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Full Length Research Paper

Studies on degradation of chlorinated aromatic hydrocarbon by using immobilized cell crude extract of *Pseudomonas aeruginosa*

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In this work *Pseudomonas aeruginosa* (NCIM-2074) has been identified as potential organism to decompose chlorobenzene by its crude extract through immobilization technique. *P. aeruginosa* was grown on chlorobenzene as sole source of carbon and energy. Chlorobenzene was used as an inducer to develop specific intracellular enzymes which will decompose chlorobenzene to non-toxic substances. Crude cell extracts produced which contains intracellular enzyme, were immobilized on sodium alginate beads. The beads were mixed with different concentration (100, 200, 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 93 to 95 percentage of chlorobenzene can be decomposed with in 10 min. The immobilized crude extracts were reused for all other experiments and found that immobilization technique can be used for higher capacity conversion for the scale up process.

Key words: Chlorobenzene, decomposition, cell crude extract, *Pseudomonas aeruginosa*, immobilization.

INTRODUCTION

Of the numerous chemical substances that enter the environment with wastewater and exhaust, great numbers are benzene derivatives and other non-polar aromatics (Walter and Hans-Joachim, 1984). The synthetic chemicals that carries chlorinated aromatic nuclei are released into the ecosystem as solvents, lubricants, insulation and hydraulic fluids, biocides, herbicides, plastics, degreesers, 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 micro-organisms (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 (Chaudhry and 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). 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 and 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

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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 chloro aromatics. 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 *Pseudomonad* sp. WR912 with 3-chloro-, 4-chloro-, and 3, 5-dichlorobenzoate as sole source of carbon and energy. 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 and Nweke, 2005). The microbial reductive dehalogenation was reported on various substances such as alkyl solvents, aryl halides, polychlorinated biphenyls and other xenobiotic compounds (Mohn et al., 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 a number of workers described microbial degradation of aromatic hydrocarbon, limited literature is available on immobilized enzymatic degradation. In the present investigation, crude cell extracts from the enrichhed strain *Pseudomonas aeruginosa* on monochlorobenzene was immobilized on sodium alginate beads and monochlorobenzene was used as substrate to study the degradation. The experiment was carried out in different concentrations of mono chlorobenzene in the immobilized beads which contains crude extracts of *P. aeruginosa*.

MATERIALS AND METHODS

Chemicals

Monochlorobenzene of 99+ (%) analytical standards was purchased from Ranbaxy Laboratory. The chemicals for the preparation of mineral inoculum medium (Ammonium sulfate, ammonium nitrate, calcium chloride, glucose, magnesium sulfate, potassium hydrogen phosphate potassium dihydrogen phosphate and sodium chloride) were purchased from Himedia Chemicals, India, and are of analytical reagent grade. The bovine serum albumin used for assay and Nutrient agar was obtained from Ranbaxy labs. Sulfuric acid and sodium hydroxide used for calibration of pH were also purchased from Himedia Chemicals. Stock solutions of chlorobenzenes dissolved in n-butanol were initially established at concentrations of 1000 ppm, and then diluted to required concentrations such as 100, 200 and 500 ppm before being used in the experiments.

Table 1. Composition of nutrient broth.

Component	Composition
Beef extract	10 g
NaCl	5 g
Peptone	10 g
Double distilled water	1 L

Table 2. Composition of nutrient medium.

Ingredient	Concentration
NH_4NO_3	1.0 g/L
$(NH_4)_2SO_4$	0.5 g/L
NaCl	0.5 g/L
K_2HPO_4	1.5 g/L
$KH_2 PO_4$	0.5 g/L
$MgSO_4.7H_2O$	0.5 g/L
$CaCl_2$	0.01 g/L
Double distilled water	1 L

Table 3. Composition of the nutrient agar.

Component	Composition
Beef extract	10 g
NaCl	5 g
Peptone	10 g
Double distilled water	1 L
Agar	20 g

Maintenance and cultivation of microorganisms

The strain *P. aeruginosa* was obtained from NCIM, Pune, India. The strain was sub cultured in nutrient broth (Table 1) and inoculated in the mineral medium (Table 2). The broth was incubated in the shaker with 135 rpm and at 37°C overnight. Sterile plates containing nutrient agar (Table 3) 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 above components were in sterile condition and were dissolved in 1000 ml of sterile distilled water. The pH of the solution was adjusted to 7.0 by using 2 N H_2SO_4 or 2 N NaOH solution. 50 ml of the medium was taken in each of 250 ml Erlenmeyer flasks and were sterilized at 1.5 kg/cm^2 (gauge) for 20 min. After cooling to room temperature, the medium was inoculated with the strains in a laminar flow chamber.

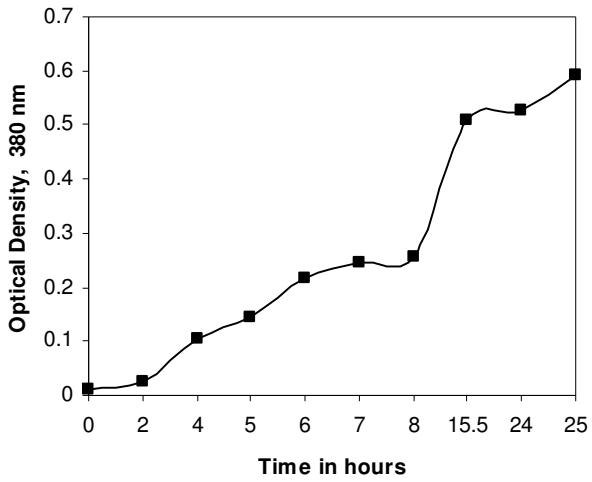

Figure 1. Growth of *Pseudomonas aeruginosa* with glucose as sole of carbon and energy.

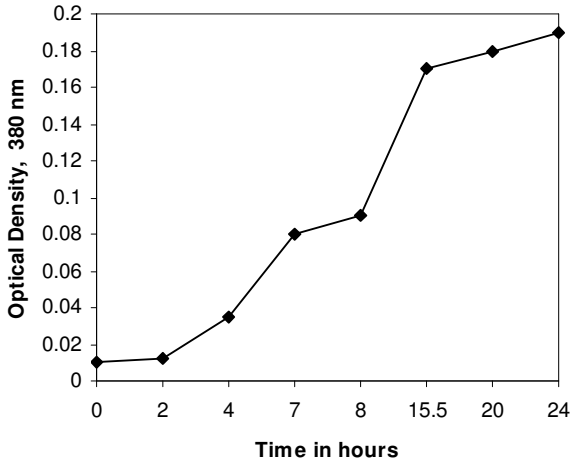

Figure 2. Growth of *Pseudomonas aeruginosa* with chlorobenzene as sole of carbon and energy.

The flasks were then incubated on a rotary shaker for 48 h at 30ºC and 135 rpm, for full growth of the strain. The growth of the strains was tested by the optical density values, the sub cultured strains were stored at 5ºC.

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 (Table 1) with known concentration of chlorobenzene in 250 ml Erlenmeyer flasks. The flasks were incubated in a rotary shaker at 135 rpm for 15 h 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. This procedure is also followed to grow *P. aeruginosa* with chlorobenzene and glucose to find out the growth pattern for different substrates.

The bacterial growth was estimated through optical density (OD) measurements using Hitachi UV 2800 UV Visible Spectrophotometer. The growth of *P. aeruginosa* was plotted as OD at 600 nm against time. After inoculation of 2% overnight inoculum into 250 ml Erlenmeyer flasks containing mineral medium with glucose, the flasks were incubated at 37°C with 135 rpm. At regular intervals (30 min), samples were collected from the flask and the optical density was measured with mineral medium as blank solution without the inoculum. The same procedure is used for growth measurements using chlorobenzene as carbon source but the time interval between measurements was increased because the growth observed was low.

Suspensions 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. For the preparation of cell extracts, cells were disrupted 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 ml^{-1}, was the crude cell extract.

Crude cell extract immobilization in sodium alginate and batch kinetic studies with immobilized cell extract

The immobilization of crude cell extract was carried out in sodium alginate solution with crude cell extract. Aliquots (4 ml) of crude cell extracts were mixed with 100 ml of 1.5 and 3% (w/v) sodium alginate solutions. 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 100 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, 7 and 8 min. The residual amount of chlorobenzene analyses were carried out using Hitachi UV 2800 UV Visible Spectrophotometer. The entire enzymatic studies were carried out at an ambient temperature. The samples were collected after every min and analyzed for residual substrate chlorobenzene concentration. The same procedure repeated to conduct experiments for 200 and 300 ppm of chlorobenzene.

RESULTS AND DISCUSSION

Growth of *Pseudomonas aeruginosa* on glucose

Figure 1 shows the growth kinetics of the strain on glucose with initial concentration of one gram per liter. The growth of organism is indirectly determined by estimation of soluble protein. After an initial lag period of 2 h, there was exponential growth up to 15.5 h with lag at 8 h. The growth of *P. aeruginosa* was measured up to 25 h.

Growth of *Pseudomonas aeruginosa* on chlorobenzene

Figure 2 shows the growth kinetics of the strain on chlorobenzene with initial concentration of 200 ppm. The growth of organism was indirectly determined by estimation of soluble protein. After an initial lag period of 2 h,

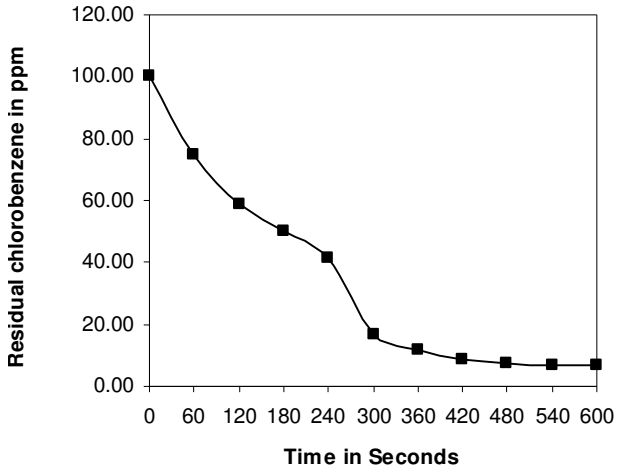

Figure 3. Degradation of chlorobenzene at 100 ppm by immobilized crude cell extract of *Pseudomonas aeruginosa*.

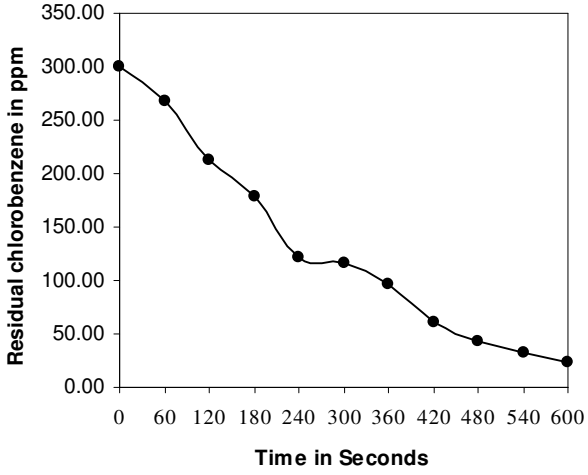

Figure 5. Degradation of chlorobenzene at 300 ppm by immobilized crude cell extract of *Pseudomonas aeruginosa*.

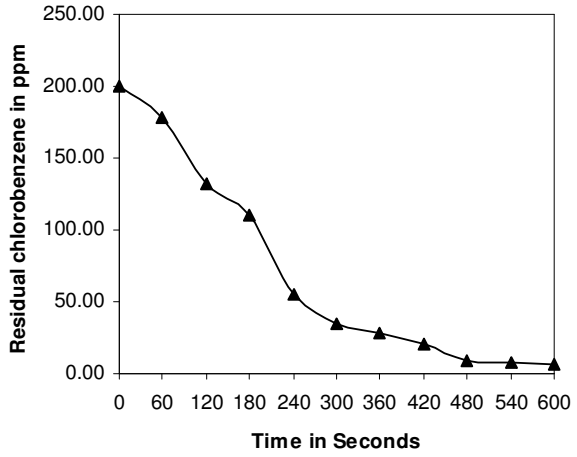

Figure 4. Degradation of chlorobenzene at 200 ppm by immobilized crude cell extract of *Pseudomonas aeruginosa*.

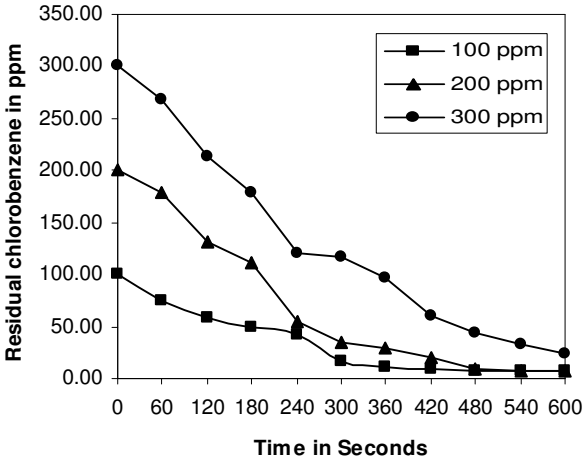

Figure 6. Degradation of chlorobenzene at 100, 200 and 300 ppm by immobilized crude cell extract of *Pseudomonas aeruginosa*.

there was exponential growth up to 15.5 h with lag at 8 h. The growth of *P. aeruginosa* was measured up to 24 h.

Degradation of chlorobenzene by crude cell extracts of *Pseudomonas aeruginosa*

The experimental results obtained in present study are discussed in this section. Figures 3 to 5 shows the time course of degradation of chlorobenzene at different concentrations by immobilized *P. aeruginosa* cell extracts. It is observed from the Figure 3 that the enzymatic decomposition of chlorobenzene with an initial concentration of 100 ppm was rapid up to 50% within 4 min. After this period, the decomposition is very slow reaching 93% for the maximum period of 10 min, whereas the microbial degradation time is 4 h for 85% (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 min may be due to formation of products that inhibits the enzymatic decomposition. The similar trend of decomposition (Figures 4 and 5) was observed for the concentration of 200 and 300 ppm with the percentage decomposition at the end 10 min is 97% for 200 ppm and 92% for 300 ppm. The reason for lower percentage decomposition at higher chlorobenzene concentration may be due to substrate inhibition. Figures 6 and 7 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

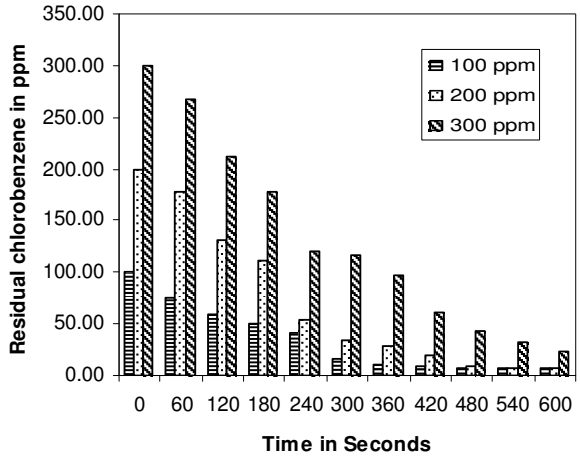

Figure 7. Degradation of chlorobenzene at 100, 200 and 300 ppm by immobilized crude cell extract of *Pseudomonas aeruginosa.*

revealed that there was no change in concentration. This proved the presence of degrading enzymes in the crude extract. We observed that a maximum of 95% of chlorobenzene can be decomposed within 10 min and without accumulation other treated waste in the environment.

Conclusion

An attempt has been made to decompose chlorobenzene by immobilized crude cell extract method. The crude cell extract were immobilized on sodium alginate to study the degradation of chlorobenzene at various initial concentrations. The degradation is much faster in the initial stages of 4 min and slows down after this period. The total reaction time is less than 10 min for the decomposition up to 93% to 97% depending on the initial monochlorobenzene 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 metabolites in *P. aeruginosa*. 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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