 minutes at 95% for 250 ppm and 96 % for 300 ppm. The reason for lower percentage of decomposition at higher concentration of chlorobenzene may be due to substrate inhibition. Figs. 5 and 6 show the comparative study of chlorobenzene degradation with different initial concentration level.

The experiment was also carried out to find the degradation of chlorobenzene by mixing with beads without crude extract. The data obtained from this experiment revealed that there was no change in concentration. This proved the presence of degrading enzymes in the crude extract. We investigated that maximum of 96 percent of chlorobenzene can be decomposed within 7 minutes without accumulation of other treated waste in the environment.

CONCLUSION

The crude cell extract were immobilized on sodium alginate to study the degradation of CB at various initial concentrations. The degradation is much faster in the initial stages of 4 minutes and slows down thereafter. The total reaction time is less than 10 minutes for the decomposition up to 86% to 96% depending on the initial chlorobenzene concentration. This method can be used effectively for industrial effluent treatment.

Another observation was made that chlorobenzene can be used to induce the production of

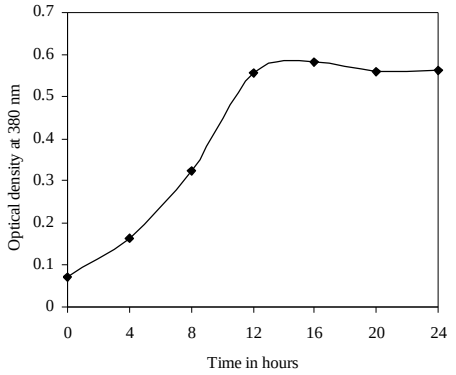

Fig. 1: Growth of *Nocardia hydrocarbonoxydans* with 2,4-dichlorophenol as sole source of carbon and energy.

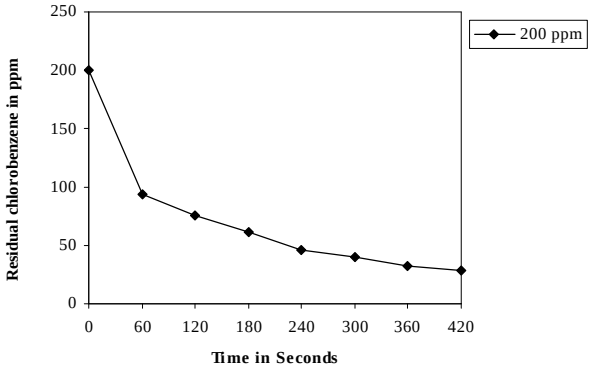

Fig. 2: Immobilized enzymatic degradation of chlorobenzene at 200 ppm.

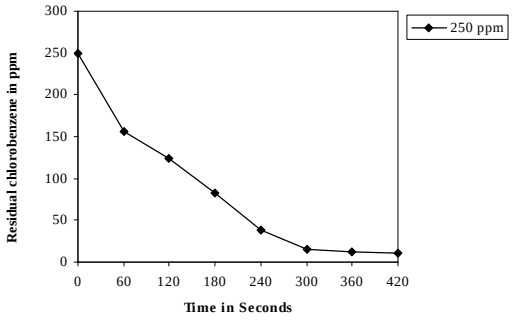

Fig. 3: Immobilized enzymatic degradation of chlorobenzene at 250 ppm.

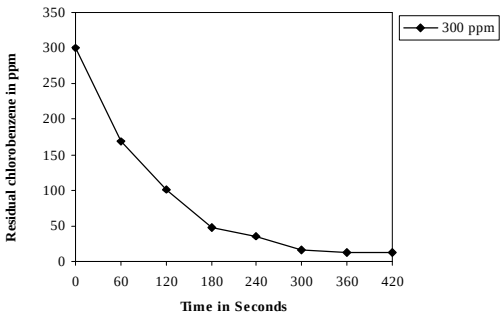

Fig. 4: Immobilized enzymatic degradation of chlorobenzene at 300 ppm.

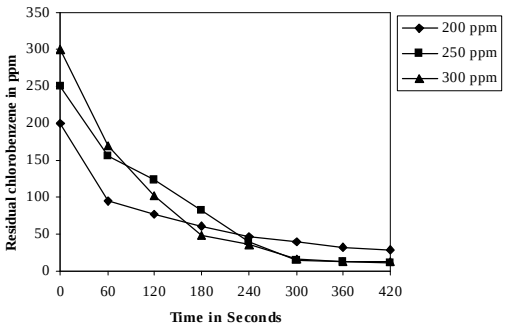

Fig. 5: Immobilized enzymatic degradation of chlorobenzene at 200, 250, 300 ppm.

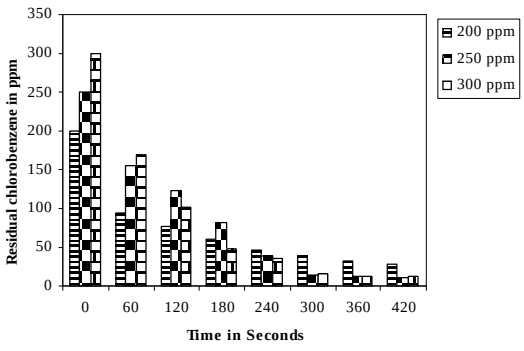

Fig. 6: Immobilized enzymatic degradation of chlorobenzene at 200, 250, 300 ppm.

metabolites in *Nocardia hydrocarbonoxydans.* These cells can further be grown on chlorobenzene, and cell extracts can be used to degrade chlorinated aromatic compounds. This novel method is very fast when compared to conventional and microbial degradation, and the process can be recycled with proper conditions.

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