

Full Length Research Paper

Biodegradation of 2,4-dichlorophenol using *Mycoplana dimorpha* extracts and evaluation of kinetic parameters

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Twenty seven combinations of process variables were developed and used to produce crude extracts of *Mycoplana dimorpha*. Crude extracts containing 2,4-dichlorophenol degrading enzymes, were immobilized on sodium alginate beads and degradation studies was conducted in a packed bed column. The rate of degradation of 2,4-dichlorophenol by immobilized crude extracts of was measured at different time intervals and it was found that 82 to 86% of 2,4-dichlorophenol can be decomposed with different initial concentrations in 30 min. The K_m and V_{max} values were determined.

Key words: 2,4-Dichlorophenol, degradation, crude extract, *Mycoplana dimorpha*, immobilization, packed bed column.

INTRODUCTION

Successful degradation of xenobiotic compounds depends upon the economically feasible process of treatment by microbes. Some aromatic compounds are toxic to weeds and pests and therefore, have been used as biocides. However, many such substances are toxic to humans and wildlife as well, and some are also resistant to biological and chemical degradation (Thakur, 2006).

The aromatic compounds express their dangerous effects on human being by impacting the hormonal arrangement, which is serious as meager amounts can have a profound effect. For instance, some polychlorinated biphenyls and DDT congeners can mimic estrogen (Toppari et al., 1996). Polybrominated diphenyl ethers and their metabolites as well as phenols have also been found to have potential endocrine disrupting properties (Meerts et al., 2001; van den Berg, 1990). Other compounds are carcinogenic; phenanthrene, benzo(a)pyrene and benzo(a)anthracene are among the polycyclic aromatic hydrocarbons (PAHs) shown to cause cancer (Samanta et al., 2002; Finlayson-Pitts and Pitts 1997). The impacts of chlorinated compounds on human being as well on environment have been studied (Leslie et al., 2004; Liu et al., 2005; Liu et al., 2004; Mortimer and Connell 1994; Thrall et al., 2004; Vorkamp et al.,

2004; Wu et al., 2006; Xue et al., 1994; Knecht and Woitowitz 2000; Kumagai and Matsunaga 1994; Kusters and Lauwerys 1990; Qian et al., 2004; and Tront et al., 2006) and the need of elimination of these toxic compounds have been emphasized.

Microbial degradation of chloro-substituted aromatics such as chlorobenzoates, chlorophenols, chlorobenzenes or chlorophenoxyacetates has been described via chlorocatechols as central intermediates, and a catechol1,2-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). Mars et al. (1997) have studied the microbial degradation of chloroaromatics and the use of the meta-cleavage pathway for mineralization of chlorinated compounds. They demonstrated the degradation studies with *Pseudomonas putida* GJ31 on toluene and chlorobenzene. The activities of enzymes of the (modified) ortho- and meta-cleavage pathways were measured in crude extracts of cells of *P. putida* GJ31 grown on various aromatic substrates.

Kim and Choi (2005) found that the isolated *Pseudomonas pickettii* from soil was able to grow in 2,4,6-trichlorophenol (2,4,6-TCP) as the sole source of carbon. *P. pickettii* DTP0602 in high-cell-density suspension cultures dechlorinated various chlorophenols (CPs). The ability of the strain DTP0602 to dechlorinate 2,4,6-TCP was induced by 2,6-dichlorophenol, 2,3,6- and 2,4,6-TCP, and 2,3,4,6-tetrachlorophenol and was repressed in

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the presence of succinate or glucose.

The enzymes used by microbes to degrade compounds like hydrocarbons occasionally have a broad spectrum of activity, allowing the degradation of similar chemicals. Thus, bacteria adapted to grow on phenol (a disinfectant) may also degrade synthetic chlorophenols such as the broad-spectrum pesticide pentachlorophenol. Bacteria that grow on toluene (a common solvent and fuel) may also attack nitrotoluenes, such as the explosive trinitrotoluene (Thakur, 2006). Bacteria from soil were isolated and adapted to metabolize various phenols when cultured in a medium containing mineral salts with phenol as the sole source of carbon. The phenol-adapted culture was subjected to respirometric tests with 104 related compounds. Significant oxidative activity was observed with 65 compounds. Dihydric phenols were oxidized while trihydric phenols were not. The addition of a chloro group increases the resistance. Overall, nitro- and chloro-substituted compounds and the benzenes were difficult to oxidize (Tabak et al., 1964).

Pseudomonas species that was able to degrade p-dichlorobenzene as the sole source of carbon and energy was isolated by selective enrichment from activated sludge. The organism also grew well on chlorobenzene and benzene. Washed cells released chloride in stoichiometric amounts from o-, m-, and p-dichlorobenzene, 2,5-dichlorophenol, 4-chlorophenol, 3-chlorocatechol, 4-chlorocatechol, and 3,6-dichlorocatechol. Initial steps in the pathway for p-dichlorobenzene degradation were determined by isolation of metabolites, simultaneous adaptation studies, and assay of enzymes in cell extracts.

Enzymes for degradation of haloaromatic compounds were induced in cells grown on chlorobenzene or p-dichlorobenzene, but not in cells grown on benzene, succinate, or yeast extract. Enzymes of the ortho pathway induced in cells grown on benzene did not attack chlorobenzenes or chlorocatechols (Spain and Nishino, 1987). *Pseudomonas* sp. strain RHO1, able to use chloro- and 1,4-dichlorobenzene as growth substrates was tested towards sensitivity against chlorobenzene. In addition to chlorobenzene, metabolites also seem to function as toxic compounds. 2-chlorophenol and 3-chlorocatechol were isolated from cell extracts. Cleavage of 3-chlorocatechol by catechol-1,2-dioxygenase seemed to be the critical step in the metabolism of chlorobenzene (Fritz et al., 1991).

Valli and Gold (1991) reported that under secondary metabolic conditions the white rot basidiomycete *Phanerochaete chrysosporium* mineralizes 2,4-dichlorophenol. The pathway for the degradation of 2,4-dichlorophenol was elucidated by the characterization of fungal metabolites and of oxidation products generated by purified lignin peroxidase and manganese peroxidase. Werlen et al. (1996) summarized the pathway for degradation of chlorinated compounds. The enzymes of the pathway, the chlorobenzene dioxygenase and the cis-chlorobenzene dihydrodiol dehydrogenase, are encoded

on a plasmid-located transposon Tn5280. The chlorobenzene dioxygenase and cis-chlorobenzene dihydrodiol dehydrogenase were capable of oxidizing 1,2-dichlorobenzene, toluene, naphthalene, and biphenyl, but not benzoate, to the corresponding dihydrodiol and dihydroxy intermediates.

Lu et al. (2006) investigated degradation of 2,4-dichlorophenol in aqueous phase by glow discharge electrolysis. It has been found that 2,4-dichlorophenol undergoes a series of intermediate step, which leads to the formation of mainly isomeric chlorophenols and aliphatic acids. These products are further oxidized, mineralized into CO₂ and chloride ion. A degradation pathway for dichlorophenol is proposed on the basis of detection of intermediate compounds. Nakagawa et al. (2006) also investigated the degradation pathways and kinetics of 2,4-dichlorophenol by an endemic soil fungus, *Mortierella* sp. (Zygomycetes) which degraded 32% within 1 h. The studies were concluded with four aromatic metabolites and two dichlorophenol degradation pathways (a hydroxylation pathway and a dechlorination pathway).

Haller and Finn (1978) have studied the degradation of p-nitrobenzoate by domestic sewage and its inhibition by benzoate. *Pseudomonas* utilized p-nitrobenzoate and benzoate by separate adaptive enzyme pathways. Counotte and Prins (1979) has presented a simple method for the calculation of kinetic parameters (K_m and V_{max}) under conditions of changing substrate concentrations. They have applied this method to detect shifts in groups involved in the utilization of a substrate in a mixed micro-bial culture. They gave the idea of modifying the kinetic equations and apply to the experimental data for the detection of required kinetic parameters. Klecka and Gibson (1981) showed that partially purified preparations of 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. Other substrates oxidized by the enzyme preparation were catechol, 4-methylcatechol, and 4-fluorocatechol. The apparent Michaelis constants for 3-methylcatechol and catechol were 10.6 and 22.0, μM , respectively. Kinetic analyses revealed that both 3-chlorocatechol and 4-chlorocatechol were noncompetitive or mixed-type inhibitors of the enzyme.

Okpokwasili and Nweke (2005) studied the microbial growth and substrate utilization kinetics. Substrate utilization results in removal of chemical contaminant increase in microbial biomass and subsequent biodegradation of the contaminant. Several microbial growth and biodegradation kinetic models have been developed, proposed and used in bioremediation schemes. Some of these models include Monod's, Andrews, Bungay's weighted model, general substrate inhibition models and some kinetic models. Most research on microbial potentials to degrade chemical pollutants has been performed on a laboratory scale.

Table 1. Composition of mineral medium used for *Mycoplana dimorpha* culture.

Ingredients	Concentration
NH ₄ NO ₃	1.0 g/L
(NH ₄) ₂ SO ₄	0.5 g/L
NaCl	0.5 g/L
K ₂ HPO ₄	1.5 g/L
KH ₂ PO ₄	0.5 g/L
MgSO ₄ .7H ₂ O	0.5 g/L
CaCl ₂	0.01 g/L
Double distilled water	1 L

Ehlers and Rose (2006) investigated during biodegradation in the anaerobic compartment, the transformation of the model contaminant, 2, 4, 6-trichlorophenol to lesser-chlorinated metabolites under sulfate-reducing conditions. In the aerobic compartment, 2,4-dichlorophenol degraded by immobilized white-rot fungi. The integrated process achieved elimination of the compound by more than 99% through fungal degradation of metabolites produced in the dechlorination stage. Sahinkaya and Dilek (2006) demonstrated two instantaneously fed sequencing batch reactors, one receiving 4-chlorophenol (4-CP) only and one receiving mixture of 4-CP and 2,4-dichlorophenol, with increasing chlorophenols concentrations in the feed. Complete degradation of chlorophenols and high-chemical oxygen demand removal efficiencies were observed throughout the reactors operation. The presence of 2,4-dichlorophenol competitively inhibited 4-CP degradation and its degradation began only after complete removal of 2,4-dichlorophenol.

The biodegradation kinetics of 2,4-dichlorophenol by culture acclimated to mixture of 4-chlorophenol (4-CP) and dichlorophenol and the culture acclimated to 4-CP only were investigated in aerobic batch reactors. The pure strains isolated from mixed cultures were found to have higher specific degradation rate of dichlorophenol compared to mixed cultures, they did not settle well under quiescent conditions (Sahinkaya and Dilek, 2007).

In the present investigation, the growth of *M. dimorpha* on 2,4-dichlorophenol as sole source of carbon, production of crude extracts containing 2,4-dichlorophenol degrading enzymes with optimization of process conditions, visualization of interactive effects of medium, inducer and inoculum and kinetic parameters (Michaelis – Menten constant (*K_m*) and reaction velocity (*V_{max}*)) were studied.

MATERIALS AND METHODS

Chemicals

2,4-dichlorophenol (DCP) of 99+% analytical standards was purchased from SRL Labs India. 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 (BSA) 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 *M. dimorpha* 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 H₂SO₄ 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/cm² (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 30°C 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 5°C.

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 2,4-dichlorophenol 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 2,4-dichlorophenol 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 (Hitachi UV 2800). The growth of *M. dimorpha* was plotted as OD at 380 nm against time.

Suspension of washed cells and cell extracts

Cells grown on 2,4-dichlorophenol 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 DCP degrading enzyme). The concentrations of protein content in the crude extracts were measured using UV Visible Spectrophotometer (Hitachi UV 2800) with the aid of standard plot of BSA at 280 nm.

Immobilization of crude extracts and batch kinetic studies in packed bed column

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) CaCl₂ solutions to form immobilized beads

Table 2. Specification of column for the for the degradation study of 2,4-dichlorophenol.

Parameter	Value
Diameter of column	3 cm
Height of column	40 cm
Capacity of column	280 ml
Beads bed height	18 cm

(3 mm diameter). After maintaining in the 2% (w/v) CaCl₂ 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.

2,4-dichlorophenol solution of 40 ppm was prepared and added (30 ml) 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 2,4-dichlorophenol was measured at 280 nm (λ_{max} of 2,4-dichlorophenol) 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 (60 and 80 ppm) of 2,4-dichlorophenol in the column. It was found that the optical density values decreased as the retention time increased.

An attempt was made to produce crude extract under different process conditions. Twenty seven set of combinations (Figure 2) 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 on sodium alginate beads as mentioned in this paper.

RESULTS AND DISCUSSION

Growth of *M. dimorpha* on 2,4-dichlorophenol

Figure 1 shows the growth kinetics of the strain on DCP with initial concentration of 200 ppm. From the graph, it was evident 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 4 h, there was exponential growth up to 16 h with lag at 12 h. The growth of *M. dimorpha* was measured up to 24 h.

Degradation of 2,4-dichlorophenol using crude extracts of *M. dimorpha* in a packed bed column

Figure 2 shows the concentration of crude extracts produced with 27 combinations of process variables and found that the maximum amount of crude extracts (1.48 mg/ml) were produced with the combination of 25 ml of medium, 2 ml of inducer and 3 ml of inoculum (Manikandan et al., 2007a).

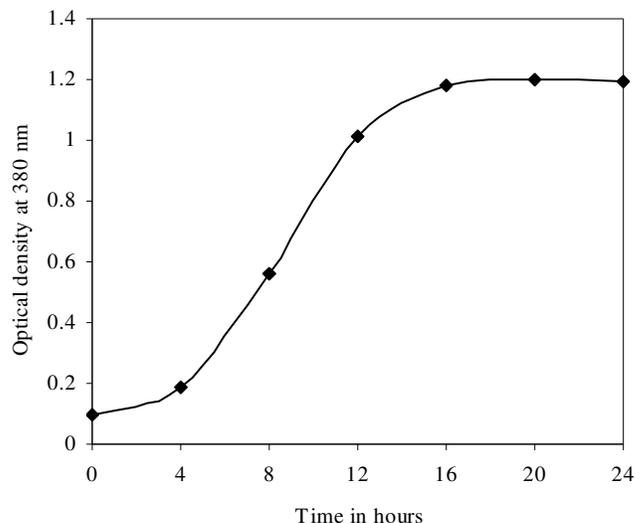


Figure 1. Growth of *Mycoplana dimorpha* on 2,4-dichlorophenol.

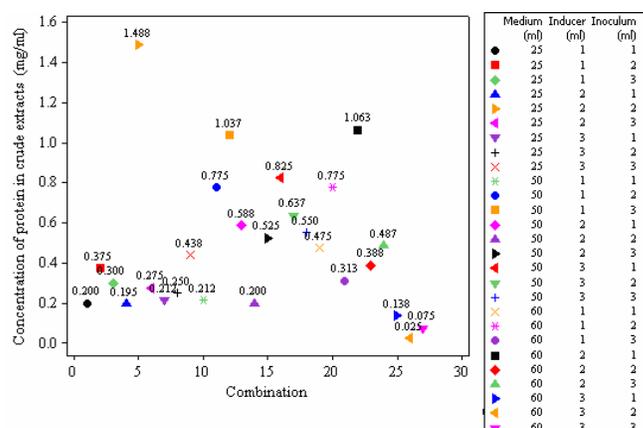


Figure 2. Scatterplot of concentration of protein (mg/ml) vs combination (crude extracts of *Mycoplana dimorpha*).

Crude extracts of 9 combinations (3, 6, 9, 12, 15, 18, 21, 24 and 27) were selected from 27 combinations based on concentrations of protein content in each inducer level to study the kinetics of degradation (Manikandan et al., 2007d). Figures 3 to 11 shows the time course of degradation of DCP at different initial concentrations with nine sets of combinations of process variables by immobilized *M. dimorpha* extracts (containing DCP degrading enzyme) in a glass column. The degradation experiment was run using three different initial concentrations of DCP (40, 60 and 80 ppm).

It is observed from the Figure 3 that the enzymatic decomposition of DCP with an initial concentration of 40 ppm was rapid up to 36% within 5 min. After this period, the decomposition is linear and reaching 54% at the end of 30 min, whereas the microbial degradation is reached 85% in 4 h (Manikandan et al., 2007c). This observation

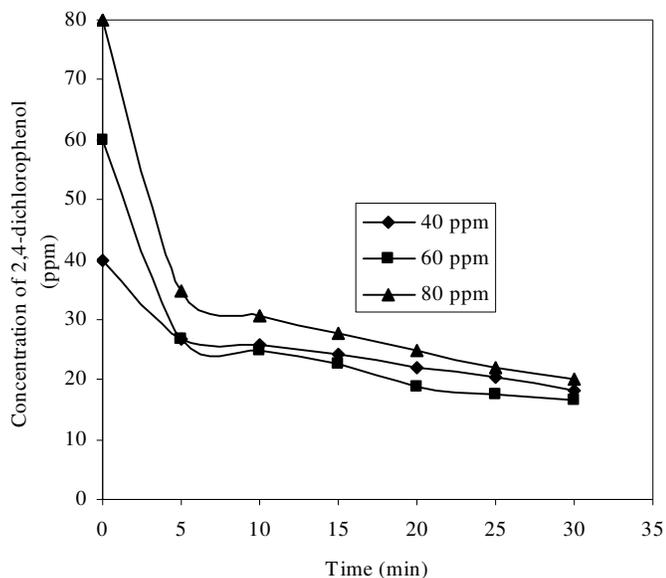


Figure 3. Degradation of 2,4-dichlorophenol by immobilized crude extracts of *Mycoplana dimorpha* (combination 3).

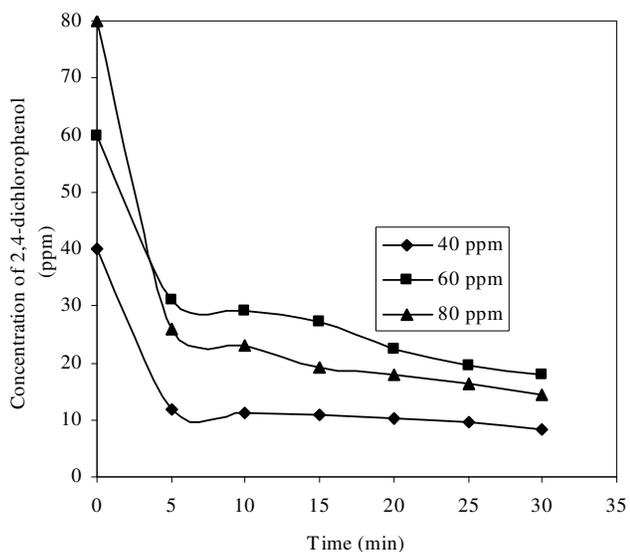


Figure 4. Degradation of 2,4-dichlorophenol by immobilized crude extracts of *Mycoplana dimorpha* (combination 6).

indicates that immobilized crude extract degradation is much faster than microbial degradation (Axcell and Geary 1973; Grund et al., 1975; Hartmann et al., 1979; Chang et al., 1992; Spiess and Gorisch 1996; Beil et al., 1997; Mars et al., 1997; Beil et al., 1998; Pollmann et al., 2001; Alfreider et al., 2003; Rapp and Gabriel-Jurgens 2003; Manikandan et al., 2007a; Manikandan et al., 2007b).

The similar trend of decomposition was observed with the initial concentrations of 60 and 80 ppm with the percentage degradation at the end of 30 min is 72% for

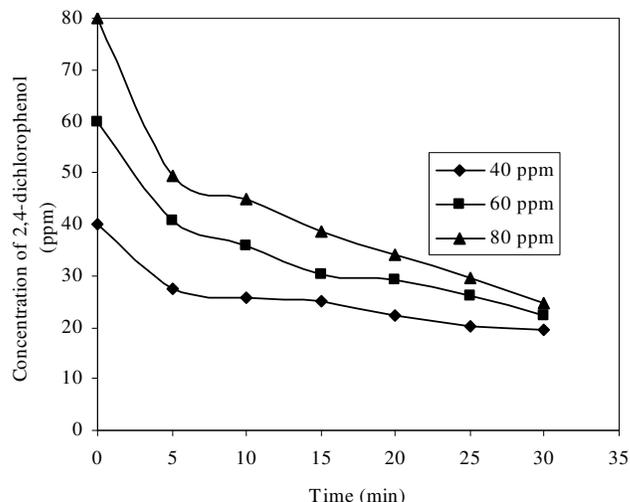


Figure 5. Degradation of 2,4-dichlorophenol by immobilized crude extracts of *Mycoplana dimorpha* (combination 9).

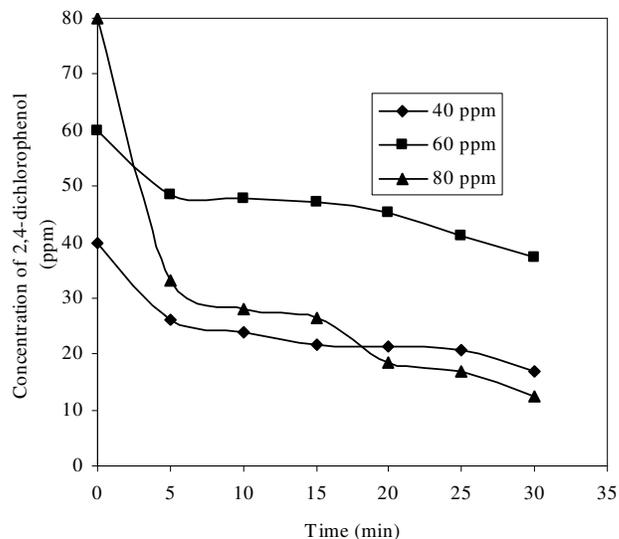


Figure 6. Degradation of 2,4-dichlorophenol by immobilized crude extracts of *Mycoplana dimorpha* (combination 12).

60 ppm and 75% for 80 ppm (Manikandan et al., 2007d).

The extracts obtained from the other combinations of the process variables were also immobilized and degradation studies were conducted (Figures 4 to 11) with different initial concentration of DCP. All the experiments results show the similar trend of degradation and proved that all nine set of combinations of variables produced DCP degrading enzymes in the extracts. Figures 12 to 17 show the comparative study of 2,4-dichlorophenol degradation in all nine experiments with different initial concentration levels. Figures 18 to 20 show the influential effects between medium, inducer and inoculum with respect to protein concentration of 27 com-

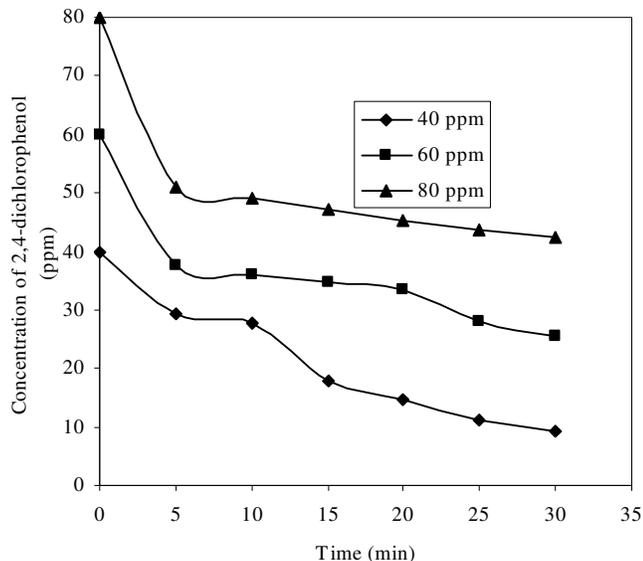


Figure 7. Degradation of 2,4-dichlorophenol by immobilized crude extracts of *Mycoplana dimorpha* (combination 15).

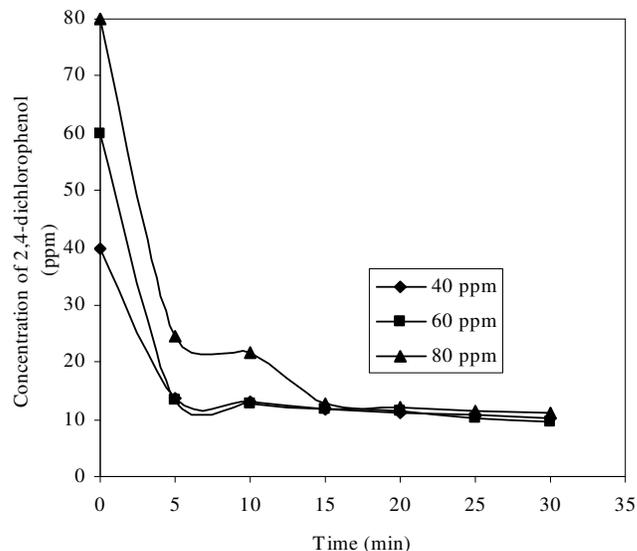


Figure 9. Degradation of 2,4-dichlorophenol by immobilized crude extracts of *Mycoplana dimorpha* (combination 21).

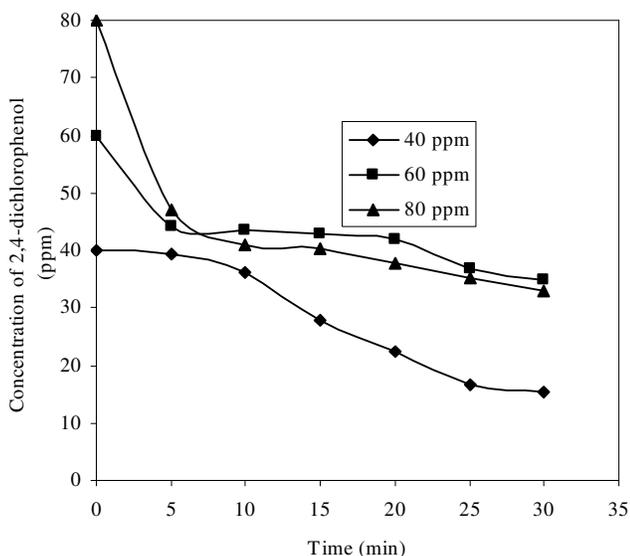


Figure 8. Degradation of 2,4-dichlorophenol by immobilized crude extracts of *Mycoplana dimorpha* (combination 15).

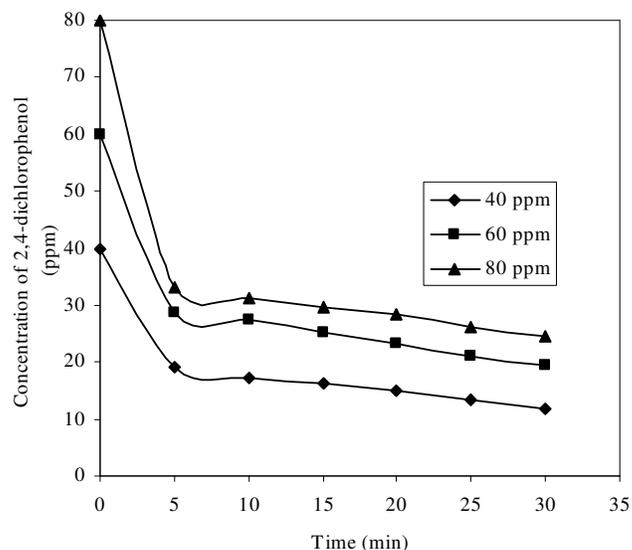


Figure 10. Degradation of 2,4-dichlorophenol by immobilized crude extracts of *Mycoplana dimorpha* (combination 24).

binations. Based on the kinetic data obtained from the experiments mentioned in the sections 3.2, the integrated form of Henri – Michaelis Menten equation has been used to find out K_m and V_{max} values and is given in Table 3.

$$\frac{2.3}{t} \log \frac{[S]_0}{[S]} = -\frac{1}{K_m} \left(\frac{[S]_0 - [S]}{t} \right) + \frac{V_{max}}{K_m}$$

Where K_m = Michaelis Menten constant ($M m^{-3}$),

V_{max} = reaction velocity ($M m^{-3} min^{-1}$), t = time (min),
 $[S]_0$ = concentration of substrate at time t_0 ($M m^{-3}$), and
 $[S]$ = concentration of substrate at any time t ($M m^{-3}$).

From the results, it is evident that the Michaelis Menten constant (K_m) value varied, while the reaction velocity was apparently same for three concentrations. The result implies that crude extracts contained more than one enzyme and mixture of enzymes was involved in degradation of DCP. The results were also compliant with that

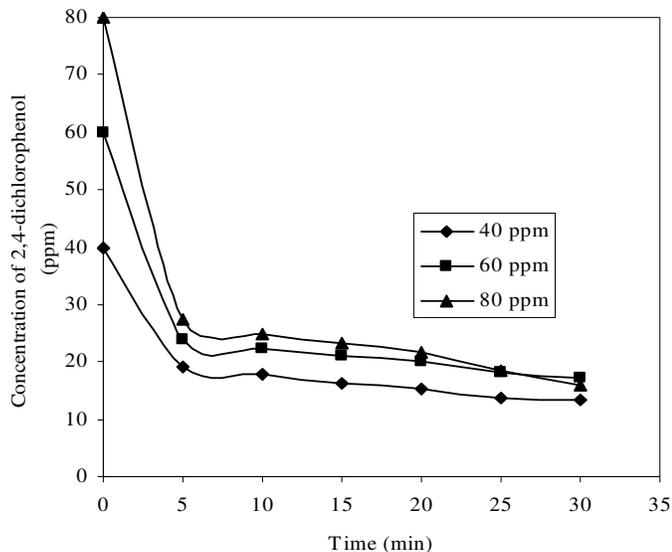


Figure 11. Degradation of 2,4-dichlorophenol by immobilized crude extracts of *Mycoplana dimorpha* (combination 27).

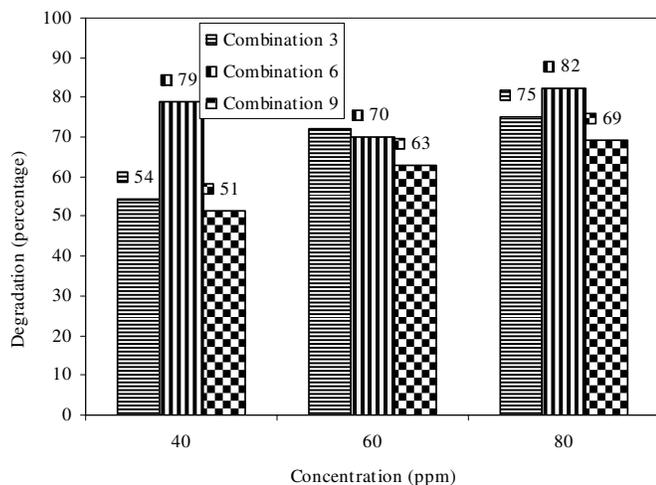


Figure 12. Degradation of 2,4-dichlorophenol by immobilized crude extracts of *Mycoplana dimorpha* (combinations 3, 6 and 9).

Ellis and Wackett (2006) who observed that in the DCP degradation pathway, multiple enzymes were involved

Conclusion

The maximum protein content (1.48 mg/ml) was observed from the combination 25 ml of medium, 2 ml of inducer and 2 ml of inoculum out of 27 combinations developed. The results show the similar trend of degradation and proved that the crude extracts from all the 9 combinations containing DCP degrading enzymes. The experiment was

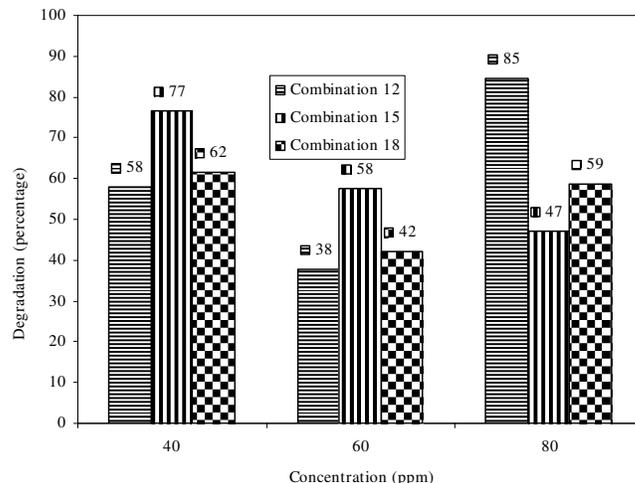


Figure 13. Degradation of 2,4-dichlorophenol by immobilized crude extracts of *Mycoplana dimorpha* (combinations 12,15 and 18).

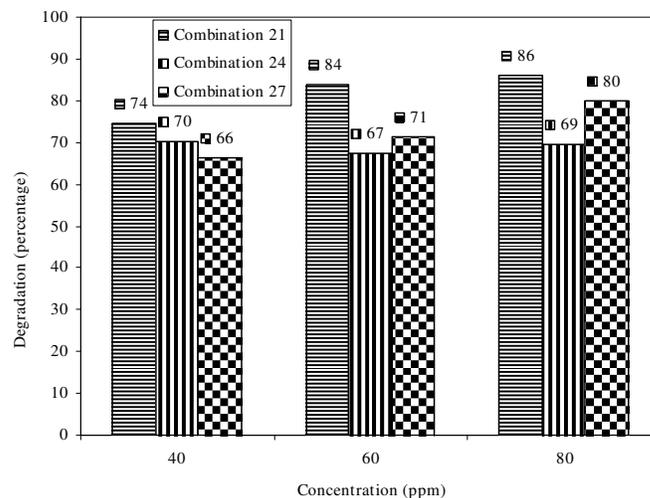


Figure 14. Degradation of 2,4-dichlorophenol by immobilized crude extracts of *Mycoplana dimorpha* (combination 21,24 and 27).

also carried out to find out the degradation of DCP separately by packing the beads (without crude extract) in the glass column. The data obtained from these experiments revealed that there was no change in concentration of DCP. This proved the presence of degrading enzymes in the crude extract. The rate of degradation of DCP with immobilized crude extracts containing degrading enzymes was observed as rapid when compared with microbial degradation and proved the presence of chlorobenzene degradable enzyme in the crude extracts of *M. dimorpha*. The experimental data were used to calculate Michaelis Menten constant (*K_m*) and reaction velocity (*V_{max}*) and confirmed the pre-

Table 3. Kinetic parameters K_m and V_{max} values obtained by degradation of DCP at different initial concentrations using crude extracts of *Mycoplana dimorpha* in a packed bed column.

Microorganism	Concentration (ppm)	K_m ($M m^{-3}$)	V_{max} ($M m^{-3} min^{-1}$)
<i>Mycoplana dimorpha</i>	80	45.45	5.15
	60	88.49	5.23
	40	31.64	5.63

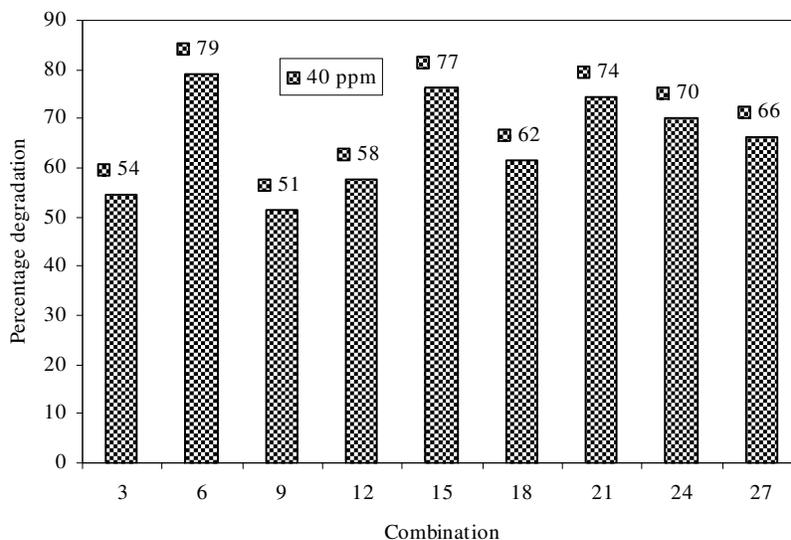


Figure 15. Degradation of 2,4-dichlorophenol using immobilized crude extracts of *Mycoplana dimorpha* (40 ppm).

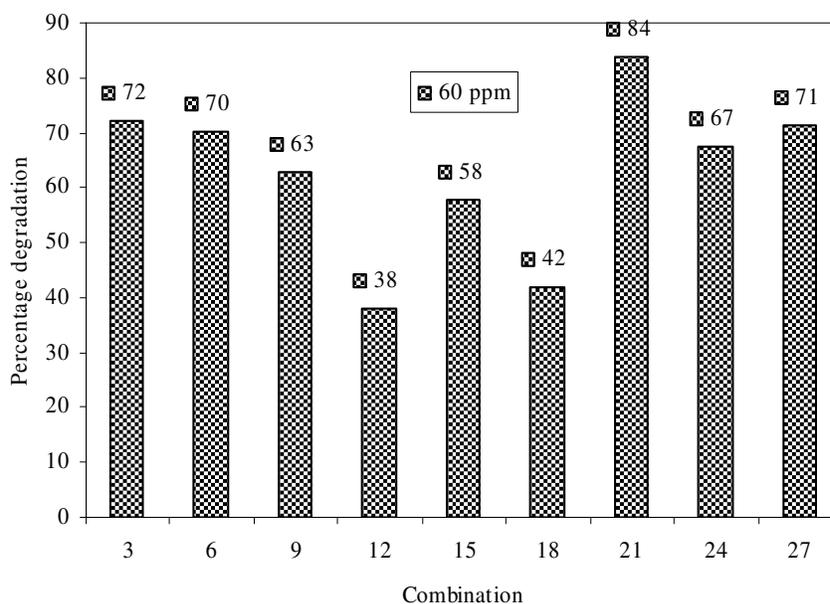


Figure 16. Degradation of 2,4-dichlorophenol using immobilized crude extracts of *Mycoplana dimorpha* (60 ppm).

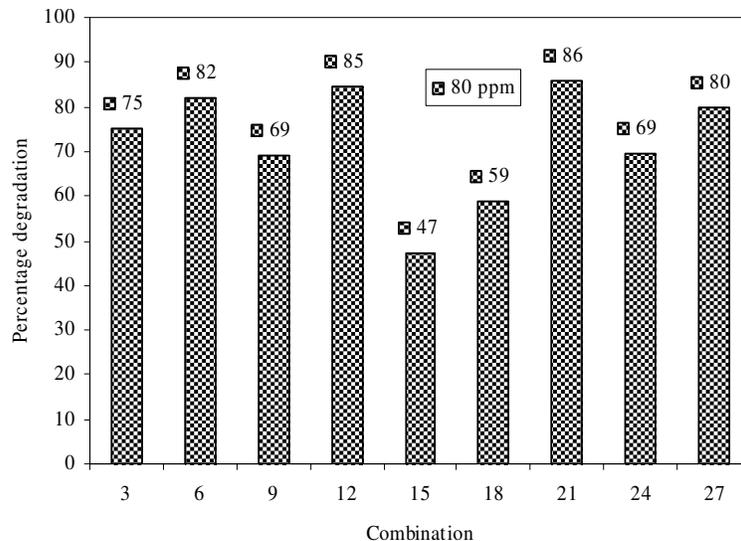


Figure 17. Degradation of 2,4-dichlorophenol using immobilized crude extracts of *Mycoplasma dimorpha* (80 ppm).

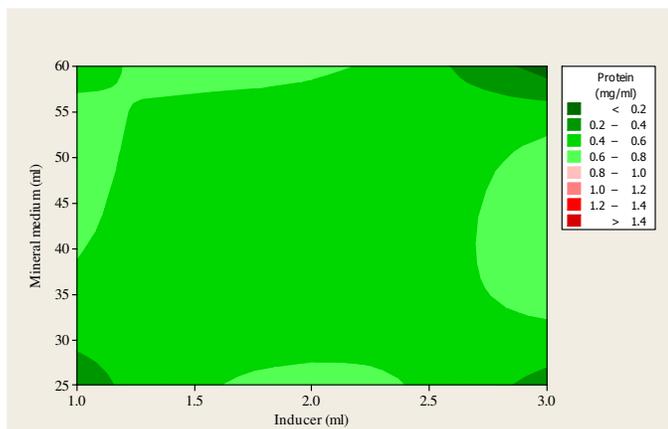


Figure 18. Contour plot of protein (mg/ml) vs mineral medium (ml), inducer (ml).

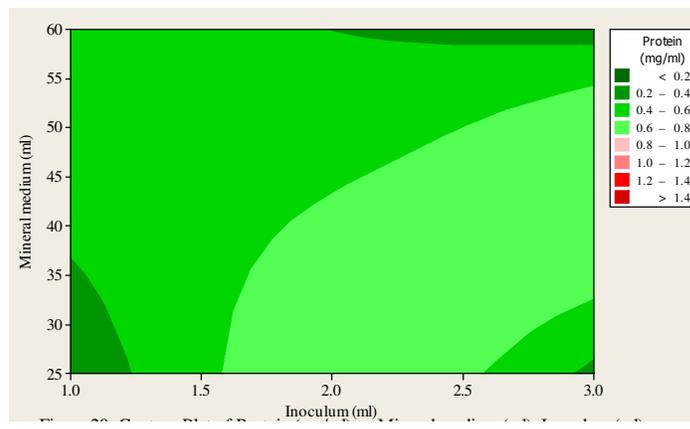


Figure 20. Contour plot of protein (mg/ml) vs inducer (ml), inoculum (ml).

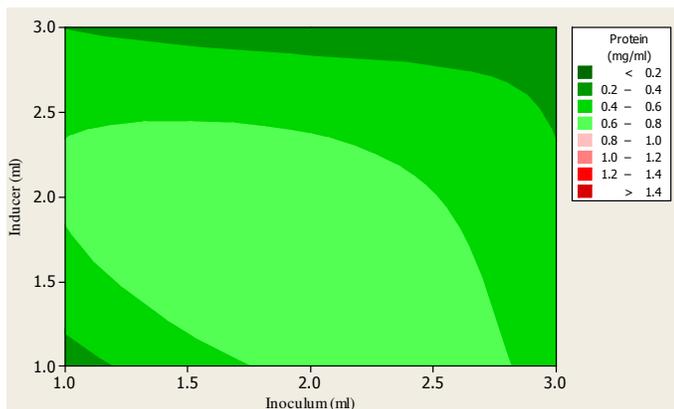


Figure 19. Contour plot of protein (mg/ml) vs inducer (ml).

sence of mixture of enzymes in the crude extracts as mentioned in the pathway which are responsible for degradation.

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