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Full Length Research Paper

Biodegradation of chlorobenzene using immobilized crude extracts in packed bed column

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Pseudomonas aeruginosa **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 substance. Crude cell extracts produced which contains intracellular enzyme, were immobilized on sodium alginate beads. The beads were packed in a glass column and added with different initial concentration (30, 40 and 50 ppm) of chlorobenzene to study the kinetics of degradation. Nine sets of combinations of process variables were developed and used to produce crude extracts keeping other parameters constant. The rate of decomposition of chlorobenzene by immobilized crude extracts was measured at different time intervals and found that 80 to 83% of chlorobenzene can be decomposed with in 35 min. The immobilized crude extracts were reused for all other experiments and found that immobilization technique can be used for higher capacity conversion of scaled up process.**

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

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, degreasers, synthetic waste products and others (Erich and Hans-Joachim, 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 and Hans-Joachim, 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 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).

The two species of *Achromobacter* were used to degrade mono- and dichlorobiphenyls, and subsequently a number of investigators have shown that axenic cul-

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Table 1. Composition of mineral 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

tures 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 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 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 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. Catechol-2,3-dioxygenase from toluene-grown cells of *P. putida* catalyzed the stoichiometric oxidation of 3-methylcatechol to 2-hydroxy-6-oxohepta-2, 4-dienoate (Klecka and Gibson, 1981)

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 and 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 *P. aeruginosa* on chlorobenzene was immobilized on sodium alginate beads and the beads were packed in a glass column to study the degradation. Nine sets of combinations of process parameters were developed to produce crude extracts. The experiment was carried out in different concentrations chlorobenzene in the immobilized beads which contains crude extracts of *P. aeruginosa*.

MATERIALS AND METHODS

Chemicals

Chlorobenzene (CB) of 99+% analytical standards was purchased from S.D. Fine- chem. Ltd The chemicals required for the preparation of mineral medium and inoculum (ammonium sulfate, ammonium nitrate, calcium chloride, glucose, magnesium sulfate, 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. Sulfuric acid and sodium hydroxide used for calibration of pH were also purchased from Himedia Chemicals.

Maintenance and cultivation of microorganism

The strain *P. aeruginosa* was obtained from NCIM, Pune, India. The strain was sub cultured in nutrient broth. The broth was incubated in the shaker with 135 rpm and at 37°C overnight. Sterile plates containing nutrient agar 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 1) of chemical substances was prepared to conduct the experiment. The pH of the mineral medium was adjusted to 7.0 by using 2 N H_2SO_4 or 2N 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 in a laminar flow chamber. 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 with known concentration of chlorobenzene in 250 ml Erlenmeyer flasks. The flasks were incubated in a rotary shaker at 135 rpm for 24 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 preparation. The bacterial growth was estimated by collecting the samples at regular intervals (30 min), through optical density (OD) measurements using spectrophotometer (Hitachi UV 2800). The growth of *P. aeruginosa* was plotted as OD at 380 nm

Table 2. Specification of column.

Parameter	Values
Diameter of column	3.1 cm
Height of column	41 cm
Capacity of column	280 ml
Beads bed height	18 cm

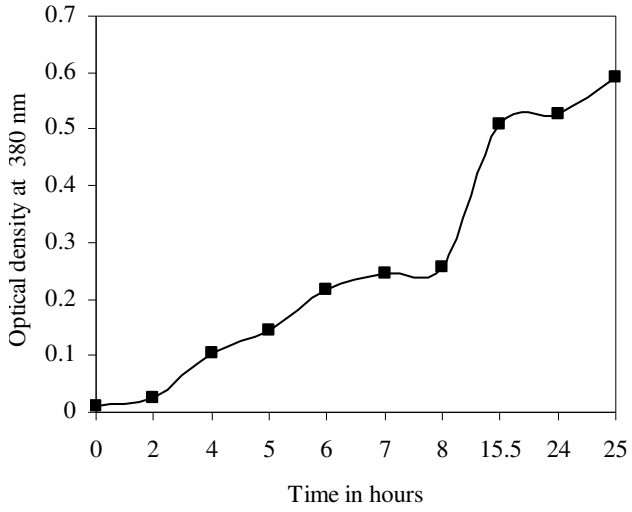

Figure 1. Growth of *Pseudomonas aeruginosa* with glucose as sole of carbon and energy.

against time.

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 ml^{-1}, was the crude cell extract (containing chlorobenzene degrading enzyme). The concentrations of protein content in the crude extracts were measured using UV Visible Spectrophotometer (Hitachi UV 2800).

Immobilization of crude cell extract in sodium alginate and batch kinetic studies of 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 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 (110 g) of beads were transferred to the specified column (Table 2) for the degradation study.

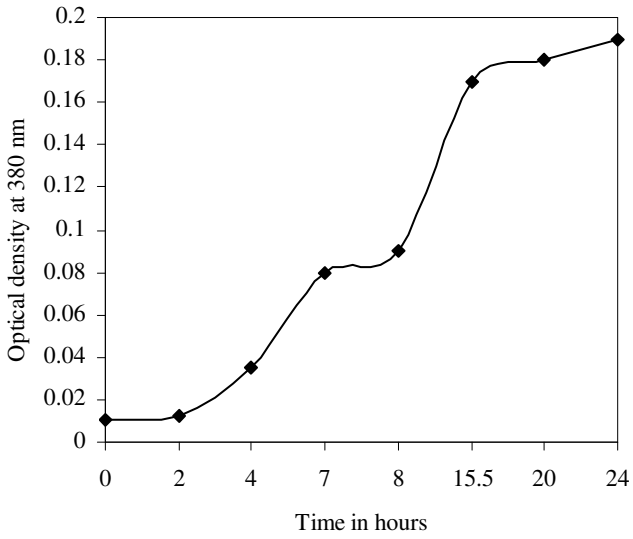

Figure 2. Growth of *Pseudomonas aeruginosa* with chlorobenzene as sole of carbon and energy.

Chlorobenzene solution of 30 ppm was prepared and added into the column so that the all the beads were submerged in it. The solution was allowed to continue in the packed bed column for 5 min and then removed from the column. The optical density of the residual amount of chlorobenzene was measured at 263 nm (λ_{max} of chlorobenzene) using UV Visible Spectrophotometer (Hitachi UV 2800). The entire enzymatic studies were carried out at an ambient temperature. The same procedure was repeated by varying the retention time (5, 10, 15, 20, 25 and 30 min) with different initial concentration (40 and 50 ppm) of chlorobenzene in the column. It was found that the optical density values decreased as the retention time increased.

An attempt has been made to produce crude extract under different process conditions. Nine set of combinations (Table 3) with 3 process variables were developed to produce crude extracts. The process variables, mineral medium, Inducer and inoculum were processed to produce extracts and immobilized sodium alginate beads as mentioned in this paper.

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. 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 the organism was indirectly determined by estimation of soluble protein. From the graph, it was evi-

Table 3. Combinations of process variables.

Combination No.	Experiment No.	Medium (ml)	Inducer (ml)	Inoculum (ml)
1	1	25	1	3
2	2	25	2	1
3	3	25	3	3
4	4	50	1	3
5	5	50	2	3
6	6	50	3	2
7	7	60	1	3
8	8	60	2	1
9	9	60	3	1

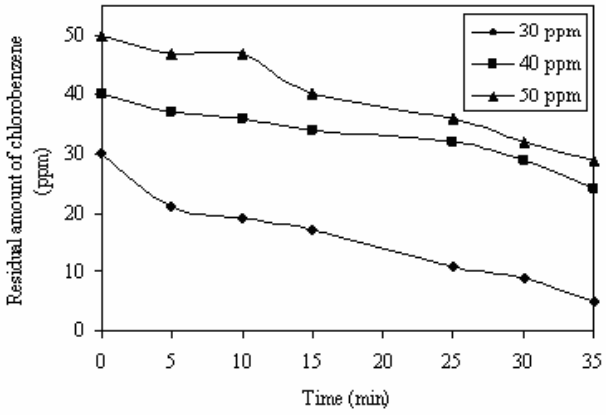

Figure 3. Degradation of chlorobenzene by immobilized crude extracts of *Pseudomonas aeruginosa* (Experiment 1).

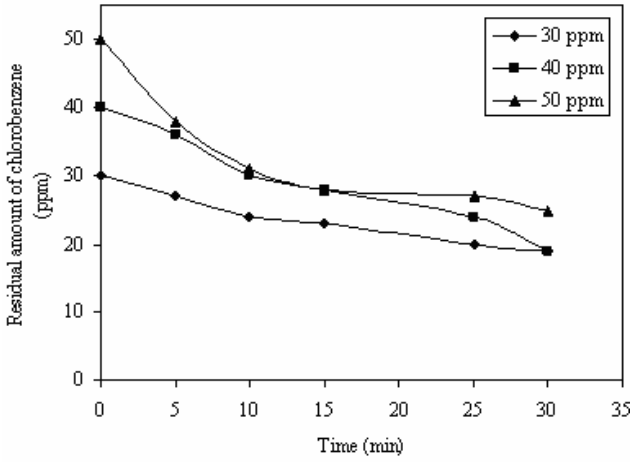

Figure 4. Degradation of chlorobenzene by immobilized crude extracts of *Pseudomonas aeruginosa* (Experiment 2).

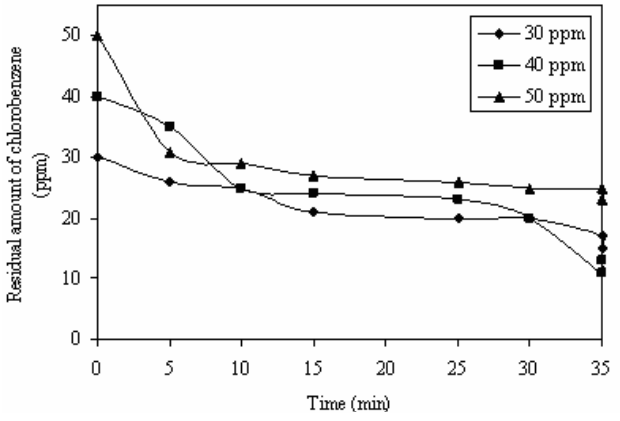

Figure 5. Degradation of chlorobenzene by immobilized crude extracts of *Pseudomonas aeruginosa* (Experiment 3).

dent that the organism takes an initial lag period during which it acclimatizes itself to the media. Then, there is an exponential growth phase with constant growth rate. A stationary phase of death rate equaling the growth was also observed after the exponential phase. 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 24 h.

Degradation of chlorobenzene in packed bed column

The experimental results obtained in present study are discussed in this section. Figure 3 to 11 shows the time course of degradation of chlorobenzene at different initial concentration with nine sets of combinations of process variables (Table 3) by immobilized *P. aeruginosa* cell extracts (containing chlorobenzene degrading enzyme) in packed bed column. The degradation experiment was run using three different initial concentrations of chlorobenzene (30, 40 and 50 ppm).

Figure 15 shows the concentration of crude extracts produced with nine set of combinations (Table 3) of process variables and found that the maximum amount of crude extracts (4.72 mg/ml) were produced with the combination of medium 50 ml inducer 3 ml and inoculum 2 ml.

It is observed from the Figure 3 (Experiment 1 and

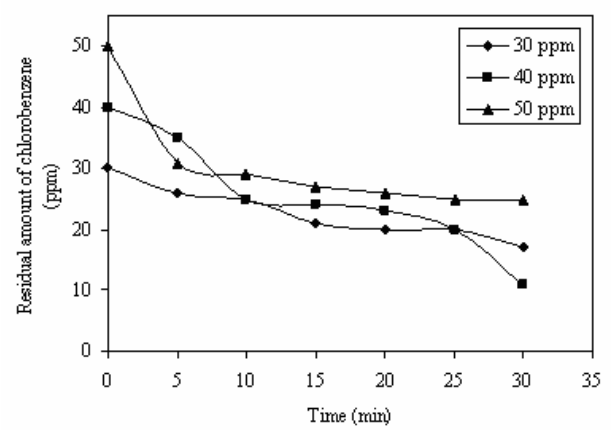

Figure 6. Degradation of chlorobenzene by immobilized crude extracts of *Pseudomonas aeruginosa* (Experiment 4).

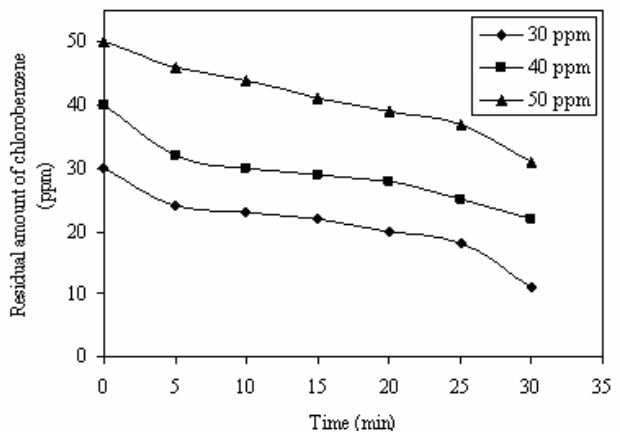

Figure 7. Degradation of chlorobenzene by immobilized crude extracts *of Pseudomonas aeruginosa* (Experiment 5).

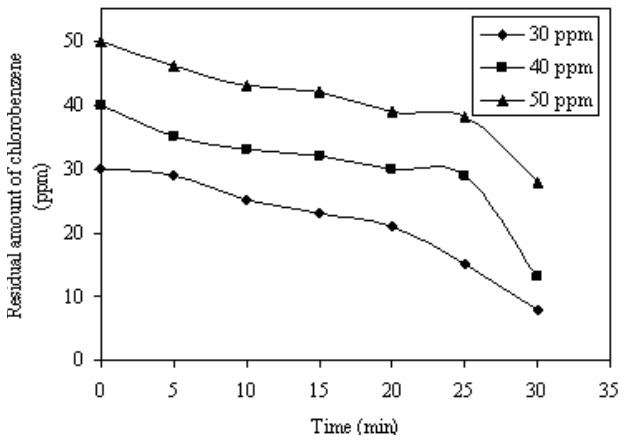

Figure 8. Degradation of chlorobenzene by immobilized crude extracts of *Pseudomonas aeruginosa* (Experiment 6).

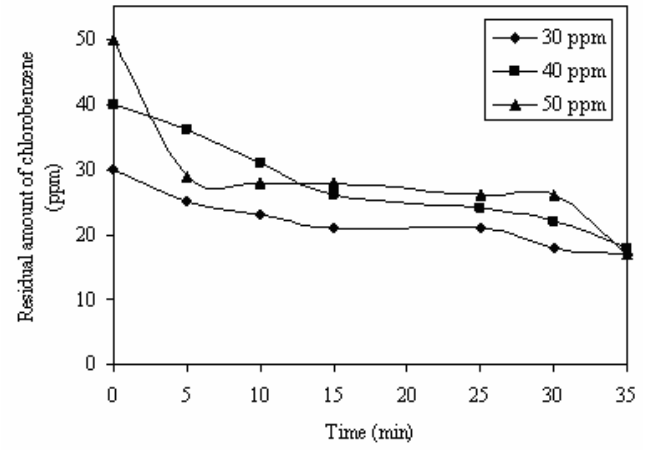

Figure 9. Degradation of chlorobenzene by immobilized crude extracts of *Pseudomonas aeruginosa* (Experiment 7).

Combination 1) that the enzymatic decomposition of chlorobenzene with an initial concentration of 30 ppm was rapid up to 13% within 5 min. After this period, the decomposition is linear and reaching 83% for the maximum period of 35min, whereas the microbial degradation time is 4 h for 85% (Manikandan et al., 2005). This observation indicates that immobilized crude extract degradation (Manikandan et al., 2007) is much faster than microbial degradation. The similar trend of decomposition was observed for the concentration of 40 and 50 ppm with the percentage decomposition at the end 35 min is 40% for 40 ppm and 46% for 50 ppm.

The extracts obtained from the other combinations of the process variable were also immobilized and conducted degradation studies (experiments 2 to 9 and Table 3) with different initial concentration of chlorobenzene. All the experiments results (Figures 4 to 11) show the similar trend of degradation and proves that all nine set of combinations of variables produced chlorobenzene

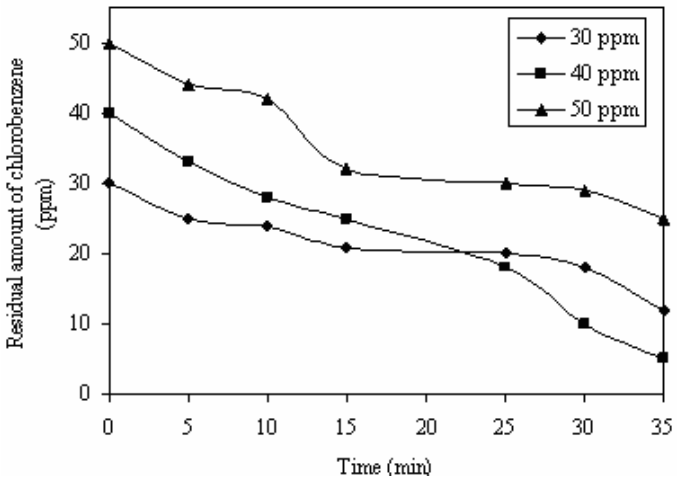

Figure 10. Degradation of chlorobenzene by immobilized crude extracts of *Pseudomonas aeruginosa* (Experiment 8).

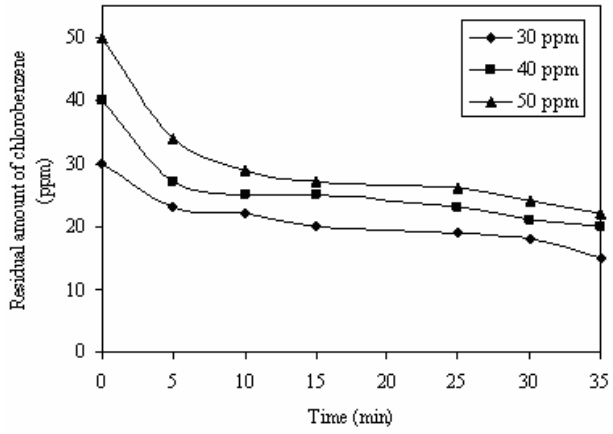

Figure 11. Degradation of chlorobenzene by immobilized crude extracts of *Pseudomonas aeruginosa* (Experiment 9).

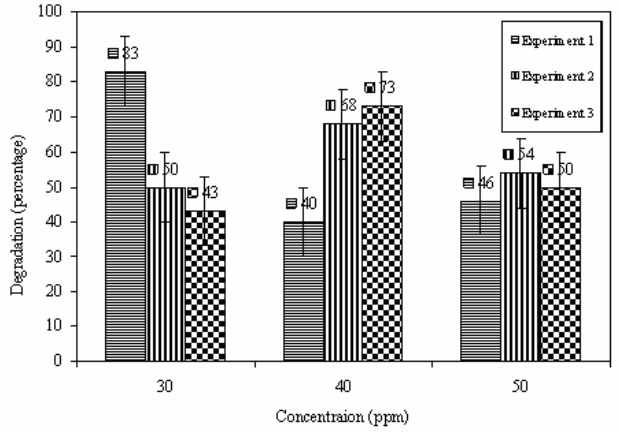

Figure 12. Degradation of chlorobenzene using immobilized crude cell extracts of *Pseudomonas aeruginosa* in packed bed column.

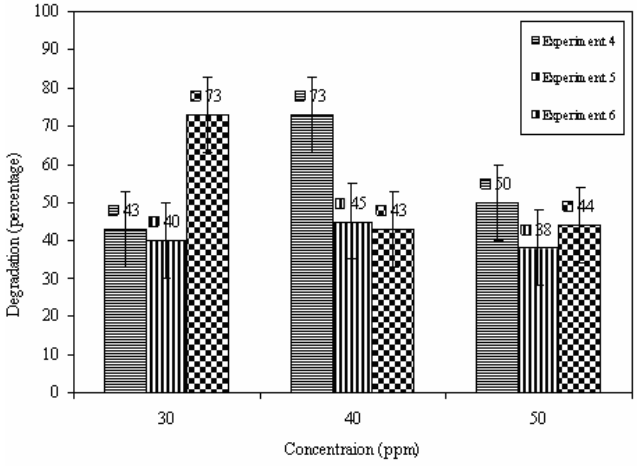

Figure 13. Degradation of chlorobenzene using immobilized crude cell extracts of *Pseudomonas aeruginosa* in packed bed column.

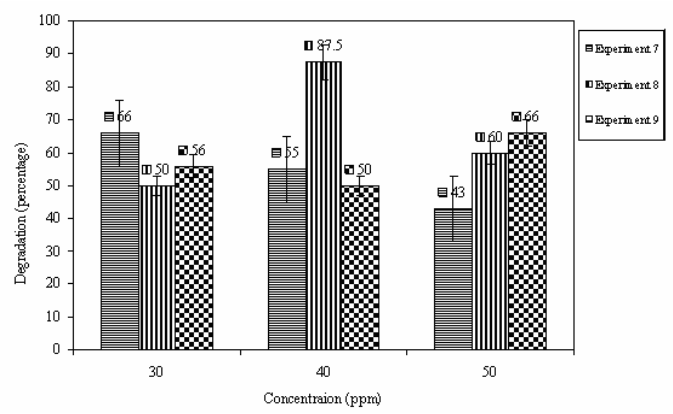

Figure 14. Degradation of chlorob enzene using immobilized crude cell extracts of *Psuedomonas aeruginosa* in packed bed column.

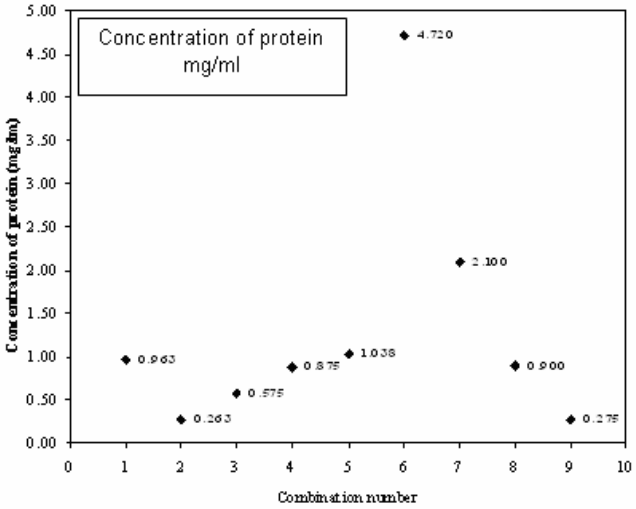

Figure 15. Scattered plot for concentration of protein with combination number.

degrading enzymes in the extracts. Figures 12 to 14 show the comparative study of chlorobenzene degradation in all nine experiments with different initial concentration level.

The experiment was also carried out to find out the degradation of chlorobenzene by packing the beads (without crude extract) in the glass column. 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 83% of chlorobenzene can be decomposed with in 35 min in the packed bed column and without accumulation other treated waste in the environment. Figure 15 shows the scattered plot for concentration of protein. The specified column for the degradation study is depicted in Figure 16, while Figure 17 shows the immobilized extracts. Figures 18 to 20 shows the influen-

Figure 16. Packed bed column with immobilized crude extracts.

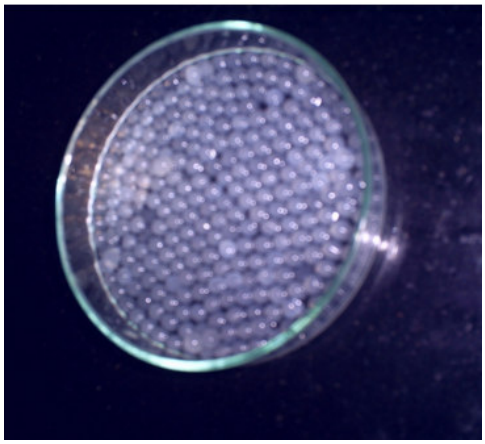

Figure 17. Immobilized beads with crude extracts.

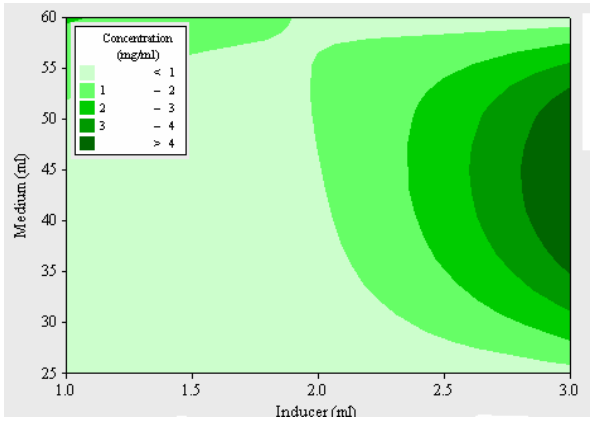

Figure 18. Contour Plot of Concentration (mg/ml) Vs Medium (ml), Inducer(ml).

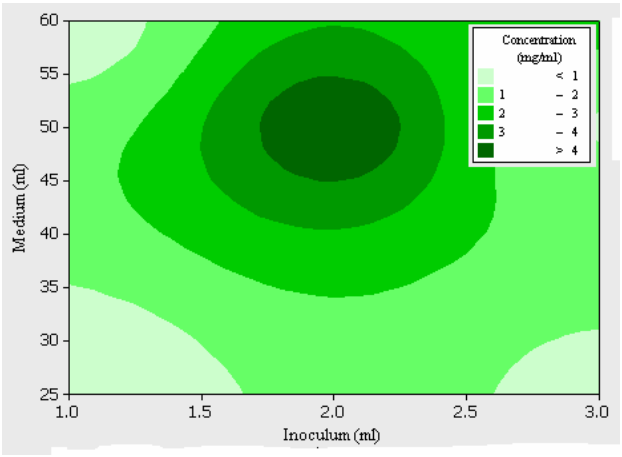

Figure 19. Contour plot of concentration (mg/ml) vs medium (ml), inoculum (ml).

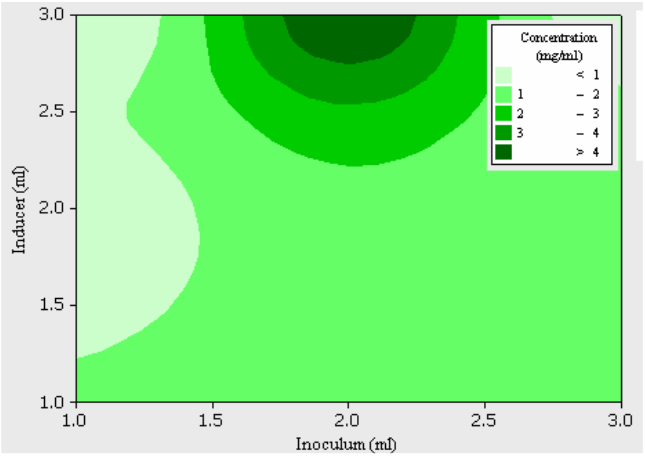

Figure 20. Contour plot of concentration (mg/ml) vs inducer (ml), inoculum (ml).

tial effect of process parameter for the production of crude extracts and this analysis can be used for the optimization of the process conditions to produce the maximum amount of crude extracts.

Conclusion

An attempt has been made to decompose chlorobenzene by immobilized crude cell extracts in packed bed column. The extracts were produced with different combinations of process variables and immobilized 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 5 min and slows linear trend after this period. The total reaction time is less than 35 min for the decomposition up to 80 to 83% 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 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 comparing with conventional and microbial degradation and the process can be recycled with proper conditions.

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