STUDIES ON BIODEGRADATION OF CHLOROPHENOLS BY *RHODOCOCCUS*

A THESIS

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By

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"STUDIES ON BIODEGRADATION OF CHLOROPHENOLS BY *RHODOCOCCUS*"**, in fulfilment of the requirement for the award of the Degree of **Doctor of Philosophy** and submitted in the **Department of Biotechnology** of the Institute is an authentic record of my own work carried out during a period from July, 1997 to October, 2002 under the supervision of **Dr. R. P. Singh, Dr.N. Shivaraman and Dr. A.K. Shrivastava.**

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other University/ Institute.

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This is to certify that the above statement made by the candidate is correct to the best of my (our) knowledge.

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ABSTRACT

The present study was carried out with the objective of isolating a potential Rhodococcal strain for the efficient degradation of wide range of chlorophenols and phenolics. Various kinetic parameters for the biological degradation of mono and dichlorophenols were evaluated by the isolated *Rhodococcus* sp. A mixed substrate study for the degradation of chlorophenol was carried out with phenol and cresols in order to simulate actual phenolic wastewater and the degradation pattern of the substrates observed. A coculture study was planned to study the microbial interaction of the isolated *Rhodococcus* strain with a fast growing bacterial population to predict the survival viability of the strain in the natural system. A *Pseudomonas* sp. being widely prevalent in natural conditions was selected for the coculture study. Biotreatment studies of synthetic wastewater containing chlorophenols were carried out by immobilizing the isolated *Rhodococcus* sp on a gravel medium in a trickling filter reactor.

A promising strain of *Rhodococcus* sp. M1 was isolated from garden soil with the capability of degrading a wide range of phenolics (phenol, o-cresol, m-cresol and p-cresol) and chlorophenols (2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol). The degradation efficiency of the strain was improved by subjecting it to induction using different aromatic compounds like toluene, benzoic acid, phenol and catechol. Benzoic acid induced culture of *Rhodococcus* sp. M1was observed to degrade chlorophenols in least time period with negligible lag.

Optimization study for temperature and pH was carried out using benzoate induced culture of *Rhodococcus* sp. M1. Optimum temperature and pH for the growth

and degradation of chlorophenol by *Rhodococcus* sp. M1 was in the range of 32 to 35°C and 7.0 to 8.0 respectively. *Rhodococcus* sp. M1 showed the ability to grow up to pH 11.5 and is thus suitable for use in the treatment of alkaline waste.

Benzoate induced culture of *Rhodococcus* sp. M1 acclimatized to respective chlorophenols was used for degradation study of chlorophenols. Complete degradation of 2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol was observed up to a concentration of 300, 100, 50 and 10 mgl^{-1} respectively. Evaluation of kinetic parameters showed inhibitory kinetics as there was decrease in specific growth rate, growth yield and substrate uptake rate for 2-chlorophenol, 4-chlorophenol and 2,4-dichlorophenol with increase in substrate concentrations.

Mixed substrate study of 2-chlorophenol by *Rhodococcus* sp. M1 in mixture with phenol and p-cresol showed simultaneous but preferential pattern of degradation of the substrates. Phenol and p-cresol was observed to be the preferred substrate to be utilized preferentially with respect to chlorophenol. Simultaneous degradation of chlorophenol could possibly be due to cometabolism resulting into transformation. Decrease in degradation rate of the substrates in mixed substrate form with respect to pure substrate degradation indicated competitive inhibition pattern of substrate utilization.

Coculture study of *Rhodococcus* sp. M1 with *Pseudomonas fluorescens* P1 showed competitive type of interaction between the populations for the degradation of 2-chlorophenol and phenol. *Pseudomonas fluorescens* P1 evolved as the dominant species during growth on 2-chlorophenol. However, the case was reversed during growth on phenol where *Rhodococcus* sp. M1 was observed to be the dominant

population. Growth on p-cresol showed neutral type of interaction between both the populations.

Biological treatment by a fixed film system using a trickling filter reactor was carried out for the treatment of 2-chlorophenol and 4-chlorophenol. Organic and hydraulic loading was optimized for the effective removal of the chlorophenols from synthetic wastewater. The trickling filter reactor was then operated at optimal organic and hydraulic loading for the treatment of 2-chlorophenol and 4-chlorophenol.

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Mandira Goswami

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LIST OF ABBREVIATIONS USED

Abs	=	Absorbance
CFU	=	Colony forming unit
COD	=	Chemical oxygen demand
GC	=	Gas chromatography
HRT	=	Hydraulic retention time
m-cresol	=	Metacresol
µg/ml	=	Micrograms per millilitre
mM	=	Milli molar
µM	=	Micro Molar
nm	=	Nano meter
o-cresol	=	Orthocresol
PCP	=	Pentachlorophenol
p-cresol	=	Paracresol
rpm	=	Rotations per minute
SRT	=	Sludge retention time
SEM	=	Scanning electron microscopy
USEPA	=	United states environmental protection agency
w/v	=	weight by volume

CHAPTER - 1

1.0 INTRODUCTION

Environmental contamination by toxic xenobiotic chemicals has become a serious global problem (Haggblom *et al.*, 1989 b). One large group of xenobiotics - the chlorinated phenols, arising primarily from agricultural and industrial sources is produced on a quantum scale (Steiert and Crawford, 1985). Chlorophenols are organic chemicals formed from phenol by substitution in the phenol ring with one or more atoms of chlorine. Nineteen congeners are possible, ranging from monochlorophenols to the fully chlorinated pentachlorophenol (WHO, 1989). Chlorophenols pose a serious environmental risk because of their acute toxicity, carcinogenicity and resistance to chemical and biological degradation (Knackmuss and Hellwig, 1978; Jin and Bhattacharya, 1997) and hence are placed in the list of priority pollutants defined by the United States Environmental Protection Agency, USEPA (Moos *et al.*, 1983; Federal Register, 1987).

Chlorophenols are introduced into the environment either through manufacturing processes or as a by-product of processes that employ chlorine or chlorinated compounds (Ryding *et al.*, 1994). Use of chlorophenols in pesticide manufacturing, chemical and petrochemical industry result in the contamination of surface water by the effluent discharged from these units (Knackmuss and Hellwig, 1978; Karns *et al.*, 1983; Lin *et al.*, 1999). Chlorophenol is formed by the production

of bleached pulp in paper mill and is discharged into water bodies with bleached kraft mill effluent (Kringstad and Lindstrom, 1984; Maltseva and Oriel, 1997). Other source of chlorophenol in water is from the treatment by chlorination of municipal and industrial water containing phenol (Kovacs *et al.*, 1984; Murin and Snoeyink, 1979). Use of chlorophenols as herbicides and biocides causes contamination of soil (Kitunen *et al.*, 1987) and its subsequent leaching leads to ground water pollution (Valo *et al.*, 1984; Goerlitz *et al.*, 1985; Jin and Bhattacharya, 1997). Soil contamination also results from accidental spills and disposal of chlorophenolic sludge (Brezny *et al.*, 1992; Brezny *et al.*, 1993). Atmospheric contamination results from air borne distribution of chlorophenols by combustion of municipal and industrial sludge containing chlorophenolic compounds (Ahling and Lindskog, 1982).

Recalcitrant nature of chlorophenols makes it very stable and persistent in soil and ground water and results in its accumulation in sediments (Schneider, 1979; Valenzuela *et al.*, 1997). Lipophilicity of these compounds leads to accumulation in the biota (Jensen, 1996). Long residence time in the system and ready accumulation in organisms indicate a possibility of long term effects (Renberg *et al.*, 1983) and result in more stringent environmental regulation and hence the need for treatment. Natural removal mechanisms of chlorophenols include volatilization and photodegradation (Riedel *et al.*, 1993; Flora *et al.*, 1994). These processes are, however, very slow and result into insignificant removal (Woods *et al.*, 1989). Hence more effective treatment technologies are desired for faster and efficient removal of chlorophenols. Major physicochemical methods employed for the removal includes incineration, oxidation and adsorption (Murin and Snoeyink, 1979). Incineration poses the chance of

production of toxic compounds like polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans from chlorophenols via fly ash catalyzed reaction at 300°C (Kanters *et al.*, 1996). Incineration has met serious public opposition because of the potentially toxic emissions and is very costly, as it requires large amounts of energy to reach the high temperature (Chen and Mulchandani, 1998). Oxidation by ozonation or breakdown by ultraviolet radiation is difficult to implement at contaminated sites (Lee and Carberry, 1992). Chemical oxidants like potassium permanganate and hydrogen peroxide (Lin *et al.*, 1996) followed by adsorption on activated carbon (Saber and Crawford, 1985) as a final clean up step may be used. However, such treatments are not cost effective. Biological removal of chlorophenols from contaminated sites and wastewater is a promising alternative receiving widespread attention (Steiert and Crawford, 1985). Biological method of removal has advantages over physicochemical treatment methods as it achieves fairly complete removal of the pollutant without the formation of undesirable byproducts and is viable cost wise.

Biological degradation of chlorophenols by the genus *Arthrobacter* (Edgehill and Finn, 1983), *Pseudomonas* (Karns *et al.*, 1983), *Flavobacterium* (Saber and Crawford, 1985), *Mycobacterium* (Haggblom *et al.*, 1988), *Alcaligenes* (Valenzuela *et al.*, 1997) and have been reported. Microorganisms belonging to the genus *Rhodococci* appear as the better agent for chlorophenol degradation due to certain advantages they possess over other microorganisms so far explored. *Rhodococci* exhibit low growth rate but have high substrate affinity and persistence (Salkinoja-Salonen *et al.*, 1991). They lack catabolite repression and in several cases, presence of additional carbon sources accelerate the metabolism of concerned compounds (Warhurst and Fewson, 1994).

Rhodococci exhibit flocculating characteristics (Finnerty, 1992), which is considered an advantage for use in biological treatment plant where good settleability of biomass is a desirable requirement.

As evident from the literature review in the succeeding chapter, degradation of chlorophenol has been reported for few species of *Rhodococci* but the substrate range for degradation was low and the time taken for degradation was significantly high. Hence there is a need to isolate potential species capable of degrading chlorophenolic compounds in less time and having wide capability to degrade related phenolic compounds. Limited information is available in the literature on the kinetics of chlorophenol degradation. However kinetic parameters are significant in any application of biodegradation to the removal of chlorinated phenols from wastewater or polluted area (Stanlake and Finn, 1982). Hence the kinetic parameters for the degradation of chlorophenolic compounds need to be evaluated. Chlorophenols in industrial effluent has been reported to be present along with phenol and cresols. Cometabolic degradation of chlorophenol in presence of phenol has been reported in literature (Hinteregger *et al.*, 1992; Hill *et al.*, 1996). Cometabolic degradation however, results in the transformation and not complete removal of the compound. Thus there is a need to study the degradation of chlorophenol in mixture with phenol and cresols. Most of the studies for the degradation of chlorophenols and phenolics have been carried out with axenic cultures. Biodegradation of chlorophenols with mixture of different pure cultures has so far not been reported. However, in natural conditions bacterial cultures are generally present in mixed culture form. Thus for the application of *Rhodococcal* cultures in bioremediation sites and waste water treatment

systems, biodegradation of chlorophenols and related phenolics needs to be studied using *Rhodococcus* along with another competing pure culture.

The present study was thus carried out having the following objectives:

1. Enrichment, isolation and identification of *Rhodococcus* cultures.
2. Screening and selection of potential *Rhodococcus* culture(s) for the degradation of chlorophenols and related phenolics.
3. Study of the degradation kinetics of chlorophenols in pure substrate form
4. Study of the degradation of chlorophenols in presence of the phenolics as mixed substrate
5. Evaluation of the degradation of chlorophenols and related phenolics by *Rhodococcus* along with another competing bacterial culture.
6. Conduct the bench scale studies based on the experimental results of the above investigations using synthetic wastewater containing chlorophenols.

2.0 *LITERATURE REVIEW*

2.1 Historical Background

Pentachlorophenol as a commercial product was first introduced in 1936 as an antiseptic. Since then the use of pentachlorophenol and other chlorophenol congeners ranging from monochlorophenol to tetrachlorophenol in industrial and agricultural sector resulted in the rise of their production at an alarming rate (Jensen, 1996). Chlorophenolic compounds were reported to be toxic acting as uncouplers of oxidative phosphorylation (Loomis, 1949). In addition to the effect on oxidative phosphorylation, chlorophenols also directly inhibit enzymes such as the P-450 complex (Wedding *et al.*, 1967). Chlorophenols were also reported to be carcinogenic and mutagenic (Fahrig *et al.*, 1978).

Microbial metabolism of pentachlorophenol was reported for the first time by fungi where detoxification of chlorophenol was due to the extracellular phenol oxidases (Lyr, 1963). The metabolism of pentachlorphenol was reported by Duncan and Deverall (1964) by a *Trichoderma* sp. (P42) and by Cserjesi and Johnson (1972) using *Trichoderma virgatum*. Chu and Kirsch (1972) isolated a bacterium belonging to a continuous flow enrichment culture that could degrade pentachlorophenol and designated it as KC3. The morphological and physiological characteristics of KC-3 suggested a relationship to the saprophytic Coryneform bacteria. An organism closely related to *Pseudomonas* sp. could completely dechlorinate pentachlorophenol at a

concentration of 0.31mM (Watanabe, 1973). Suzuki (1977) isolated a *Pseudomonas* that rapidly mineralized pentachlorophenol.

Historical methods for the treatment of chlorophenol waste include activated carbon adsorption and land farming. The former method only transferred the chlorophenols to charcoal, while in the traditional method of land farming, only the bioavailable fractions could be removed. (Parker and Jenkins, 1986; Harmsen 1991; Mueller *et al.*, 1991a).

2.2 Toxicity of chlorophenols

The toxicity of chlorophenols depends on the number and position of the chlorine atom on the phenol ring (Ravanel *et al.*, 1985; Cascorbi and Aklers, 1989). The site of action of chlorophenols is the lipid matrix and the membrane bound proteins; chlorophenols targetting the lipid matrix cause lipid membrane disruptions (Jensen, 1996), while, chlorophenols at high concentrations inhibit the cytochrome P-450 complex (Wadding *et al.*, 1967, Ahlborg and Thunberg, 1980).

Studies have shown chlorophenols to be carcinogenic, based on the mutagenicity screening tests (Kozak *et al.*, 1979; Boyd *et al.*, 1983; WHO, 1989). Human exposure to chlorophenols result from consumption of contaminated drinking water, inhalation of air contaminated by emissions from combustion of wastes and occupational contact with chlorophenols. Chlorophenols are irritating to skin and mucous membranes of upper respiratory tract, besides causing severe eye irritation and lacrimation. Pentachlorophenol was observed to cause liver injury (Wang *et al.*, 2001). 2-chlorophenol causes lung, liver and kidney damage; muscular weakness,

gastroenteritis, edema of the lung, injury of pancreas and spleen, nervous disorder, skin eruptions and is a general protoplasmic poison (WHO, 1989). Chlorophenols undergo transformation *in vitro* to polychlorinated dibenzo-p-dioxins and dibenzofurans as reported specifically for the 2,4,5, 2,3,4,6 and pentachlorophenols, the reaction was catalyzed by a myeloperoxidase recovered from human leucocytes (Wittsiepe *et al.*, 2000).

Killing of the fish have been reported as a result of pentachlorophenol spills, some of which have also involved tetrachlorophenol (WHO, 1989). Pentachlorophenol has been shown to cause acute toxicity to fish at a concentration of 0.6 mg l^{-1} (Edgehill and Finn, 1983). Chlorophenols (4-monochlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol) generally impaired the algal primary production, reproduction and altered algal species composition (WHO, 1989). Chlorophenols at concentrations greater than 2.02mM and 0.02mM, inhibited photosynthesis and protein synthesis respectively in the algae, *Scenedesmus obliquus* (Senger and Ruhl, 1980). Exposure to high concentrations of chlorophenols (4-monochlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol) have also been reported to reduce the zooplankton biomass production (WHO, 1989).

2.3 Sources of chlorophenols in the environment

Chlorinated phenols in the wastewaters result primarily from the manufacture of chlorophenols and compounds in which chlorophenols are used as intermediates (Bhandari *et al.*, 1996; Jensen 1996). The industrial units using and subsequently discharging chlorophenolic compounds in their effluents have been detailed in Table 1.

The biocidal property of higher chlorophenols (pentachlorophenol and tetrachlorophenol) accounts for their use as preservatives in wood, paper, paint, textile, and leather industry (Moos *et al.*, 1983; Muller and Caillard, 1986; Flora *et al.*, 1994; Bhandari *et al.*, 1996). The lower substituted chlorophenols (monochlorophenols, dichlorophenols and trichlorophenols) are used as intermediates in the manufacturing of herbicidal phenoxy acids, in the synthesis of dyes, phenolic resins and as solvent in petrochemical industries and in oil refineries (Lin *et al.*, 1999). Typical composition of wastewater from herbicide manufacturing units revealed monochlorophenolic concentrations to be 100 mg l^{-1} (Bond and Straub, 1974).

In paper industry, chlorophenolic compounds are formed in kraft process from bleaching of wood pulp using chlorine and chlorine dioxide and extraction with alkali (Kringstad and Lindstrom, 1984; Paasivirta *et al.*, 1985). The filtrates from chlorination and extraction stages are dominant source of chorinated phenols and are discharged with the bleached kraft mill effluent into recipient waters (Taghipour and Evans, 1996). Lindstrom and Nordin (1978) identified and listed 14 chlorinated phenols found in the phenol fraction of bleach plant effluent. Conventional chlorine bleaching of kraft pulp has been estimated to produce approximately 100 to 300 g of chlorinated phenolic compounds per ton of pulp (Jokela and Salkinoja-Salonen, 1992). Chlorophenols also result from chlorination of water supplies containing phenol (Jensen 1996, Bhandari *et al.*, 1996).

Table 1. Industries using and discharging chlorophenolic compounds.

Sl.No	Industries using chlorophenols	Type of chlorophenol compound released in the effluents
1	**Paint industry**- as polymer intermediate for fire-retardant varnishes **Textile industry**- for cotton fabric treatment to provide rot resistance **Coal handling and processing industry**-as ingredient for coal processing **Chlorophenol production industry**-as an intermediate for further chlorination to 2,4-dichlorophenol, 2,4,6–trichlorophenol, and pentachlorophenol	2-chlorophenol
2	**Dyes, Pharmaceuticals and Chlorophenol production industries**	4-chlorophenol
3	**Pesticide industry**-as an intermediate for production of Sessone, Nitrofen, Nemacide, Genite EM-923, mothproofing, miticide,as intermediate for production of 2,4-D and other herbicides **Polyester film industry**-as raw material for polyester films **Pharmaceutical industry**- as ingredient of antiseptics	2,4-dichlorophenol
4	**Pesticide industry**- as intermediate in manufacture of 2,4,5-T and related herbicides, fungicide, bactericide, algicide **Soap industry**- as germicides and ingredients of germicidal soaps	2,4,5-trichlorophenol
5	**Textile, Wood, Leather industries** as preservatives **Pesticide, soap industries**	2,4,6-trichlorophenol
6	**Rubber, Plywood industries**-as preservative for latex and as preservative in glue for plywood respectively **Wood industry**- as fungicide and bactericide for wood preservation	2,3,4,5,6-tetrachlorophenol
7	**Wood industry** –as preservation of wood	Pentachlorophenol

Source: US EPA (1980)

Soil treated with pesticide is a source of chlorophenol contamination as it is formed as intermediate metabolites during the microbiological degradation of 2,4-dichlorophenoxyacetic acid and 2,4,5 trichlorophenoxyacetic acid, lindane and benzene hexachloride (Crawford *et al.*, 1983; Haggblom and Valo, 1995). Runoffs from these soils find its way into adjacent water bodies and ground water resulting in chlorophenol contamination (Baker *et al.*, 1980; Jin and Bhattacharya, 1997).

2.4 Physicochemical methods of treatment of chlorinated phenols

The long persistence of chlorophenols in the environment and their toxicity necessitates faster and effective removal of chlorophenolic compounds from environment. Various abiotic methods are in use for removal of chlorophenols. Physicochemical methods employed for handling high concentrations of chlorinated phenols are presented in Table 2 and include adsorption by activated carbon (Murin and Snoeyink, 1979), ultrafiltration, reverse osmosis, irradiation, incineration, ozonation, electrochemical oxidation and chemical decomposition (Lin *et al.*, 1999).

Activated carbon used as an adsorbent is an expensive method and the spent carbon must either be regenerated or incinerated (Murin and Snoeyink, 1979). Ultrafiltration and reverse osmosis achieve separation of chlorophenols from the effluent but not complete removal (Taghipour and Evans, 1996). Irradiation is an effective process for treatment of chlorophenol containing wastewater and sludge. Pentachlorophenol (PCP) concentration was reduced by 40-45% after 12 h of irradiation. Gamma irradiation was used with 76% efficiency for the removal of organic halogen from PCP (Taghipour and Evans, 1996). Irradiation, however, in

certain cases produces toxic intermediates. One of the primary modes of treatment of chlorophenol containing sludge is by incineration. Incineration however results in the emission of toxic compounds like chlorinated dibenzo-dioxins (PCDDs) and dibenzofurans (PDBFs) which is formed from chlorophenol at elevated pressure and temperature (Renberg *et al.,* 1983; Boyd and Shelton, 1984; Jensen, 1996). Oxidation process such as ozonation, though effective, could not be implemented at the contaminated site (Lee and Carberry, 1992). Also ozonated intermediates of oxidized chlorophenols was shown to be highly toxic. Formation of chlorocatechols, chloromuconic acids and hydroxylated chlorinated dimeric compounds were detected in ozonation of chlorophenols. These chlorinated byproducts may cause greater toxicity than the parent chlorophenols (Shang *et* al., 2002). Ozonation also results in the formation of oxalic acid as final product in case of pentachlorophenol (Kim and Moon, 2000). Electrochemical oxidation of 4-chlorophenol by anode made of titanium sheet mesh coated with Ti/Tio(2) and RuO(2) film has been proposed for chlorinated phenols but is hampered by anode fouling (Rodgers *et al.*, 1999; Azzam *et al.*, 2000). Chemical process of treatment is primarily by oxidation of chlorophenol with hydrogen peroxide and Fenton's reagent (Lee and Carberry, 1992; Lu, 2000; Yeh *et al.*, 2002). Chemical degradation of 2,4-dichlorophenol by Fenton's agent resulted in chlorocatechol formation (Schlosser *et al.,* 2000).

Physical process like adsorption by activated carbon though aims at high treatment efficiency, results only in phase shift and not complete removal of chlorinated phenols (Murin and Snoeyink, 1979). Another major drawback of physical methods is the formation of toxic intermediates which causes environmental concern as

in the case of treatment of chlorophenolic compounds by incineration, irradiation and ozonation (Boyd and Shelton, 1984; Lee and Carberry, 1992). Also the high cost of these processes limit its use at the treatment site (Kim and Maier, 1986).

Table 2. Physicochemical process of treatment of chlorinated phenols

Sl. No.	Treatment Methods
1.	Adsorption by activated carbon
2.	Ultrafiltration
3.	Reverse osmosis
4.	Irradiation
5.	Incineration
6.	Ozonation
7.	Electrochemical oxidation
8.	Chemical decomposition (Hydrogen peroxide and Fentons Reagent)

2.5 Microbial degradation of chlorinated phenols by microorganisms other than *Rhodococcus*

In order to overcome the drawbacks of the physicochemical methods of treatment, biological ways of degradation of the chlororphenols has emerged as a more successful alternative. Different groups of microorganisms have been reported to degrade chlorophenols and are summarized in Table 3.

Table 3. Microorganisms involved in chlorophenol degradation

Microorganisms	Type of chlorophenol degraded	References
Alcaligenes	4-chlorophenol, 2,4-dichlorophenol, 2,6-dichlorophenol, 2,4,6-trichlorophenol	Pemberton et al., 1979; Koh et al., 1997;Valenzuela et al., 1997; Steinle et al., 1998
Arthrobacter	Pentachlorophenol	Stanlake and Finn, 1982; Edgehill and Finn, 1983
Azotobacter	Trichlorophenol	Maltseva and Oriel, 1997
Flavobacterium	Pentachlorophenol, 2,3,4,6-tertrachlorophenol, 2,3,5,6-tertrachlorophenol, 2,3,6-trichlorophenol, 2,4,6-trichlorophenol and 2,6-dichlorophenol	Saber and Crawford, 1985; Steirt et al., 1987; Topp et al., 1988
Nocardia	2,4,5-trichlorophenol, 2,4,6-trichlorophenol, 2,4-dichlorophenol	Maltseva and Oriel, 1997
Pseudomonas	4-chlorophenol, 2-chlorophenol, 2,3-dichlorophenol, 2,4-dichlorophenol, 2,5-dichlorophenol, 2,3,4-trichlorophenol, 2,4,5-trichlorophenol, 2,3,4,6-tetrachlorophenol, 2,3,5,6-tetrachlorophenol, pentachlorophenol	Knackmuss and Hellwig, 1978; Karns et al, 1983; Radehaus and Schmidt, 1992; Lee et al., 1994; Aranda et al., 1999; Wang et al., 2000; Farrell and Quilty, 2002

Pseudomonas sp. B13, isolated from sewage was reported to degrade 4-chlorophenol as the sole source of carbon and energy after being enriched on 3-chlorobenzoate. It was grown on 4-chlorophenol up to a substrate concentration of 0.15 mM. Degradation was inhibited at higher (> 1mM) concentration of 4-chlorophenol

(Knackmuss and Hellwig, 1978). Resting cells of *Pseudomonas* sp. B13 that had been grown on phenol or 4-chlorophenol readily co-oxidized the isomeric chlorophenols (Knackmuss and Hellwig, 1978). Complete degradation of 2-chlorophenol and 3-chlorophenol up to a concentration of 1.56 mM and 4-chlorophenol up to 2.34 mM was observed by *Pseudomonas putida* CP1 (Farrel and Quilty, 2002). *Pseudomonas putida* and *Pseudomonas testosteroni* isolated from the effluent of a petroleum wastewater treatment plant was able to degrade 2-chlorophenol and 2,4-dichlorophenol (Lee *et al.*, 1994). Karns *et al* (1983) reported the degradation and dechlorination of 2,3-dichlorophenol, 2,4-dichlorophenol, 2,5-dichlorophenol, 2,3,4-trichlorophenol, 2,4,5-trichlorophenol, 2,3,4,6-tetrachlorophenol, 2,3,5,6-tetrachlorophenol and pentachlorophenol by 2,4,5-trichlorophenoxyaceticacid induced resting cell suspensions of *Pseudomonas cepacia* AC1100. *Pseudomonas* sp. strain O1 and *Pseudomonas* sp. strain O2 were able to remove 2,4,6-trichlorophenol only in presence of phenol (Wang *et* al., 2000). *Pseudomonas paucimobilis* S37 was able to degrade 2,4,6-trichlorophenol up to 0.1 mM. Degradation in presence of glucose was enhanced (Aranda *et al.*, 1999). Radehaus and Schmidt (1992) isolated a *Pseudomonas* sp. strain RA2 that was capable of mineralizing pentachlorophenol up to a concentration of 160 mg l^{-1}.

Alcaligenes eutrophus JMP134 was able to degrade 2,4-dichlorophenol. The *tfdb* gene encode the oxidation of the ring and the plasmid pJP4 contains the *tfdcdef* genes which encode the complete chlorocatechol pathway downstream from the central metabolite, 3,5-dichlorocatechol (Pemberton *et al.*, 1979). *Alcaligenes eutrophus* JMP134 (pJP4) showed removal of 2,4,6-trichlorophenol (100 mg l^{-1}) in 6 days with

93% efficiency in presence of high level of degradable organic matter. *Alcaligenes eutrophus* JMP134 (pJP4) was reported to remove 20 mg l^{-1} of 4-chlorophenol and 2,4,6-trichlorophenol present in mixture with 75% and 55.5% efficiency from bleached kraft mill effluent in 6 days in presence of degradable organic matter (Valenzuela *et al.*, 1997). *Alcaligenes eutrophus* JMP222 cured of plasmid pJP4 was able to dehalogenate 2,4-dichlorophenol when grown on o-cresol (Koh *et al.*, 1997). *Ralstonia* sp. strain RKI (formerly *Alcaligenes*) completely degrades 2,6-dichlorophenol up to a concentration of 1200μM under oxic condition with stoichiometric release of chloride (Steinle *et al.*, 1998) 4-chlorphenol was found to be utilized as a cometabolite in presence of phenol by *Alcaligenes eutrophus* (Hill *et al.*, 1996).

Azotobacter sp. strain GP1 was capable of degrading up to 100 mgl^{-1} of trichlorophenol (Maltseva and Oriel, 1997). Alkalophilic and slightly halophilic *Nocardioides* sp. strain M6 was found to degrade 2,4,6-trichlorophenol, 2,4,5-trichlorophenol and 2,4-dichlorophenol up to concentrations of 50 mgl^{-1} in high density culture (Maltseva and Oriel, 1997).

Pentachlorophenols were reported to be degraded by the strains of *Flavobacterium*. Saber and Crawford (1985) isolated 40 pentachlorophenol (PCP) mineralizing strains of *Flavobacterium* from different pentachlorophenol contaminated sites and all the strains were reported to be utilizing PCP up to 20 mgl^{-1} of pentachlorophenol (PCP) in the growth media. Metabolism of PCP by *Flavobacterium* sp. was facilitated by supplementary carbon source. Sodium glutamate and glucose was reported to be used as the supplementary carbon source (Topp *et al.*, 1988). *Flavobacterium* cells that had been induced for PCP degradation, dechlorinated and

mineralized 2,3,4,6, and 2,3,5,6-tetrachlorophenol; 2,3,6 and 2,4,6-trichlorophenol and 2,6-dichlorophenol. The ability of the *Flavobacterium* sp. to dechlorinate and mineralize chlorophenols was related to the chlorine ring substitution patterns of specific compounds. Chlorophenols with chlorine substituted at 2 and 6 positions were completely dechlorinated and mineralized by *Flavobacterium* sp. (Steirt *et al.*, 1987).

Complete mineralization of pentachlorophenol by five strains of *Arthrobacter* was reported (Stanlake and Finn, 1982). An *Arthrobacter* strain (ATCC 33790) was found to be capable of utilizing pentachlorophenol as sole source of carbon and energy up to 300 mg l^{-1} (Edgehill and Finn, 1983).

Ryding *et al.* (1994) had investigated the chlorophenol degradation by the toluene enriched aerobic mixed culture and showed rapid degradation of 2,4-dichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol.

Enzyme activities for catabolism of PCP and other chlorinated phenols by *Flavobacterium* are reported to be under the control of an inducible enzyme system (Steiert and Crawford, 1985). PCP degradation enzymes were induced when *Flavobacterium* cells were grown in presence of 2,4,6-trichlorophenol and 2,3,5,6-tetrachlorophenol (Steiert *et al.*, 1987). In the degradation of pentachlorophenol by a *Flavobacterium* sp., tetrachlorohydroquinone formed after hydroxylation was reductively dechlorinated through trichlorohydroquinone and 2,6 dichlorohydroquinone. The pathway for complete dechlorination was not elucidated (Steirt and Crawford, 1986). Enzymes involved in the degradation process of 2,4,5-trichlorophenol or pentachlorophenol by *Pseudomonas cepacia* were also inducible and

were repressed during growth in the presence of alternate carbon source (Karns *et al.*, 1983).

2.6 Biological degradation of chlorophenols by *Rhodococcus*

Biological degradation of aromatics by the eubacterial genus *Rhodococcus* is receiving much attention in recent times. The versatility and robustness of this genus in natural environment and their wide variety of metabolic capabilities can cause it to replace the most predominantly used genus *Pseudomonas* in the biological treatment system (Warhurst and Fewson, 1994). *Rhodococci* have been found to be more effective in degrading chlorophenols in natural conditions than other chlorophenol degrading bacteria (Valo *et al.*, 1990).

2.6.1 General biology of the genus *Rhodococcus*

Rhodococci have a wide distribution in nature and are found in soil, marine, fresh water habitats and from soil contaminated with petroleum compounds (Dobrovolskaya and Tretyakova, 1991; Maeda *et a*l, 1995). The *Rhodococci* are aerobic, gram positive, non-motile, mesophilic, nocardioform actinomycetes with a growth cycle ranging from cocci or short rods to more complex growth phases that form filaments with short projections, elementary branching and in some species extremely branched hyphae (Goodfellow, 1989). *Rhodococci* are partially acid fast with an oxidative type of metabolism and grow well in standard laboratory media. Some strains require thiamine when grown on chemically defined media (Starr *et al.*, 1981). *Rhodococci* are characterized by pigmented colony forms (red, orange and pink). Pigmentation is believed to be due to the presence of carotenoids (Takaichi *et*

al., 1990). *Rhodococci* have been identified and classified based on their distinctive cell wall composition which consists of mycolic acid (32 to 36 carbon atoms), tuberculostearic acid, menaquinones with nine isoprenoid units and one hydrogenated double bond (Finnerty, 1992). *Rhodococci* are allochthonous organisms that are characterized by low growth rate but high substrate affinity and persistence in environment (Salkinoja-Salonen *et al.*, 1991). *Rhodococci* unlike *Pseudomonas* do not exhibit catabolite repression, which causes suppression in the breakdown of target organic substrate in presence of easily degradable substrate. In several cases Rhodococcal biotransformation is accelerated by provision of additional feedstock, and also by cosubstrates (Warhurst and Fewson, 1994). Extracellular flocculant was produced by *Rhodococcus erythropolis* grown on 1% glucose, urea and yeast extract under conditions of low aeration and pH 8.5 to 9.5 at 30°C. The flocculant of *Rhodococcus erythropolis* is able to remove coloring material from an aqueous solution in addition to the flocculation of suspended solids (Kurane *et al.*, 1986). The ability of *Rhodococci* for high persistence in the environment even under starvation conditions, lack of catabolite repression and flocculating ability makes it ideal for use in bioremediation of contaminated sites and biological treatment of wastewater (Briglia *et al.*, 1990; Warhurst and Fewson, 1994).

2.6.2 Biodegradation of chlorophenols by *Rhodococcus*

Biological degradation of chlorophenols by pure cultures of *Rhodococcus* has been reported. *R. erythropolis* 1CP can grow on 4-chlorophenol (0.1 g l^{-1}) and 2,4-dichlorophenol (0.05 g l^{-1}) as sole source of carbon and energy. Co-substrates such as glucose and maltose accelerate the breakdown of chlorophenols (Gorlatov *et al.*, 1989;

Gorlatov and Golovleva, 1992). Chlorobenzene induced cells of a *Rhodococcus opacus* strain GM-14 was reported to degrade monochlorophenol. The organism was reported to utilize 2-chlorophenol, 3-chlorophenol and 4-chlorophenol (Zaitsev *et al.*, 1995). Phenol induced cultures of *Rhodococcus rhodochrous* and *Rhodococcus* species strain P1 and AN 117 were shown to transform 10 μmoles of 3-chlorophenol, 4-chlorophenol, 2,3-dichlorophenol and 3,4- dichlorophenol into chlorocatechol (Haggblom *et al.*, 1989).

The ability of pure culture of *Rhodococcus chlorophenolicus* to mineralize chlorinated phenols has been intensively studied. (Apajalahati *et al.*, 1986; and Apajalahati and Salkinoja-Salonen, 1986). *Rhodococcus chlorophenolicus* degrades a series of chlorinated phenols ranging from dichlorophenols to pentachlorophenols (Apajalahati *et al.*, 1986). *R. cholorophenolicus* (DSM 43826) utilized pentachlorophenol as the sole source of carbon and energy (Apajalahati and Salkinoja-Salonen, 1987a). *Rhodococcus* sp. strain CP-2 (DSM4598) isolated from chlorophenol contaminated soil mineralizes pentachlorophenol and other polychlorinated phenols (Haggblom *et al.*, 1989 a). *Rhodococcus* sp. strain CG-1 degraded pentachlorophenol, 2,3,4,6-tetrachlorophenol, 2,3,5,6-tetrachlorophenol, 2,3,5-trichlorophenol, 2,3,6-trichlorophenol, 2,4,6-trichlorophenol and 3,4-dichlorophenol (Allard *et al.*, 1987). *Rhodococcus percolatus* was able to mineralize 2,4,6-trichlorophenol (Briglia *et al.*, 1990).

Metabolism of monochlorinated phenols was reported to proceed through chlorocatechol in *Rhodococcus* sp. AN 117 with dechlorination occuring only after the

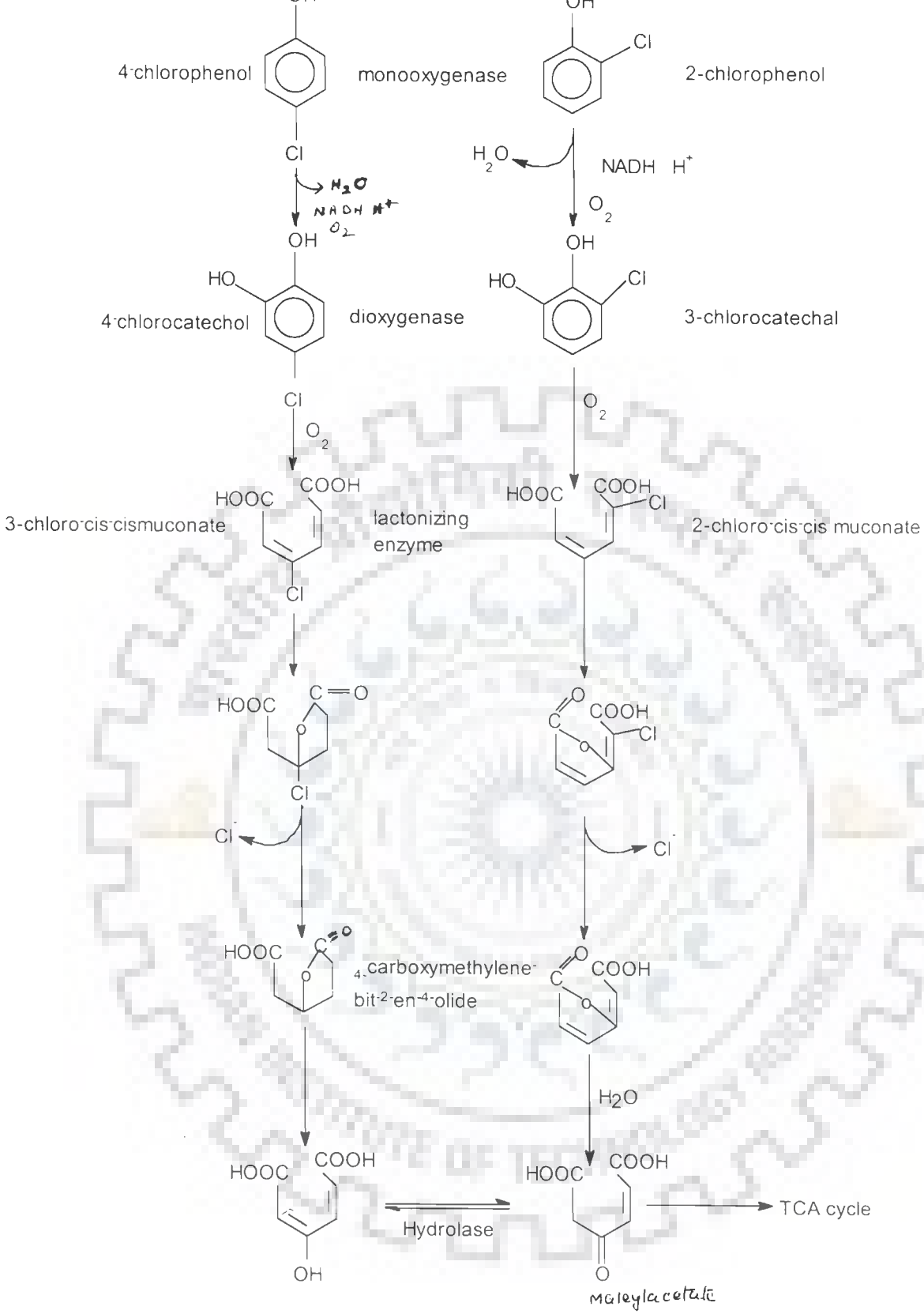


Figure 1. Orthocleavage pathway for the degradation of 2-chlorophenol and 4-chlorophenol (Steirt and Crawford, 1985)

ring is cleaved (Janke *et al.*, 1989). The complete degradation pathway of monochlorinated phenol is shown in Figure 1. The enzyme catechol 1,2 dioxygenase involved in the degradation of monochlorophenol and dichlorophenol has been purified from *Rhodococcus erythropolis* 1CP (Maltseva *et al.*, 1991).

Degradation of pentachlorophenol involves removal of all the chlorines in the phenol ring before the ring is cleaved (Apajalahati and Salkinoja-Salonen, 1987b). *R. cholorophenolicus* PCP-1 initiates pentachlorophenol degradation by hydrolytic parahydroxylation which is expressed by inducible enzyme systems (Reiner *et al.*, 1978; Haggblom *et al.*, 1986; Apajalahati and Salkinoja-Salonen, 1987). The tetrachlorohydroquinone formed in parahydroxylation were further degraded through hydrolytic dechlorination and three reductive dechlorination, producing 1,2,4-trihydroxybenzene (Apajalahati, and Salkinoja-Salonen, 1987). The dechlorination is undertaken by two enzyme activities, first dechlorinating para-hydroxylation by a membrane-bound, FAD and NADPH dependent enzyme, then dechlorination by a soluble enzyme (Uotila *et al.*, 1991). Similar pathway of pentachlorophenol degradation, involving parahydroxylation followed by a second hydroxylation and three reductive dechlorination have been reported for *Rhodococcus* sp. CP-2 as shown in Figure 2 (Apajalahati and Salkinoja-Salonen, 1987 a and b).

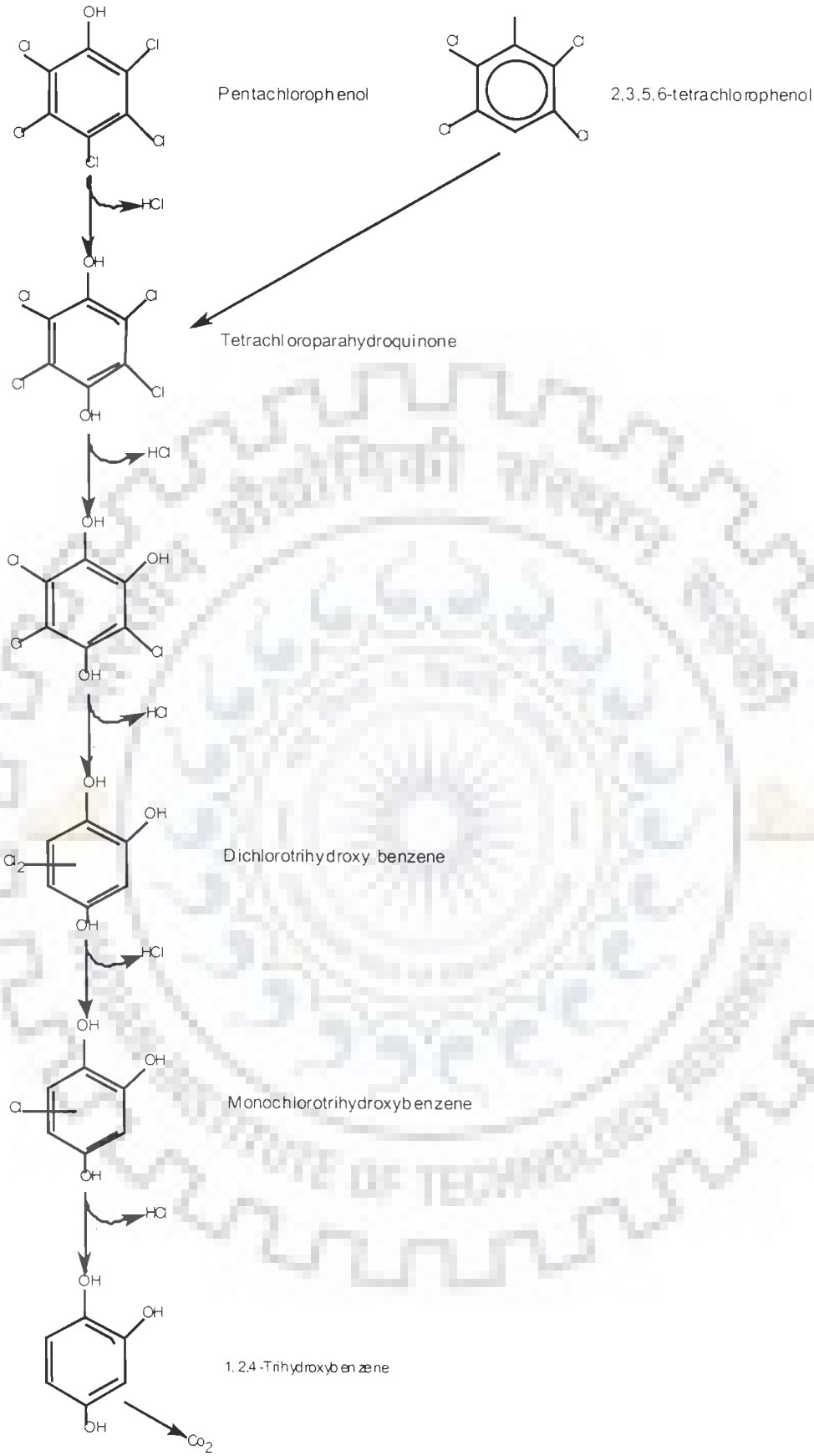


Figure 2. Pathway for the degradation of Pentachlorophenol and tetrachlorophenol (Apajalahati and Salkinoja-Salonen, 1987 a, b)

2.7 Microbial degradation of chlorophenol in mixed substrate

In most of the contaminated site and industrial effluent, chlorophenols rarely occurs as a pure compound but more often in a mixture with other pollutants making it important to understand the microbial degradation of this compound in mixtures (Gu and Korus, 1995; Hill *et al.*, 1996). In the utilization of multiple substrates, degradation patterns include diauxie or sequential utilization (Monod, 1949; Gaudy *et al.*, 1963; Kim and Maier, 1986; Rozich and Calvin, 1986), simultaneous utilization (Law and Button, 1977; Moos *et* al., 1983; Deshpande *et al.*, 1987) and competitive inhibition (Strand *et al.*, 1990; Reardon *et al.*, 2000).

In diauxic growth one substrate is utilized preferentially and completely before acting on the other. Diauxic growth has also been attributed to catabolite inhibition or catabolite repression (Klecka and Maier, 1988). Sequential removal was demonstrated in a study by Deshpande and Chakrabarti (1988) for resorcinol and catechol from binary substrate systems containing resorcinol and meta-aminophenol, and catechol and resorcinol respectively by the activated sludge acclimated to the respective mixed feed. Preferential utilization of substrate was also reported for a mixture of pentachlorophenol and phenol by enrichment culture. Phenol degradation was observed when pentachlorophenol was exhausted by the culture (Klecka and Maier, 1988). Diauxic growth pattern was observed for a mixture of 2-chlorophenol and 4-chlorphenol by *Alcaligenes sp.* A7-2, where mineralization of 4-chlorphenol was nearly complete before the degradation of 2-chlorphenol started (Menke and Rehm, 1992). Similar pattern of utilization was reported for a mixture of phenol and acetate by pure cultures of *Alcaligenes eutrophus*. Acetate was the preferred substrate. The

specific rate of phenol consumption was considerably decreased though not abolished. Similarly in the mixture of benzoate and phenol, benzoate was found to completely block the catabolism of phenol. Phenol was metabolized only after benzoate was totally exhausted which is a typical case of diauxic pattern of growth (Ampe *et al.*, 1998).

Simultaneous utilization pattern has also been well documented for a mixture of carbohydrates (Yoon *et al.*, 1977), glucose and amino acids (Law and Button, 1977), benzoic acid and glucose (Zollinger, 1996), metanitrobenzene and resorcinol (Deshpande and Chakrabarti, 1988). Aniline and lactate were used simultaneously by *Pseudomonas* strain K1 (Konopka *et al.*, 1989). Shivaraman (1992) showed simultaneous utilization of phenol and resorcinol by *Candida tropicalis*. Similar results were obtained with *Alcaligenes faecalis* for a mixed system of p-cresol and phenol. Binary mixture of 4-chlorophenol and phenol was observed to be transformed simultaneously by *Alcaligenes sp.* A7-2 (Menke and Rehm, 1992). Simultaneous utilization of substrate was noted for a mixture of dichlorophenol, trichlorophenol and pentachlorophenol by a *Flavobacterium* sp. (Gu and Korus, 1995). However both dichlorophenol and trichlorophenol lowered the cell growth and pentachlorophenol degradation rate. Simultaneous utilization of pentachlorophenol and 2,4,5-trichlorophenol was observed for enrichment culture by Klecka and Maier (1998).

2.8 Biological treatment of chlorophenol

Aerobic treatment of chlorinated phenols by biological reactors has been investigated by few researchers. Suspended growth system for the degradation of chlorophenol has been evaluated by few workers. Etzel and Kirsch (1974) reported

99% pentachlorophenol removal in case of wood treatment wastewater from wood preservation process containing 20 to 60 mg l^{-1} of pentachlorophenol by a mixed aerobic suspension culture at hydraulic retention time of 6 h to 12 h. Degradation of PCP by a microbial consortium in a continuously stirred tank reactor was reported by Moos *et al.* (1983) with maximum mineralization occurring at about 350 μg l^{-1}. PCP degradation in a continuous laboratory scale activated sludge system with PCP concentration varying between 1 and 120 mg l^{-1} was studied in acclimated systems with and without the addition of *Arthrobacter* sp. strain ATCC 33790. Hydraulic retention time ranged from 8.9 to 10.4 h and a mean cell residence time of 6.2 days was maintained. *Arthrobacter* amended system showed a brief improved response to shock loads as compared to the acclimated system without the specific strain (Rochkind-Dubinsky *et al.*, 1987). The fate of pentachlorophenol in bench scale, continuous flow, and activated sludge reactor was studied at sludge retention time (SRT) of 10 to 15 days. Reduction of PCP concentration was observed from 0.1 to 12 mg l^{-1} to less than 10 μg/l (Melcer and Bedford, 1988). Pentachlorophenol degradation has been examined by Nyholm *et al.* (1992) in a laboratory scale activated sludge reactor fed with synthetic sewage under various operating conditions. The bioreactor was spiked with 5 to 1000 μg PCP l^{-1}. Biodegradation exceeded 80% when the sludge age was greater than 10 to 15 days.

Use of fixed film system for the treatment of chlorophenolic waste has also been reported. A packed bed fermentor using lecaton (light expanded clay aggregate) particles as the support material was seeded with *Alcaligenes* sp. A7-2 and was used to treat municipal water contaminated with 4-chlorophenol. The water was

decontaminated at a rate of 300 µmol/l/h (Westmeier and Rehm, 1987). Tokuz (1989) studied the treatment of wastewater with PCP concentration of 1 to 4 mg l^{-1} using rotating biological contactors with 52.6% efficiency. Continuous flow fluidized bed reactor with spherical silica based micro carriers for cell immobilization showed 92.5% removal when used to treat contaminated ground water with pentachlorophenol loading rate of 217 g/m^3/day and a hydraulic retention time (HRT) of 5h (Puhakka and Jarvinen, 1992). At an hydraulic retention time of 5 h and polychlorophenol loading rate of 445 mg l^{-1}, day, 99.7% of chlorophenol removal was reported using aerobic fluidized bed reactor systems (Maklen *et al.*, 1993). An aerobic fluidized bed reactor was evaluated for the treatment of contaminated groundwater containing 2,4,6-trichlorphenol, 2,3,4,6-tetrachlorophenol and pentachlorophenol employing sand, volcanite and diatomaceous earth as biomass carriers. The volcanite reactor had the highest biomass accumulation where as the sand reactor had the lowest biomass during the treatment of chlorophenols (Melin *et al.*, 1998). The capacity to degrade 2,6-dichlorophenol was examined in a fixed bed reactor. *Ralstonia* sp. Strain RK 1 (formerly *Alcaligenes*) mineralized 2.6-dichlorophenol at the concentration of up to 740 µM in continuous reactor (Steinle *et al.*, 1998).

A number of studies have looked at the application of anaerobic treatment systems for biodegradation of chlorinated phenols. Most of the anaerobic treatment units were fed with alternate carbon source. Krumme and Boyd, (1988) used anaerobic upflow bioreactors to treat monochlorophenolic isomers with substrate conversion efficiency >90% at substrate loading rate of up to 20 mg/l/day at hydraulic retention time (HRT) of 2-4 days. Wood *et al.* (1989) studied chlorophenol degradation in an

upflow anaerobic sludge blanket reactor in presence of readily biodegradable organic compounds. 2,4,6-trichlorophenol; 2,3,4,5-tetrachlorophenol and pentachlorophenol at concentration 0.1 to 1 mg l^{-1} were converted to lesser-chlorinated compounds at HRT of 13 h. Treatment of chlorinated phenols with anaerobic fluidized granular activated carbon showed removal efficiency of 99% for 2-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol at sludge mean residence time of 60-100 days in presence of acetate and phenol (Flora *et al.*, 1994).

CHAPTER-3

3.0 *MATERIALS AND METHODS*

3.1 MATERIALS

3.1.1 Chemicals

All the chemicals used for reagent and media preparations were of analytical grade (AR) of highest purity and were commercially available. For the preparations of microbiological media, biochemicals of Hi-Media, India were used. Cycloheximide was purchased from Sigma Chemicals, U.S.A. Inorganic and organic chemicals used in the study were procured from E-Merck, Germany, Glaxo India Ltd. and Ranbaxy fine chemicals Ltd. India.

3.1.2 Microbiological media

3.1.2.1 M3 medium as described in Prokaryotes (Starr *et al.*, 1981) was used for enrichment and isolation of *Rhodococcus* sp. Composition of the M3 medium is given in Table 4.

3.1.2.2 M3 medium as described above was supplemented with 1.8% (w/v) agar (Hi Media Agar type I) to make M3 agar medium.

3.1.2.3 Modified M3 medium was used for screening, induction, optimization, kinetic, mixed substrate and mixed culture studies. The composition of the modified M3 medium is presented in Table 5.

3.1.2.4 DSM-65 medium was used for the growth of *Rhodococcus* sp. (Table 6).

3.1.2.5 *Pseudomonas* isolation agar was used for the isolation of *Pseudomonas* sp. The composition is described in Table 7.

3.1.2.6 Nutrient broth was used for growth of the cultures for biochemical characterization for identification of the culture. The composition of the nutrient broth medium is presented in Table 8.

3.1.2.7 Nutrient agar was used in the study of colony characteristics for identification and maintenance of the cultures. Nutrient agar was prepared by adding 2% (w/v) agar to the nutrient broth.

3.1.2.8 Luria broth was used to study the morphological changes (viewed microscopically) of *Rhodococcus* sp. The composition of the medium is described in Table 9.

3.1.2.9 Acetamide agar medium was used for the differentiation of *Rhodococcus* sp. in coculture experiment with *Pseudomonas* sp. The composition of the medium is given in Table 10.

Table 4. Composition of M3 medium (Prokaryotes. 1981)

S.No.	Constituents	Amount (gl^{-1})
1	KH_2PO_4	0.466
2	Na_2HPO_4	0.732
3	KNO_3	0.01
4	NaCl	0.29
5	$MgSO_4.7H_2O$	0.1
6	$CaCO_3$	0.02
7	$FeSO_4.7H_2O$	0.0002
8	$ZnSO_4.7H_2O$	0.00018
9	$MnSO_4.4H_20$	0.00002
10	Sodium Propionate	0.2
	pH was set to 7.0	

Cycloheximide and thiamine hydrochloride, sterilized by membrane filtration was added to the autoclaved and cooled medium to give final concentrations of 50 mgl^{-1} and 4 mgl^{-1} respectively.

Table 5. Composition of modified M3 medium

S.No.	Constituents	Amount (gl^{-1})
1	KH_2PO_4	0.466
2	Na_2HPO_4	0.732
3	KNO_3	0.01
4	$MgSO_4.7H_2O$	0.1
5	$CaCO_3$	0.02
6	$FeSO_4.7H_2O$	0.0002
7	$ZnSO_4.7H_2O$	0.00018
8	$MnSO_4.4H_20$	0.00002
	pH was set to 7.0	

Cycloheximide and thiamine hydrochloride, sterilized by membrane filtration was added to the autoclaved and cooled medium to give final concentrations of 50 mgl^{-1} and 4 mgl^{-1} respectively.

Table 6. Composition of DSM-65 medium (Haggblom *et al.*, 1989)

S.No.	Constituents	Amount (gl^{-1})
1	Malt extract	4.0
2	Yeas extract	4.0
3	Glucose	4.0
	pH was set to 7.2	

Table 7. Composition of *Pseudomonas* isolation agar

S.No.	Constituents	Amount (gl^{-1})
1	Malt extract	20.0
2	$MgCl_2$	1.4
3	K_2SO_4	10.0
4	Glycerol	20.0*
5	Irgasan	0.025
6	Agar	13.6
	pH was set to 7.2	

* Volume in ml

Table 8. Composition of Nutrient agar medium

S.No.	Constituents	Amount (gl^{-1})
1	Peptone	5.0
2	Yeast extract	3.0
3	NaCl	5.0
4	Agar	20.0
	pH was set between 6.8 to 7.2	

Table 9. Composition of Luria broth (Lanyi, 1987)

S.No.	Constituents	Amount (gl^{-1})
1	Tryptone	10.0
2	Yeast extract	5.0
3	NaCl	5.0
	pH was set to 7.3	

Table 10. Composition of Acetamide agar medium (Lanyi, 1987)

S.No.	Constituents	Amount (gl^{-1})
1	Acetamide	10.0
2	NaCl	5.0
3	K_2HPO_4	1.39
4	KH_2PO_4	0.73
5	Phenol red	3.0*
6	Agar	20.0
	pH was set to 7.0	

*Volume in ml of 4% (w/v) aqueous solution of phenol red

3.1.3 BUFFERS

3.1.3.1 Sodium phosphate buffer (0.24 M Na_2HPO_4 and 0.02 M NaH_2PO_4) containing glutarladehyde solution (2.5% w/v) was used for scanning electron micrograph of bacterial culture.

3.1.3.2 (0.2 M) Potassium phosphate buffer (pH 7.2) was used for preparation of cell suspension.

3.1.4 Trickling filter unit

A circular unit of perspex glass sheet of the following specifications was used to fabricate a trickling filter unit:

Diameter	: 30.0 cm
Circumference	: 94.0 cm
Height	: 28.8 cm
Total volume of the reactor	: 20.3 l
Total working volume of the reactor	: 14.49 l
Void volume of the reactor	: 6.1 l
Packed volume of the reactor	: 8.39 l
Total surface area of the reactor	: 2714.33 cm^2
Area of the reactor in touch with the column	: 1932.1 cm^2
Cross sectional area of the reactor	: 706.85 cm^2

Gravel of size 4.5 cm were used to fill up the unit to give a column height of 20.5 cm. Peristaltic pump (Watson and Marlow) was used for feeding the synthetic waste into the unit. Carboys of 30 l capacity were used for containing the synthetic waste and collecting the effluent. Composition of the synthetic waste is as described in Table 11.

Table 11. Composition of synthetic waste for trickling filter reactor

Constituents	Amount (gl^{-1})
Chlorophenols : 2-chlorophenol/ 4-chlorophenol	0.01 to 0.2 / 0.01 to 0.1
Potassium di-hydrogen phosphate	0.009
Di-sodium hydrogen phosphate	0.014
Potassium nitrate	0.05

3.2 METHODS

3.2.1 Enrichment and Isolation of *Rhodococcus* sp.

3.2.1.1 Sources for isolation of *Rhodococcus* sp.

Microorganisms belonging to the genus *Rhodococcus* were isolated from soil samples collected from garden, compost, pond sediment, and oil-contaminated sites viz. petrol pump, garage and gas station within the National Environmental Engineering Research Institute premises.

3.2.1.2 Enrichment in selective media for isolation of *Rhodococcus* sp.

Samples used as inoculum for enrichment were processed following the procedure described in Prokaryotes (Starr *et al.*, 1981). The samples, suspended in 25 ml distilled water were put under shaking (rpm) in a 250 ml Erlenmeyer flask at room temperature in a shaker for 30 minutes. The suspension was then allowed to settle for 10 minutes. The supernatant (15 ml) was added to an equal volume of sodium hydroxide (8% w/v), and the mixture was shaken for 10 minutes before centrifugation at 5000 x g for 15 minutes. The residue was suspended in M3 medium specified for isolation of *Rhodococcus* (Prokaryotes, 1981). 50 ml of M3 medium was taken in a 250 ml Erlenmeyer flask and kept at 120 rpm for incubation at room temperature. Aliquots of 10 ml from existing flasks were transferred into 40 ml of fresh M3 medium once a week for two months.

3.2.1.3 Acclimatization of the enrichment culture to 2-chlorophenol

Cultures thus isolated were subsequently exposed to 2-chlorophenol by gradually decreasing the sodium propionate (carbon source in M3 medium) concentration from 200 to 0 mg l^{-1}. Simultaneously, 2-chlorophenol concentration was increased from 10 mg l^{-1} to a final concentration of 50 mg l^{-1} at 10 mg l^{-1} increment. Samples showing increase in turbidity of the medium indicated positive growth and were used for isolation of the pure cultures. These samples were plated on M3 agar medium and incubated at 32°C for one to two weeks for the isolation of pure cultures. Pure cultures thus obtained were designated as M1, M2, M3, M4, M5 and maintained on chlorophenol (50 mgl^{-1}) containing M3 agar slants stored at 4°C.

3.2.2 Enrichment and isolation of *Pseudomonas* sp.

Pseudomonas sp. was isolated from soil sample by using Pseudomonas isolation agar (Table 7). Pure culture isolated on *Pseudomonas* isolation agar was designated as strain P1. The isolate was maintained on nutrient agar slants and stored at 4°C.

3.2.3 Identification of the isolates

Pure cultures isolated on M3 agar medium and the strain P1 were identified based on the classification scheme described in Bergey's Manual of Systematic Bacteriology (Goodfellow, 1989). Isolates were identified on the basis of colony, cell morphological, physiological and biochemical characterization. The purity of the cultures were checked by plating on nutrient agar plate before the detailed examination

of the isolates for identification.

3.2.3.1 Colony and cell morphological characterization of the isolates

Colony characteristics of the isolates were studied on nutrient agar plates. Colony size, form, elevation, surface and margin were noted.

Cell morphology of the cultures isolated on M3 agar medium was studied by subjecting the cultures to scanning electron microscopy. Morphology of the cells at different stages of growth was observed by growing the culture in Luria broth. Observation was made at periodic intervals by a light microscope (Olympus, BX-50) at 800 x.

The isolates were subjected for gram staining and the motility of the isolates was examined by the hanging drop method.

3.2.3.1.1 Scanning electron microscopy of the isolates

The culture was smeared on a cover slip in a uniform layer and heat fixed. The coverslip was then dipped overnight in the glutaraldehyde (2.5%) phosphate buffer for fixation followed by dehydration in graded series of ethanol. Ethanol treatment was initiated by treatment with 30% alcohol in which coverslip was dipped for 10 minutes. This was followed by treatment with 50%, 75%, and 90% of alcohol respectively for a period of 10 minutes for each gradation of alcohol. Finally the coverslip was dipped in absolute alcohol for 30 minutes. The cover slip was then coated with osmium tetrachloride and then subjected to scanning electron microscopy (– 250 MKB from Cambridge instruments, USA).

3.2.3.2 Physiological and biochemical characterization of the isolates

Physiological and biochemical characterization of the isolates was based on standard biochemical tests. Specifications for different media used in the biochemical tests and the detailed procedure for the tests were adopted from Methods in Microbiology (Norris and Swain, 1971) and Methods in Microbiology (Lanyi, 1987).

- Catalase activity was determined by hydrogen peroxide treatment
- Starch hydrolysis was observed on starch agar plates by treating it with iodine after incubation.
- Caseinase activity of the isolates was studied on caseinase agar plate.
- Sugar fermentation tests for acid or gas production were carried out in peptone water broth with different sugars at the concentration of 0.1% (w/v).
- Tests for glucose oxidation or fermentation were carried out in oxidation-fermentation medium (Hugh and Leifson's medium).
- Urease activity of the isolates was determined by Christensen's urea agar slant.
- Indole production by the culture in tryptone water was detected with Kovac's reagent.
- Methyl red – Voges Proskauer tests were carried out on glucose-phosphate broth.
- Simmon's citrate agar was used to test the ability of the isolate to utilize citrate as the sole source of carbon and energy.
- Nitrate reduction by the isolate was tested on nitrate broth.
- Oxidase activity of the culture was tested by tetramethyl-p-pheylene diamine hydrochloride.
- Hydrolysis of gelatin was observed by adding 4 % gelatin to nutrient broth.

- Gluconate broth was used to detect production of 2 – Ketogluconate.
- Deamination of phenylalanine to phenylpyruvic acid was detected using phenylalanine agar.
- Production of lipase was detected by growth on tryptone soy agar to which Tween – 80 has been added.
- Growth in presence of NaCl was observed by growing the cultures in mineral salt medium containing yeast extract (100 mgl^{-1}) and 5% NaCl (w/v).

3.2.4 Screening of the *Rhodococcus* isolates for phenol, cresols and chlorophenols degradation

Isolates belonging to the genus *Rhodococcus* were screened for phenol, cresols (ortho, meta and para) and chlorophenols (2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol) degradation. The phenolics and chlorophenols were added at an initial concentration of 100 mgl^{-1} as the only carbon source in 50 ml of modified M3 medium in a 250 ml Erlenmeyer flask and kept in a rotary shaker (120 rpm) at 32°C. Inoculated control devoid of organic carbon sources mentioned above was used as the biological control. Chemical control in which organism was not inoculated was kept to check for any abiotic loss of the substrates. Suitable aliquots of cell suspensions of the *Rhodococcus* isolates were added to the medium to give the initial Absorbance value at 540 nm from 0.01 to 0.02. Aliquots of the samples (5 ml) were taken at periodic intervals for the measurement of cell growth and estimating substrate concentration. The preparation of the cell suspension, stock solutions of the substrates and determination of cell growth are as described below:

3.2.4.1 Preparation of cell suspension

Rhodococcus isolates were grown in DSM-65 medium in a rotary shaker (120 rpm) at room temperature. Cells were harvested after 24 h under aseptic condition by centrifugation at 5,000× g for 20 minutes, washed twice with 0.2 M sterile phosphate buffer (pH 7.2) and resuspended in the same buffer.

3.2.4.2 Estimation of cell growth

The cell growth was estimated by measuring the absorbance at 540 nm by a Perkin-Elmer Spectrophotometer. Absorbance values were correlated with cell dry weight by using a standard curve in which absorbance was plotted against cell weight. For the measurement of the cell dry weight suitable volume of the culture fluid was filtered through pre-weighed 0.45-µm, 47 mm Millipore membrane filter and dried at 105°C for 1 hour. The difference between the initial weight of filter paper and the weight after drying gave the cell dry weight.

3.2.4.3 Preparation of the stock solution of substrates

Stock solutions (1% w/v) of phenol and cresols (ortho, meta and para) were prepared in double distilled water. Stock solutions of chlorophenols (2-chlorophenol, 4-chlorophenpol, 2,4-dichlorophenol and 2,4.6-trichlorophenol) of similar strength of (1%, w/v) were prepared in 1 N NaOH and the pH of the solution was adjusted to 7.5. The solutions were sterilized by filtering it through 0.45 µm, 47 mm Millipore membrane filters.

3.2.5 Induction of *Rhodococcus* sp. M1 for 2-chlorophenol degradation

Rhodococcus sp. M1 selected on the basis of screening experiments was subjected to induction by different inducers with the objective of reducing the lag period for 2-chlorophenol degradation. *Rhodococcus* sp. M1 was grown in 100 ml of modified M3 medium in 500 ml Erlenmeyer flasks containing different inducers such as toluene, benzoic acid, phenol and catechol at 100 mgl^{-1} individually one at a time. Stock solutions (1%, w/v) of toluene, benzoic acid, phenol and catechol were prepared and sterilized by membrane filter. Inoculum was prepared as described in section 3.2.4.1 and was added to the medium so as to give initial absorbance of 0.01 to 0.02 at 540 nm. The inoculated flasks were incubated on a rotary shaker (120 rpm) at 32°C. Aliquots (5 ml) were collected at periodic intervals for measuring the absorbance (540 nm) using a spectrophotometer (Perkin Elmer, U.S.A.). The values thus obtained recorded the cell growth at different time intervals.

3.2.6 Degradation of 2-chlorophenol by induced cells of *Rhodococcus* sp. M1

Rhodococcus sp. M1 was grown in 100 ml of modified M3 medium containing any one of the inducers, benzoic acid, phenol, toluene or catechol at 100 mgl^{-1} in 500 ml Erlenmeyer flasks. Cells grown on toluene, benzoic acid, phenol or catechol were harvested in their logarithmic phase after 32 h, 36 h, 44 h and 52 h respectively by centrifugation. The cell pellet was washed with 0.2 M phosphate buffer (pH 7.2) and suspended in the same buffer. Inoculum thus prepared was added to 50 ml of modified M3 medium containing 2-chlorophenol (100 mgl^{-1}) to give an initial absorbance value of 0.01 to 0.02 at 540 nm. Multiple flasks were setup for each experiment as one flask was sacrificed at each sampling period. Flasks were subjected to shaking conditions in

a rotary shaker (120 rpm) at 32°C for incubation. Samples were collected and evaluated at periodic intervals for 2-chlorophenol and chloride concentrations. The cell growth was determined by evaluating the absorbance at 540 nm for the samples.

3.2.7 Optimization of temperature and pH for chlorophenol degradation by *Rhodococcus* sp. M1

Benzoic acid induced cells were acclimatized to 2-chlorophenol by inoculating into modified M3 medium containing 2-chlorophenol at a concentration of 100 mg l^{-1} and incubating in at 32°C under shaking condition at 120 rpm. Cells grown on 2-chlorophenol were harvested by centrifugation after 18 h and washed with 0.2 M phosphate buffer (pH 7.2) before being used as inoculum for optimization studies. Optimization of temperature and pH conditions for the degradation of chlorophenols by *Rhodococcus* sp. M1 was carried out using 2-chlorophenol as the substrate.

3.2.7.1 Optimization of temperature

2-chlorophenol was added to an initial concentration of 100 mg l^{-1} in 50 ml of modified M3 medium taken in 250 ml Erlenmeyer flask. Inoculum was added to give an initial absorbance value between 0.01 to 0.02 at 540 nm. The experimental flasks were kept in incubators set at 10, 15, 20, 25, 30, 35, 40 and 45°C. One experimental set up was kept in the refrigerator to note the growth at 4°C. Samples were taken at an interval of 4 h for estimating 2-chlorophenol concentration and growth. Chemical and biological controls were kept for each experimental set up at the above mentioned temperature range.

3.2.7.2 Optimization of pH

2-chlorophenol was added to an initial concentration of 100 mg l^{-1} in 50 ml of modified M3 medium taken in 250 ml Erlenmeyer flask. The pH of the medium was adjusted at a desired value by addition of 0.1 N sodium hydroxide and 0.02 N sulfuric acid and varied from 5 to 12 at an increment of 0.5. Inoculum was added to give an initial absorbance value between 0.01 to 0.02 at 540 nm. The experimental flasks were subjected to shaking conditions in a rotary shaker (120 rpm) at 32°C. Chemical and biological control were kept for each pH condition. Samples were collected at periodic intervals to estimate the 2-chlorophenol concentrations and growth in the sample.

3.2.8 Degradation Kinetics of Chlorophenols by *Rhodococcus* sp. M1

Kinetic studies for the degradation of different chlorophenols, 2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol was done. 50 ml of the modified M3 medium taken in a 250 ml Erlenmeyer flask was added with the above mentioned chlorophenols as the substrates. Study with 2-chlorophenol was carried out at the concentrations i.e. 10, 25, 50, 100, 150, 200, 250, 300, 350 and 400 mg l^{-1}. Similarly 4-chlorophenol was added at 10, 25, 50, 75, 100, 150 and 200 mg l^{-1} in the medium. Concentrations used for 2,4-dichlorophenol were 10, 25, 40, 50 and 100 mg l^{-1}, whereas 10, 25 and 50 mg l^{-1} of the 2,4,6-trichlorophenol were used. Benzoic acid induced culture of *Rhodococcus* sp. M1, acclimatized to 2-chlorophenol was harvested after 18 h by centrifugation and washed twice with 0.2 M phosphate buffer (pH 7.2) for the study of 2-chlorophenol degradation. The cells were then suspended in the same

buffer and were used as the inoculum. Similarly benzoic acid induced cells of *Rhodococcus* sp. M1 acclimatized to respective chorophenols for the study of their degradation kinetics, was used as inoculum. Appropriate volume of the inoculum was added to the medium to give the initial absorbance value of 0.01 to 0.02 at 540 nm. The experimental flasks were incubated in a rotary shaker (120 rpm) at 32°C. Multiple numbers of flasks were incubated for each concentration of the substrates as one flask was sacrificed for each sampling point. Chemical and biological controls were kept for all the experimental set up. Periodic sampling was done to evaluate the substrate concentration, cell growth and chloride. COD of the samples was also estimated.

3.2.9 Degradation of mixed substrate consisting of 2- chlorophenol, phenol and p-cresol as the constituents by *Rhodococcus* sp. M1

3.2.9.1 Degradation of phenol and p-cresol by *Rhodococcus* sp. M1

Degradation of phenol was studied at the concentrations of 50 and 100 mg l^{-1}, added to modified M3 medium. *Rhodococcus* sp. M1 grown in DSM-65 medium washed twice with 0.2 M phosphate buffer (pH 7.2) was used as the inoculum, for the 50 ml of medium added with defined concentrations of phenol. Reaction was incubated at 32°C in shaking condition (120 rpm) and samples were collected for measuring the phenol levels and cell growth. Similar experimental set up was used to study the degradation of p-cresol added at 50 and 100 mg l^{-1} concentrations.

3.2.9.2 Degradation of mixed substrates by *Rhodococcus* sp. M1

Mixed substrate study was carried out with binary mixtures containing 2-chlorophenol (100 mg l^{-1}l) along with phenol (50 mg l^{-1}) and 2-chlorophenol (100 mg l^{-1}) along with p-cresol (50 mg l^{-1}). For the tertiary mixture, 2-chlorophenol, phenol and p-cresol were taken together at a concentration of 50 mg l^{-1} each. Concentrations of individual substrate selected in the present study were based on an effort to simulate actual waste water.

The above mentioned combinations were added in 100 ml of modified M3 medium in a 500 ml Erlenmeyer flask and the benzoic acid induced cells of *Rhodococcus* sp. M1 acclimatized to a mixture of 2-chlorophenol, phenol and p-cresol was used as the inoculum. The inoculum was added in the medium to give an initial absorbance of 0.01 to 0.02 at 540 nm. The medium was incubated on a rotary shaker (120 rpm) at 32°C. Multiple flasks were setup for each experiment as one flask was sacrificed at each sampling point. Samples were evaluated at periodic intervals for growth, substrate concentration, chloride and COD. The different phenolics were estimated by gas chromatography.

3.2.10 Degradation of 2-chlorophenol, phenol and p-cresol by mixed cultures of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1

3.2.10.1 Degradation of phenol, p-cresol and 2-chlorophenol by *Pseudomonas fluorescens* P1

Degradation of phenol and p-cresol by *Pseudomonas fluorescens* P1 was studied for the concentration of 100 mg l^{-1}. The substrates were added individually to 50 ml of

modified M3 medium in a 250 ml Erlenmeyer flask. The culture was grown in nutrient broth and harvested after 18 h by centrifugation. The cell pellet was washed twice with phosphate buffer and was suspended in the same buffer. The inoculum thus prepared was added to the medium to give initial absorbance of 0.01 to 0.02 at 610 nm. The experimental flasks were incubated in a rotary shaker (120 rpm) at 32°C. The degradation level of phenol and p-cresol was detected by analysing samples collected at periodic intervals. Cell growth of the culture was measured at 610 nm.

To study the 2-chlorophenol degradation, the organism was subjected for benzoic acid induction by growing in benzoic acid. The cells were then harvested after 24 h by centrifugation. The cell pellet was washed with 0.2 M phosphate buffer (pH 7.2) and inoculated in M3 medium containing 100 mg l^{-1} of 2-chlorophenol. Incubation was carried out at 32°C in a gyratory shaker. Sampling was done at periodic intervals to estimate 2-chlorophenol, chloride and cell growth at 610 nm.

3.2.10.2 Degradation of chlorophenol, phenol and p-cresol by the mixed cultures of *Rhodococcus* sp M1 and *Pseudomonas fluorescens* P1

Modified M3 medium (100 ml) containing 100 mg l^{-1} of 2- chlorophenol was taken in a 500 ml Erlenmeyer flask. Benzoic acid enriched cells of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 aclimatized to 2-chlorophenol and washed with 0.2 M phosphate buffer (pH 7.2) was used as the inoculum. Appropriate inoculum of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 was added to the modified M3 medium so as to give initially identical CFU/ml (Colony Forming Unit) for both the populations. For equating the CFU/ml values with absorbance suitable aliquots of both

the cultures were used for measuring the absorbance (540 nm for *Rhodococcus* sp. M1 and 610 nm for *Pseudomonas*) and a plot was prepared in which absorbance was plotted against CFU/ml. The medium was incubated under shaking conditions (120 rpm) at 32°C. Suitable aliquots (5 ml) of the samples taken at regular intervals were subjected for the estimation of 2-chlorophenol and also for the enumeration of viable cell counts for both *Rhodococcus* sp M1 and *Pseudomonas fluorescens* P1.

Similar experimental set up was used for the study of phenol (100 mg l^{-1}) and p-cresol (100 mg l^{-1}) degradation individually by the mixed cultures of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1. *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 was grown in DSM-65 medium and nutrient broth respectively. The cells harvested after 24 h by centrifugation and acclimatized to phenol or p-cresol was washed twice with 0.2 M phosphate buffer (pH 7.2). Sampling was done at periodic intervals for the enumeration of viable cell count for both *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 and for the estimation of phenol and p-cresol.

3.2.10.2.1 Determination of viable cell count

The spread plate method was used for enumeration of viable cell count in the sample. 1ml of sample was subjected to 10 fold serial dilutions in sterile dilution blank. Dilution blank was prepared as per the procedure mentioned in Standard Methods for the Examination of Water and Wastewater treatment (APHA, 20th edn., 1998). 1.25 ml of 0.25 M stock phosphate buffer (pH 7.2) was diluted to 1 liter for the preparation of dilution blank. Sample (1 ml) diluted within the range 10^{-2} to 10^{-9} were plated on

nutrient agar plate (Table 8) and acetamide agar plates (Table 10) with the help of a glass spreader. The plates were incubated at 30°C for 48 – 72 h and the colonies developed were counted. Colonies on the nutrient agar plate gives the total colony count for both *Rhodococcus* sp M1 and *Pseudomonas fluorescens* P1 and the colonies on acetamide agar plate gives the colony count for only *Rhodococcus* sp. M1. *Rhodococcus* sp. M1 was plated on both nutrient agar and acetamide agar medium separately to evaluate the colony count on both the medium. Similar count was observed on both the plates. However, incubation period on nutrient agar and acetamide agar was 48 and 72 h, respectively. The colony count for *Pseudomonas fluorescens* P1 showing negative growth on acetamide (Goodfellow 1989) was evaluated by deducting the *Rhodococcal* count on acetamide agar plate from total count on nutrient agar plate.

3.2.11 Bench scale study for degradation of 2-chlorophenol and 4-chlorophenol by a trickling filter reactor

Synthetic waste (Table 11) containing monochlorophenolic isomers (2-chlorophenol / 4-chlorophenol) individually were fed into the trickling filter unit by a peristaltic pump (Watson and Marlow). The liquid (synthetic waste) was uniformly distributed through a perforated distribution arm over the surface of the packed bed column. The distribution arm was uniformly rotated using a electric motor (Adept instrument Motors, Pune, India) at the speed of 5 rpm. The gravel medium was seeded with *Rhodococcus* sp. M1, acclimatized to 2-chlorophenol or 4-chlorophenol.

3.2.11.1 Optimization of organic loading for 2-chlorophenol and 4-chlorophenol

For studying the optimal organic loading rate, the concentration of 2-chlorophenol and 4-chlorophenol individually were varied keeping the flow rate constant at 30 ml/minute. The concentration of 2-chlorophenol in the synthetic waste was varied from 10 to 200 mg l^{-1} to give an organic loading rate of 0.0518 to 1.037 g COD/l-day and the concentration of 4-chlorophenol was varied from 10 to 150 mg l^{-1} for an organic loading rate of 0.0518 to 0.778 g COD/l-day. Grab samples of influent and effluent were collected at an interval of 6h and monitored for pH, substrate and chloride.

3.2.11.2 Optimization of hydraulic loading for degradation of 2-chlorophenol and 4-chlorophenol

For optimizing the hydraulic loading for the treatment of 2-chlorophenol, the flow rate of the trickling filter unit was varied from 30 to 60 ml/minute at a constant organic loading rate of 0.259 g COD/l-day. Optimization of hydraulic loading rate for effective removal of 4-chlorophenol was carried out in the similar flow rate range of 30 to 60 ml/minute keeping the organic loading rate constant at 0.129 g COD/l-day. Grab samples of influent and effluent were taken at an interval of 6h from the trickling filter unit and monitored for pH, the substrates concentration and chloride.

3.2.11.3 Operation of trickling filter reactor at optimal organic and hydraulic loading for the treatment of 2-chlorophenol and 4-chlorophenol

Synthetic waste of optimal organic loading for 2- chlorophenol (0.259 g COD/l-day) and 4-chlorophenol (0.129 g COD/l-day) individually was fed in the trickling filter reactor at the optimal flow rate of 30 ml/min. Grab samples of effluent were collected at one hour interval and composited for six hours before subjecting it to analysis for pH, substrate, chloride, suspended solids and COD.

3.2.12 Analytical Methods

3.2.12.1 Estimation of phenol, cresols and chlorophenols

Suitable aliquots of sample (5 ml) were clarified by filtration (through membrane filter) and estimated by a Perkin-Elmer spectrophotometer (Model Lambda 900). Phenol, cresols (ortho, meta and para), 2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol concentrations were determined by measuring the absorbance at their respective λmax value (Table 14). Concentrations of the substrates were calculated from the standard calibration curve obtained by plotting various concentrations of these phenolics against their absorbance at their respective λmax value. Terminal set of degradation assay samples which showed no chlorophenols by UV-Visible spectrophotometer were subjected to finer analysis by gas chromatography to confirm the presence of any trace amount of chlorophenols left.

Table 12. λ_{max} values for phenol, cresols and chlorophenols

Substrate	λmax (nm)
Phenol	270
o-cresol	271
m-cresol	272
p-cresol	277
2-chlorophenol	273
4-chlorophenol	280
2,4-dichlorophenol	306
2,4,6-trichlorophenol	313

3.2.12.2 Estimation of phenol, cresols and chlorophenols by Gas Chromatography

Gas chromatograph (GC) of Perkin-Elmer-Autosystem (Software LC1002, attached to Okidata microline 320, 9 pin printer) was used for the estimation of the phenolics. The GC was equipped with a CP SIL 8 CB capillary column (Chrompack-length 25 m; and inner diameter 0.32 mm) and a flame ionization detector. Hydrogen was used as the carrier gas at 4.5 psi. Injector and detector temperature was at 300°C. The initial oven temperature was 125°C and then raised to 180°C at the rate of 4°C/minute. From 180°C the temperature was raised to 250°C at the rate of 10°C/m and kept isothermal at 250°C for 2 minutes.

Samples were removed aseptically for estimation and filtered through 0.45 μm Millipore filter paper for clarification. Cell free medium was then extracted with

hexane. 20 ml of samples were taken and acidified with hydrochloric acid to pH<2.0. The samples were then extracted three times with 5 ml of hexane and passed through sodium sulfate column. The volume of the hexane extract was reduced to 1 ml by passing nitrogen gas through it. 1µl of the hexane extract was then injected into gas chromatograph column.

3.2.12.3 Estimation of chloride

Chloride was estimated by silver nitrate method specified in 407 A of Standard Methods for the Examination of Water and Wastewater (APHA, 20th Ed, 1998). Chloride estimation was done by taking suitable volume of the sample (30 ml to 40 ml) and titrating it with silver nitrate (0.0141 M) using potassium chromate as indicator. For certain samples with lower concentration of chloride, volume of sample taken for estimation was 100 ml.

3.2.12.4 Determination of Chemical Oxygen Demand (COD)

COD was determined by the dichromate reflux method as described in 508 H of Standard Methods for the Examination of Water and Wastewater (APHA, 20th Ed, 1998). Sample was refluxed in a strongly acidic solution (concentrated H_2SO_4) with a known excess of (0.25 N) potassium dichromate ($K_2Cr_2O_7$). After digestion, the remaining unreduced $K_2Cr_2O_7$ was titrated with ferrous ammonium sulfate (0.25 M) using ferroin indicator to determine the amount of $K_2Cr_2O_7$ consumed. The oxidizable organic matter was calculated in terms of oxygen equivalent.

3.2.12.5 Estimation of suspended solids

100 ml of the sample was taken and filtered through preweighed glass fibre filter paper (Whatman) and dried for 1 h in an oven at 105°C. The weight of the filter paper was taken after drying. The difference between the initial weight of the filter paper and weight after drying showed the amont of suspended solids in the sample.

3.2.13 Presentation of data

The values presented for screening, optimization, and kinetic studies were the mean values of six sets of observations. Values presented for the mixed substrate and mixed culture studies were the average of five sets of observations. Values presented for the optimization of organic loading were the average of 7 days of observation for each concentration of substrate, similarly the values presented for the optimization of flow rate were the average of 7 days of observation for each flow rate. Values presented for the operation of trickling filter reactor at optimal organic and hydraulic loading for the treatment of 2-chlorophenol and 4-chlorophenol were the average of data collected at an interval of 6 h for over a period of one month.

3.2.13.1 Evaluation of growth yield (Schlegel *et al.*, 1990)

Growth yield was calculated from the increase in cell biomass and the amount of substrate consumed and determined from the following equation:

$Y = dX/dS$

where Y is the growth yield calculated from maximum bacterial growth

X is the cell biomass produced (mg)

S is the substrate consumed (mol)

Growth yield was expressed as (dry weight (mg)/mol of substrate used)

3.2.13.2 Determination of specific growth rate (μ) (Schlegel *et al*,. 1990)

Logarithmic value of bacterial cell mass was plotted on the ordinate against the time on abscissa and gives an exponential curve. Specific growth rate (μ) was calculated from the slope of the curve using the following equation:

$$\mu = \log x_t - \log x_o / \log e(t - t_o)$$

Where loge=0.43429

x_0 and x_t are bacterial cell concentrations at the times t_0 and t,

Specific growth rate was expressed as t^{-1} (where t is time)

3.2.13.3 Determination of doubling time (t_d) (Schlegel *et al*,. 1990)

The time required for the doubling of bacterial cell mass is referred to as the doubling time. It is related to specific growth rate (μ) and is calculated from the following equation:

$$t_d = \ln 2 / \mu$$

Value of $\ln 2$: 0.693

μ: specific growth rate of the bacterial cells

3.2.13.4 Determination of substrate utilization rate (SUR) (Lee and Carberry, 1992)

The substrate utilization rate was calculated from the change in substrate concentration over time divided by the microbial cell concentration, as follows:

- SUR = 1/x.ds/dt

  x = microbial concentration (mg/l)

  ds/dt = rate of substrate uptake (mg/l/h)

CHAPTER - 4

4.0 RESULTS

4.1 Enrichment and Isolation of *Rhodococcus* sp.

Enrichment was carried out with the aim of isolating microorganisms belonging to the genus *Rhodococcus*. It was carried out in the selective M3 medium containing sodium propionate as the carbon source as specified in Prokaryotes (Starr *et* al., 1981) for *Rhodococcus*. Substrate specific enrichment on chlorophenol was not carried out during the initial stages of enrichment as the first objective was to isolate a *Rhodococcus* sp. Samples used as inoculum for enrichment were from the sites as described in methods (Chapter 3). Enrichment sources showing positive growth, indicated by the enhancement in turbidity of the medium are presented in Table 13. Positive growth was noted for all the enrichment samples except for Soil-III. Samples showing growth were subjected to chlorophenol acclimatization. Sodium propionate used as the carbon source in M3 medium was gradually reduced and replaced by 2-chlorophenol. 2-chlorophenol concentrations in the M3 medium were increased stepwise up to a maximum concentration of 50 mgl^{-1}, completely replacing sodium propionate in the process. Pure cultures were then isolated from the enrichment samples by plating on M3 agar medium containing 50 mgl^{-1} of 2-chlorophenol and incubating at 32°C for one to two weeks. Five isolates were obtained from different sources and are designated as M1, M2, M3, M4 and M5 (Table 14). The isolates were subjected to colony, cell morphological, physiological and biochemical characterization for identification.

Table 13. Different sources for enrichment in M3 medium for isolation of *Rhodococcus* sp.

Sl. No.	Sources	Growth in M3 medium
1.	Soil – I (Garden soil)	+
2.	Soil – II (Oil contaminated soil from petrol pump)	+
3.	Soil – III (Oil contaminated soil from gas station)	-
4.	Soil – IV (Oil contaminated soil from garage)	+
5.	Compost	+
6.	Sediment (from oxidation pond)	+

+, Presence of growth; –, Absence of growth

Table 14. Pure cultures isolated from different sources after enrichment

Sl. No.	Sources	Isolates
1.	Soil – I	M1
2.	Soil-II	M2
3.	Soil-IV	M3
4.	Compost	M4
5.	Pond sediment	M5

4.2 Identification of the Isolates

The pure cultures isolated from enrichment procedure were subjected to identification based on their colony, cell morphological, physiological and biochemical characteristics.

4.2.1 Colony characteristics of the isolates

Colony characteristics of the cultures were studied on nutrient agar plate and M3 agar plate. Colonies appearing on nutrient agar plate after 48 h of incubation is

Plate 1: The colony characteristics of *Rhodococcus* sp. M1 on nutrient agar plate.

The colony characteristics of the isolate M2, M3, M4 and M5 are similar to that of M1.

A – B: Showing pigmented colony of *Rhodococcus* sp. M1

A

B

shown in Plate 1. Longer incubation period of one week was required for the growth of the culture on M3 agar plate. However, colony characteristics were observed to be similar in both nutrient agar and M3 agar plates. Colony characteristics of all the isolates when grown on nutrient agar plate have been summarized in Table 15. All the isolates were characterized by pigmented colony forms. Pale orange, pink and red colored colonies were observed. Pigmented colony of the isolate M1 is shown in Plate 1. Decreased pigmentation was observed on M3 agar plates. Margin characteristics of the cultures changed with age from entire to irregular filamentous form. Surface characteristics of the colonies were also observed to change with age from moist to dry. Pigmentation was observed to enhance with the age of the culture. Colonies of all the isolates were characterized by elevated center. The margin of the colonies was observed to be flattened and less dense than the center.

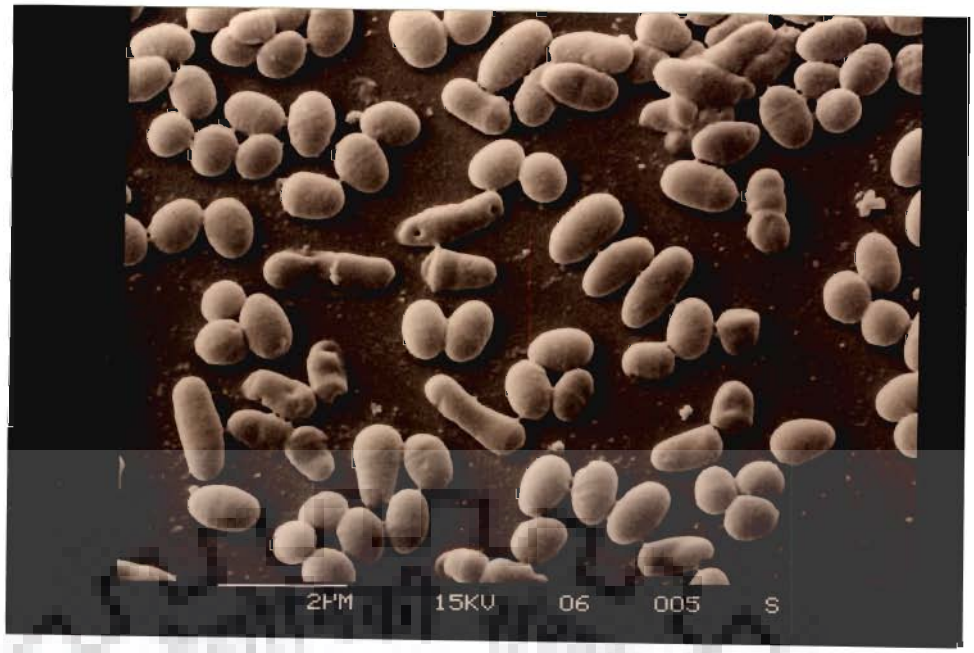
4.2.2 Cell morphological characteristics of the isolates

The isolates were shown to have pleomorphic cells varying from cocci to rods forming filaments (Table 16). The pleomorphic nature of the cells is evident from the study of the cells by scanning electron microscopy. The scanning electron micrographs of the isolates are shown in Plate 2. To further confirm the pleomorphic nature of the cells, growth cycle of the isolates was studied by growing on Luria broth and making periodic observations. Cell morphology at different stages of growth is shown in Plate 3. Coccus shaped cells was observed after 16h of growth in Luria broth. Growth of the cells after 24 h of incubation resulted in rod shaped cells. The cells at this stage showed a tendency to agglomerate. Further growth results in the elongation of cells to form filaments. Filamentous growth of the cells and the tendency of the cells to clump

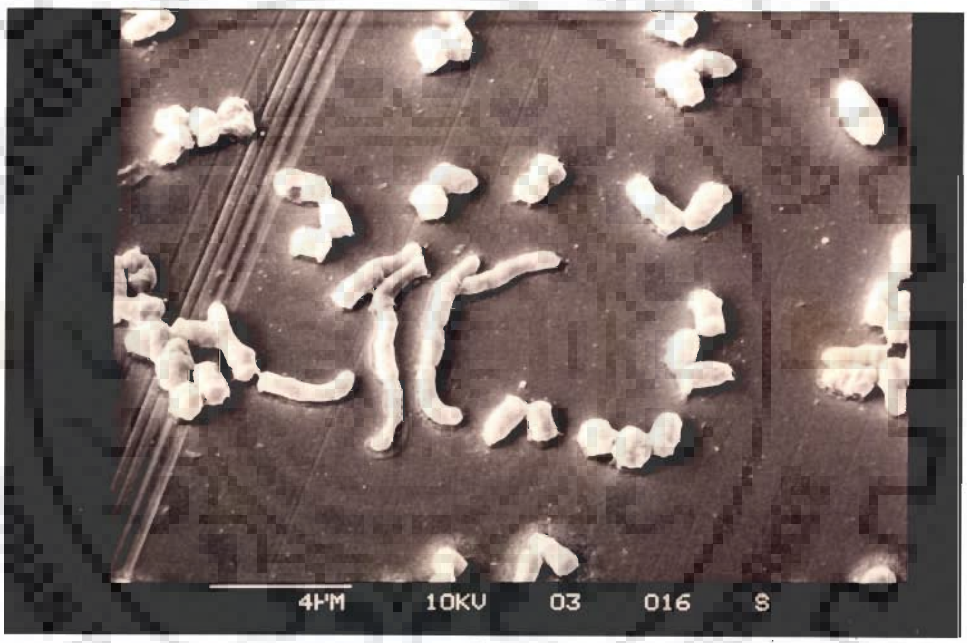
Plate 2: Scanning electron micrograph of *Rhodococcus* sp. M1 showing pleomorphic cells varying from cocci to rods. Similar morphological characteristics was observed for the isolate M 2, M3, M4 and M5

A – B: Showing pleomorphic cells of *Rhodococcus* sp. M1

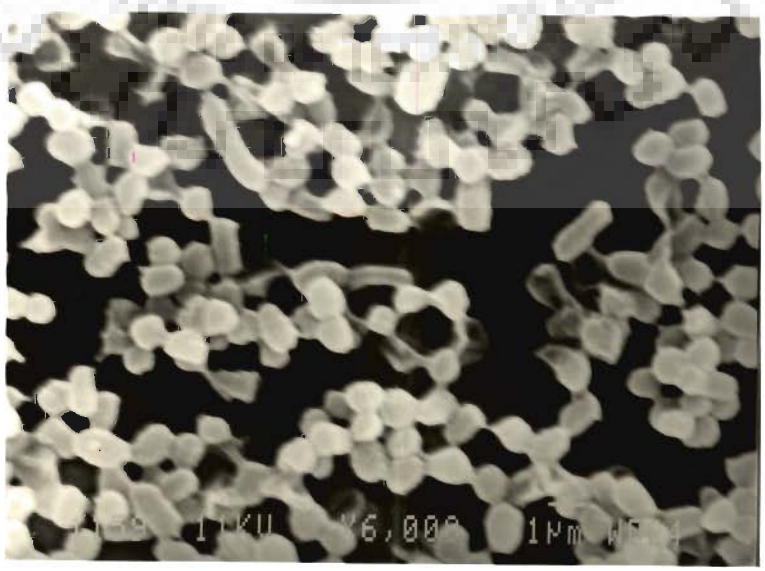
C: Showing agglomeration of cells in *Rhodococcus* sp. M1



A



B



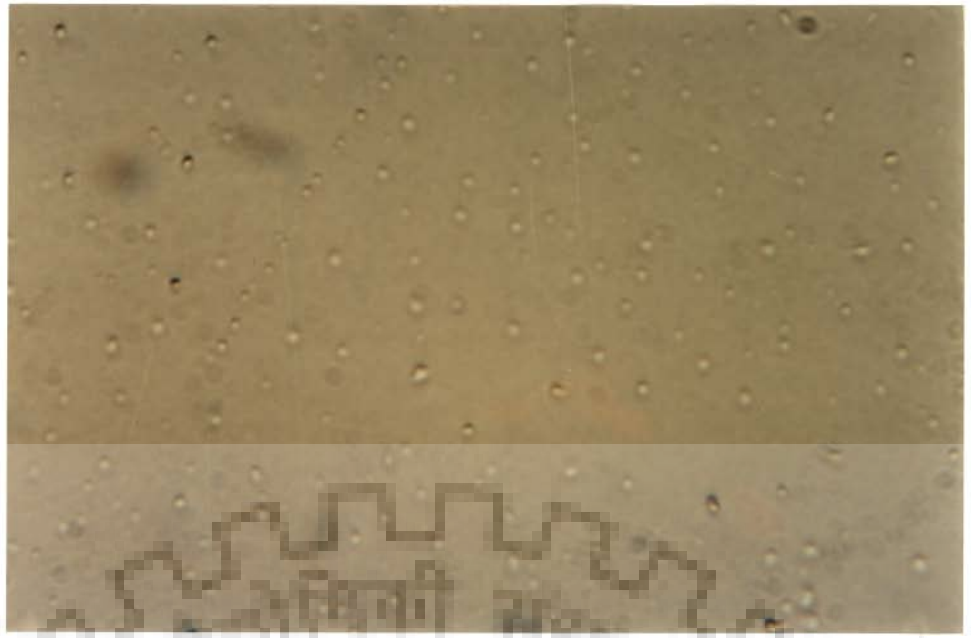
C

together results into a pellicle structure after 72 h of growth. The tendency of the cells to agglomerate and formation of pellicle structure leads to flocculating growth of the cultures in liquid medium.

The isolates were tested positive for gram staining reaction and were shown to be non-motile by using the hanging drop method (Table 16).

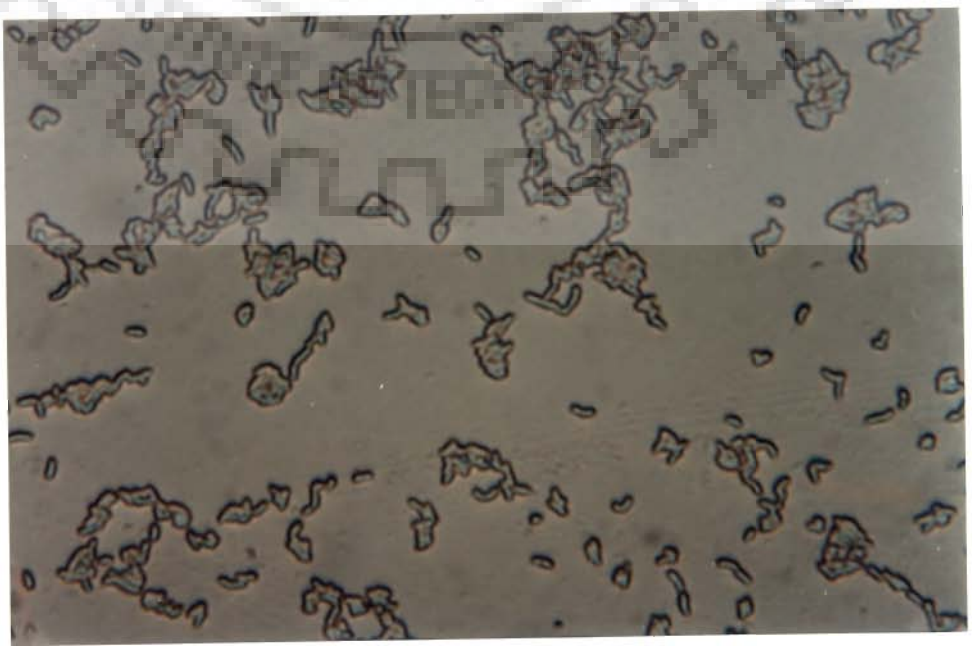
Table 15. Colony characteristics of the isolates in nutrient agar medium

Sl. No.	Isolates	Colony characteristics
1.	M1	Small circular colonies with entire margin at young stage, margin serrated and filamentous in old cultures, surface butyrous, smooth and moist, becomes dry in old cultures, center of colony elevated, margin flattened and less dense than center, opaque. Colonies pigmented pale orange.
2.	M2	Small circular colonies with entire margin at young stage, margin serrated and filamentous in old cultures, surface butyrous, smooth and moist, becomes dry in old cultures, center of colony elevated, margin flattened and less dense than center, opaque. Colonies pigmented orange.
3.	M3	Small circular colonies with entire margin at young stage, margin serrated and filamentous in old cultures, surface butyrous, smooth and moist, becomes dry in old cultures, center of colony elevated, margin flattened and less dense than center, opaque. Colonies pigmented pink
4.	M4	Small circular colonies with entire margin at young stage, margin serrated and filamentous in old cultures, surface butyrous, smooth and moist, becomes dry in old cultures, center of colony elevated, margin flattened and less dense than center, opaque. Colonies pigmented pink
5.	M5	Small circular colonies with entire margin at young stage, margin serrated and filamentous in old cultures, surface butyrous, smooth and moist, becomes dry in old cultures, center of colony elevated, margin flattened and less dense than center, opaque. Colonies pigmented red

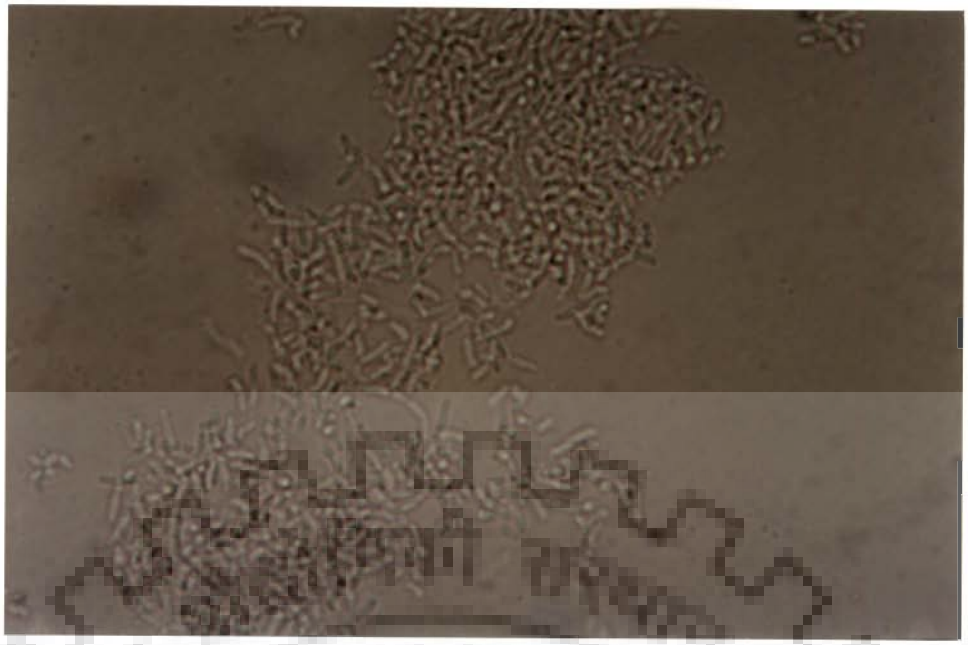


A

B



C



D

E

Table 16. Morphological characteristics of the isolates

Sl. No.	Isolates	Cell shape	Gram staining	Motility
1	M1	Cocci to rod forming filaments	Positive	Non-motile
2	M2	Cocci to rod forming filaments	Positive	Non-motile
3	M3	Cocci to rod forming filaments	Positive	Non-motile
4	M4	Cocci to rod forming filaments	Positive	Non-motile
5	M5	Cocci to rod forming filaments	Positive	Non-motile

4.2.3 Physiological and biochemical characterization of the isolates

Biochemical properties of the isolates were studied on the basis of standard biochemical tests and are presented in Table 17. Utilization of sugars by the isolates has been summarized in Table 18. Acid production was observed for dextrose, fructose, sucrose, maltose, trehalose, sorbitol and mannitol. The isolates when grown in glucose oxidation fermentation medium were capable of oxidizing glucose, but fermentation of glucose was not observed.

The isolates, based on the colony, morphological characteristics and biochemical properties of the isolates and correlating it with the properties described in Bergey's Manual of Systematic Bacteriology (1989) and Prokaryotes (1981), were identified as *Rhodococcus* sp.

Table 17. Physiological and biochemical properties of the isolates

Biochemical tests	Isolates				
	M1	M2	M3	M4	M5
Catalase test	+	+	+	+	+
Caseinase hydrolysis	-	-	-	-	-
Starch hydrolysis	+	+	-	+	+
Gelatin liquefaction	-	-	-	-	-
Nitrate reduction	+	+	+	+	+
Urease production	+	+	+	+	+
Oxidase test	-	-	-	-	-
Tween-80 hydrolysis	+	-	-	+	-
Phenylalanine deaminase test	+	-	-	-	-
Indole production	-	-	-	-	-
Methyl red test	+	+	+	+	+
Voges Proskauer test	-	-	-	-	-
Gluconate oxidation	-	-	-	-	-
Citrate utilization	+	+	-	+	+
Acetamide utilization	+	+	-	+	+
Growth on 5% Nacl	+	-	+	+	-

+, Positive; -, Negative

Plate 3: Growth cycle of *Rhodococcus* sp. M1. Coccus shaped cells was observed after 16h of growth followed by rod shaped cells after 24 h of incubation. The cells at this stage showed agglomeration. Further growth results in the elongation of cells to form filaments.

A - Growth after 16h

B - Growth after 24 h

C - Growth after 36 h

D - Growth after 48 h

E - Growth after 72 h

Table 18. Utilization of sugars by the isolates

Sugars	Isolates				
	M1	M2	M3	M4	M5
Arabinose	-	-	-	-	-
Rhamnose	-	-	-	-	-
Xylose	-	-	-	-	+
Dextrose	+	+	+	+	+
Galactose	-	-	-	-	-
Mannose	-	-	+	+	-
Fructose	+	+	+	+	+
Sucrose	+	+	+	+	+
Maltose	+	+	+	+	+
Lactose	-	-	-	-	-
Cellobiose	-	-	-	-	-
Melibiose	-	-	-	-	-
Trehalose	+	+	+	+	+
Raffinose	-	+	-	-	-
Inulin	-	-	-	-	-
Adonitol	-	-	-	-	-
Sorbitol	+	+	-	+	+
Dulcitol	-	-	-	-	-
Mannitol	+	+	-	+	+
Salicin	-	-	-	-	-
Inositol	-	-	-	-	-

+, Production of acid; -, No production of acid

4.3 Screening of the *Rhodococcus* isolates for phenol, cresols and chlorophenols degradation

The isolates obtained from different enrichment sources and identified as *Rhodococcus* sp., were screened for their ability to degrade chlorophenols. Chlorophenols most often in the industrial effluents and contaminated sites have been reported to be present along with phenol and cresols. Hence the isolates along with their ability to utilize chlorophenols were also screened for their capability to degrade phenol and cresols. Modified M3 medium containing phenol, cresols (ortho, meta, and para), 2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol as the sole source of carbon and energy were used for the growth of the isolates. Substrates taken in each case was 100 mgl^{-1}. Screening for the degradation of substrates was based on substrate utilization and increase in growth. Substrate removal efficiency for phenol and cresols (ortho, meta and para) by the isolates was observed after 72 h (Table 19). Growth of the organism was noted after a period of 72 h and summarized in Table 20. The isolate M1 was capable of utilizing phenol and all the cresols. Isolate M5 was also observed to grow on phenol and all the cresols but the extent of degradation was lower than that of M1.

Screening of the isolates for chlorophenols degradation was carried out similarly by measuring substrate degradation and increase in growth. Compared to phenol and cresols, considerably longer incubation period of 30 days was required for the removal of chlorophenols at a concentration of 100 mgl^{-1} by the isolates. Substrate removal and growth were thus noted after 30 days and the results are presented in Tables 21 and 22 respectively. Results indicated that isolate M1 and M4 were capable

of degrading all the chlorophenols tested, which include 2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol. *Rhodococcus* isolate M1 however, was observed more effective in degrading the chlorophenols as compared to isolate M4. These results therefore indicated *Rhodococcus* isolate M1 appeared having a better ability for degrading a variety of phenolics and therefore was selected for further studies.

Table 19. Biodegradation potential of phenol and cresols by *Rhodococcus* isolates

Rhodococcus Isolates	% Degradation			
	Phenol	o-cresol	m-cresol	p-cresol
M1	+++++	+++	+++	+++++
M2	-	-	-	-
M3	++++	++	-	+++
M4	++++	-	-	-
M5	++++	+	+++	+++

Concentration of substrates taken was 100 mgl^{-1}, degradation was observed after 72 h; % Degradation: +, up to 20%; ++, 20% to 40%; +++, 40% to 60%; ++++, 60% to 80%; +++++, 80% to 100%; -, Nil

Table 20. Growth of the *Rhodococus* isolates on phenol and cresols

Rhodococcus Isolates	Growth			
	Phenol	o-cresol	m-cresol	p-cresol
M1	++++++	+++	+++	+++++
M2	-	-	-	-
M3	++++	++	-	+++
M4	+++++	-	-	-
M5	++++	+	+++	+++

Concentration of substrates taken was 100 mgl^{-1}, Growth was observed after 72h; Growth at 540nm: +, (Abs) 0.01 to 0.05; ++, (Abs) 0.05 to 0.1; +++, (Abs) 0.1 to 0.15; ++++, (Abs) 0.15 to 0.2; +++++, (Abs) 0.2 to 0.25; ++++++, (Abs) 0.25 to 0.3; -, No growth

Table 21. Biodegradation potential of chlororphenols by *Rhododcoccus* isolates

Rhodococcus Isolates	% Degradation			
	2-chlorophenol	4-chlorophenol	2,4-dichlorophenol	2,4,6-trichlorophenol
M1	++++	++	+	+
M2	+++	+	-	-
M3	++++	+	-	-
M4	+++	+	+	-
M5	+++	-	-	-

Concentration of substrates taken was 100 mgl^{-1}, Degradation was observed after 30 days; % Degradation of substrates: +, up to 20%; ++, 20% to 40%; +++, 40% to 60%; ++++, 60% to 80%; +++++, 80% to 100%; -, Nil

Table 22. Growth of the *Rhodococus* isolates on chlorophenols

Rhodococcus Isolates	Growth			
	2-chlorophenol	4-chlorophenol	2,4-dichlorophenol	2,4,6-trichlorophenol
M1	+++	+	+	+
M2	++	+	-	-
M3	+++	+	-	-
M4	++	+	+	+
M5	++	-	-	-

Concentration of substrates taken was 100 mgl^{-1}, Growth was observed after 30 days; Growth at 540 nm: +, (Abs) up to 0.04; ++, (Abs) 0.04 to 0.08; +++, (Abs) 0.08 to 0.12; ++++, (Abs) 0.12 to 0.16; -, No growth

4.4 Induction of *Rhodococcus* sp. M1 for 2-chlorophenol degradation

Rhodococcus sp. M1 was identified as the most promising culture among the five isolates as per initial analyses and was used for further study. Out of the four chlorophenols (2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol) used in the initial screening, maximum degradation was observed for 2-chlorophenol by the isolate M1 (Table 21). However degradation of 2-chlorophenol (100 mgl^{-1}) was observed to commence after a lag period of 5 days and required an incubation period of 45 days for the complete removal of the substrate (Figure 3). In order to reduce the lag period and the extended period of degradation, *Rhodococcus* sp. M1 was subjected to induction. Some of the chemical factors that were previously observed to induce the degradation of chlorophenols such as toluene, phenol and also

the other structurally related substrates such as benzoic acid and catechol were used. The *Rhodococcus* sp. M1 was inoculated in the modified M3 medium containing either toluene, benzoic acid, phenol or the catechol as the substrate (100 mgl^{-1}). A normal level of growth was observed following an initial lag period of 4 to 8 h for all the substrates used (Figure 4).

Figure 3. **DEGRADATION OF 2-CHLOROPHENOL (100 mgl^{-1}) BY UNINDUCED CELLS OF *Rhodococcus* sp. M1.**

Cells grown on DSM-65 medium were harvested after 24 h and used as inoculum. The study was carried out in modified M3 medium at 32°C under shaking conditions (120 rpm). 2-chlorophenol, chloride and growth were estimated at an interval of 5 days as described in methods.

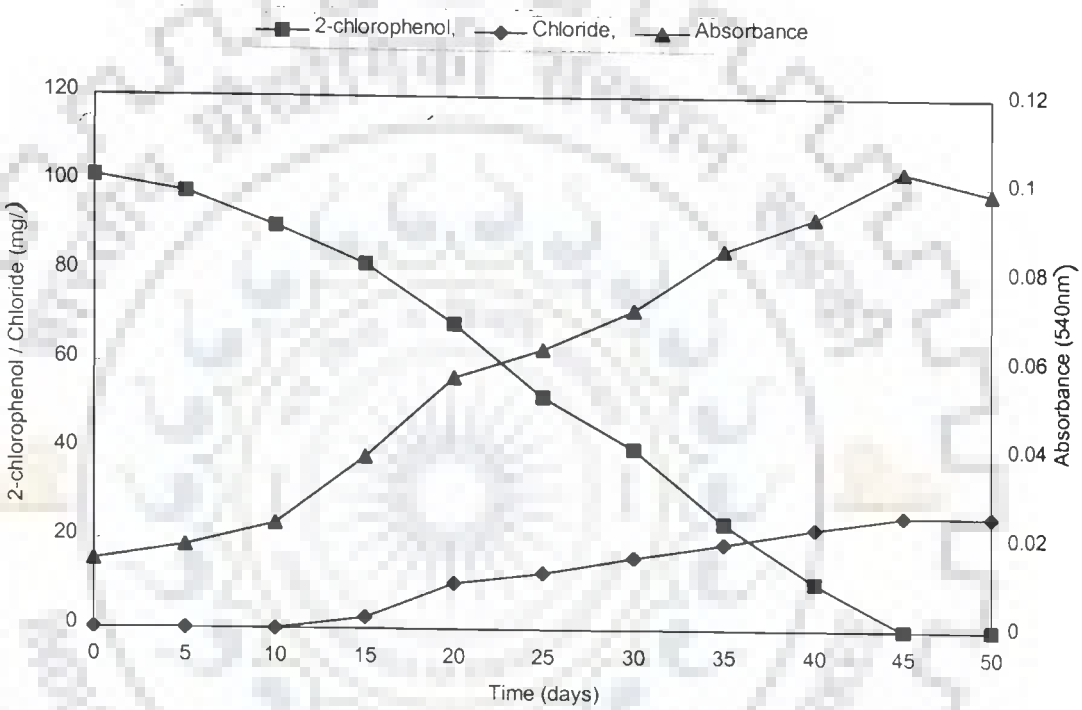

2-chlorophenol
Chloride
Absorbance
120
100
80
60
40
20
0
2-chlorophenol / Chloride (mg/l)
0.12
0.1
0.08
0.06
0.04
0.02
0
Absorbance (540nm)
0
5
10
15
20
25
30
35
40
45
50
Time (days)

Figure 3

Figure 4. GROWTH CURVE OF *Rhodococcus* sp. M1 ON BENZOIC ACID, TOLUENE, PHENOL AND CATECHOL

Inoculum was prepared by growing *Rhododcoccus* sp M1 in DSM-65 medium and harvesting by centrifugation after 24 h. Modified M3 medium-containing either benzoic acid, toluene, phenol or catechol of concentration 100 mgl^{-1} was used for the study. The study was carried out at 32°C under shaking condition (120 rpm). Sampling for the estimation of cell growth at an absorbance of 540 nm was done at an interval of 4 h.

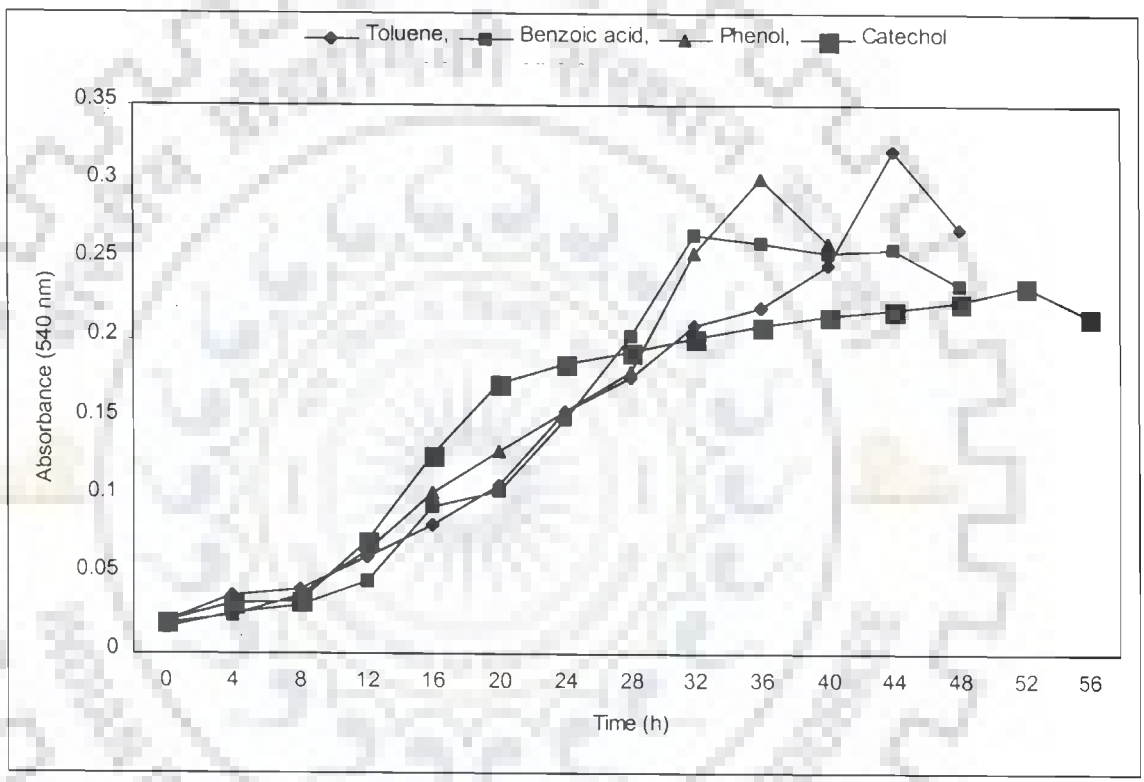

Toluene,
Benzoic acid,
Phenol,
Catechol
Absorbance (540 nm)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
Time (h)

Figure 4

4.5 Degradation of 2-chlorophenol by induced cells of *Rhodococcus* sp. M1

Rhodococcus sp. M1 enriched on the inducers as mentioned earlier was harvested during the logarithmic phase of growth and employed for the degradation of 2-chlorophenol at the concentration of 100 mgl^{-1}. Complete degradation of 2-chlorophenol was observed in 30 days by the toluene enriched cells of *Rhodococcus* sp M1 (Figure 5), whereas benzoic acid induced cells of *Rhodococcus* sp M1 resulted into complete removal of 2-chlorophenol in 48 h (Figure 6). Phenol and catechol induced cells required respectively the longer duration of 35 and 45 days for complete removal of 2-chlorophenol (Figure 7 and 8). Benzoic acid induced cells of *Rhodococcus* sp M1 since, required remarkably shorter period for 2-chlorophenol removal, hence this culture was selected for further studies with respect to optimal temperature and pH determinations for degradation and kinetics of degradation for chlorophenol.

Figure 5. DEGRADATION OF 2-CHLOROPHENOL BY TOLUENE INDUCED CELLS OF *Rhodococcus* sp. M1.

Toluene enriched cells were harvested after 44 h in their exponential phase of growth and used for 2-chlorophenol degradation. Modified M3 medium containing 100 mgl^{-1} of 2-chlorophenol was used. Degradation studies were carried out at 32°C in a rotary shaker at 120 rpm. Samples were collected at an interval of 5 days, 2-chlorophenol and chloride were estimated and cell growth was monitored as described in methods.

Figure 6. 2-CHLOROPHENOL DEGRADATION BY BENZOIC ACID INDUCED CELLS OF *Rhodococcus* sp. M1.

Benzoic acid enriched cells were harvested after 32 h in exponential phase of growth and used for 2-chlorophenol degradation. The other conditions were as described for Figure 5 except that samples were collected at an interval of 8 h.

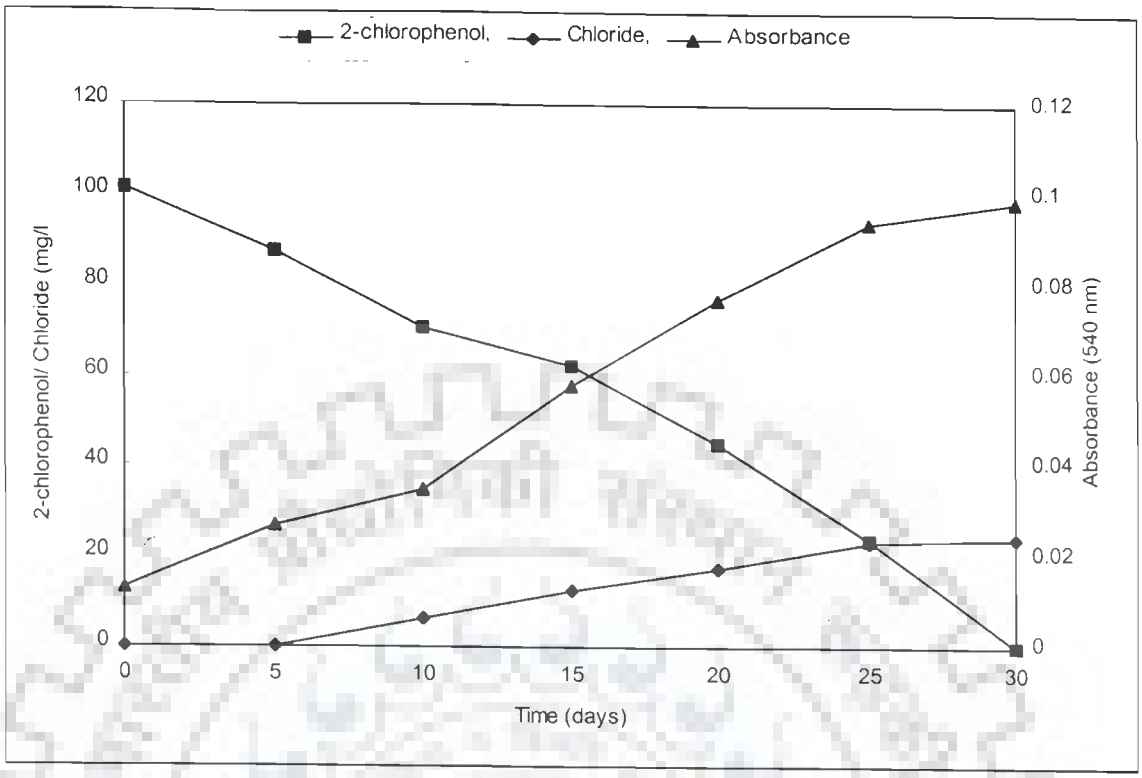


Figure 5

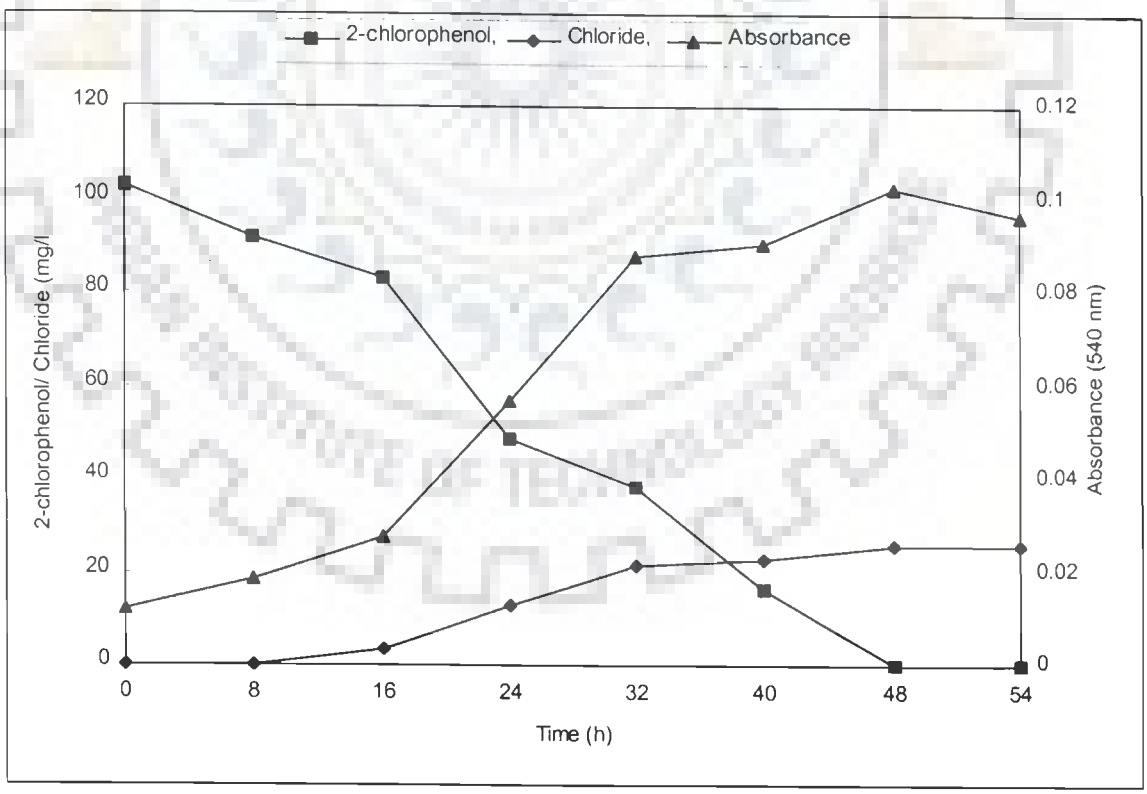


Figure 6

Figure 7. 2-CHLOROPHENOL DEGRADATION BY PHENOL INDUCED CELLS OF *Rhodococcus* sp. M1.

Phenol enriched cells were harvested after 36 h in their exponential phase o growth and used for 2-chlorophenol degradation. The other conditions were as described for Figure 5.

Figure 8. 2-CHLOROPHENOL DEGRADATION BY CATECHOL INDUCED CELLS OF *Rhodococcus* sp. M1.

Catechol enriched cells were harvested after 52 h in their exponential phase of growth and tested for 2-chlorophenol degradation. The other conditions were as described for Figure 5.

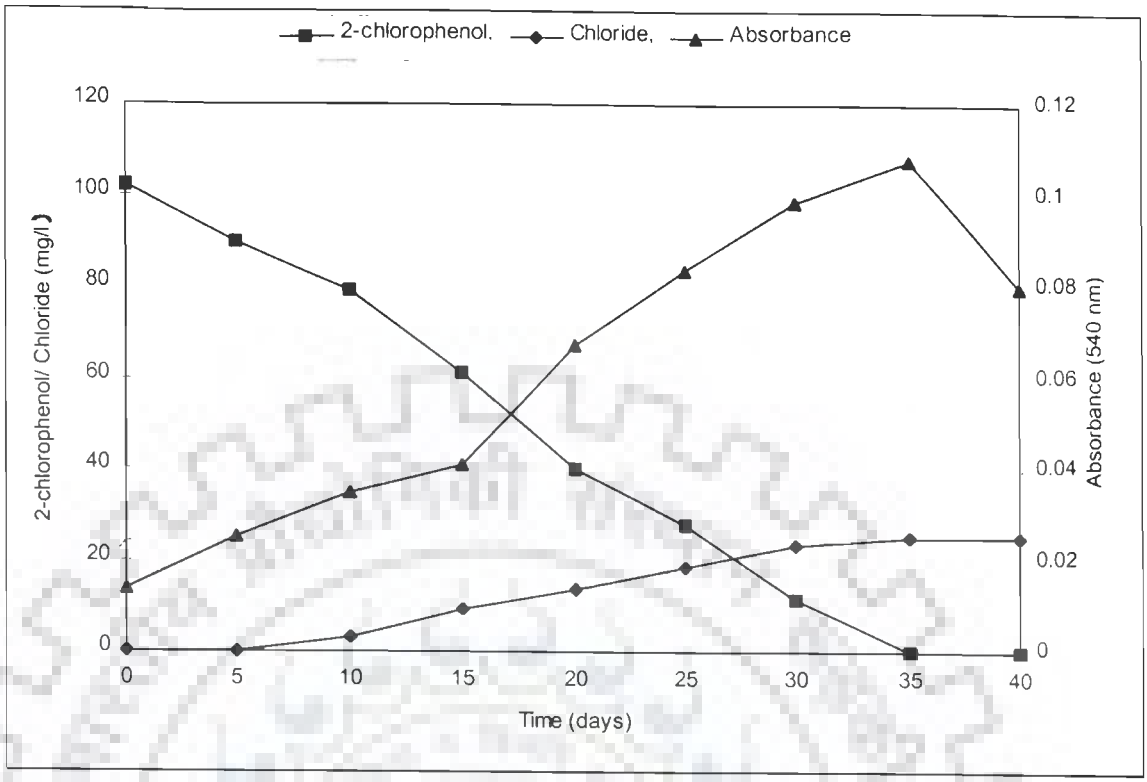


Figure 7

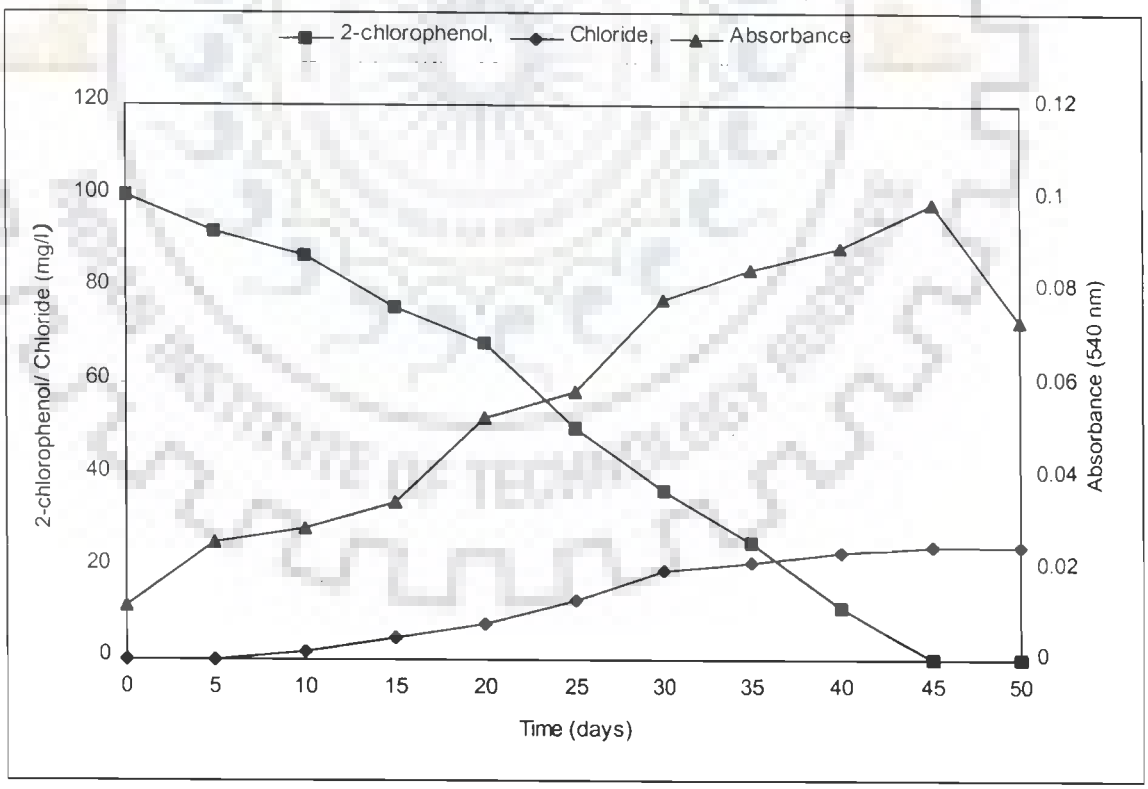


Figure 8

4.6 Optimization of temperature and pH for chlorophenol degradation by *Rhodococcus* sp. M1

Benzoic acid induced culture of *Rhodococcus* sp. M1 was acclimatized to 2-chlorophenol and subsequently used for the optimization of temperature and pH using 2-chlorophenol as the substrate

4.6.1 Optimization of temperature

Degradation of 2-chlorophenol (100 mgl^{-1}) was studied in the temperature range of 4 to 45°C (Figure 9). Percentage reduction of substrate was computed and growth was estimated after an incubation period of 24 h. Maximum rate of degradation and growth was observed in the temperature range of 32 to 35°C.

4.6.2 Optimization of pH

Degradation of 2-chlorophenol was studied for the pH values in the range of 5.0 to 12.0 at a substrate concentration of 100 mgl^{-1} (Figure 10). Degradation of the substrate and growth below pH 6.5 was observed to be negligible. The organism was observed to grow up to a pH value of 11.5. The optimum pH range for the growth of the organism was observed to be in the range of 7.0 to 8.0.

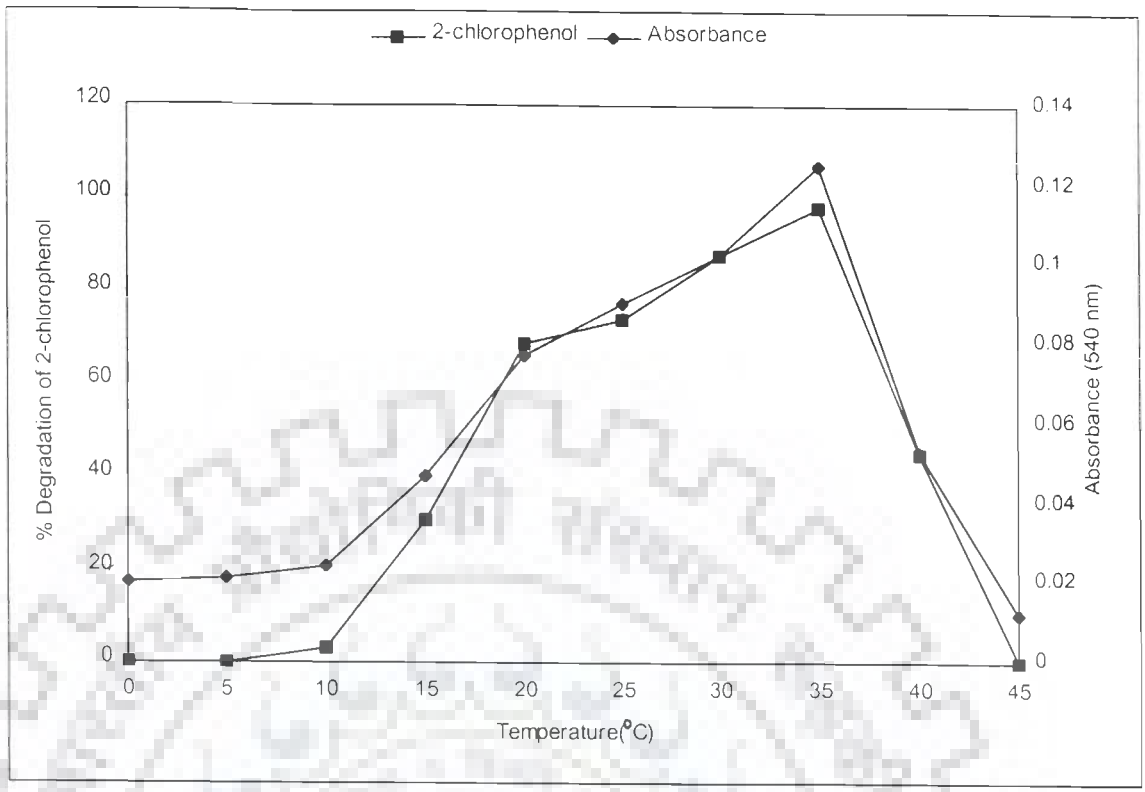


Figure 9

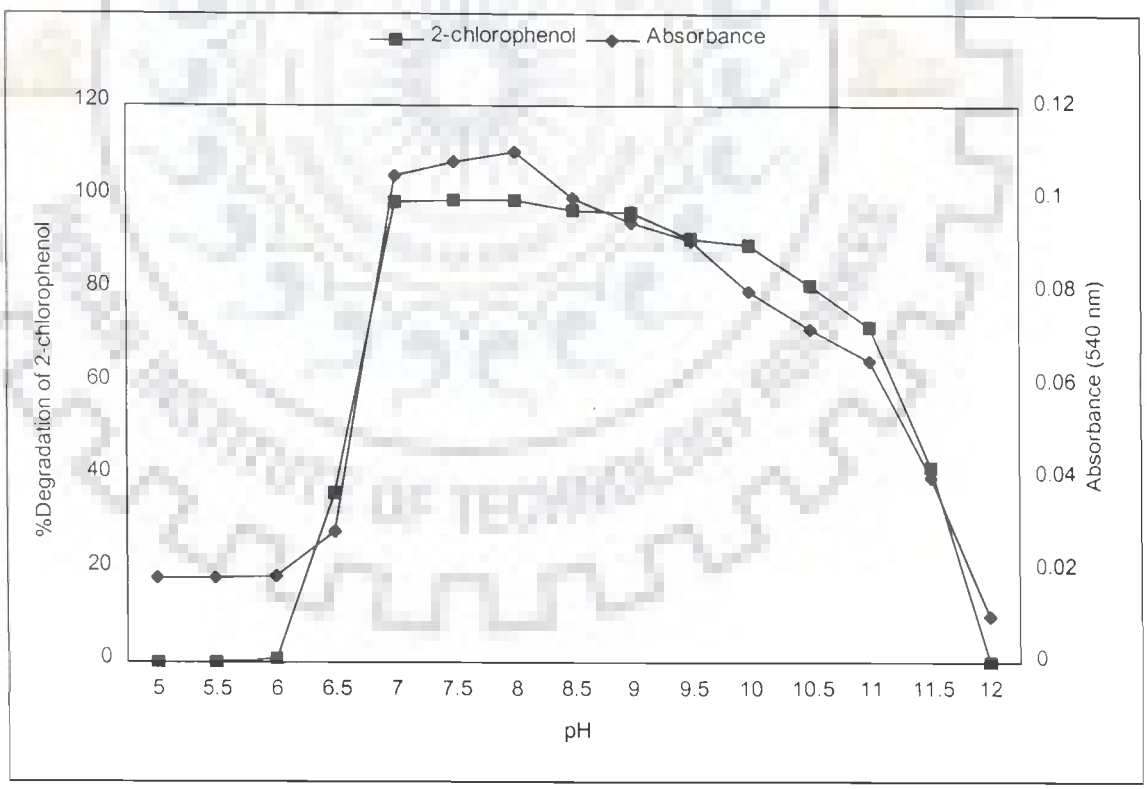


Figure 10

4.7 Kinetics of different chlorophenol degradation by *Rhodococcus* sp. M1

The kinetics of chlorophenol degradation was studied by acclimatizing the benzoic acid induced cells of *Rhodococcus* sp. M1 to respective chlorophenols.

4.7.1 Degradation of chlorophenols

4.7.1.1 Degradation of monochlorophenols

4.7.1.1.1 Degradation of 2-chlorophenol

Degradation of 2-chlorophenol was studied in modified M3 medium containing no other carbon source. The study was carried out for 2-chlorophenol concentrations ranging from 10 to 400 mgl^{-1} (Figure 11 a – j). The organism was found to degrade 2-chlorophenol up to a concentration of 300 mgl^{-1}. Rapid degradation of 2-chlorophenol was observed up to a substrate concentration of 100 mgl^{-1} (Figure 11 a – d) and complete removal of 2-chlorophenol (100 mgl^{-1}) was observed within 18 h of incubation (Figure 11 d). Increase in substrate concentration indicated the requirement of longer period for degradation (Figure 11 e - h). Rate of degradation was observed to increase with initial substrate concentration and reaches a maximum when the substrate concentration is 100 mgl^{-1} after which a sudden decline in the rate was observed (Table 23). Release of chloride was found to be more than 90% of the theoretical value for 2-chlorophenol concentration up to 250 mgl^{-1} as evident from Figure 11 a - g. Chloride released however decreases to 82.65% of the theoretical amount when the substrate concentration was increased to 300 mgl^{-1} (Figure 11 h). Release of chloride was observed to be initiated during the exponential phase of the growth of the culture on 2-chlorophenol. Substrate at further higher concentration of 350 mgl^{-1}, led to its

degradation that started after a lag period of 6 days. Substrate removal at this concentration was found to be only 48% (Figure 11 i). Release of chloride and growth was negligible at this concentration. Medium was observed developing brown colouration. No growth and degradation were observed at 2-chlorophenol concentration of 400 mgl^{-1} (Figure 11 j). The medium containing 2-chlorophenol at 400 mgl^{-1} was observed to develop brown coloration. Control assays without substrate showed no growth, and sterile control showed no abiotic loss of substrate.

Residual soluble COD was estimated for the degradation of 2-chlorophenol in the concentration range of 10 to 300 mgl^{-1}. Results showed no detectable soluble COD for substrate concentration up to 250 mgl^{-1}. Residual soluble COD of 60.8 mgl^{-1} was observed when 300 mgl^{-1} of 2-chlorophenol was degraded.

Figure 11 a. DEGRADATION OF 2-CHLOROPHENOL AT 10 mgl^{-1} BY *Rhodococcus* sp. M1.

Benzoic acid enriched cells acclimatized to 2-chlorophenol was used as the inoculum. The study was carried out at 32°C, in modified M3 medium under shaking condition (120 rpm). The estimation for 2-chlorophenol, chloride and growth were performed as described in methods.

Figure 11 b. DEGRADATION OF 2-CHLOROPHENOL AT 25 mgl^{-1} BY *Rhodococcus* sp. M1

Conditions for the study were as described for Figure 11 a.

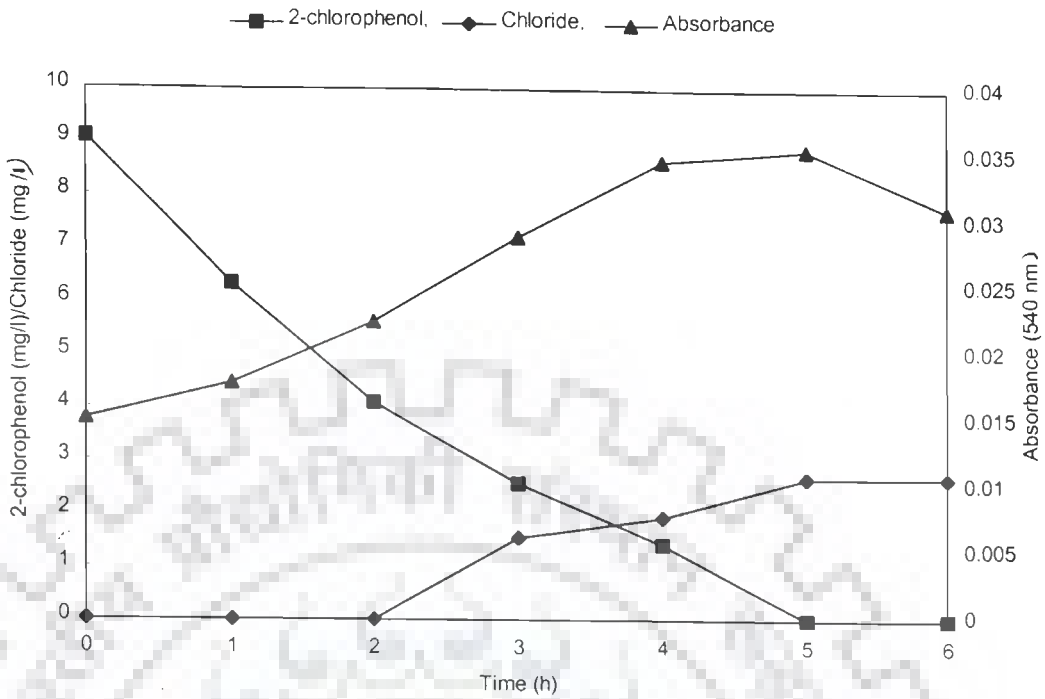


Figure 11 a

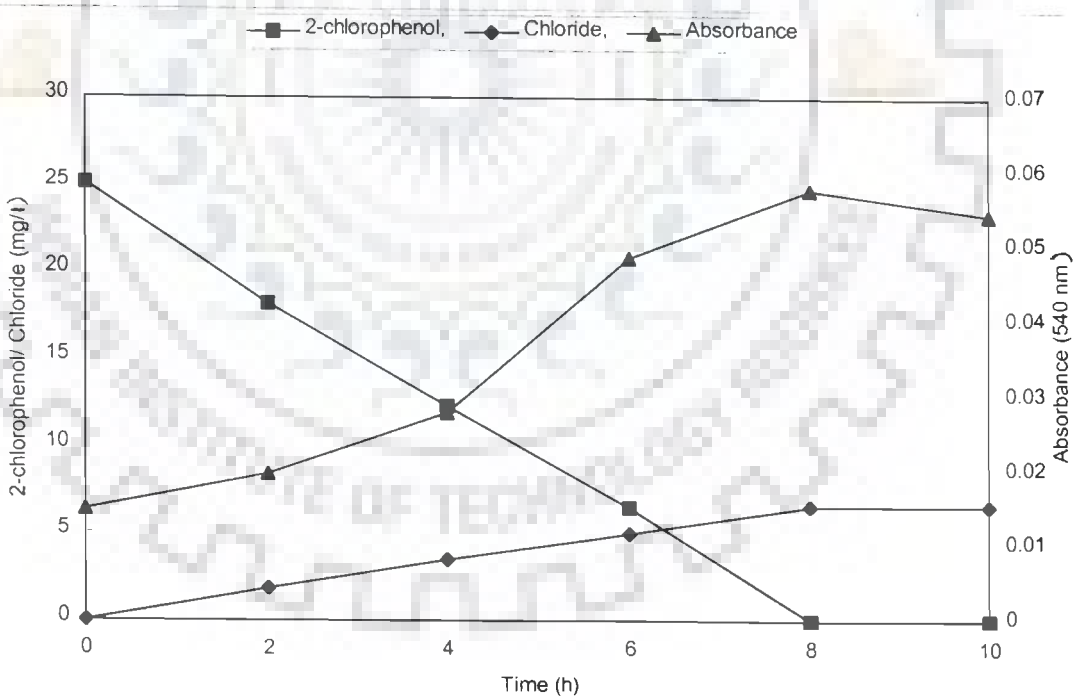


Figure 11 b

Figure 11 c. DEGRADATION OF 2-CHLOROPHENOL AT 50 mgl^{-1} BY *Rhodococcus* sp. M1

Conditions for the study were as described for Figure 11 a.

Figure 11 d. DEGRADATION OF 2-CHLOROPHENOL AT 100 mgl^{-1} BY *Rhodococcus* sp. M1.

Conditions for the study were as described for Figure 11 a.

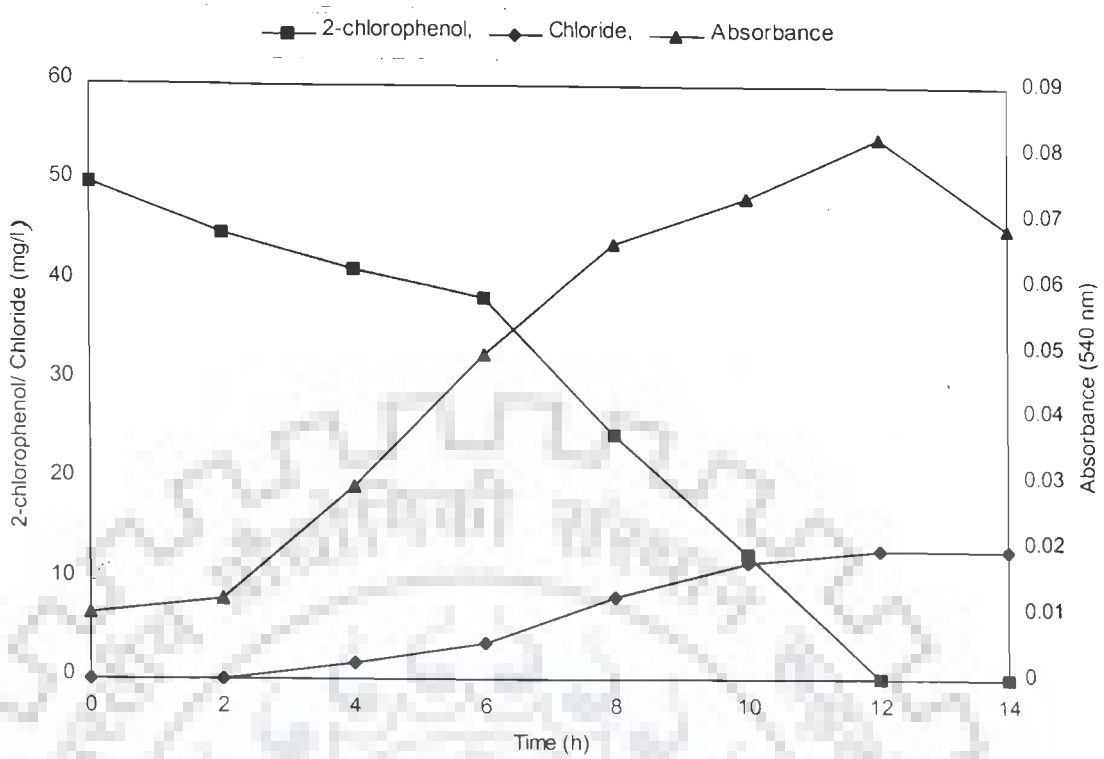


Figure 11 c

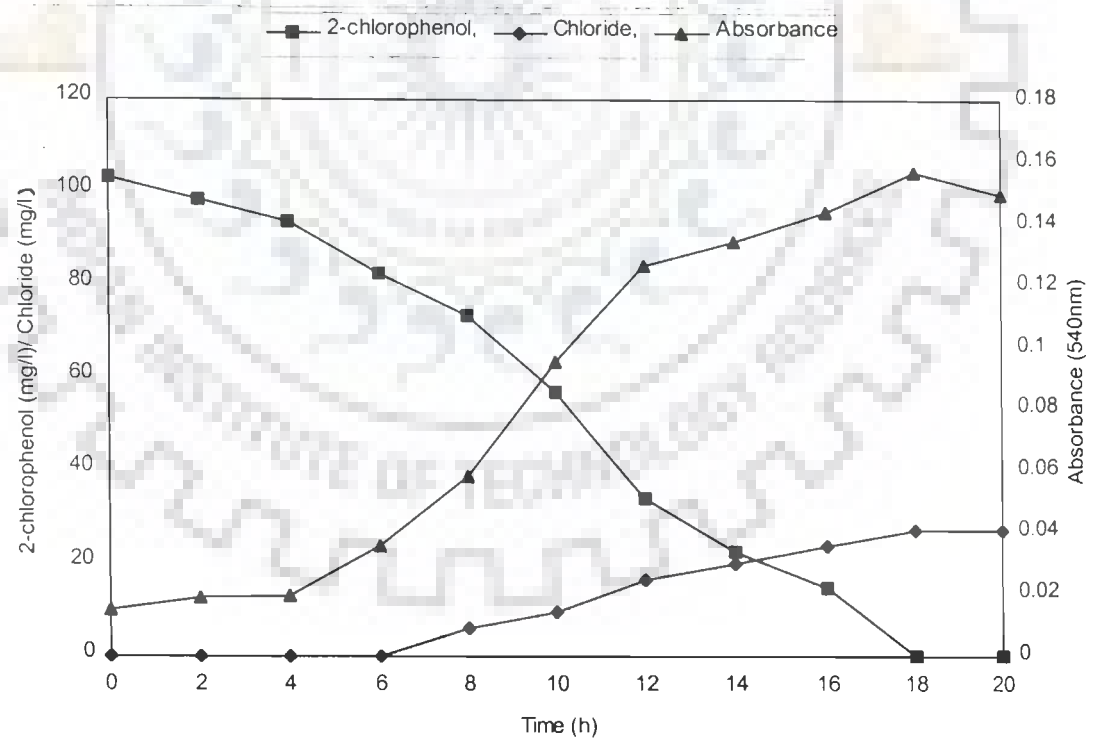


Figure 11 d

Figure 11 e. DEGRADATION OF 2-CHLOROPHENOL AT 150 mgl^{-1} BY *Rhodococcus* sp. M1

Conditions for the study were as described for Figure 11 a.

Figure 11 f. DEGRADATION OF 2-CHLOROPHENOL AT 200 mgl^{-1} BY *Rhodococcus* sp. M1

Conditions for the study were as described for Figure 11 a.

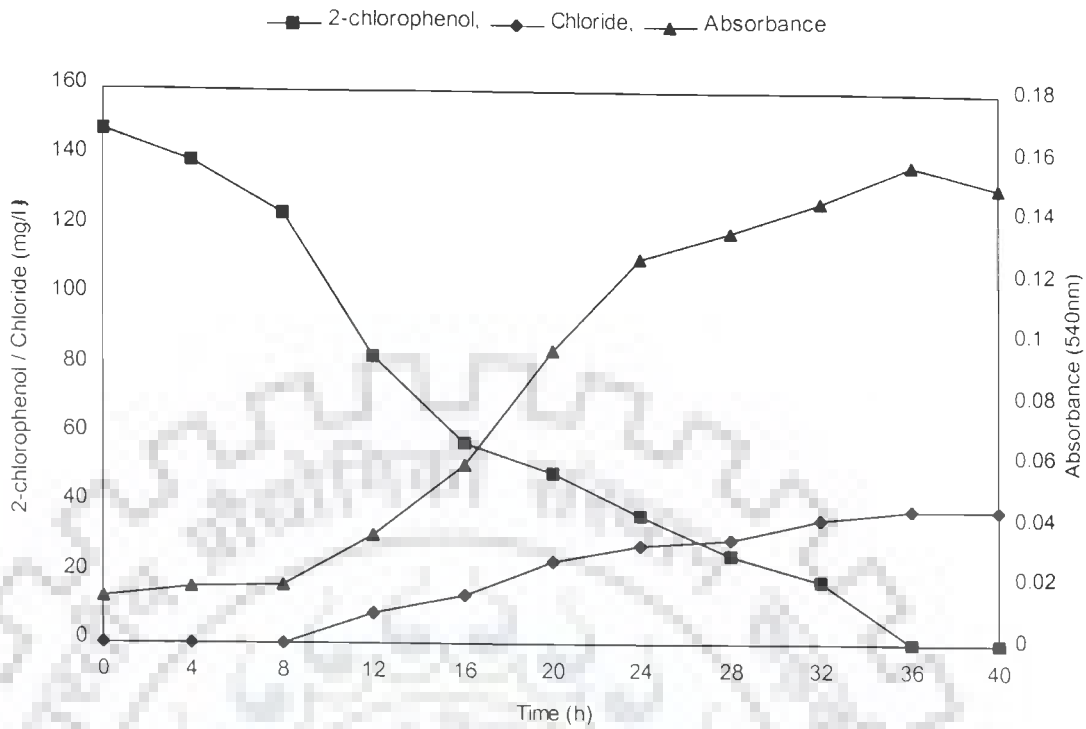


Figure 11 e

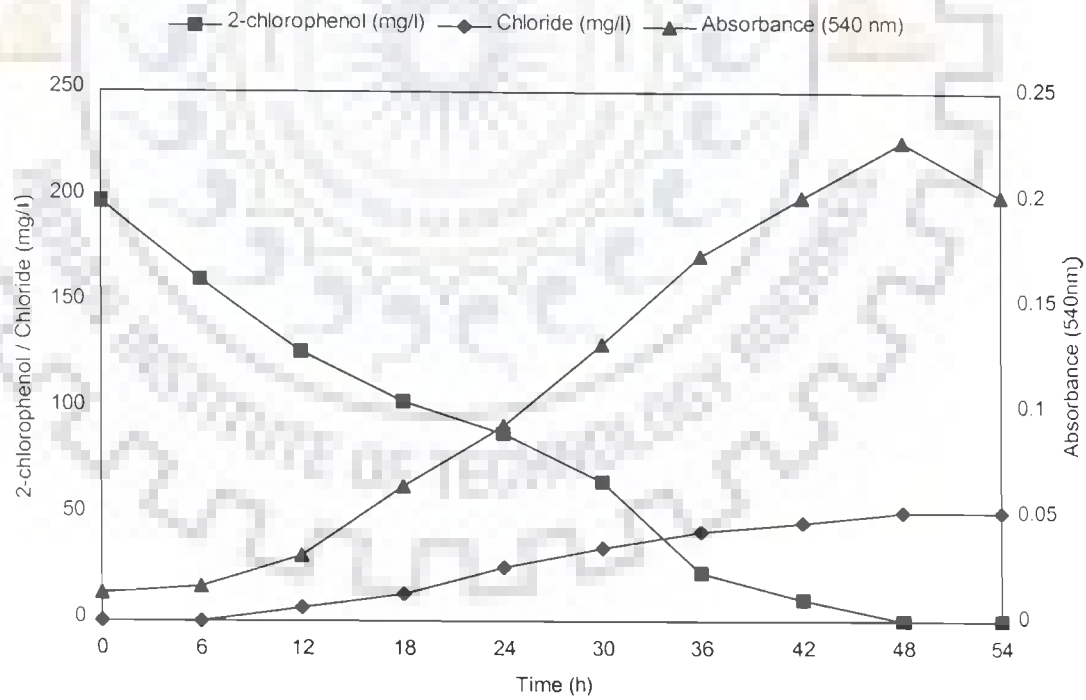


Figure 11 f

Figure 9. ANALYSIS OF TEMPERATURE FOR 2-CHLOROPHENOL DEGRADATION BY *Rhodococcus* sp. M1

Benzoic acid enriched cells acclimatized to 2-chlorophenol was used as the inoculum. Inoculum was added in the modified M3 medium containing 2-chlorophenol of concentration 100 mgl^{-1}. The study was carried out in the temperature range of 4°C to 45°C. Percentage degradation of 2-chlorophenol and growth at absorbance of 540 nm was estimated after 24 h of incubation.

Figure 10. OPTIMIZATION OF pH FOR THE GROWTH OF *Rhodococcus* sp. M1 ON 2-CHLOROPHENOL.

The study was carried out in the pH range of 5 to 12 at 32°C under shaking condition (120 rpm). Other conditions for the experiment were same as described for Figure 9.

Figure 11 g. **DEGRADATION OF 2-CHLOROPHENOL AT 250 mgl^{-1} BY *Rhodococcus* sp. M1**

Conditions for the study were as described for Figure 11 a.

Figure 11h. **DEGRADATION OF 2-CHLOROPHENOL AT 300 mgl^{-1} BY *Rhodococcus* sp. M1**

Conditions for the study were as described for Figure 11 a.

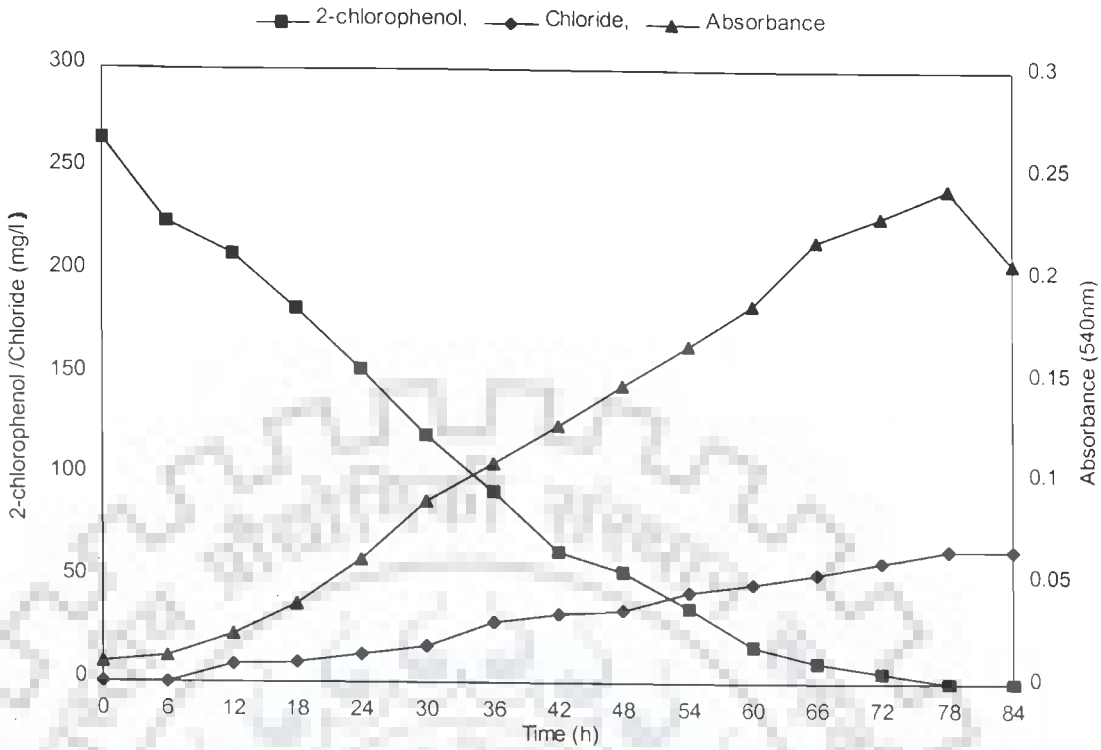


Figure 11 g

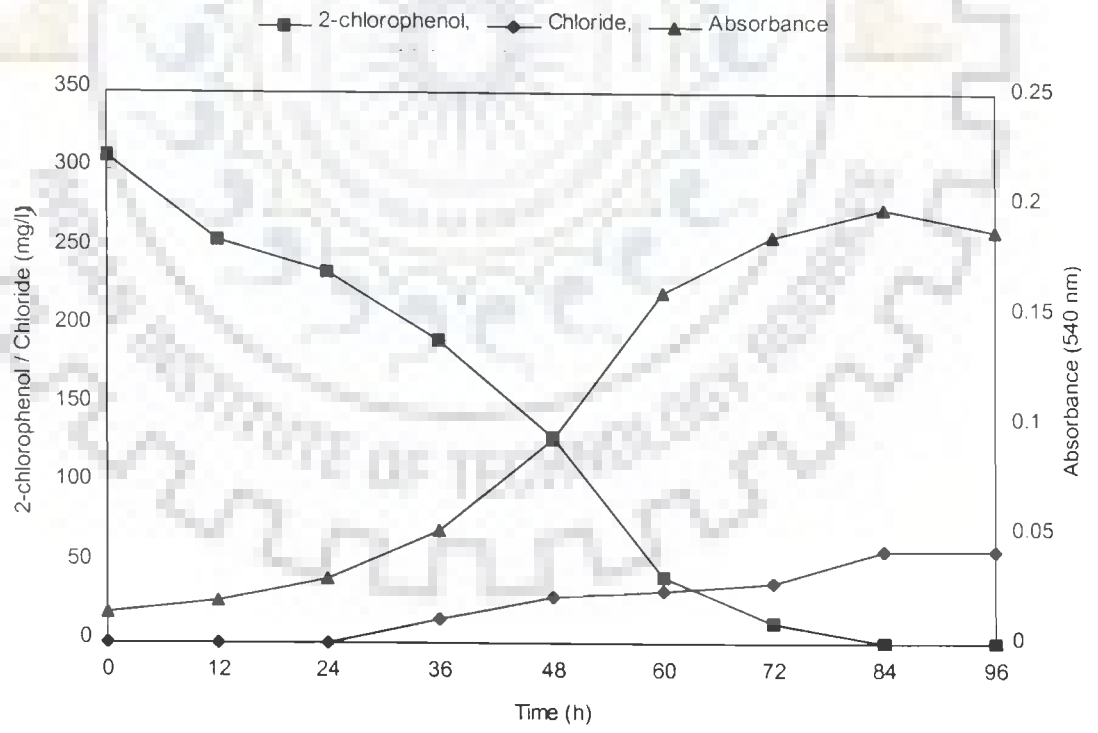


Figure 11 h

Figure 11 i. DEGRADATION OF 2-CHLOROPHENOL AT 350 mgl^{-1} BY *Rhodococcus* sp. M1

Conditions for the study were as described for Figure 11 a.

Figure 11 j. DEGRADATION OF 2-CHLOROPHENOL AT 400 mgl^{-1} BY *Rhodococcus* sp. M1

Conditions for the study were as described for Figure 11 a.

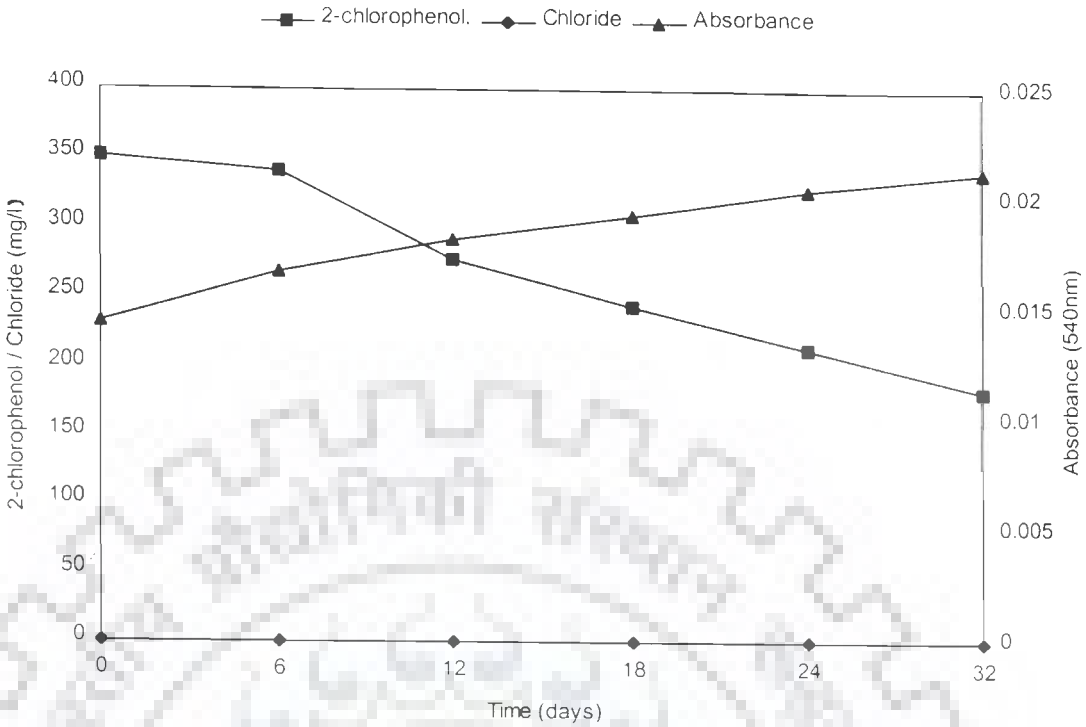


Figure 11 i

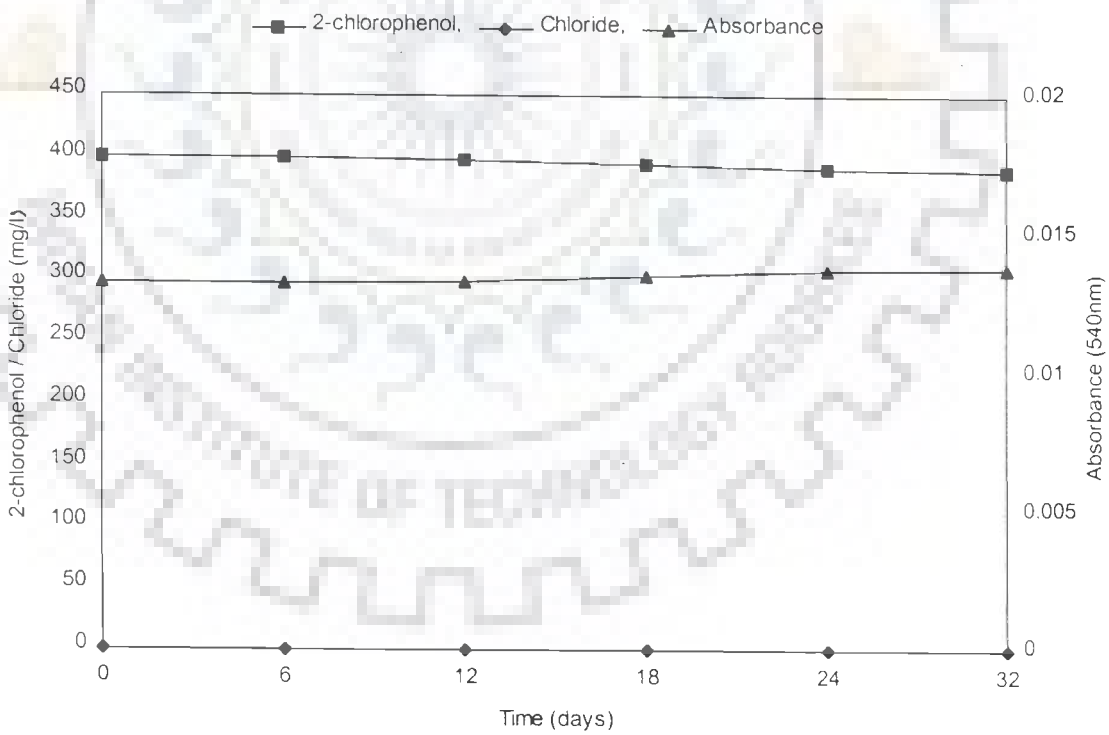


Figure 11 j

4.7.1.1.2 Degradation of 4-chlorophenol

Degradation of 4-chlorophenol was studied by *Rhodococcus* sp. M1 for the substrate concentrations in the range of 10 to 200 mgl^{-1} (Figure 12 a - g). Complete degradation of 4-chlorophenol was observed up to the substrate concentrations of 100 mgl^{-1} however; the time required for complete removal was observed to be increasing upon increasing the substrate concentration (Figure 12 a – e). Rate of degradation showed gradual increase with increase in substrate concentration and reached a maximum for substrate concentration of 50 mgl^{-1} after which there was a slow decline (Table 23). Chloride was released in the exponential phase of growth of culture and was equivalent to more than 90% of the theoretical value for initial substrate concentration up to 75 mgl^{-1}. 4-chlorophenol when added to a further concentration of 150 mgl^{-1} appeared to undergo a partial degradation (43.27 %) after a considerable higher incubation period of 14 days (Figure 12 f). Chloride released was negligible. No growth was observed at 4-chlorophenol concentration of 200 mgl^{-1} (Figure 12 g).

Degradation of 4-chlorophenol up to a concentration of 75 mgl^{-1} showed no detectable soluble COD. Removal of 100 mgl^{-1} of 4-chlorophenol resulted in residual soluble COD of 16.4 mgl^{-1}.

Figure 12 a. DEGRADATION OF 4-CHLOROPHENOL AT 10 mgl^{-1} BY *Rhodococcus* sp. M1

Benzoic acid enriched cells acclimatized to 4-chlorophenol was used as the inoculum. The study was carried out at 32°C, in modified M3 medium under shaking condition (120 rpm). The estimation for 4-chlorophenol, chloride and growth were performed as described in methods.

Figure 12 b. DEGRADATION OF 4-CHLOROPHENOL AT 25 mgl^{-1} BY *Rhodococcus* sp. M1

Conditions for the study were as described for Figure 12 a.

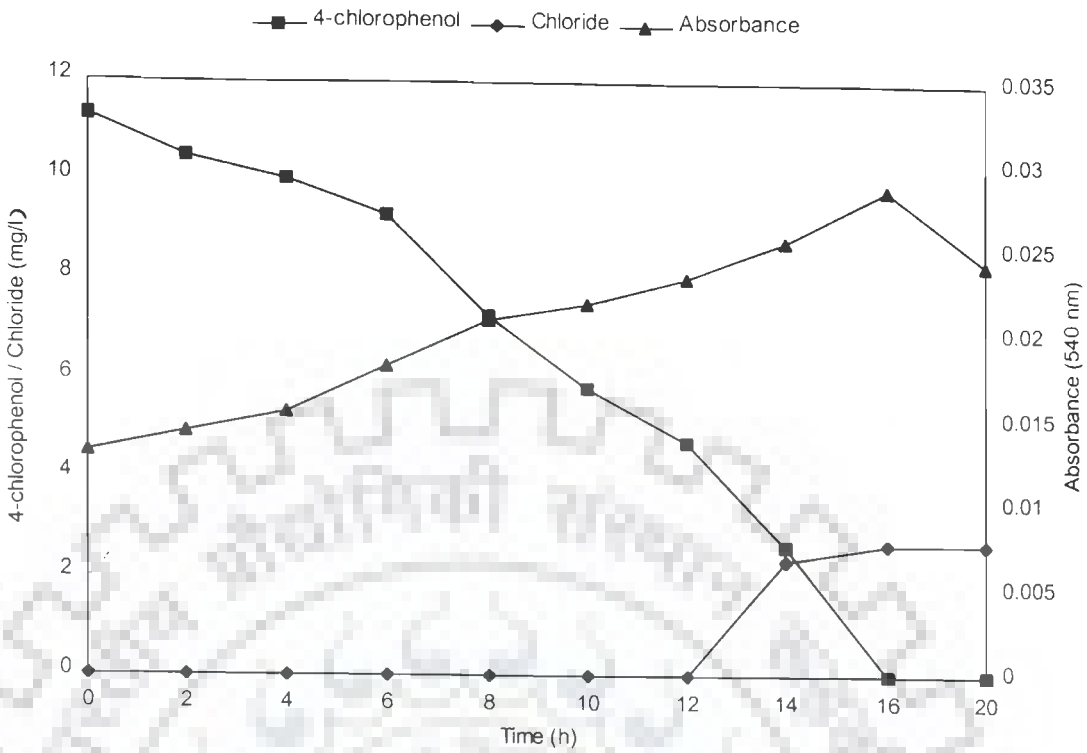


Figure 12 a

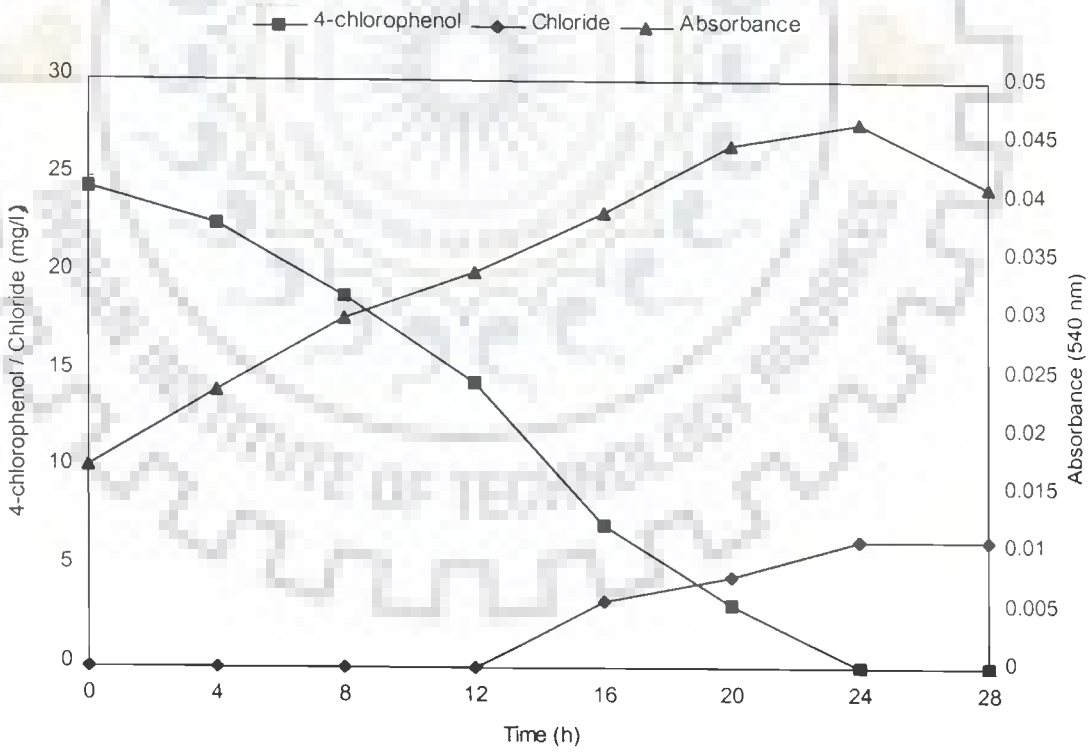


Figure 12 b

Figure 12 c. DEGRADATION OF 4-CHLOROPHENOL AT 50 mgl^{-1} BY *Rhodococcus* sp. M1

Conditions for the study were as described for Figure 12 a.

Figure 12 d. DEGRADATION OF 4-CHLOROPHENOL AT 75 mgl^{-1} BY *Rhodococcus* sp. M1

Conditions for the study were as described for Figure 12 a.

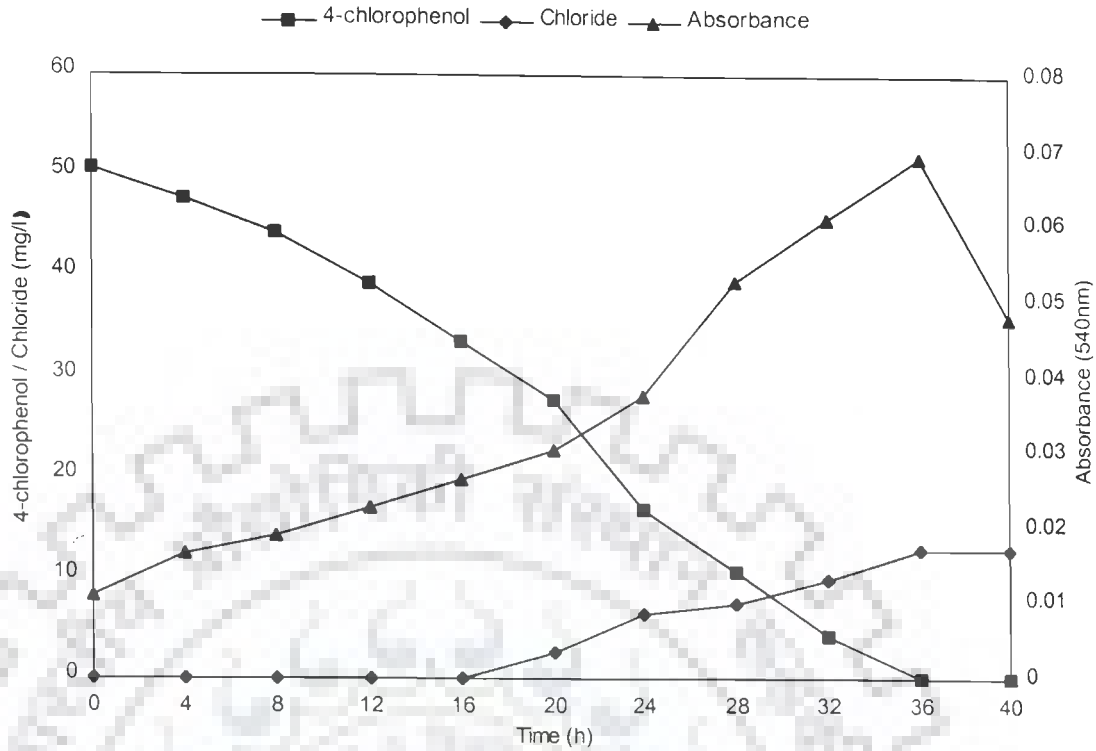


Figure 12 c

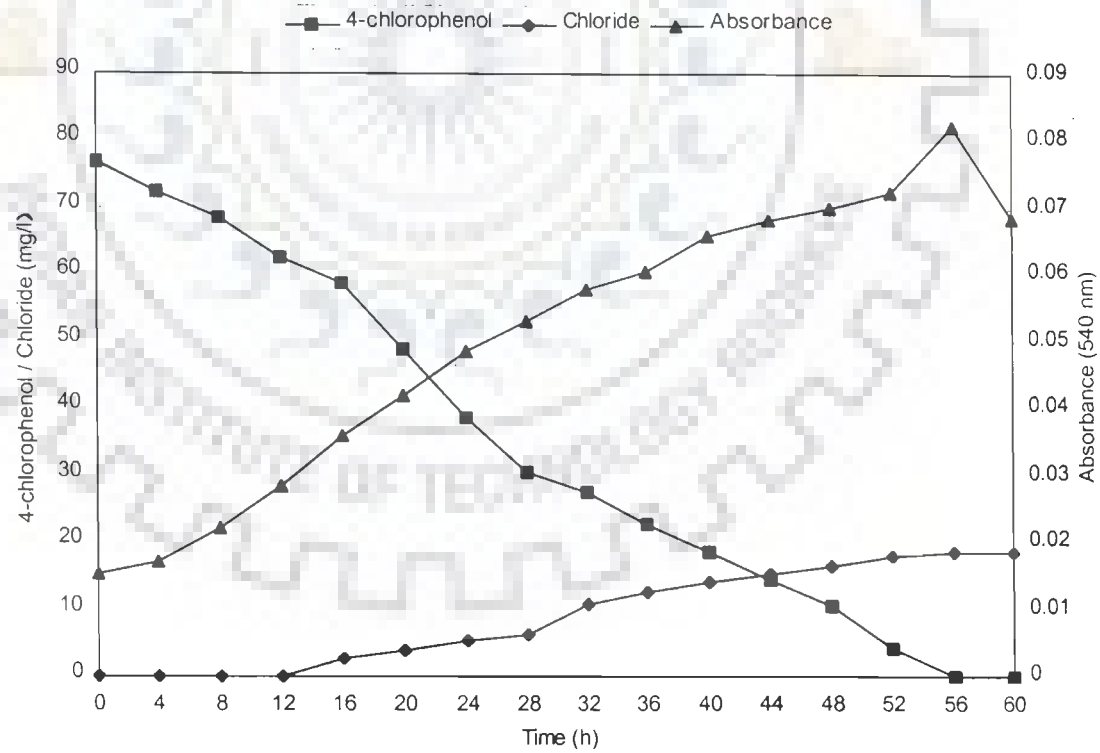


Figure 12 d

Figure 12 e. DEGRADATION OF 4-CHLOROPHENOl AT 100 mgl^{-1} BY *Rhodococcus* sp. M1

Conditions for the study were as described for Figure 12 a.

Figure 12 f. DEGRADATION OF 4-CHLOROPHENOL AT 150 mgl^{-1} BY *Rhodococcus* sp. M1

Conditions for the study were as described for Figure 12 a.

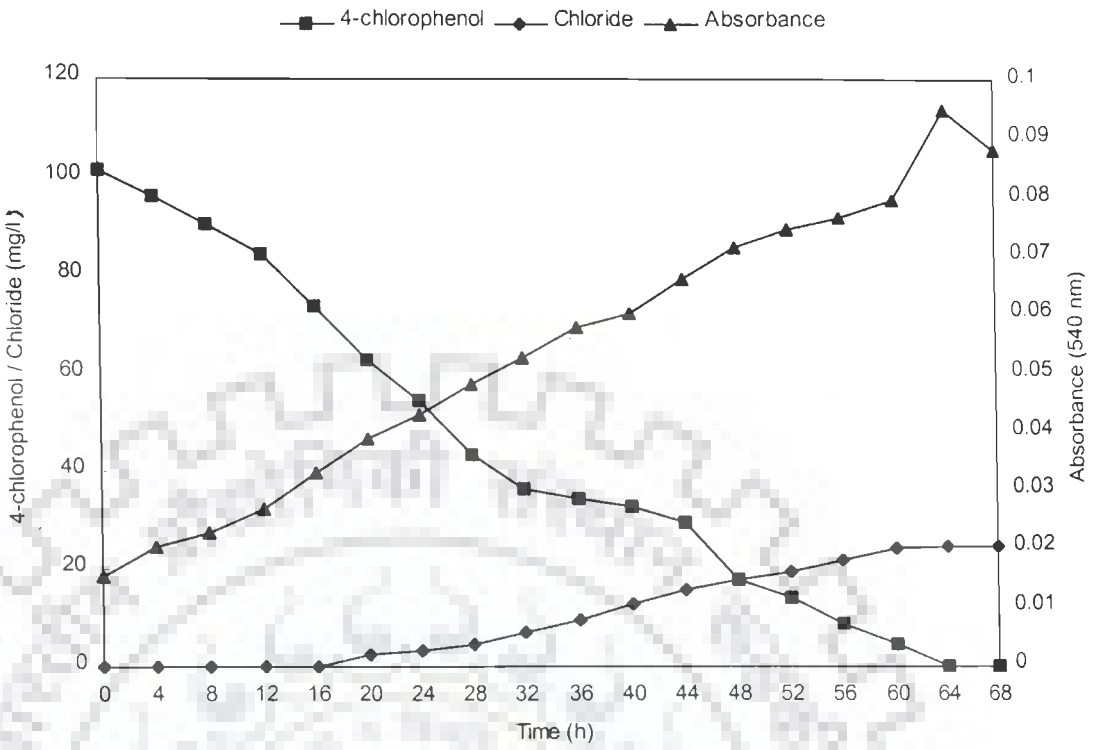


Figure 12 e

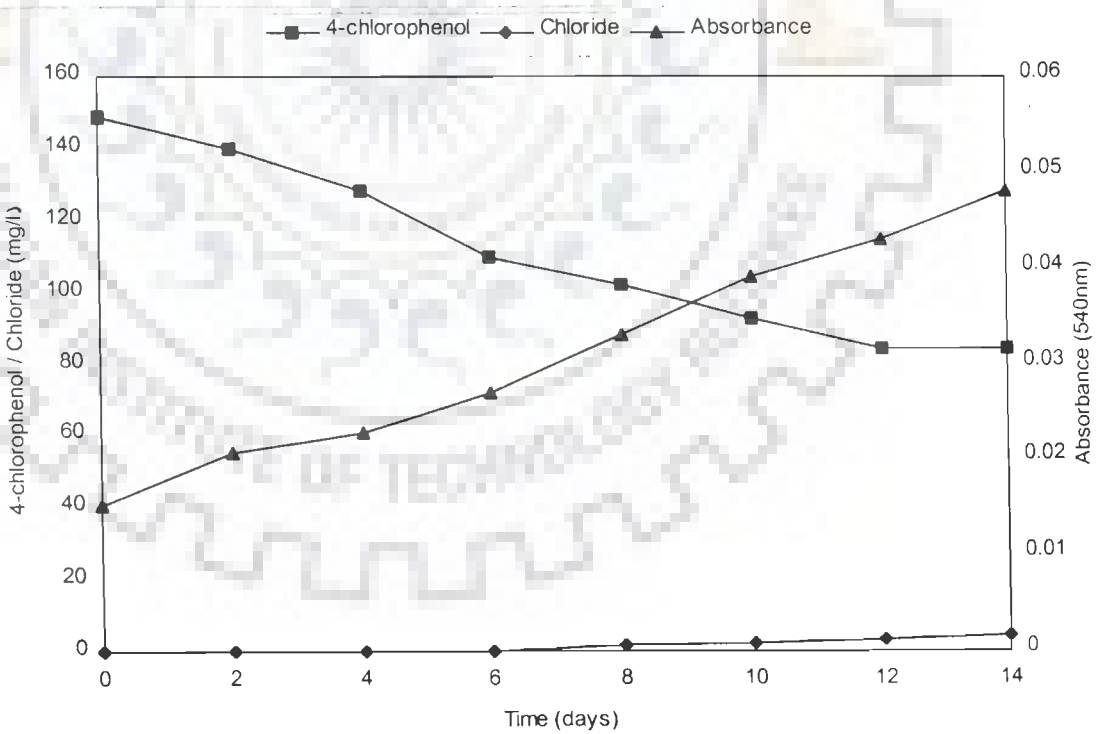


Figure 12 f

Figure 12 g. DEGRADATION OF 4-CHLOROPHENOL AT 200 mgl^{-1} BY *Rhodococcus* sp. M1

Conditions for the study were as described for Figure 12 a.

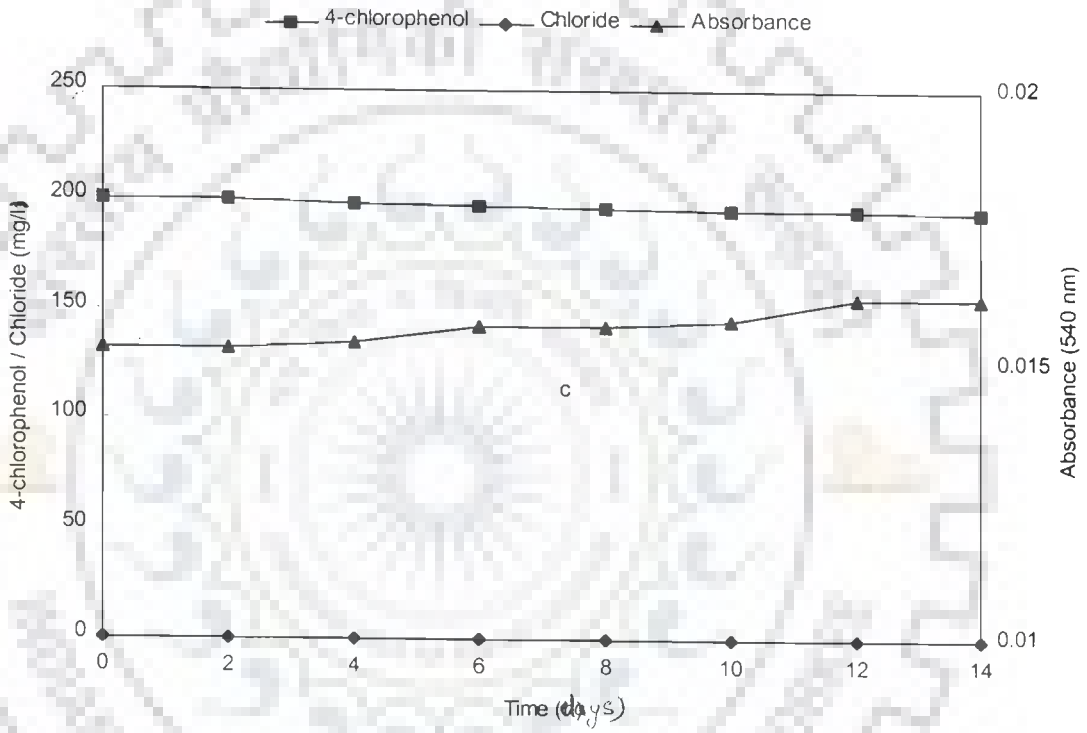

4-chlorophenol
Chloride
Absorbance
250
200
150
100
50
0
0.02
0.015
0.01
4-chlorophenol / Chloride (mg/l)
Absorbance (540 nm)
c
0
2
4
6
8
10
12
14
Time (days)

Figure 12 g

4.7.1.2 Degradation of 2,4-dichlorophenol

Degradation of 2,4-dichlorphenol by *Rhodococcus* sp. M1 was studied for concentration range of 10 - 100 mgl^{-1} (Figure 13 a - e). Complete removal of substrate was observed for 2,4-dichlorophenol concentrations of up to 50 mgl^{-1}; a total period of 144 h was required for complete degradation of 2,4-dichlorophenol when the same was added to a concentration of 50 mgl^{-1} (figure 13 d). Rate of degradation was observed to be highest for substrate concentration of 10 mgl^{-1} and was observed to decrease with further increase in initial substrate concentration (Table 23). Release of chloride was less than 80% of the theoretical value for substrate concentration up to 50 mgl^{-1}. Chloride was released in the exponential phase of growth of the organism. No growth and release of chloride was observed for substrate concentration of 100 mgl^{-1} (Figure 13 e). Degradation of substrate up to the concentration of 25 mgl^{-1} showed no residual soluble COD. Degradation of 40 mgl^{-1} and 50 mgl^{-1} of 2,4-dichlorophenol resulted in residual soluble COD of 28 mgl^{-1} and 47 mgl^{-1} respectively.

Table 23. Rate of degradation of monochlorophenols and dichlorophenol by benzoate-induced *Rhodococcus* sp. M1

Concentrations (mg l^{-1})	Rate of degradation (mg l^{-1} h^{-1})		
	2-chlorophenol	4-chlorophenol	2,4-dichlorophenol
10	1.31	0.74	0.44
25	3.02	1.31	0.41
40	-	-	0.38
50	5.27	1.74	0.34
75	-	1.59	-
100	9.8	1.38	-
150	4.32	-	-
200	4.13	-	-
250	4.04	-	-
300	3.95	-	-

-, Not tested

Figure 13 a. DEGRADATION OF 2,4-DICHLOROPHENOL AT 10 mgl^{-1} BY *Rhodococcus* sp. M1

Benzoic acid enriched cells acclimatized to 2,4-dichlorophenol was used as the inoculum. The study was carried out at 32°C, in modified M3 medium under shaking condition (120 rpm). The estimation for 2,4-dichlorophenol, chloride and growth were performed as described in methods.

Figure 13 b. DEGRADATION OF 2,4-DICHLOROPHENOL AT 25 mgl^{-1} BY *Rhodococcus* sp. M1

Conditions for the study were as described for Figure 13 a.

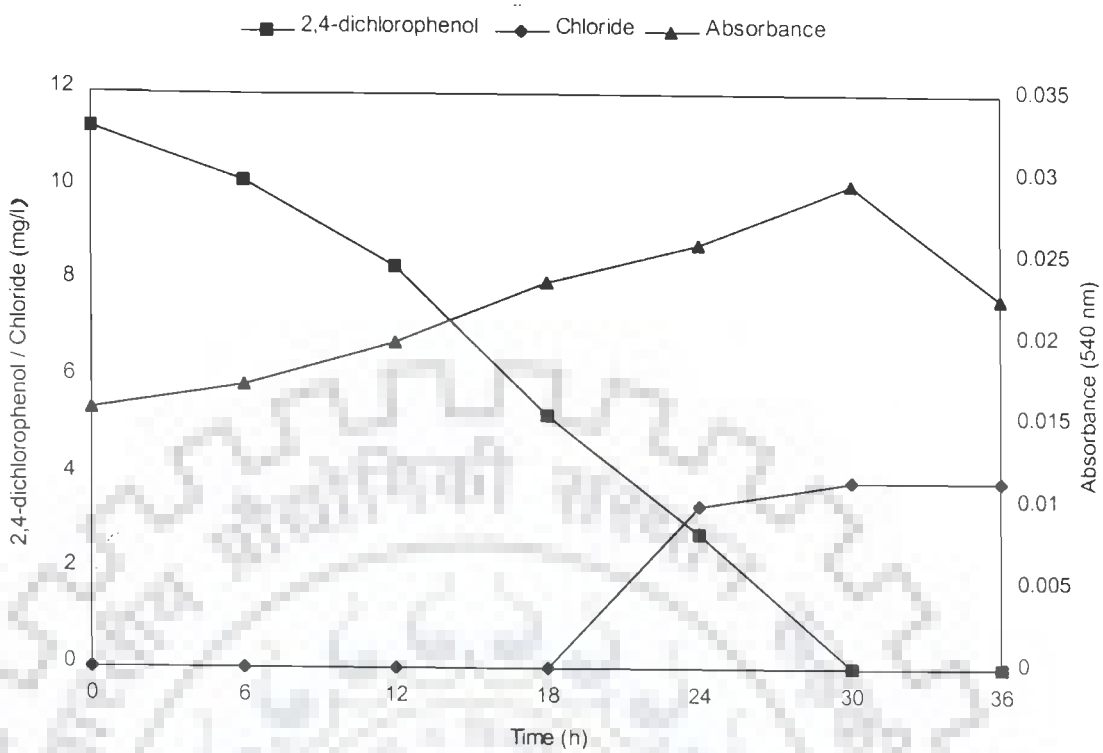


Figure 13 a

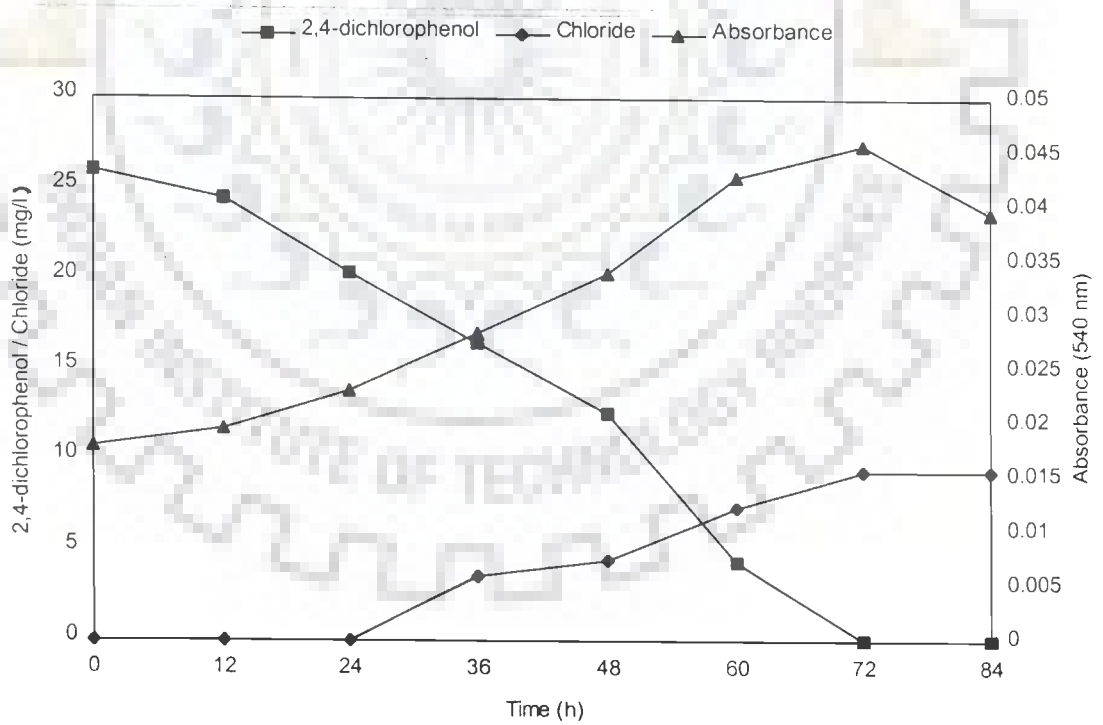


Figure 13 b

Figure 13 c. DEGRADATION OF 2,4-DICHLOROPHENOL AT 40 mgl^{-1} BY *Rhodococcus* sp. M1

Conditions for the study were as described for Figure 13 a.

Figure 13 d. DEGRADATION OF 2,4-DICHLOROPHENOL AT 50 mgl^{-1} BY *Rhodococcus* sp. M1

Conditions for the study were as described for Figure 13 a.

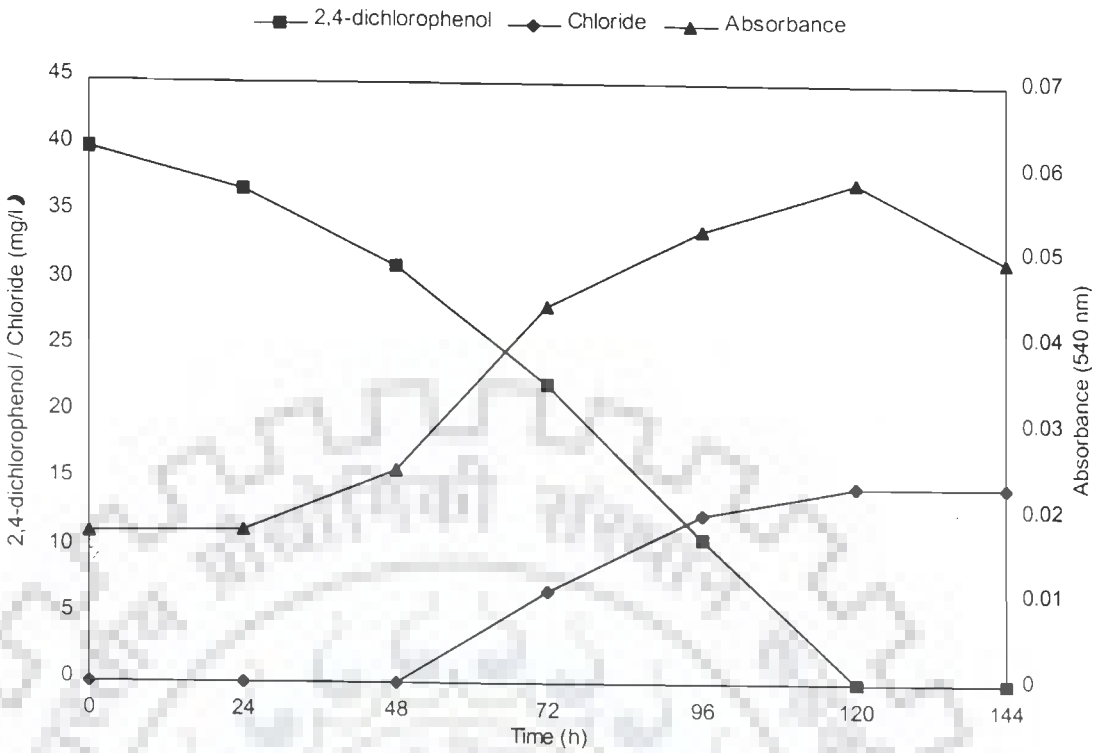


Figure 13 c

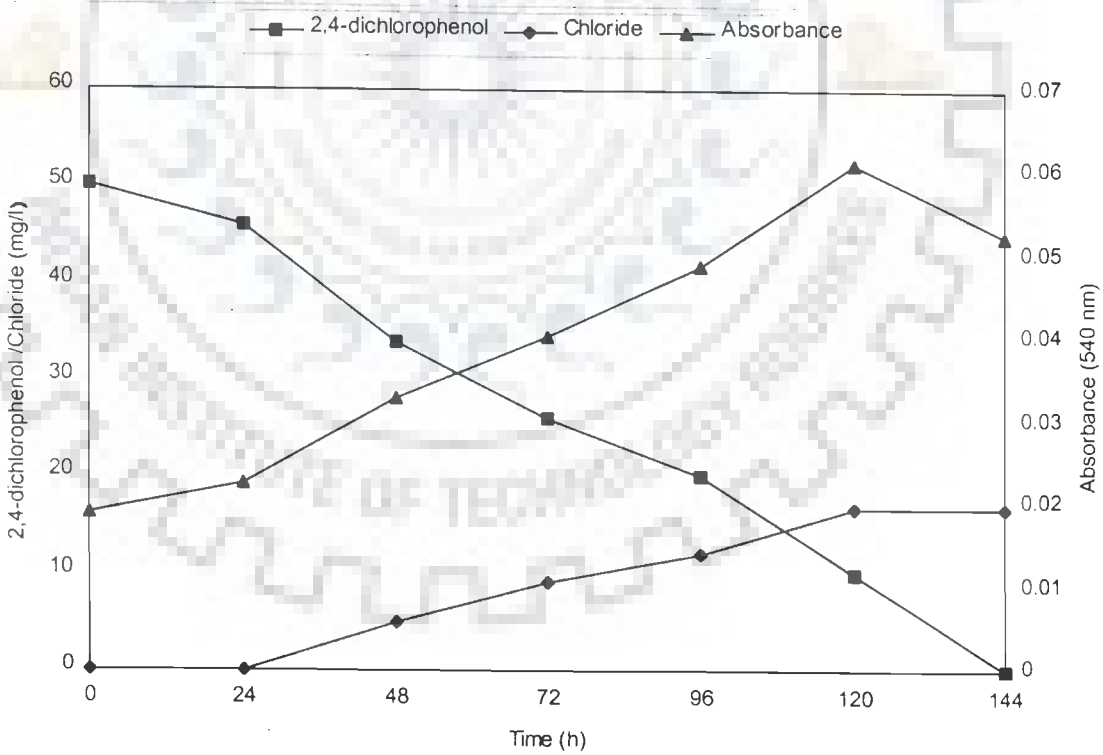


Figure 13 d

Figure 13 e. DEGRADATION OF 2,4-DICHLOROPHENOL AT 100 mgl^{-1} BY *Rhodococcus* sp M1

Conditions for the study were as described for Figure 13 a.


2,4-dichlorophenol
Chloride
Absorbance (540 nm)
120
100
80
60
40
20
0
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
2
4
6
8
10
2,4-dichlorophenol / Chloride (mg/l)
Absorbance (540 nm)
Time (days)

Figure 13 e

4.7.1.3 Degradation of 2,4,6-trichlorophenol

Degradation of 2,4,6-trichlorophenol was studied at the substrate concentrations of 10 - 50 mgl^{-1} (Figure 14 a - c). Complete degradation was observed after 48 h of incubation for the substrate concentration of 10 mgl^{-1} (Figure 14 a). Lower levels of degradation (60.47%) was observed at higher 2,4,6-trichlorophenol concentration (25 mgl^{-1}) (Figure 14 b). Chloride released was observed respectively to be 79.77% and 57.07% of the theoretical value for substrate concentrations of 10 mgl^{-1} and 25 mgl^{-1}. No growth and release of chloride was observed for 2,4,6-trichlorophenol concentration of 50 mgl^{-1} (Figure 14 c).

Figure 14 a. DEGRADATION OF 2,4,6-TRICHLOROPHENOL AT 10 mgl^{-1} BY *Rhodococcus* sp. M1

Benzoic acid enriched cells acclimatized to 2,4,6-trichlorophenol was used as the inoculum. The study was carried out at 32°C, in modified M3 medium under shaking condition (120 rpm). The estimation for 2,4,6-trichlorophenol, chloride and growth were performed as described in methods.

Figure 14 b. DEGRADATION OF 2,4,6-TRICHLOROPHENOL AT 25 mgl^{-1} BY *Rhodococcus* sp.M1

Conditions for the study were as described for Figure 14 a.

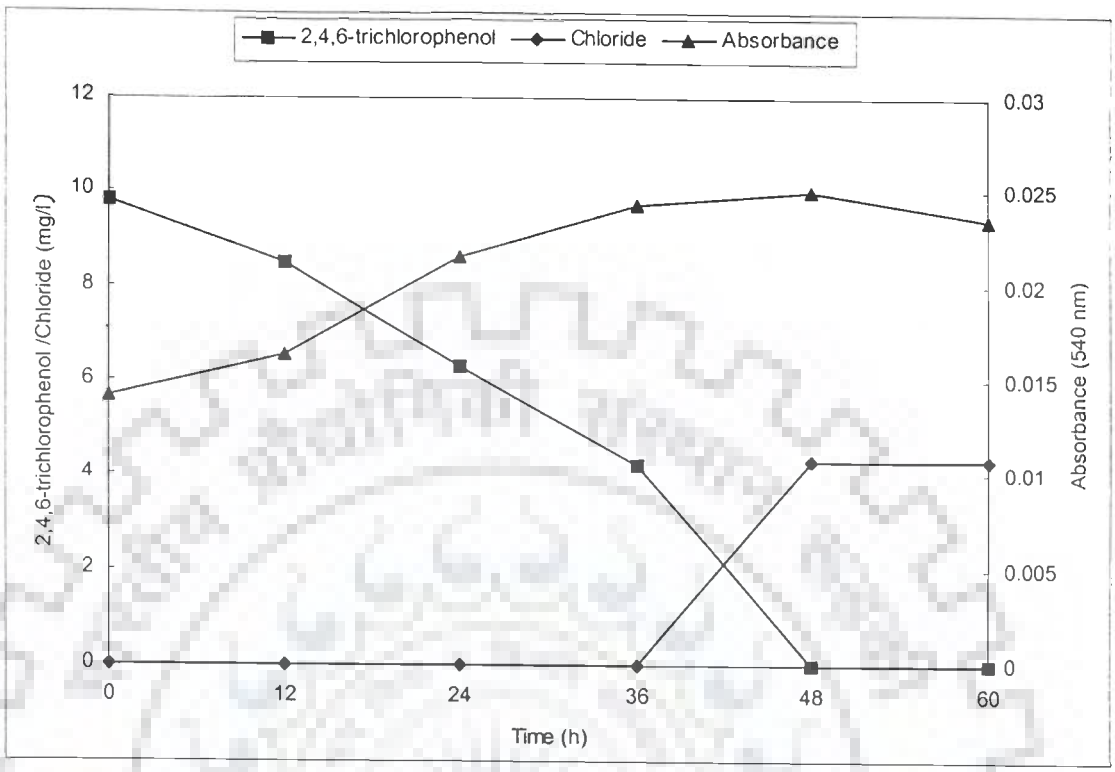


Figure 14 a

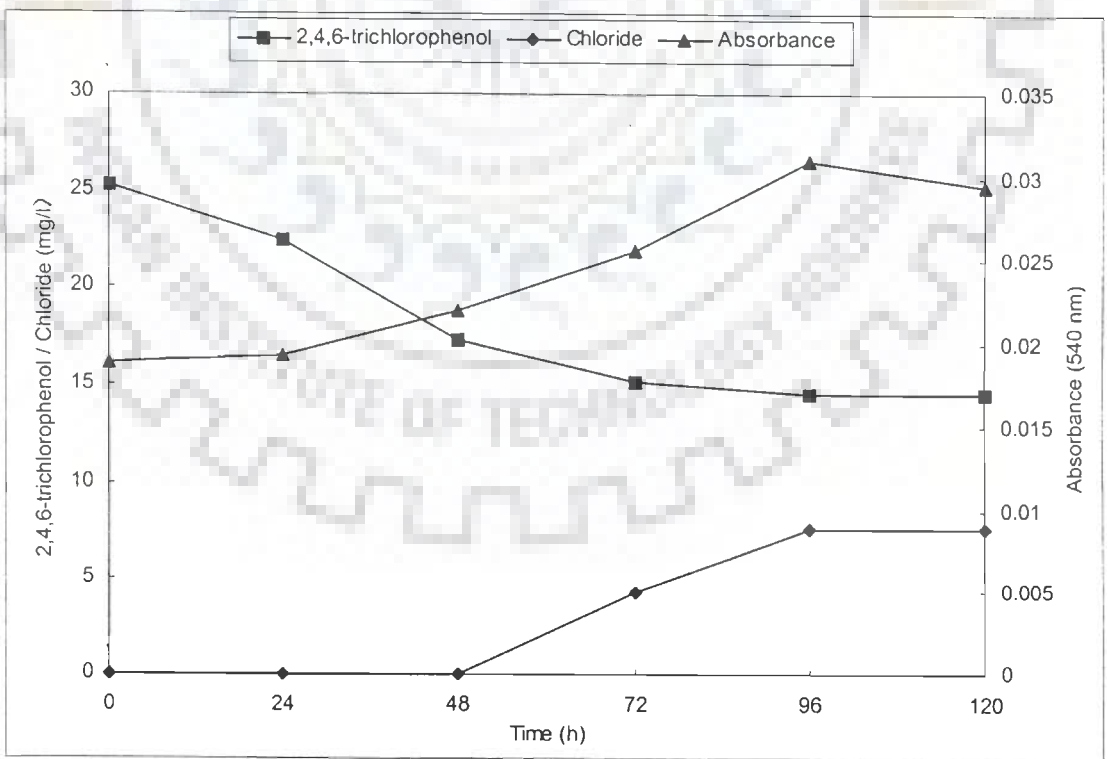


Figure 14 b

Figure 14 c. DEGRADATION OF 2,4,6-TRICHLOROPHENOL AT 50 mgl^{-1} BY *Rhodococcus* sp.M1

Conditions for the study were as described for Figure 14 a.

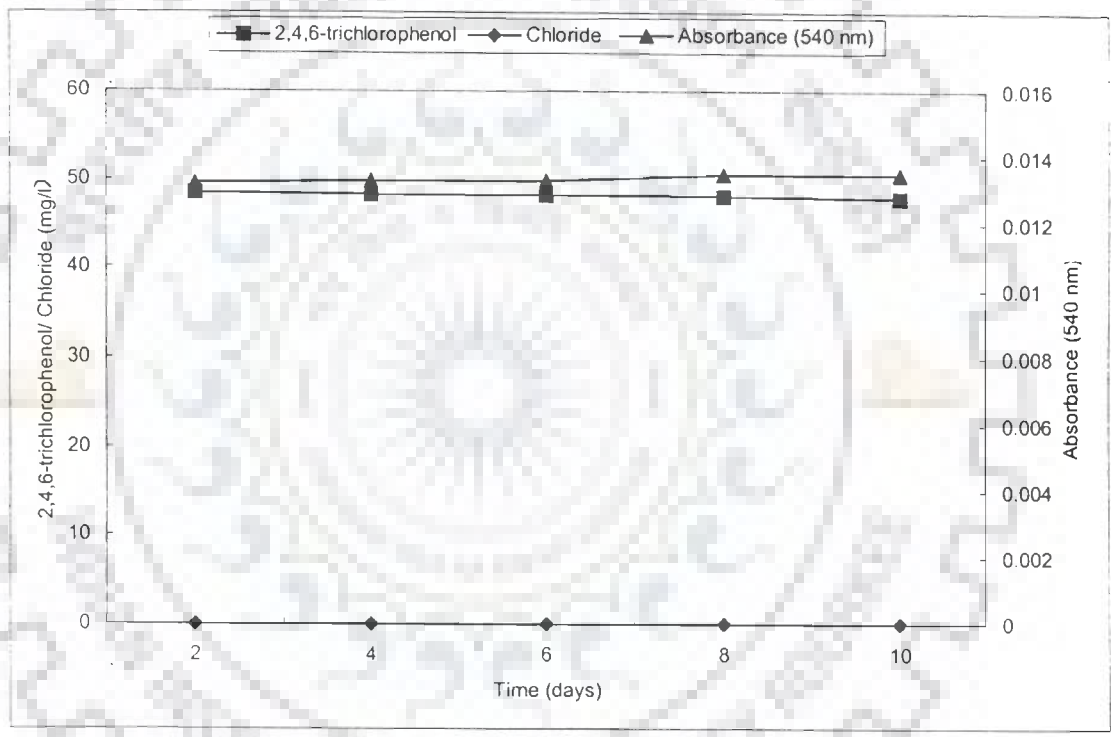

2,4,6-trichlorophenol
Chloride
Absorbance (540 nm)
2,4,6-trichlorophenol/ Chloride (mg/l)
Absorbance (540 nm)
Time (days)
60
50
40
30
20
10
0
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
2
4
6
8
10

Figure 14 c

4.7.2 Evaluation of kinetic parameters for degradation of chlorophenols

4.7.2.1 Monochlorophenol degradation

4.7.2.1.1 Degradation of 2-chlorophenol

Growth yield of *Rhodococcus* sp. M1 on 2-chlorophenol, evaluated from the increase in cell mass and the amount of substrate consumed was in the range of 0.087 to 0.026 mg mol^{-1} (Table 24). Growth yields decreased with increasing substrate concentration. Substrate utilization rate (SUR) was computed from change in substrate concentration over time and microbial cell mass as per the equation in section 3.2.13.4. SUR was found to decrease with increasing substrate concentration and was in the range of 0.267 to 0.059 mg/mg.h (Table 24). Specific growth rate (μ) of *Rhodococcus* sp. M1 for varying 2-chlorophenol concentrations (10 - 300 mgl^{-1}) was observed to be in the range of 0.221 to 0.043 h^{-1} (Table 24). The value of μ when plotted against concentration was found to drop linearly with increasing substrate concentration (Figure 15). The graph was plotted on the basis of regression equation and correlation coefficient was also computed and was found to be 0.9348. The intercept of the line gives a maximum specific growth rate (μ_{max}) of 0.248 h^{-1}. The slope of the line gives an inhibition parameter (Ki_I) of 354.28 mgl^{-1} (Hill *et al.*, 1996). The value of Ki_I is the maximum substrate concentration beyond which no growth should be observed. Doubling time (t_d) of the organism is related to μ and is calculated from equation 3.2.13.3. Doubling time (t_d) of the organism at non-inhibitory concentration of 2-chlorophenol was evaluated to be 3.13 h (Table 27).

Table 24. Analysis of Kinetic parameters for 2-chlorophenol degradation at different concentrations of the substrate

2-chlorophenol (mgl^{-1})	μ (h^{-1})	Growth yield ($mg\ mol^{-1}$)	SUR (mg/mg.h)
10	0.221	0.087	0.267
25	0.228	0.074	0.218
50	0.224	0.062	0.173
100	0.211	0.061	0.119
150	0.123	0.056	0.063
200	0.078	0.046	0.058
250	0.047	0.040	0.043
300	0.043	0.026	0.059

μ, Specific growth rate of the culture

SUR, Substrate Utilization Rate

Figure 15. **SPECIFIC GROWTH RATE OF *Rhodococcus* sp. M1 PLOTTED AGAINST CONCENTRATIONS OF 2-CHLOROPHENOL**

The line gives the maximum specific growth rate μ_{max} and the inhibition parameter K_{I}

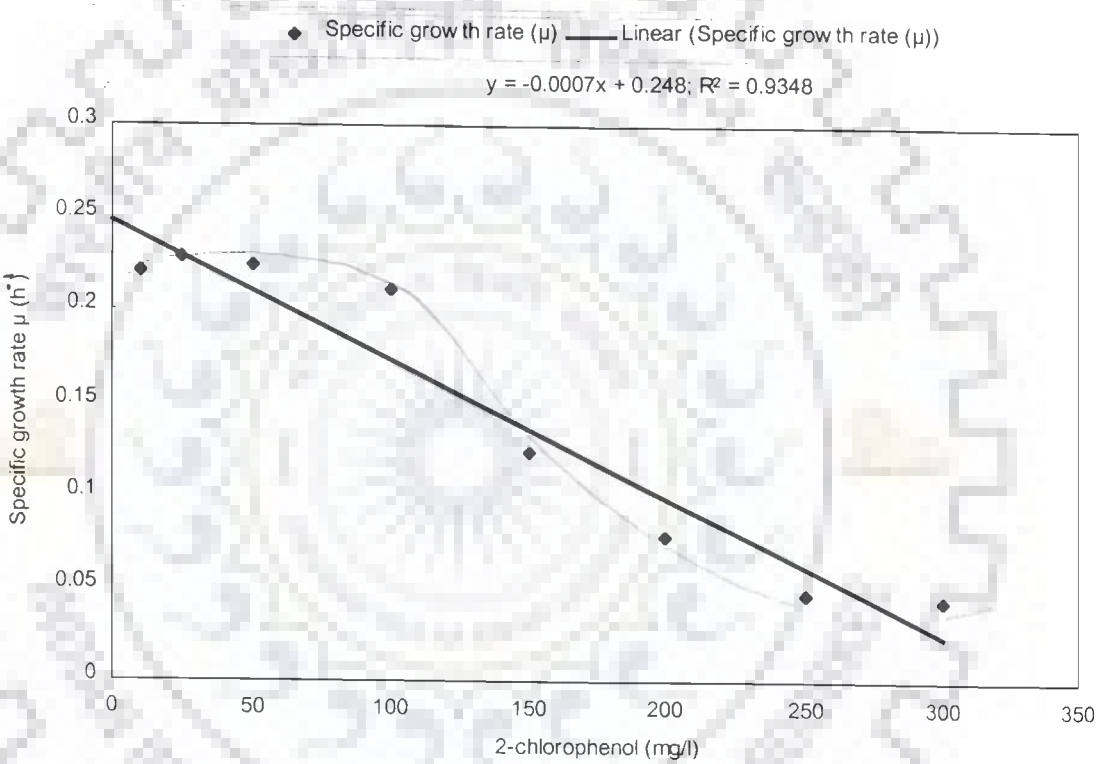

Specific grow th rate (μ)
Linear (Specific grow th rate (μ))
y = -0.0007x + 0.248; R² = 0.9348
Specific growth rate μ (h⁻¹)
0.3
0.25
0.2
0.15
0.1
0.05
0
0
50
100
150
200
250
300
350
2-chlorophenol (mg/l)

Figure 15

4.7.2.1.2 Degradation of 4-chlorophenol

Growth yield of *Rhodococcus* sp. M1 decreases with increase in 4-chlorophenol concentration and is in the range 0.065 to 0.0337 mg mol^{-1} (Table 25). Substrate uptake rate decreases with increase in substrate concentration and is in the range 0.136 to 0.059 mg/mg.h (Table 25). Specific growth rate μ of *Rhodococcus* sp. M1 on 4-chlorophenol for concentrations 10 to 100 mg l^{-1} was found to be in the range 0.0481 to 0.024 h^{-1} (Table 25). The value of μ on 4-chlorophenol was thus found to be significantly less than that reported on 2-chlorophenol. A linear fall in the specific growth rate was observed when plotted against substrate concentration (Figure 16). The graph was plotted on the basis of regression equation and correlation coefficient was found to be 0.9835. Maximum specific growth rate (μ_{max}) of the culture is evaluated from the intercept of this line and was 0.0493 h^{-1}. The slope of the line gives an inhibition parameter (Ki_1) of 164.33 mg l^{-1}. Batch studies as earlier using 4-chlorophenol as the substrate substantiated the value of Ki_1, as growth was observed to be inhibited beyond 100 mg l^{-1} of substrate. Doubling time of the organism at non-inhibitory concentration of the substrate was found to be 16.6 h (Table 27).

Table 25. Analysis of Kinetic parameters for 4-chlorophenol degradation at different concentrations of the substrate

4-chlorophenol (mgl^{-1})	μ (h^{-1})	Growth yield (mg mol^{-1})	SUR (mg/mg.h)
10	0.0481	0.065	0.136
25	0.041	0.050	0.126
50	0.037	0.048	0.089
75	0.03	0.037	0.061
100	0.024	0.0337	0.059

μ, Specific growth rate of the culture

SUR, Substrate Utilization Rate

Figure 16. SPECIFIC GROWTH RATE OF *Rhodococcus* sp. M1 PLOTTED AGAINST CONCENTRATIONS OF 4-CHLOROPHENOL

The line gives the maximum specific growth rate μ_{max} and the inhibition parameter K_{i1}

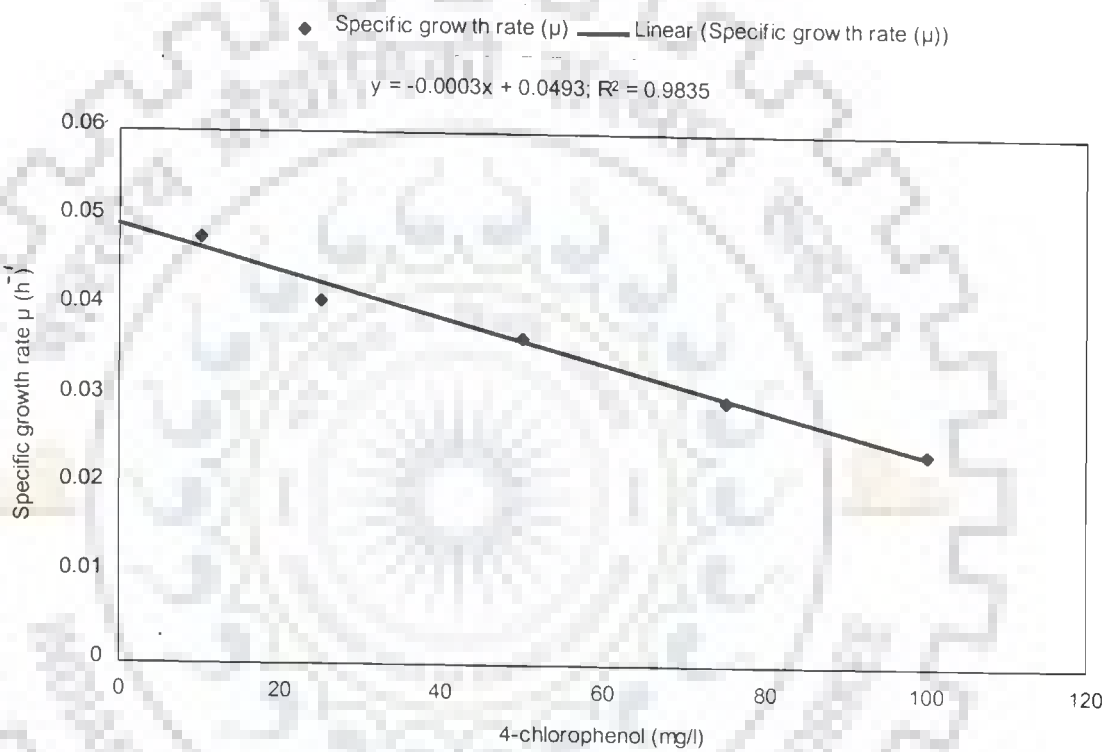

Specific grow th rate (μ)
Linear (Specific grow th rate (μ))
y = -0.0003x + 0.0493; R² = 0.9835
Specific growth rate μ (h⁻¹)
0.06
0.05
0.04
0.03
0.02
0.01
0
0
20
40
60
80
100
120
4-chlorophenol (mg/l)

Figure 16

4.7.2.2 Degradation of 2,4-dichlorophenol

The growth yield of *Rhodococcus* sp. M1 decreased with increase in substrate concentration and was in the range 0.065 to 0.045 mg mol^{-1} at the substrate concentration of 10 to 50 mg l^{-1} (Table 26). Substrate uptake rate was significantly lower than that on monochlorophenol and was in the range 0.082 to 0.024 mg/mg.h (Table 26). Specific growth rate μ of *Rhodococcus* sp. M1 on 2,4-dichlorophenol of concentration (10 to 50 mg l^{-1}) was observed to be 0.022 to 0.01 h^{-1} (Table 26) which was considerably lower than that for monochlorophenol. The specific growth rate (μ) was observed to drop linearly with substrate concentration (Figure 17). The graph, plotted on the basis of regression equation and correlation coefficient computed, was 0.9687. Maximum specific growth rate (μ_{max}) evaluated from the intercept of this line was detected to be 0.0244 h^{-1}. The slope of the line gives an inhibition parameter of Ki_1 of 81.3 mg l^{-1}. This Ki_1 value was in accordance with the degradation analysis in which growth was inhibited at a substrate concentration of 75 mg l^{-1}. The cells show a high doubling time of 37.41 h even at non-inhibitory concentrations of the substrate (Table 27).

Table 26. Analysis of Kinetic parameters for 2,4-dichlorophenol degradation at different concentrations of the substrate

2,4-dichlorophenol (mgl^{-1})	μ (h^{-1})	Growth yield (mg mol^{-1})	SUR (mg/mg.h)
10	0.022	0.065	0.082
25	0.0162	0.058	0.038
40	0.014	0.053	0.027
50	0.010	0.045	0.024

μ, Specific growth rate of the culture

SUR: Substrate Utilization Rate

Table 27. Doubling time of *Rhodococcus* sp. M1 on chlorophenols

Substrate	t_d (h)
2-chlorophenol	3.13
4-chlorophenol	16.6
2,4-dichlorophenol	37.41

t_d, Doubling time

Figure 17. **SPECIFIC GROWTH RATE OF *Rhodococcus* sp. M1 PLOTTED AGAINST CONCENTRATIONS OF 2,4-DICHLOROPHENOL**

The line gives the maximum specific growth rate μ_{max} and the inhibition parameter K_{II}.

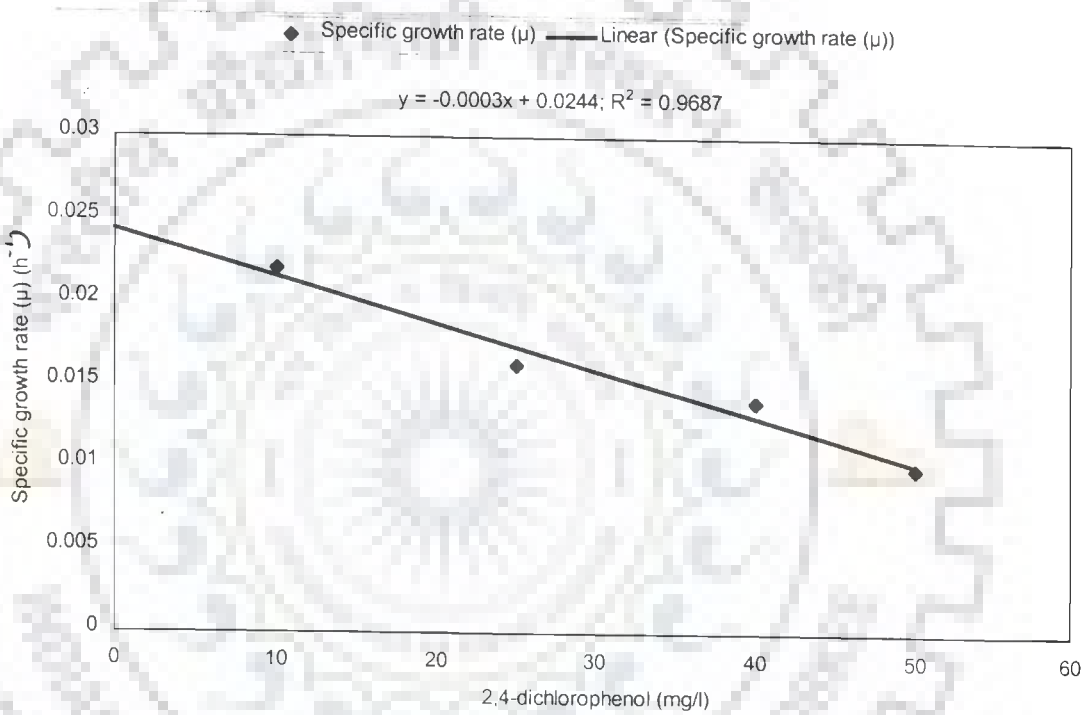

Specific growth rate (μ)
Linear (Specific growth rate (μ))
y = -0.0003x + 0.0244; R² = 0.9687
Specific growth rate (μ) (h⁻¹)
0.03
0.025
0.02
0.015
0.01
0.005
0
0
10
20
30
40
50
60
2,4-dichlorophenol (mg/l)

Figure 17

4.8 Degradation of mixed substrates by *Rhodococcus* sp. M1 using 2-chlorophenol, phenol and p-cresol as the components

Industrial synthesis of chlorinated phenols result into the wastewater which often contains phenol along with chlorinated phenols. In addition the effluents from pesticide formulation plants are reported to contain cresols and phenol along with chlorophenols. The degradation of chlorophenol in mixed substrate form with phenol and cresol was thus investigated by *Rhodococcus* sp. M1.

4.8.1 Degradation of mixture of phenol and 2-chlorophenol

Degradation of binary substrate consisting of phenol (50 mg l^{-1}) and 2-chlorophenol (100 mg l^{-1}) by *Rhodococcus* sp. M1 was investigated. Degradation of phenol (50 mg l^{-1}) and 2-chlorophenol (100 mg l^{-1}) when utilized as sole substrate was achieved within 14 h (Figure 18) and 18 h (Figure 11 d) respectively. Degradation of the mixed substrate consisting of 2-chlorophenol and phenol was evaluated by gas chromatographic analysis and is presented in Figure 19. Degradation of both the substrates was delayed when the compounds are utilized in mixture. A simultaneous, but preferential pattern of utilization was observed for the mixture (Figure 20). Phenol was the preferred substrate and was completely removed in 48 h. Levels of 2-chlorophenol was reduced to 53.2 mg l^{-1} in 48 h. Degradation of 2-chlorophenol had resulted into the release of 5.4 mg l^{-1} of chloride in 48 h (Figure 20). Complete removal of 2-chlorophenol was achieved within 96 h and was supported by the release of equimolar amount of chloride. Residual soluble COD was observed to be 139.2 mg l^{-1} after 48 h and resulted into sharp decline following 48 h (Figure. 21). Negligible

residual COD of 10.44 mg l^{-1} was observed after 96 when 2-chlorophenol was completely removed. Rate of degradation of phenol decreased drastically from 4.7 mg l^{-1} h^{-1} when used as pure substrate to 1.66 mg l^{-1} h^{-1} when used in mixture with 2-chlorophenol. Similarly the rate of degradation of 2-chlorophenol as pure substrate was 9.8 mg l^{-1} h^{-1} and that decreased in mixture with phenol to 1.04 mg l^{-1} h^{-1} (Table. 28).

Growth was initiated without any apparent lag period. The growth curve showed a typical pattern with two peaks after 48 h and 96 h. Increase in the cell growth was observed to show gradual increase giving the first peak after phenol is completely consumed in 48 h. The cell growth then shows slight decline followed by increase in growth to give another peak after 96 h when 2-chlorophenol is completely degraded (Figure 20). Specific growth rate of the culture on mixture of 2-chlorophenol and phenol showed a much lower value of 0.03 h^{-1} as compared to 2-chlorophenol (0.211 h^{-1}) and phenol (0.163 h^{-1}) when utilized as pure substrates (Table. 29).

Figure 18. DEGRADATION OF PHENOL BY *Rhodococcus* sp. M1

The study was carried out in modified M3 medium containing phenol (50 mg l^{-1}) at 32°C under shaking condition (120rpm). Inoculum was prepared by growing the culture in DSM-65 medium and washing with the phosphate buffer. The culture was then grown in phenol of the same concentration before using it for the present study. Estimation of phenol and growth was measured as described in methods.

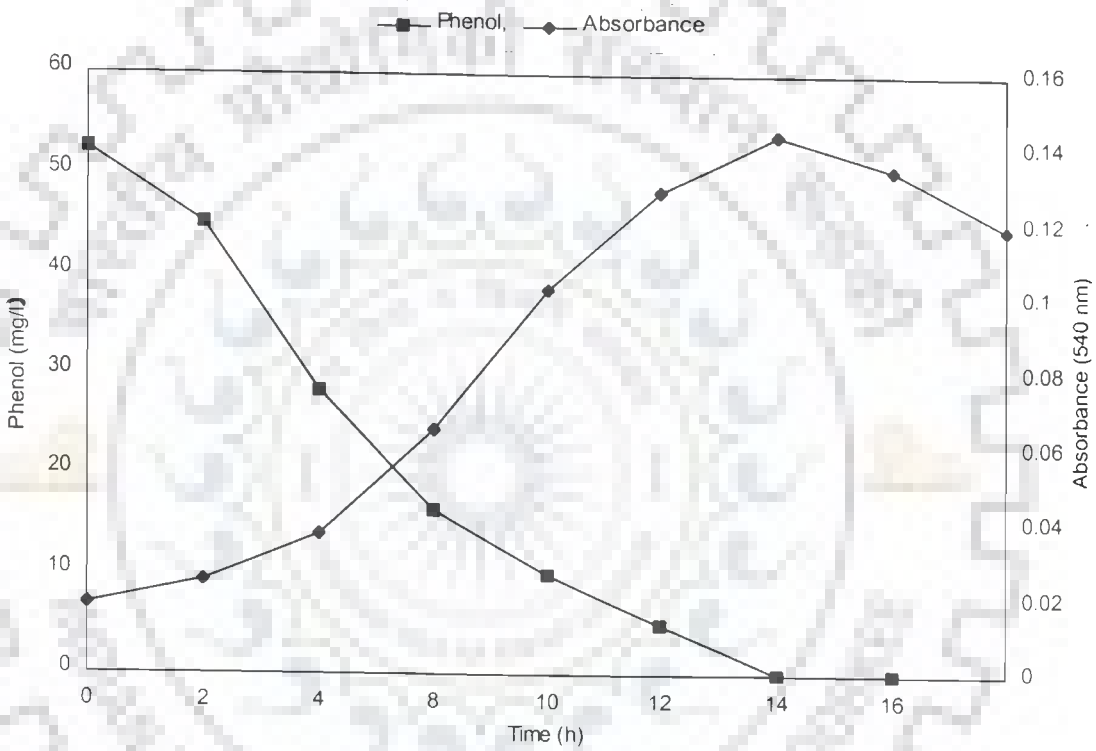

Phenol,
Absorbance
60
50
40
30
20
10
0
Phenol (mg/l)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Absorbance (540 nm)
0
2
4
8
10
12
14
16
Time (h)

Figure 18

Figure 19. GAS CHROMATOGRAPHIC ANALYSIS OF DEGRADATION OF MIXED SUBSTRATE OF 2-CHLOROPHENOL (100 mg l^{-1}) AND PHENOL (50 mg l^{-1})

The Gas chromatograph was equipped with a CP SIL 8 CB capillary column (Chrompack- length 25 m; and inner diameter 0.32 mm) and a flame ionization detector. Hydrogen was used as the carrier gas at 4.5 psi. Injector and detector temperature was at 300°C. The initial oven temperature was 125°C and then rose to 180°C at the rate of 4°C/min. From 180°C the temperature was raised to 250°C at the rate of 10°C/min and kept isothermal at 250°C for 2 minutes. Sample was treated as described in methods before injecting into gas chromatograph column.

Figure 19 A. GAS CHROMATOGRAPHIC ANALYSIS OF MIXTURE OF 2-CHLOROPHENOL (100 mg l^{-1}) and phenol (50 mg l^{-1}) at 0 h.

Figure 19 B. GAS CHROMATOGRAPHIC ANALYSIS OF DEGRADATION OF MIXTURE OF 2-CHLOROPHENOL (100 mg l^{-1}) AND PHENOL (50 mg l^{-1}) AFTER 24 h.

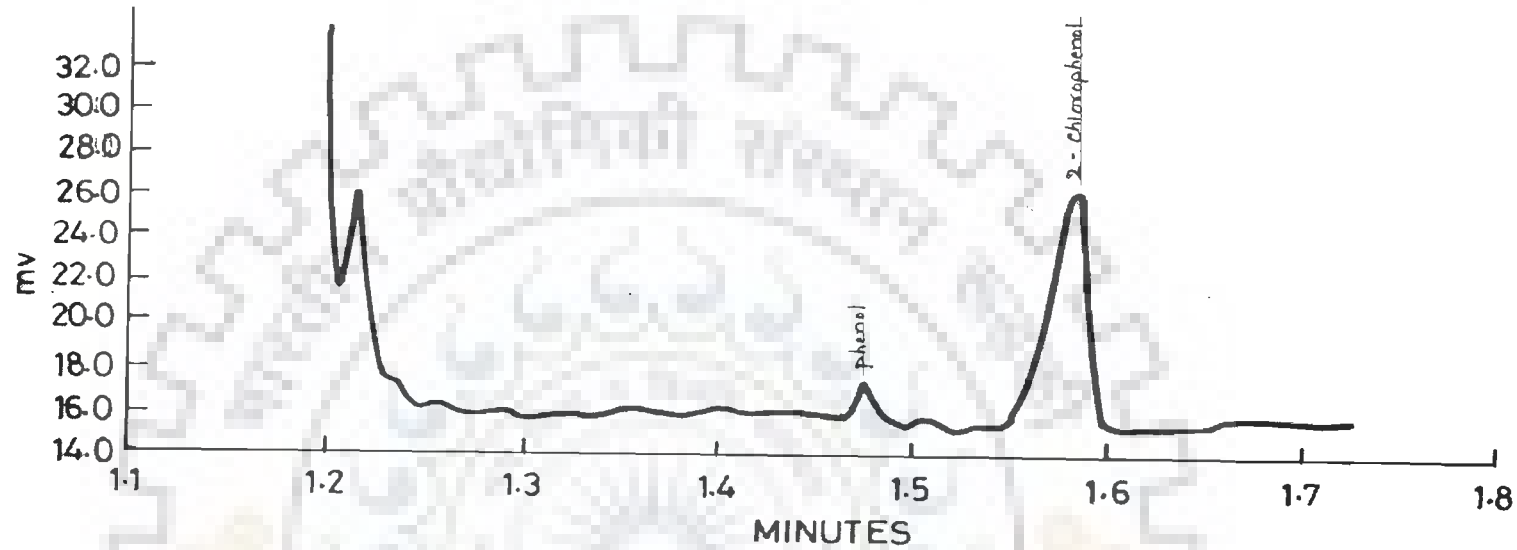


Figure 19 A

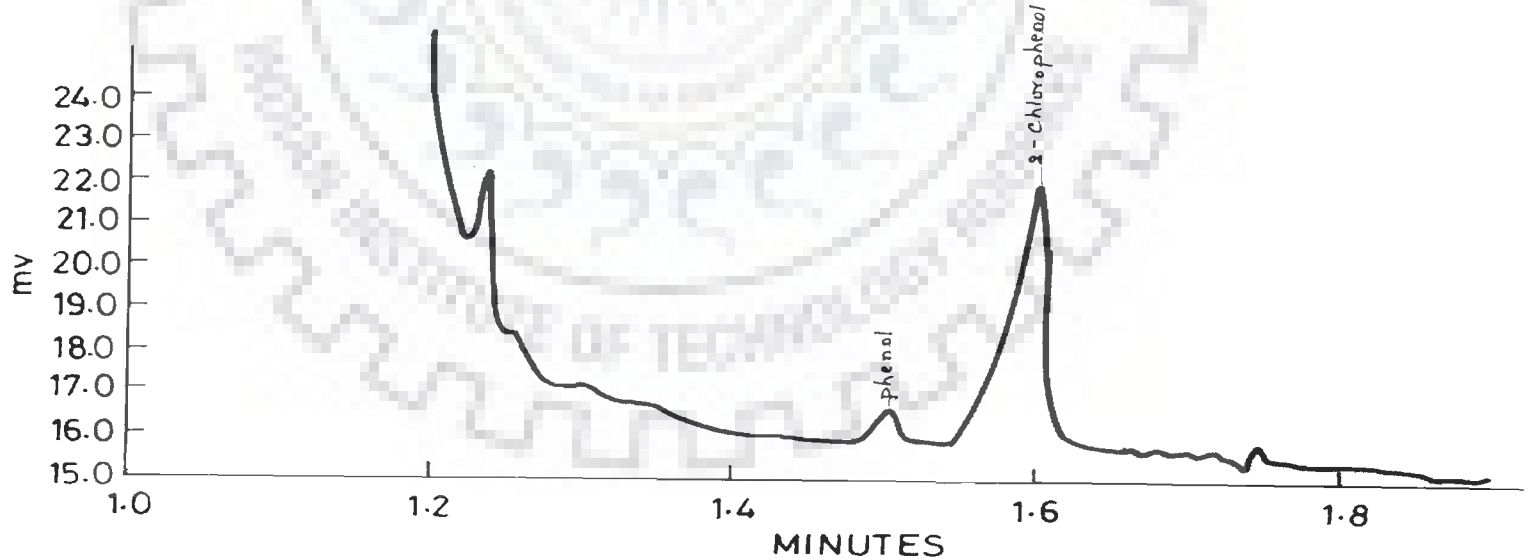


Figure 19 B

Figure 19 C. GAS CHROMATOGRAPHIC ANALYSIS OF DEGRADATION OF MIXTURE OF 2-CHLOROPHENOL (100 mg l^{-1}) AND PHENOL (50 mg l^{-1}) AFTER 48 h.

Figure 19 D. GAS CHROMATOGRAPHIC ANALYSIS OF DEGRADATION OF MIXTURE OF 2-CHLOROPHENOL (100 mg l^{-1}) AND PHENOL (50 mg l^{-1}) AFTER 72 h.

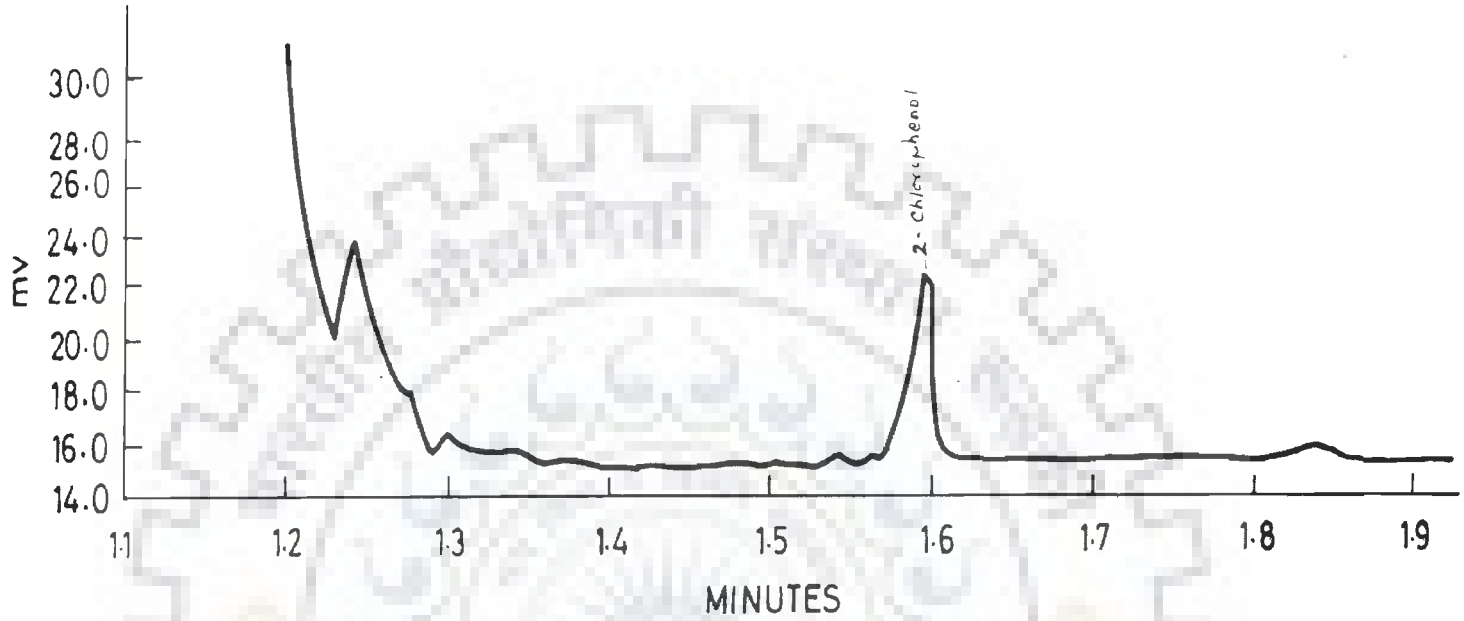


Figure 19 C

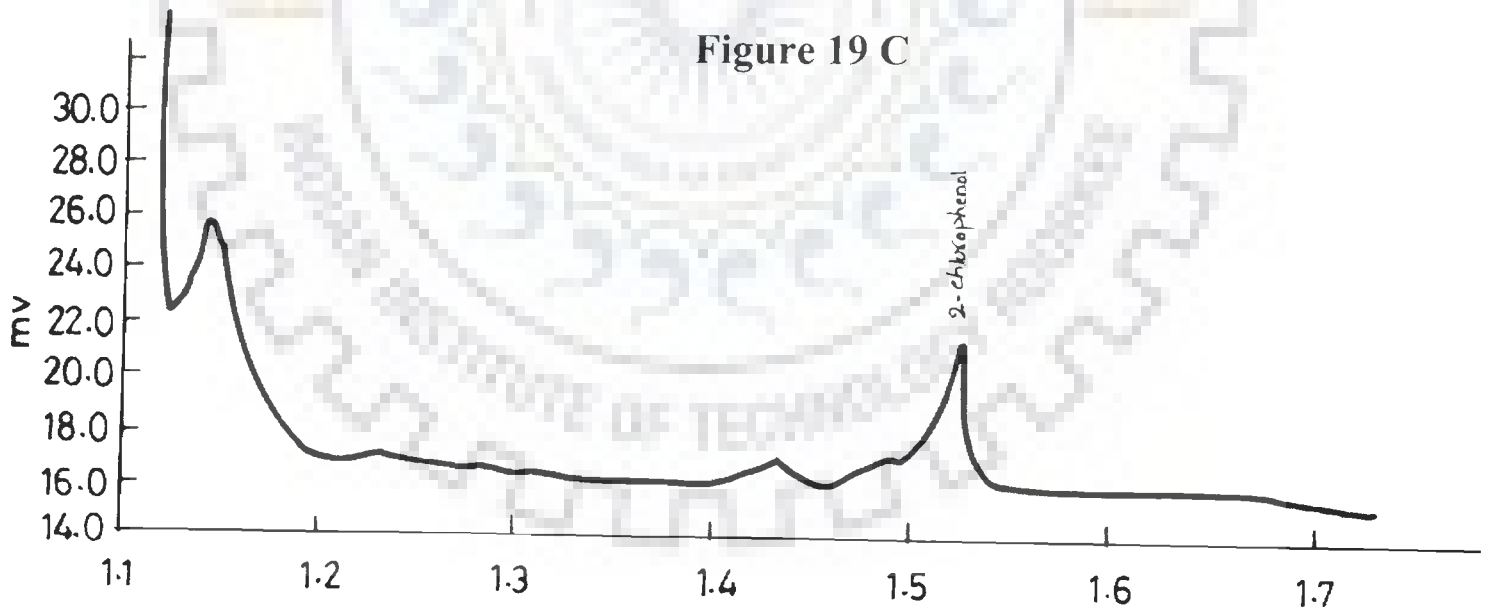


Figure 19 D

Figure 19 E. GAS CHROMATOGRAPHIC ANALYSIS OF DEGRADATION OF MIXTURE OF 2-CHLOROPHENOL (100 mg l^{-1}) AND PHENOL (50 mg l^{-1}) AFTER 96 h.

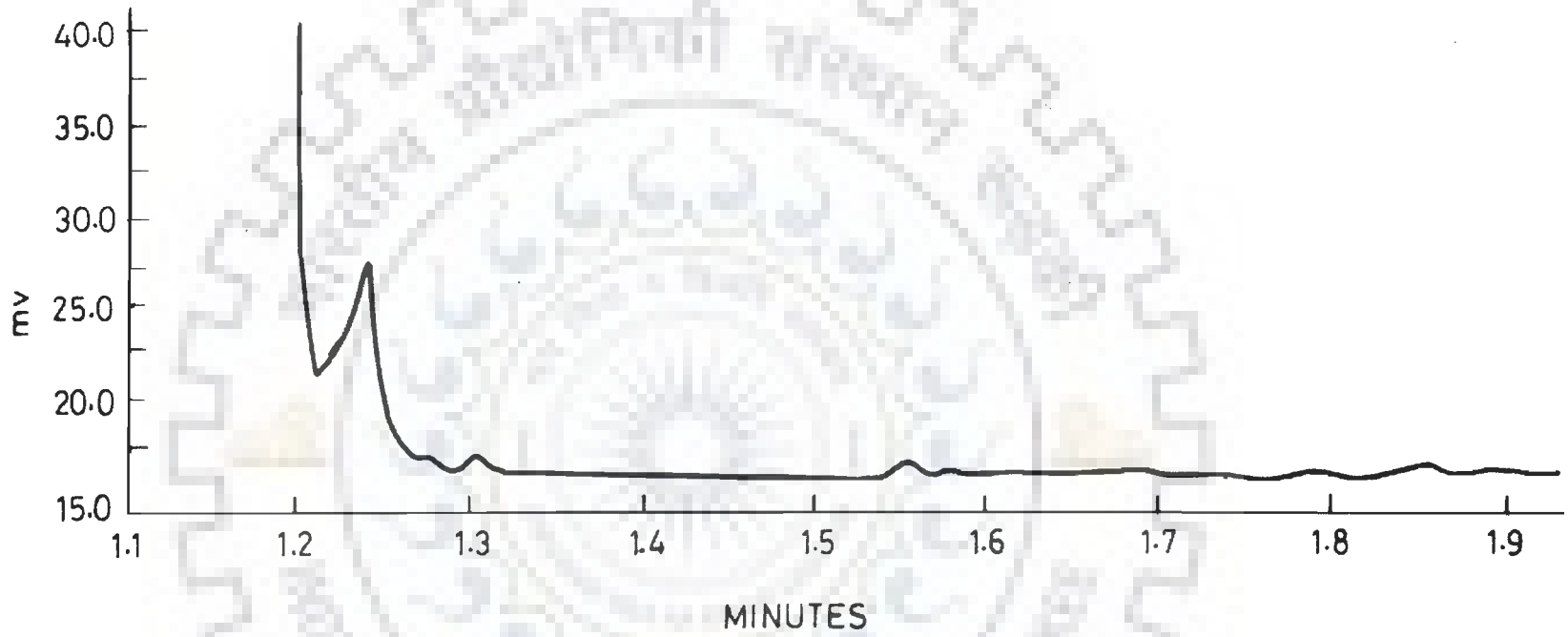


Figure 19 E

Figure 20. DEGRADATION OF MIXED SUBSTRATES CONSISTING OF 2-CHLOROPHENOL AND PHENOL BY *Rhodococcus* sp. M1

Concentrations of 2-chlorophenol and phenol were respectively 100 mgl^{-1} and 50 mgl^{-1}. The study was carried out in modified M3 medium at 32°C under shaking condition (120 rpm). Benzoic acid induced cells of *Rhodococcus* sp. M1 acclimatized to a mixture of 2-chlorophenol and phenol was used as the inoculum. Substrate concentration, chloride and cell growth was estimated as described in methods.

Figure 21. RESIDUAL SOLUBLE COD FOR THE DEGRADATION OF MIXED SUBSTRATE CONSISTING OF PHENOL AND 2-CHLOROPHENOL BY *Rhodococcus* sp. M1

Concentrations of phenol and 2-chlorophenol used for the study respectively were 50 mgl^{-1} and 100 mgl^{-1}. COD was estimated as described in methods.

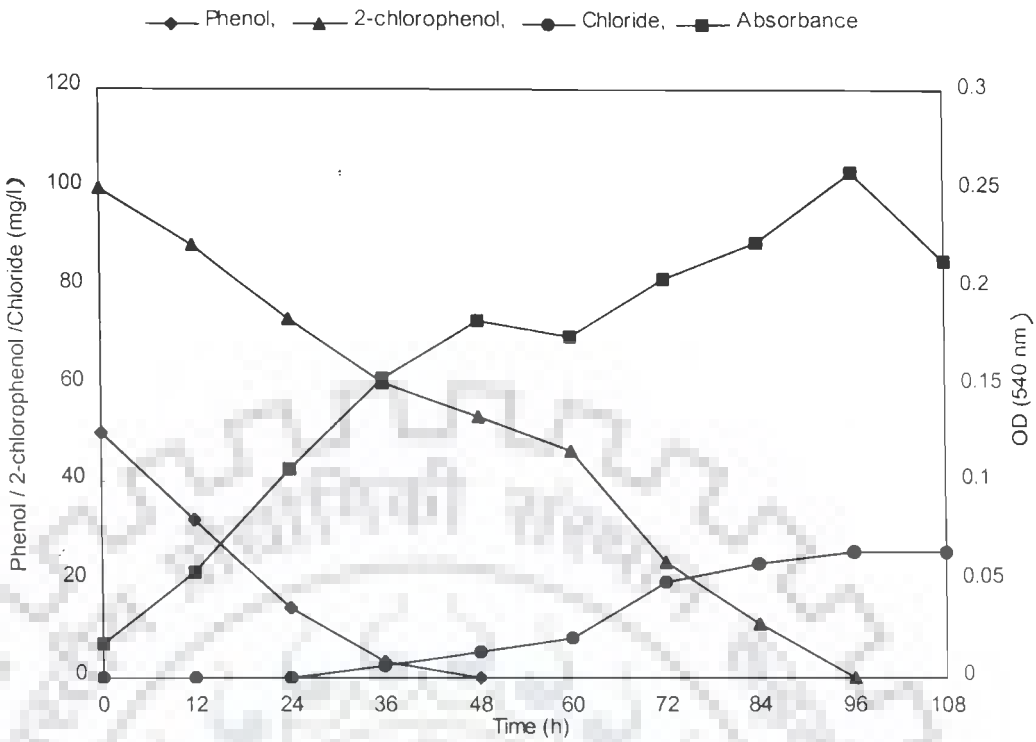


Figure 20

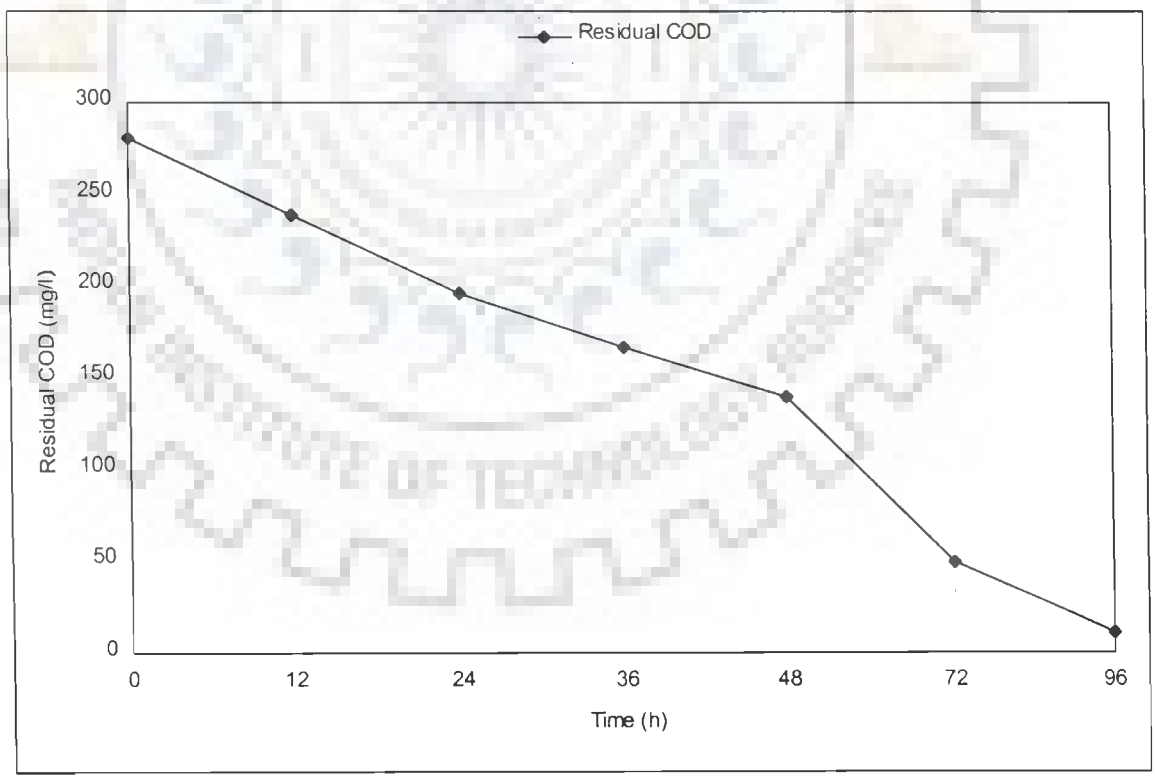


Figure 21

4.8.2 Degradation of mixture of p-cresol and 2-chlorophenol

Degradation of p-cresol (50 mg l^{-1}) by *Rhodococcus* sp. M1 was observed in 12h (Figure 22) when utilized as single substrate. 2-chlorophenol (100 mg l^{-1}) as reported in section 4.7 was observed to be removed in 18 h (Figure 11 d). However 2-chlorophenol (100 mg l^{-1}) when used in combination with p-cresol (50 mg l^{-1}) had resulted into delayed degradation of both the substrate as compared to the degradation of these substrates when utilized as sole carbon source. Degradation of the binary mixture of 2-chlorophenol and p-cresol was studied by gas chromatographic analysis and is shown in Figure 23. Mixture of 2-chlorophenol and p-cresol showed simultaneous but preferential pattern of degradation with p-cresol being removed in 36 h and 2-chlorophenol after 48 h of incubation (Figure 24). Gradual removal of 2-chlorophenol up to 42.6 mg l^{-1} was observed in 36h after which there was a linear fall showing complete degradation of 2-chlorophenol after 48 h. Release of chloride was observed after a period of 18 h (Figure 24). Chloride formed after 36 h was 11.5 mg l^{-1}. Release of chloride after 48 h when 2-chlorophenol was completely removed was equivalent to the expected theoretical amount (Figure 24). Residual soluble COD of 100.92 mg l^{-1} was observed after 36 h and that decreased to a negligible level of 5.22 mg l^{-1} after 48 h (Fig 25). Rate of degradation of p-cresol decreased from 5.2 mg l^{-1} h^{-1} to 1.83 mg l^{-1} h^{-1} when used as pure substrate and in the binary mixture with 2-chlorophenol respectively. Rate of degradation of 2-chlorophenol in mixture with p-cresol is 2.08 mg l^{-1} h^{-1} (Table 28).

Gradual increase in growth without any notable lag phase was observed up to 42 h followed by sharp increase to yield a pronounced peak at 48 h (Figure 24). Specific

growth rate of the organism in the mixture of 2-chlorophenol and p-cresol was 0.055 h^{-1}, which was significantly lower compared to specific growth rate of p-cresol (0.178 h^{-1}) when utilized singly (Table 29).

Figure 22. DEGRADATION OF P-CRESOL BY *Rhodococcus* sp. M1

The study was carried out in modified M3 medium containing p-cresol (50 mg l^{-1}) at 32°C under shaking condition (120rpm). Inoculum was prepared by growing the culture in DSM-65 medium and washing with the phosphate buffer. The culture was then grown in p-cresol of the same concentration before using it for the present study. Estimation of p-cresol and growth was measured as described in methods.

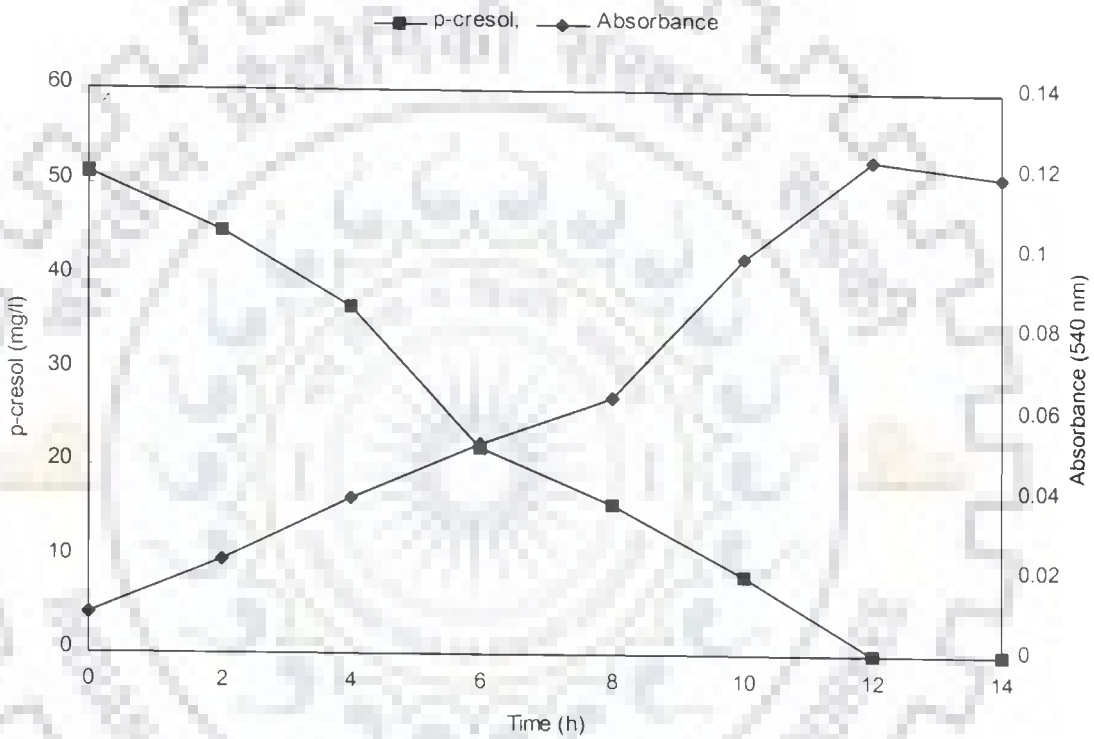

p-cresol
Absorbance
60
50
40
30
20
10
0
p-cresol (mg/l)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Absorbance (540 nm)
0
2
4
6
8
10
12
14
Time (h)

Figure 22

Figure 23. GAS CHROMATOGRAPHIC ANALYSIS OF DEGRADATION OF MIXED SUBSTRATE OF 2-CHLOROPHENOL (100 mg l^{-1}) AND P-CRESOL (50 mg l^{-1})

The Gas chromatograph column specification and operational conditions are as described for Figure 19.

Figure 23 A. GAS CHROMATOGRAPHIC ANALYSIS OF MIXTURE OF 2-CHLOROPHENOL (100 mg l^{-1}) AND P-CRESOL (50 mg l^{-1}) AT 0 h.

Figure 23 B. GAS CHROMATOGRAPHIC ANALYSIS OF DEGRADATION OF MIXTURE OF 2-CHLOROPHENOL (100 mg l^{-1}) AND P-CRESOL (50 mg l^{-1}) AFTER 24 h.

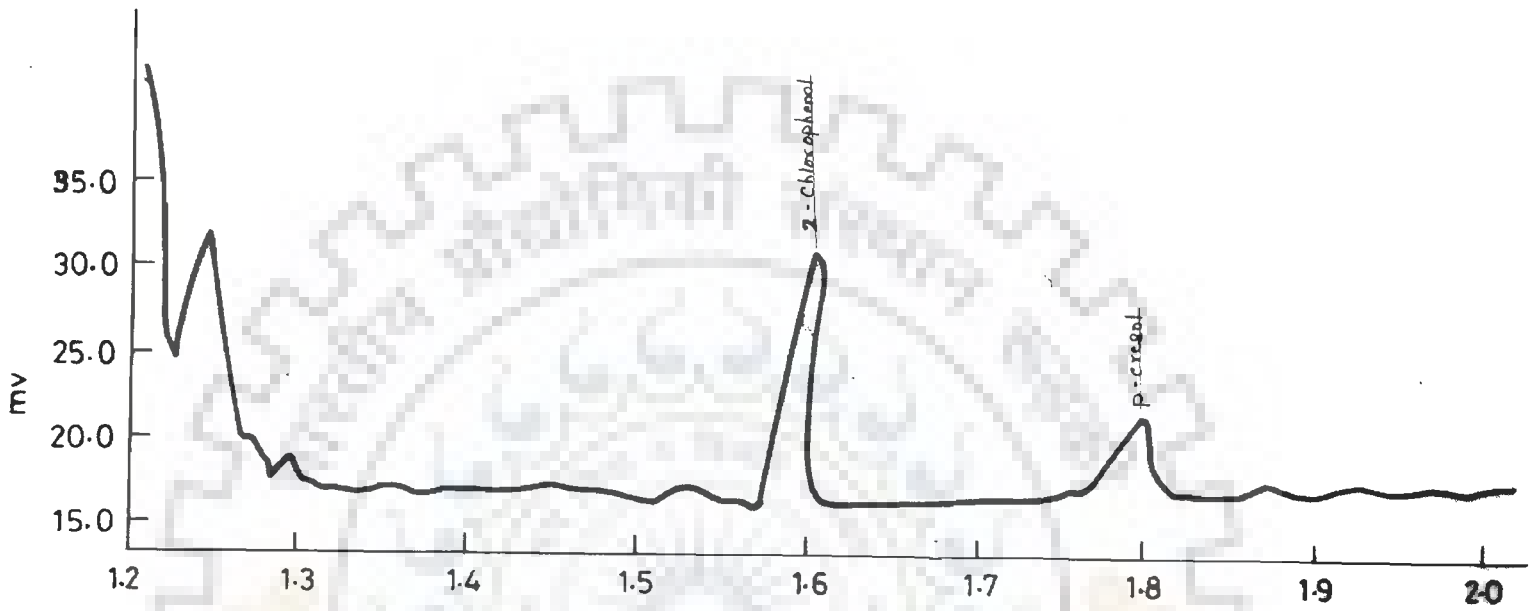


Figure 23 A

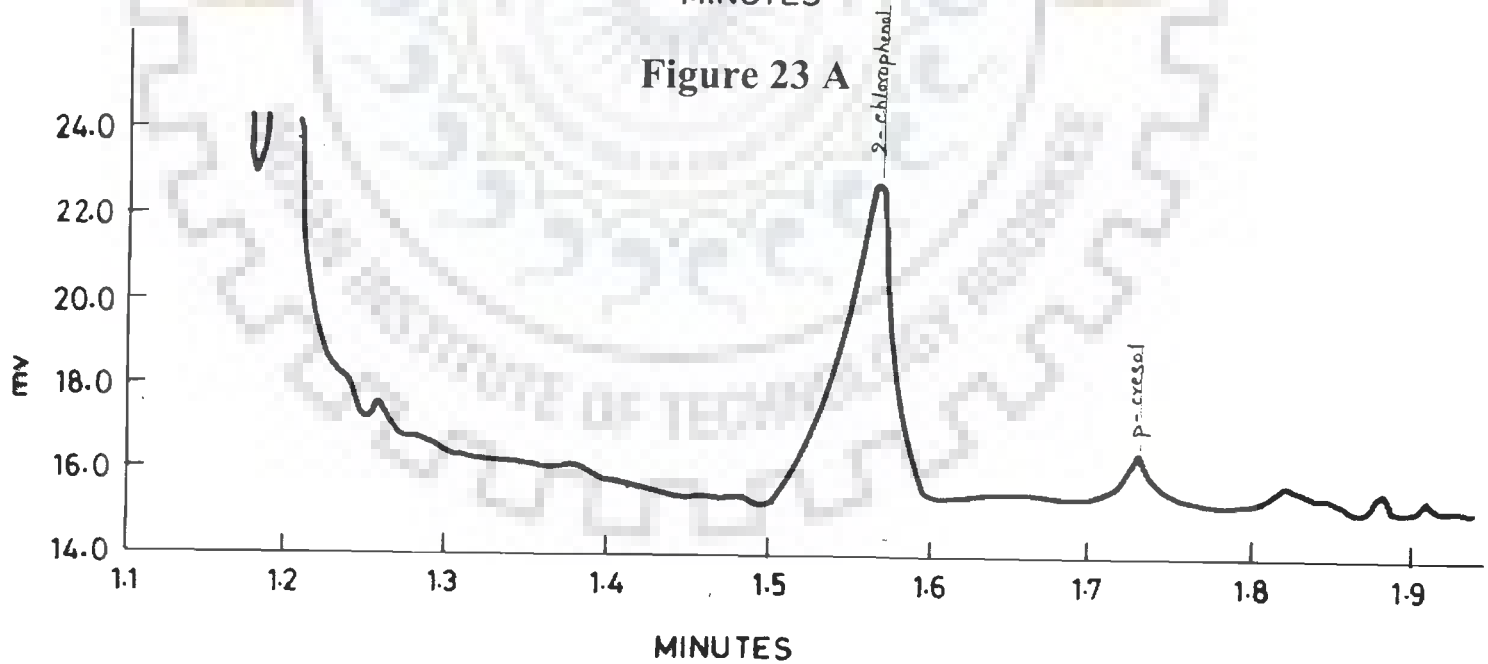


Figure 23 B

Figure 23 C. GAS CHROMATOGRAPHIC ANALYSIS OF DEGRADATION OF MIXTURE OF 2-CHLOROPHENOL (100 mg l^{-1}) AND P-CRESOL (50 mg l^{-1}) AFTER 36 h.

Figure 23 D. GAS CHROMATOGRAPHIC ANALYSIS OF DEGRADATION OF MIXTURE OF 2-CHLOROPHENOL (100 mg l^{-1}) AND P-CRESOL (50 mg l^{-1}) AFTER 48 h.

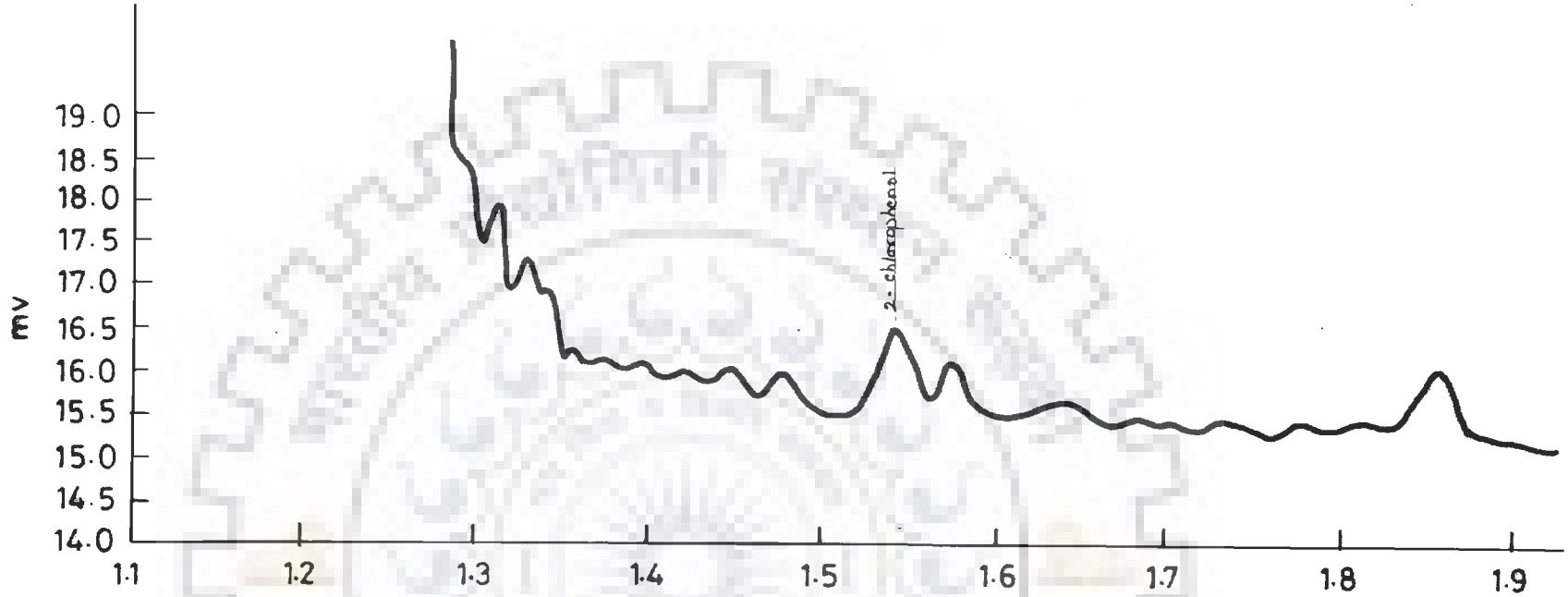


Figure 23 C

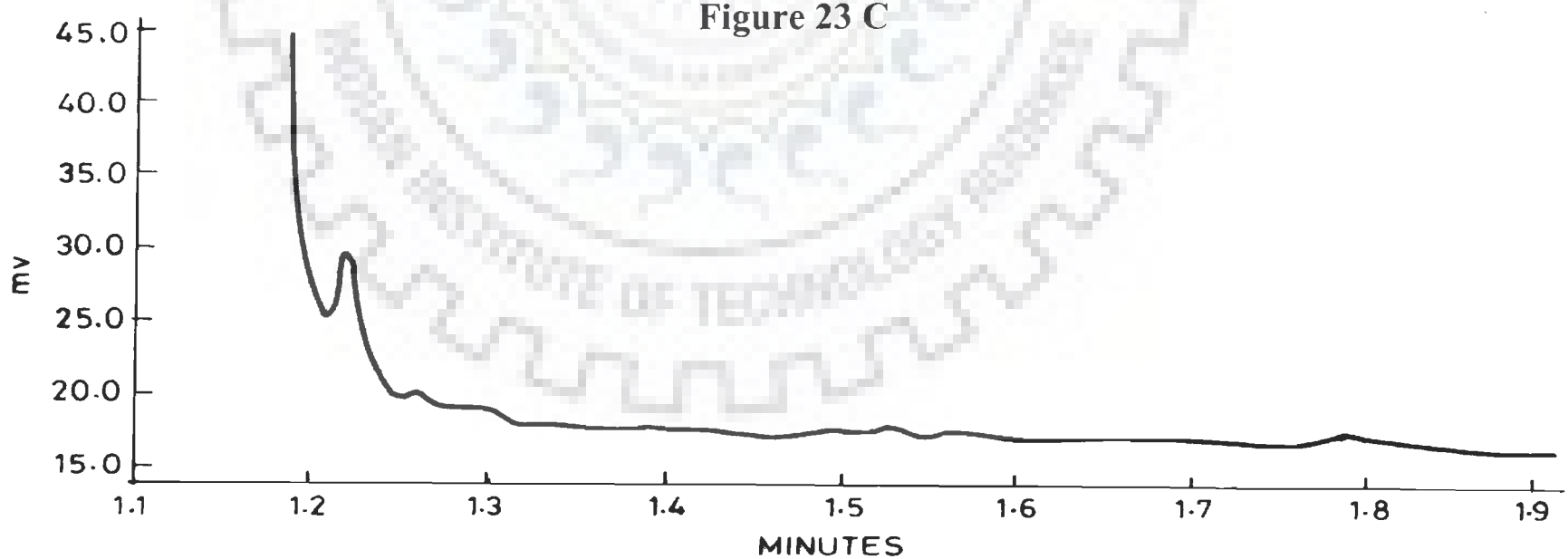


Figure 23 D

Figure 24. DEGRADATION OF MIXED SUBSTRATES CONSISTING OF 2-CHLOROPHENOL AND P-CRESOL BY *Rhodococcus* sp. M1

Concentrations of 2-chlorophenol and p-cresol were respectively 100 mgl^{-1} and 50 mgl^{-1}. The study was carried out in modified M3 medium at 32°C under shaking condition (120 rpm). Benzoic acid induced cells of *Rhodococcus* sp. M1 acclimatized to a mixture of 2-chlorophenol and p-cresol was used as the inoculum. Substrate concentration, chloride and cell growth was estimated as described in methods.

Figure 25. RESIDUAL SOLUBLE COD FOR THE DEGRADATION OF MIXED SUBSTRATE CONSISTING OF P-CRESOL AND 2-CHLOROPHENOL BY *Rhodococcus* sp. M1

Concentrations of p-cresol and 2-chlorophenol used for the study respectively were 50 mgl^{-1} and 100 mgl^{-1}. COD was estimated as described in methods.

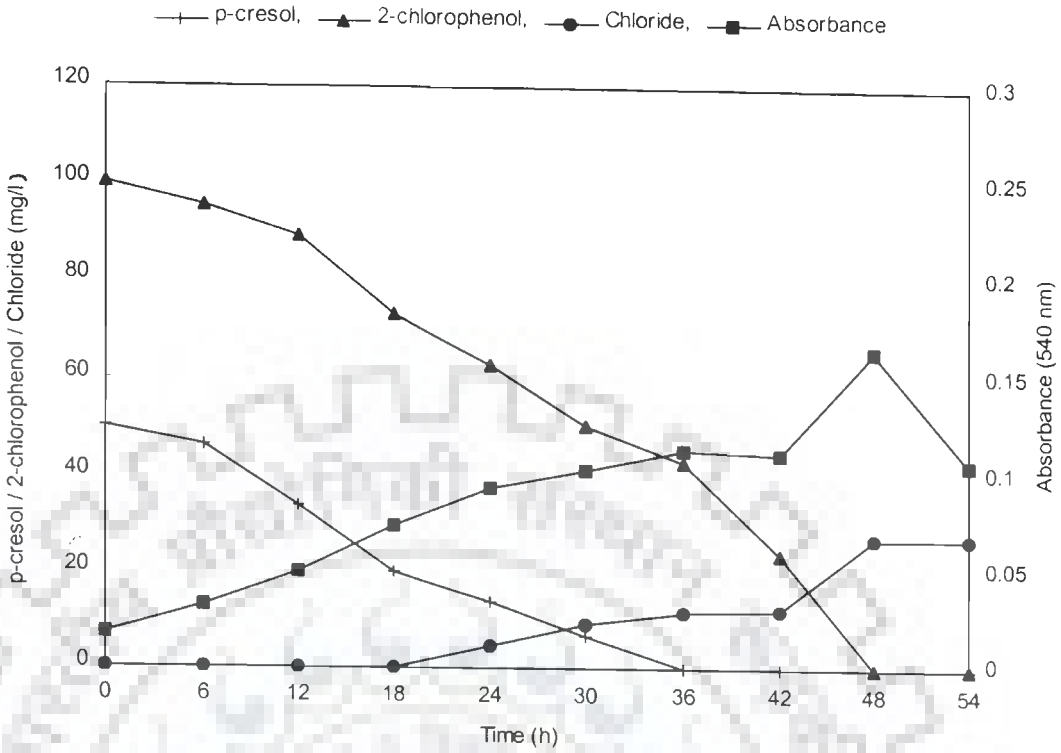


Figure 24

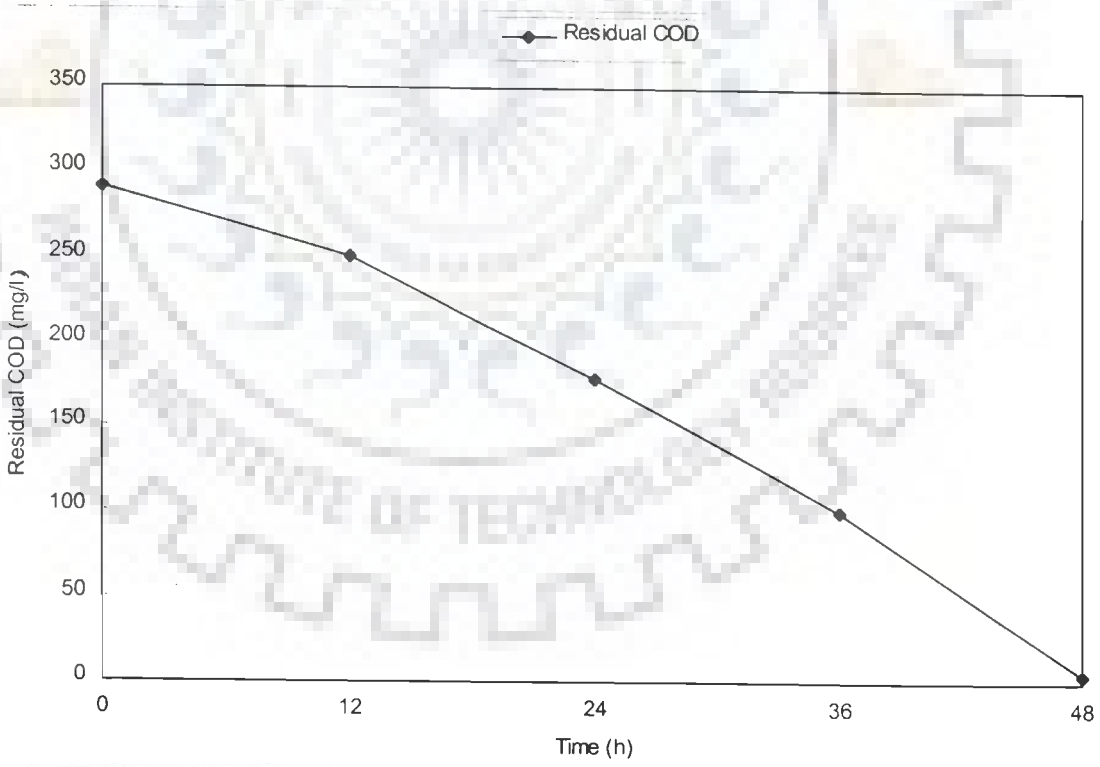


Figure 25

4.8.3 Degradation of mixture of substrates consisting of phenol, p-cresol and 2-chlorophenol

Studies on degradation of the mixture of phenol, p-cresol and 2-chlorophenol at 50 mg l^{-1} each by *Rhodococcus* sp. M1 was carried out by gas chromatographic analysis and is presented in Figure 26. Preferential pattern of utilization of the substrate was observed for the tertiary mixture containing phenol, p-cresol and 2-chlorophenol. Removal of phenol was observed after 72 h followed by utilization of p-cresol within 96 h, whereas complete degradation of 2-chlorophenol was observed after a period of 120 h. Degradation of all the three substrates when used as a mixture was delayed as compared to when these substrates were used as sole substarte or as the binary mixtures. Though gradual removal of 2-chlorophenol was observed during initial period however, detectable levels of chloride were released only after 72 h (Figure 27). 96% of the theoretical amount of chloride was observed to be released after 120 h when 2-chlorophenol was completely degraded. Residual soluble COD after the removal of phenol was observed to be 145.25 mg l^{-1}. COD was further reduced to 57.24 mg l^{-1} in 96 h when p-cresol was completely degraded. Removal of 2-chlorophenol after 120 h resulted in no detectable residual COD (Figure 28). Rate of degradation of all the substrates declined significantly compared to their rate of degradation as single substrates or as in binary mixtures. Rate of degradation of 2-chlorophenol, phenol and p-cresol in this case were 0.33 mg l^{-1} h^{-1}, 1.25 mg l^{-1} h^{-1} and 0.46 mg l^{-1} h^{-1} respectively (Table 28).

Growth on the mixture of substrates started after an initial lag phase of 12 h. Growth curve is biphasic giving first peak after 96 h. followed by a sharp decline in

growth and then gradual increase to give the second peak at 120 h (Figure 27). Specific growth rate of the organism on mixture of 2-chlorophenol phenol and p-cresol was 0.035 h^{-1} which was significantly lower compared to the growth rate of these substrates as single substrate or in binary substrate mixtures (Table 29).

Figure 26. GAS CHROMATOGRAPHIC ANALYSIS OF DEGRADATION OF MIXED SUBSTRATE OF 2-CHLOROPHENOL (50 mg l^{-1}), PHENOL (50 mg l^{-1}) AND P-CRESOL (50 mg l^{-1})

The Gas chromatograph column specification and operational conditions are as described for Figure 19.

Figure 26 A. GAS CHROMATOGRAPHIC ANALYSIS OF MIXTURE OF 2-CHLOROPHENOL (50 mg l^{-1}), PHENOL (50 mg l^{-1}) AND P-CRESOL (50 mg l^{-1}) AT 0 h.

Figure 26 B. GAS CHROMATOGRAPHIC ANALYSIS OF MIXTURE OF 2-CHLOROPHENOL (50 mg l^{-1}), PHENOL (50 mg l^{-1}) AND P-CRESOL (50 mg l^{-1}) AFTER 24 h.

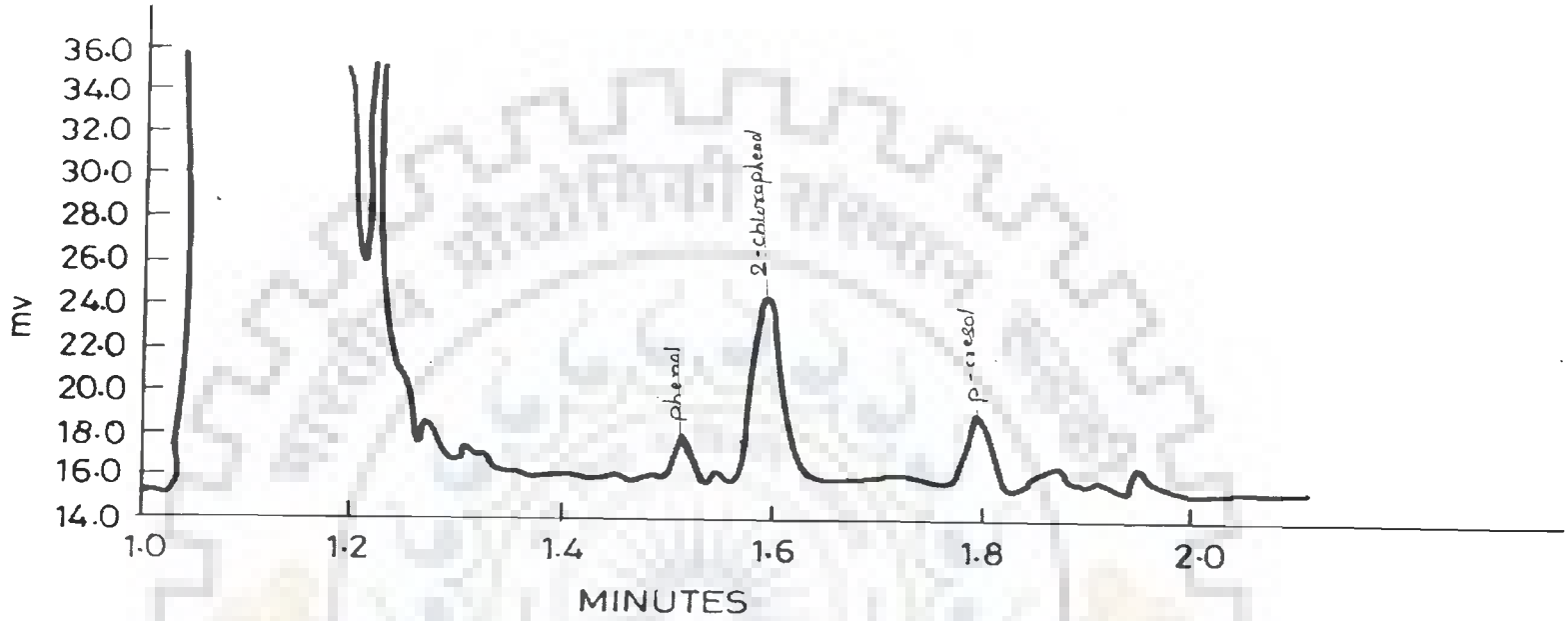


Figure 26 A

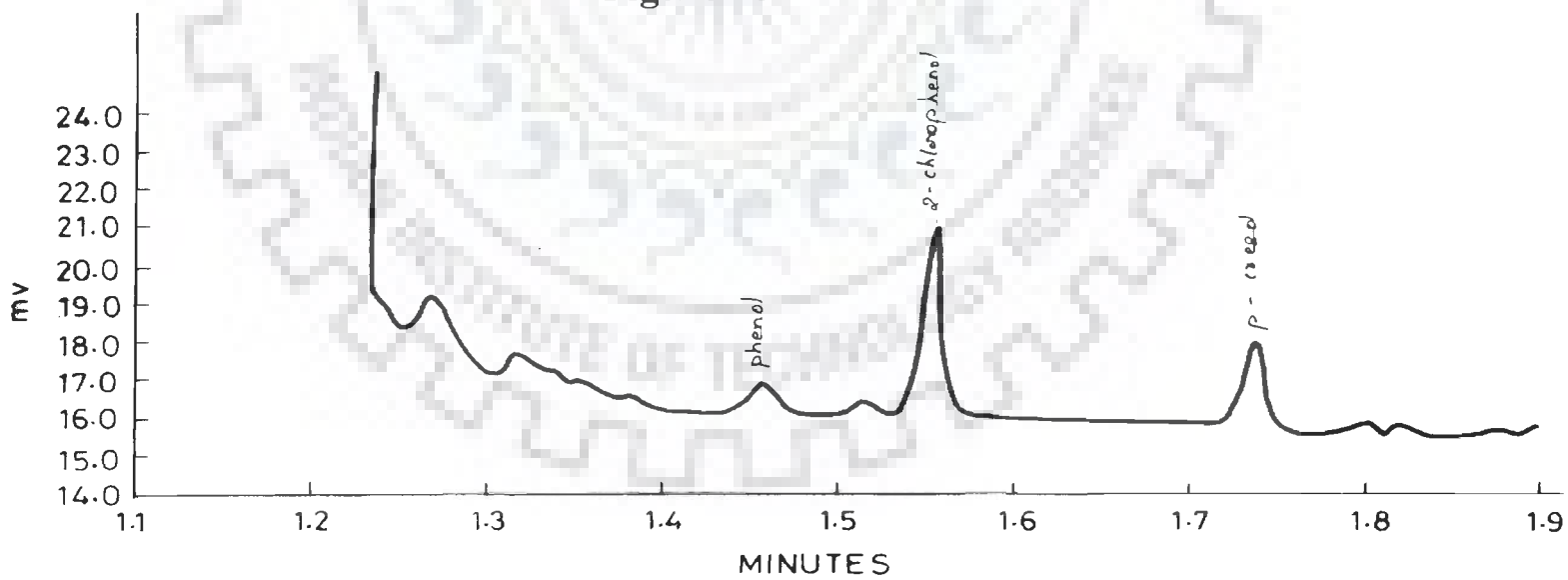


Figure 26 B

Figure 26 C. GAS CHROMATOGRAPHIC ANALYSIS OF MIXTURE OF 2-CHLOROPHENOL (50 mg l^{-1}), PHENOL (50 mg l^{-1}) AND P-CRESOL (50 mg l^{-1}) AFTER 48 h.

Figure 26 D. GAS CHROMATOGRAPHIC ANALYSIS OF MIXTURE OF 2-CHLOROPHENOL (50 mg l^{-1}), PHENOL (50 mg l^{-1}) AND P-CRESOL (50 mg l^{-1}) AFTER 72 h.

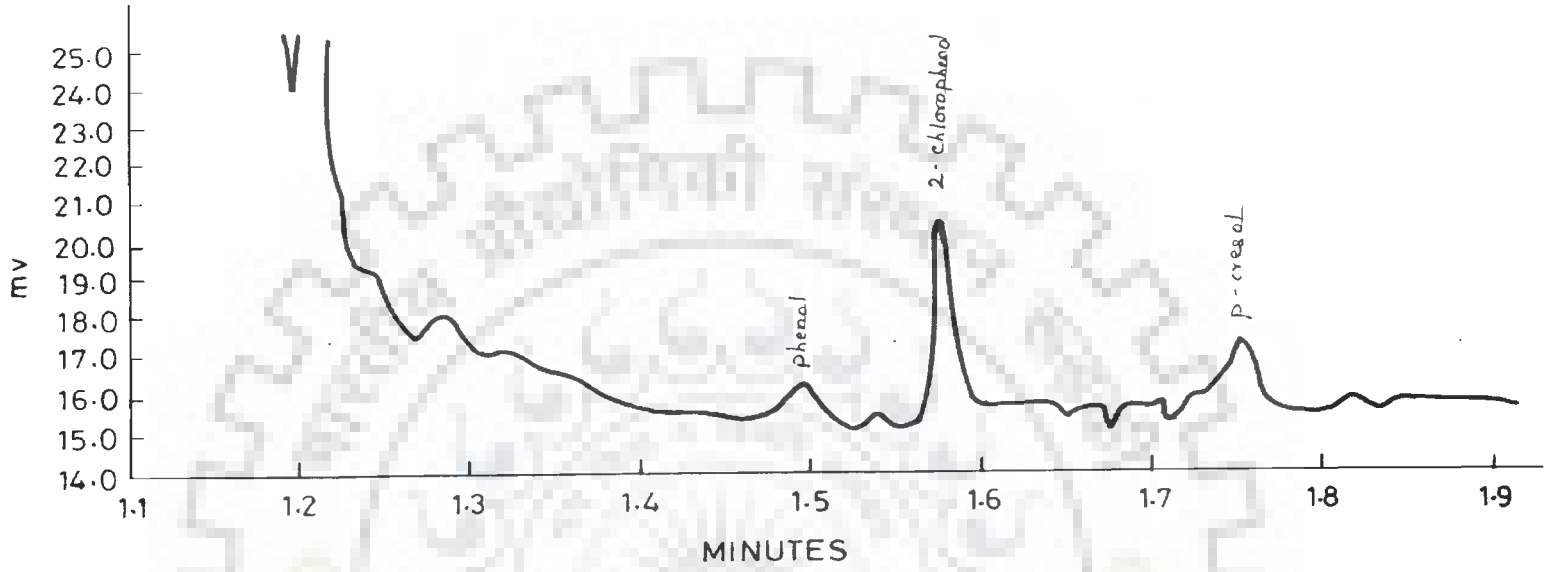


Figure 26 C

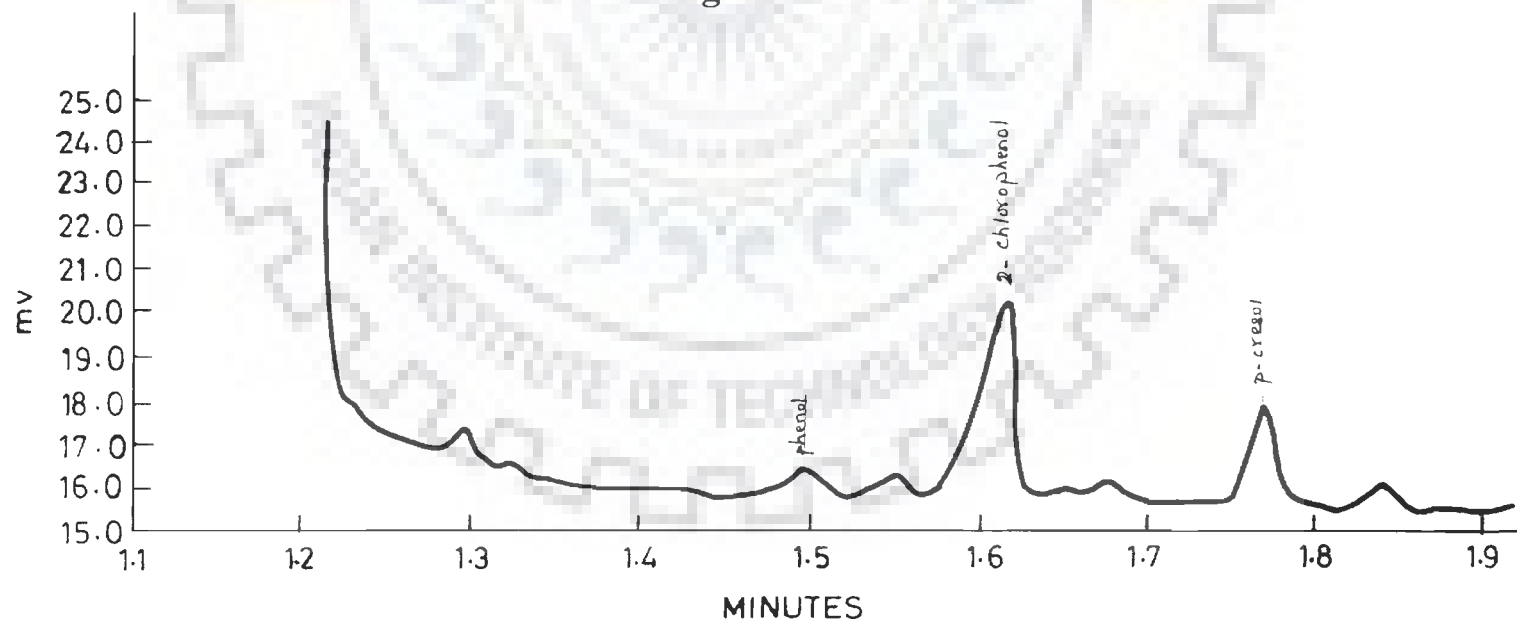


Figure 26 D

Figure 26 E. GAS CHROMATOGRAPHIC ANALYSIS OF MIXTURE OF 2-CHLOROPHENOL (50 mg l^{-1}), PHENOL (50 mg l^{-1}) AND P-CRESOL (50 mg l^{-1}) AFTER 96 h.

Figure 26 F. GAS CHROMATOGRAPHIC ANALYSIS OF MIXTURE OF 2-CHLOROPHENOL (50 mg l^{-1}), PHENOL (50 mg l^{-1}) AND P-CRESOL (50 mg l^{-1}) AFTER 108 h.

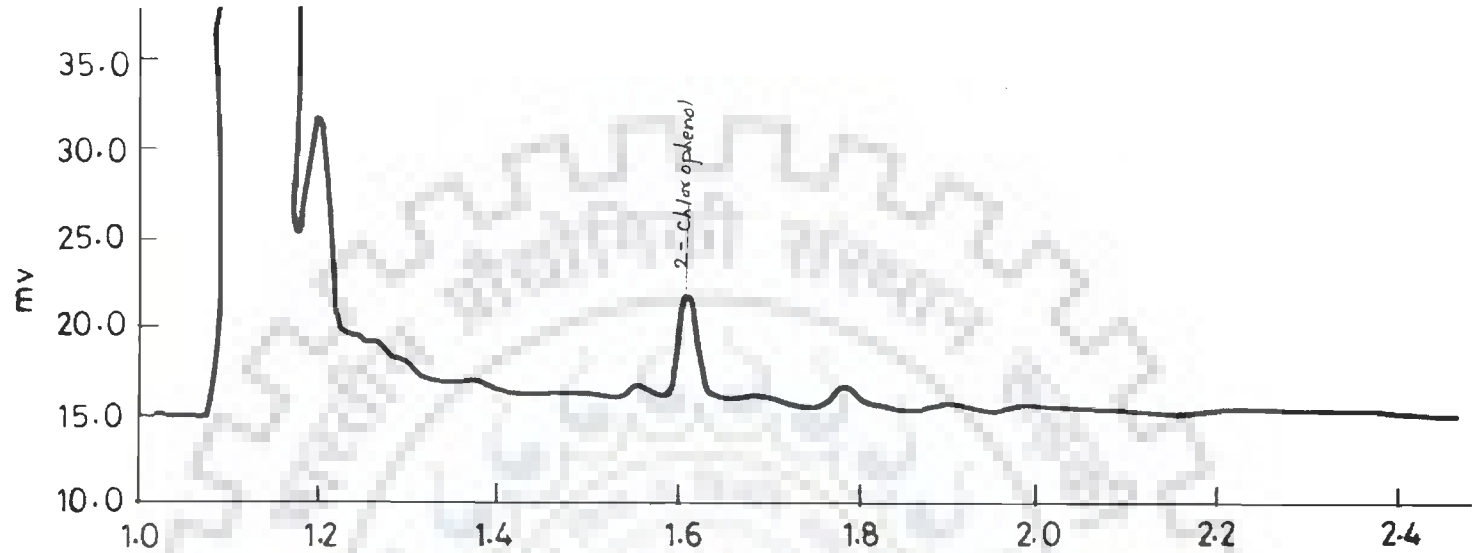


Figure 26 E

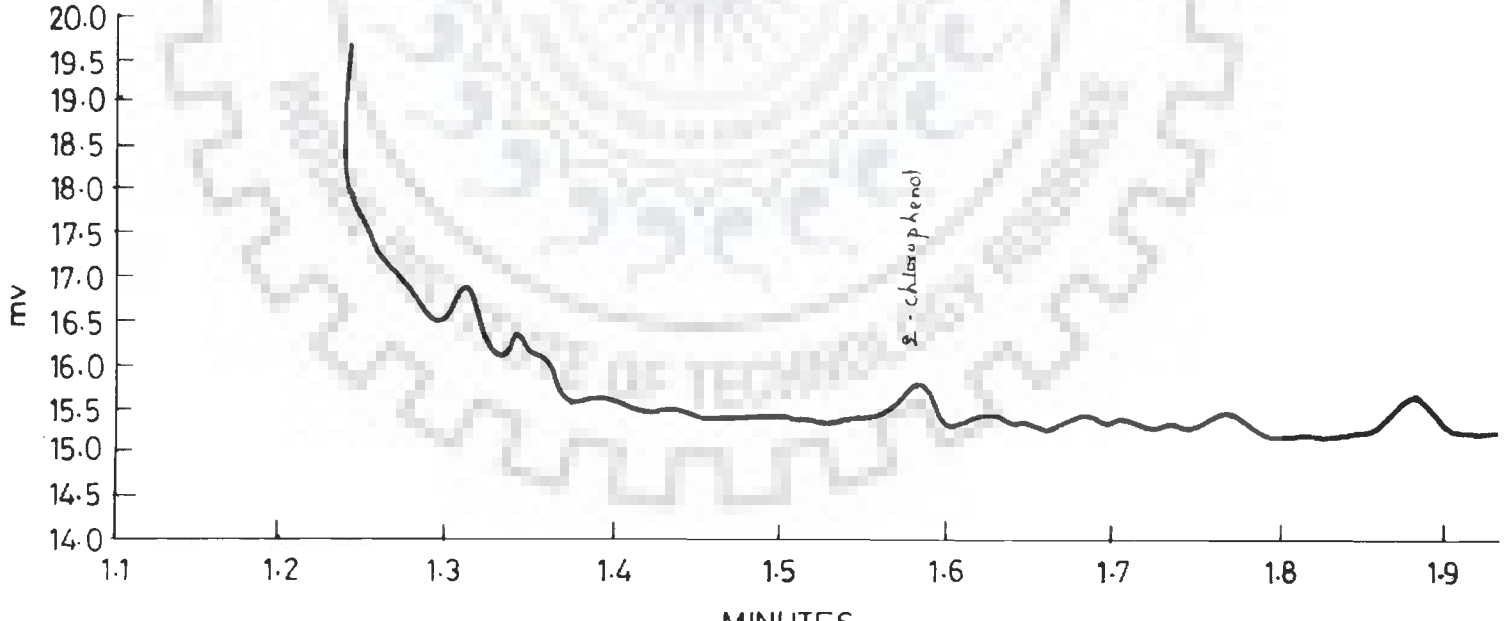


Figure 26 F

Figure 26G. GAS CHROMATOGRAPHIC ANALYSIS OF MIXTURE OF 2-CHLOROPHENOL (50 mg l^{-1}), PHENOL (50 mg l^{-1}) AND P-CRESOL (50 mg l^{-1}) AFTER 120 h.

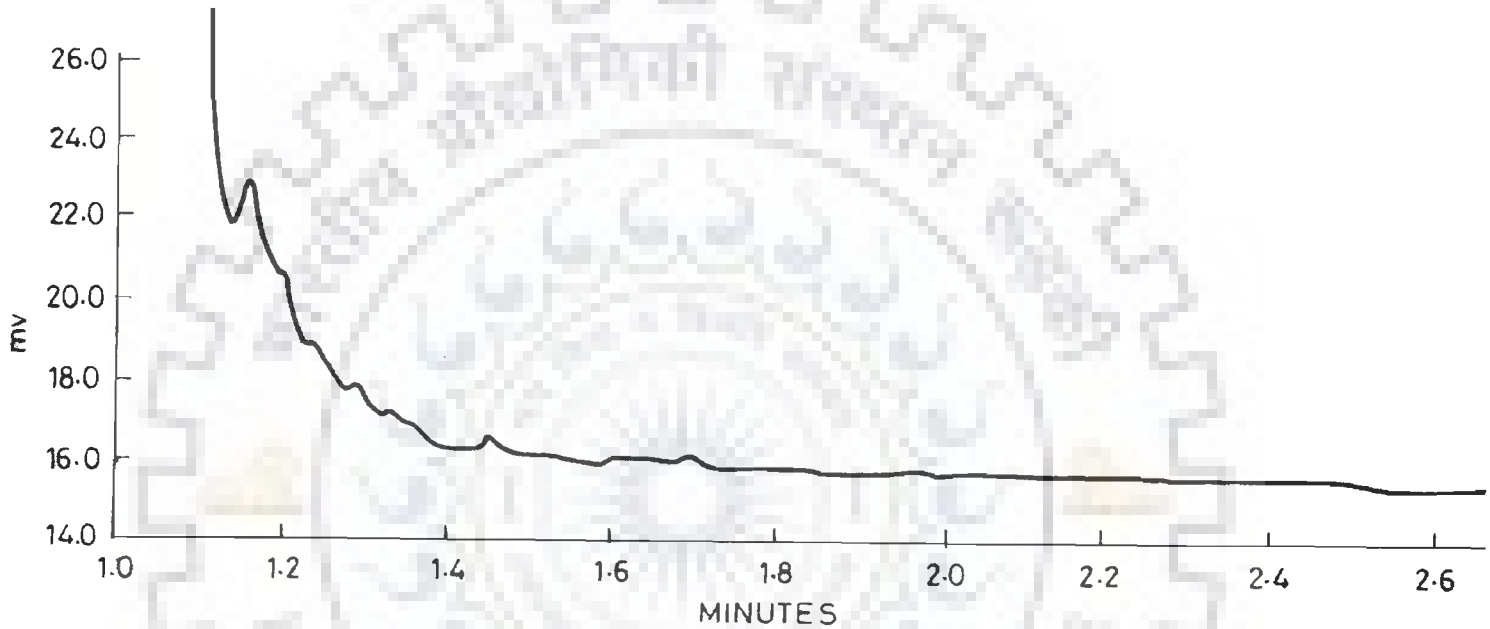


Figure 26 G

Figure 27. DEGRADATION OF MIXTURE OF SUBSTRATES CONSISTING OF 2-CHLOROPHENOL, PHENOL AND P-CRESOL BY *Rhodococcus* sp. M1

Concentrations of 2-chlorophenol, phenol and p-cresol were 50 mgl^{-1} each. The study was carried out in modified M3 medium at 32°C under shaking condition (120 rpm). Benzoic acid induced cells of *Rhodococcus* sp. M1 acclimatized to a mixture of 2-chlorophenol, phenol and p-cresol was used as the inoculum. Substrate concentration, chloride and cell growth was estimated as described in methods.

Figure 28. RESIDUAL SOLUBLE COD FOR THE DEGRADATION OF MIXED SUBSTRATES CONSISTING OF 2-CHLOROPHENOL, PHENOL AND P-CRESOL BY *Rhodococcus* sp. M1

Concentrations of 2-chlorophenol, phenol and p-cresol used for the study were 50 mgl^{-1} each. COD was estimated as described in methods.

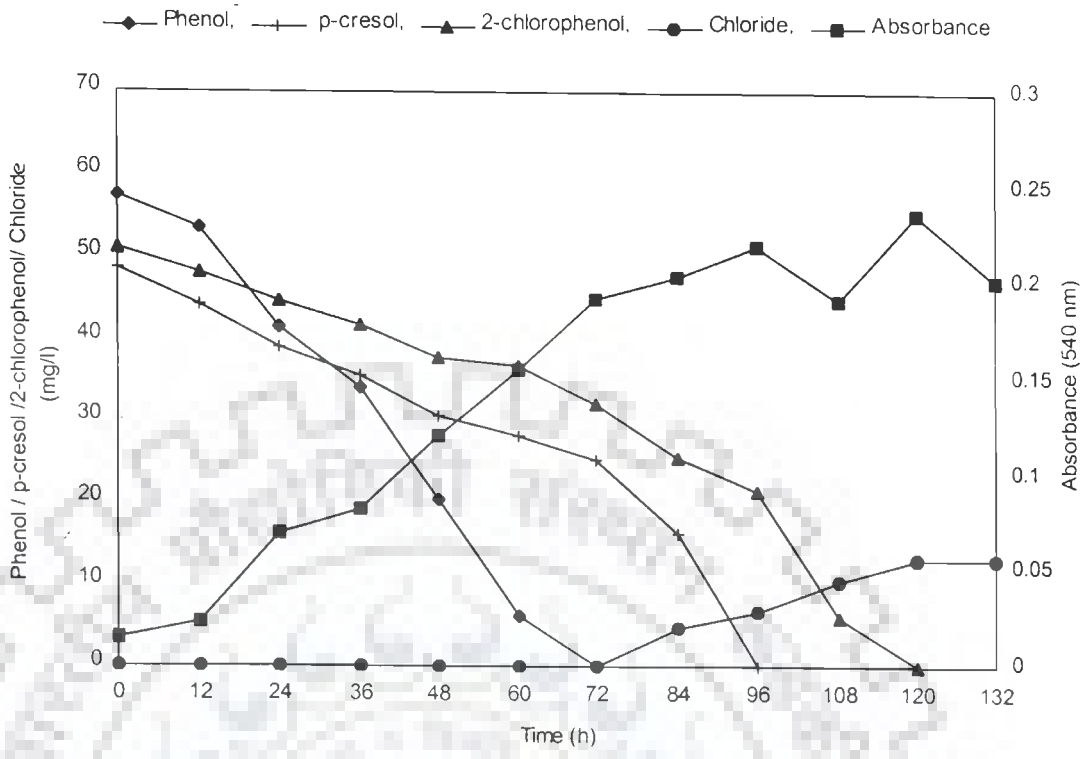


Figure 27

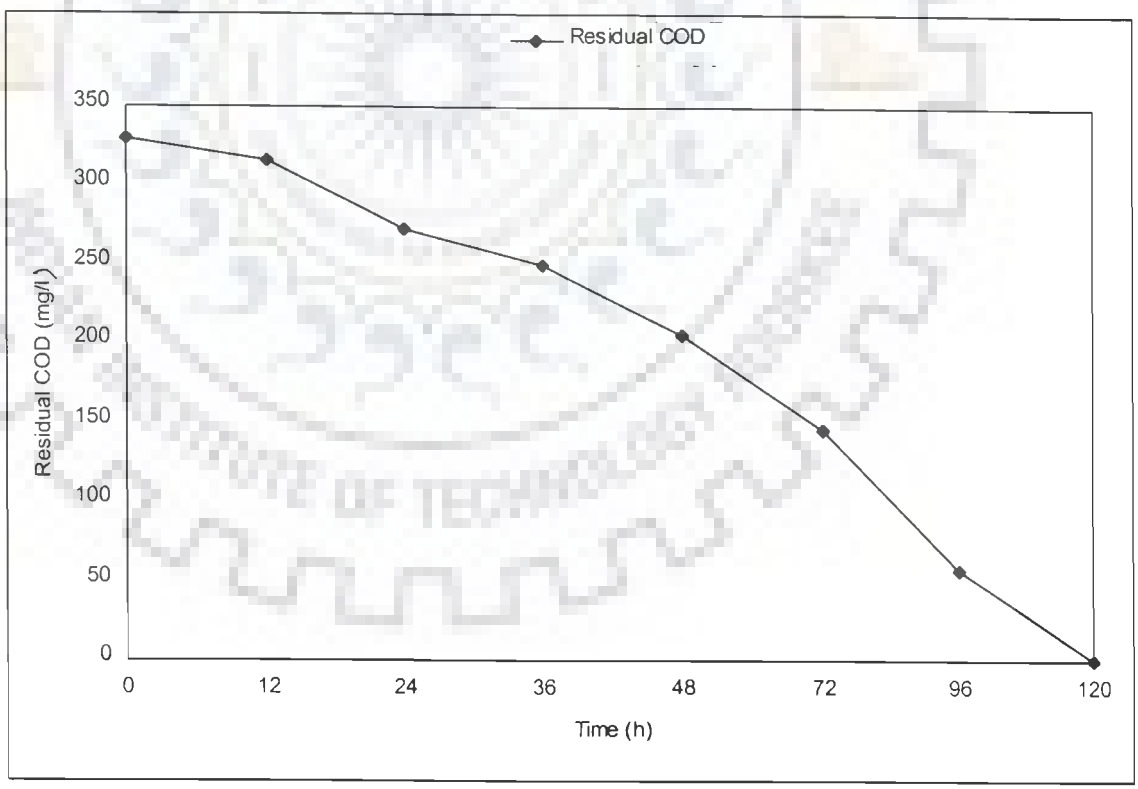


Figure 28

Table 28. Rate of degradation of 2-chlorophenol, phenol and p-cresol as pure substrates and in mixed substrate form by *Rhodococcus* sp. M1.

Substrates	Rate of degradation ($mg\ l^{-1}\ h^{-1}$)
Pure substrates	
a. 2-chlorophenol (50 $mg\ l^{-1}$)	5.27
b. 2-chlorophenol (100 $mg\ l^{-1}$)	9.8
c. phenol (50 $mg\ l^{-1}$)	4.7
d. p-cresol (50 $mg\ l^{-1}$)	5.2
Mixed substrates	
a. 2-chlorophenol (100 $mg\ l^{-1}$) and phenol (50 $mg\ l^{-1}$):	
(i) phenol	1.66
(ii) 2-chlorophenol	1.04
b. 2-chlorophenol (100 $mg\ l^{-1}$) and p- cresol (50 $mg\ l^{-1}$):	
(i) p-cresol	1.83
(ii) 2-chlorophenol	2.08
c. 2-chlorophenol (50 $mg\ l^{-1}$), phenol (50 $mg\ l^{-1}$) and p-cresol (50 $mg\ l^{-1}$):	
(i) phenol	1.25
(ii) p-cresol	0.46
(iii) 2-chlorophenol	0.33

Table 29. Specific growth rate of *Rhodococcus* sp. M1 on 2-chlorophenol, phenol and p-cresol as pure substrate and mixed substrate.

Sl. No	Substrates	μ (h^{-1})
1.	2-chlorophenol (50 mg l^{-1})	0.224
2.	2-chlorophenol (100 mg l^{-1})	0.211
3.	phenol (50 mg l^{-1})	0.163
4.	p-cresol (50 mg l^{-1})	0.178
5.	2-chlorophenol (100 mg l^{-1}) and phenol (50 mg l^{-1})	0.030
6.	2-chlorophenol (100 mg l^{-1}) and p- cresol (50 mg l^{-1})	0.055
7.	2-chlorophenol (50 mg l^{-1}), phenol (50 mg l^{-1}) and p-cresol (50 mg l^{-1})	0.035

μ, Specific growth rate of the culture

4.9 Degradation of 2-chlorophenol and phenolics by mixed culture of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1

In bioremediation sites and wastewater treatment systems microorganisms are reported to coexist with other microorganisms. *Pseudomonas* being one of the most widely prevalent organisms was selected for the coculture study. Degradation of 2-chlorophenol, phenol and p-cresol was thus studied by *Rhodococcus* sp. M1 in mixed culture form alongwith a *Pseudomonas* species isolated from the soil.

The pure colony of the isolated *Pseudomonas* sp. was designated as P1 and examined for colony, cell morphological, physiological and biochemical characteristics. The colony and cell morphological characterization of the isolate P1 is presented in Table 30. Physiological and biochemical characteristics of the isolate P1 is presented in Table 31 and the utilization of sugars by the isolate P1 is presented in Table 32. The guanine plus cytosine (G+C) content of DNA of the isolate P1 was estimated by modified method of Marmur (1961) and was found to be 60.3 Mol % G+C. The isolate therefore based on colony, cell morphological, physiological, biochemical characteristics and the G+C value was identified to be *Pseudomonas fluorescens*.

Table 30. Colony and morphological characteristics of isolate P1

Colony and morphological parameters	Characteristics
Colony characteristics	Small dirty white colonies with entire margin, surface smooth and glistening, opaque, low convex
Pigment production	Nutrient agar medium coloration changes to bluish green
Growth in liquid media	Suspended
Shape	Bacilli
Gram staining	Negative
Motility	Highly motile

Table 31. Physiological and biochemical characteristics of the isolate P1

Parameters	Characteristics
Catalase test	+
Oxidase test	+
Casein hydrolysis	+
Starch hydrolysis	-
Gelatin liquefaction	+
Nitrate reduction	-
Urease production	+
Tween 80 hydrolysis	-
Phenylalanine deaminase test	-
Indole production	-
Methyl red test	-
Voges Proskauer test	-
Gluconate oxidation	+
Citrate utilization	+
Acetamide utilization	-
Arginine dihydrolase	+
Poly- ß-hydroxybutyrate accumulation	-
Growth at 4°C	+
Growth at 41°C	-
Mol % G+C	60.3

+, Positive; -, Negative

Table 32. Utilization of sugars by the isolate P1

Sugars	Utilization
Arabinose	+
Rhamnose	-
Xylose	+
Dextrose	+
Galactose	+
Mannose	+
Fructose	+
Sucrose	+
Maltose	-
Lactose	-
Cellobiose	-
Melibiose	-
Trehalose	+
Raffinose	-
Inulin	-
Adonitol	-
Sorbitol	+
Dulcitol	-
Mannitol	+
Salicin	-
Inositol	+

+, Production of acid; -, No production of acid

4.9.1 Degradation of 2-chlorophenol by mixed cultures of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1

Rhodococcus sp. M1 was found to degrade 100 mg l^{-1} of 2-chlorophenol in 18 h (Fig 11 d). *Pseudomonas fluorescens* P1 after induction with benzoic acid was shown to degrade 2-chlorophenol (100 mg l^{-1}) in 48 h (Figure 29). Consequently higher specific growth rate was observed for axenic culture of *Rhodococcus* sp. M1 as compared to *Pseudomonas fluorescens* P1 (Table 37) on 2-chlorophenol.

2-chlorophenol (100 mg l^{-1}) was treated by mixed cultures of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1. Complete degradation of the substrate by the mixed culture was observed in 48 h (Figure 30). Lower rate of degradation of 2-chlorophenol was observed by mixed culture and by *Pseudomonas fluorescens* P1 as compared to degradation by pure culture of *Rhodococcus* sp. M1 (Table 36).

Viable count in terms of colony forming unit/ml (CFU/ml) for both the cultures degrading 2-chlorophenol was monitored at 12 h interval (Table 33). The total count of both the species as CFU/ml was taken on a nutrient agar plate. The total count of *Rhodococcus* sp. M1 as CFU/ml was evaluated by plating the sample on acetamide agar plate. Colony count of *Pseudomonas fluorescens* P1 as CFU/ml was evaluated by deducting the colony count of *Rhodococcus* sp. M1 (as estimated from acetamide agar plate) from the total colony count on the nutrient agar plate. Initial count for *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 was 6.0×10^2 CFU/ml and 5.0×10^2 CFU/ml respectively. Gradual increase in the count of both the populations was observed. Total count for *Pseudomonas fluorescens* P1 was 1.10×10^7 CFU/ml after 48 h when the substrate was completely utilized, whereas *Rhodococcus*

sp. M1 was observed to have a lower count of $5x10^5$ CFU/ml after similar period of incubation (Table 33). Evaluation of total count for both the species thus shows *Pseudomonas fluorescens* P1 to be the dominant species.

Table 33. Growth behaviour of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 as mixed culture in presence of 2-chlorophenol as the substrate

Time (h)	Total colony count (CFU/ml)		Colony count of *Rhodococcus* sp. M1 (CFU/ml)		Colony count of *Pseudomonas fluorescens* P1 (CFU/ml)	
	Range	Average	Range	Average	Range	Average
0	$9.0x10^2 - 1.4x10^3$	$1.1x10^3$	$3.0x10^2 - 1x10^3$	$6.0x10^2$	$3x10^2 - 7x10^2$	$5.0x10^2$
12	$1.1x10^4 - 2.0x10^4$	$1.52x10^4$	$1.1x10^3 - 5.0x10^3$	$2.0x10^3$	$1.2x10^4 - 1.7x10^4$	$1.5x10^4$
24	$8.5x10^4 - 2.5x10^5$	$1.27x10^5$	$3.0x10^3 - 7.5x10^3$	$7.0x10^2$	$9.0x10^4 - 1.5x10^5$	$1.2x10^5$
36	$1.1x10^6 - 2.0x10^6$	$1.82x10^6$	$1.13x10^5 - 2x10^5$	$1.2x10^5$	$1.3x10^6 - 2.0x10^6$	$1.7x10^6$
48	$1.05x10^7 - 1.8x10^7$	$1.15x10^7$	$2.0x10^5 - 7.0x10^5$	$5.0x10^5$	$9.5x10^6 - 2.0x10^7$	$1.1x10^7$

Initial concentration of the substrate was 100 mg l^{-1}
Values of viable plate count for *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 are average of five sets of experiment

Figure 29. DEGRADATION OF 2-CHLOROPHENOL ($100mgl^{-1}$) BY *Pseudomonas fluorescens* P1

The study was carried out in modified M3 medium at 32°C under shaking condition (120 rpm). Inoculum of *Pseudomonas fluorescens* P1 was prepared by growing the culture in nutrient broth. The culture was then given benzoate induction before acclimatizing it to 2-chlorophenol. Substrate concentration, chloride and cell growth was estimated as described in methods.

Figure 30. 2-CHLOROPHENOL DEGRADATION BY THE MIXED POPULATION OF *Rhodococcus* sp. M1 AND *Pseudomonas fluorescens* P1

The study was carried out in modified M3 medium containing 2-chlorophenol (100 mg l^{-1})at 32°C under shaking condition (120 rpm). Benzoic acid induced cells of *Rhodococcus* sp. M1 and *Psedomonas fluorescens* P1, acclimatized to 2-chlorophenol was used as the inoculum. Appropriate inoculum of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 was added to the modified M3 medium so as to give initially identical CFU/ml (Colony Forming Unit) for both the populations. Substrate concentration was estimated as described in methods.

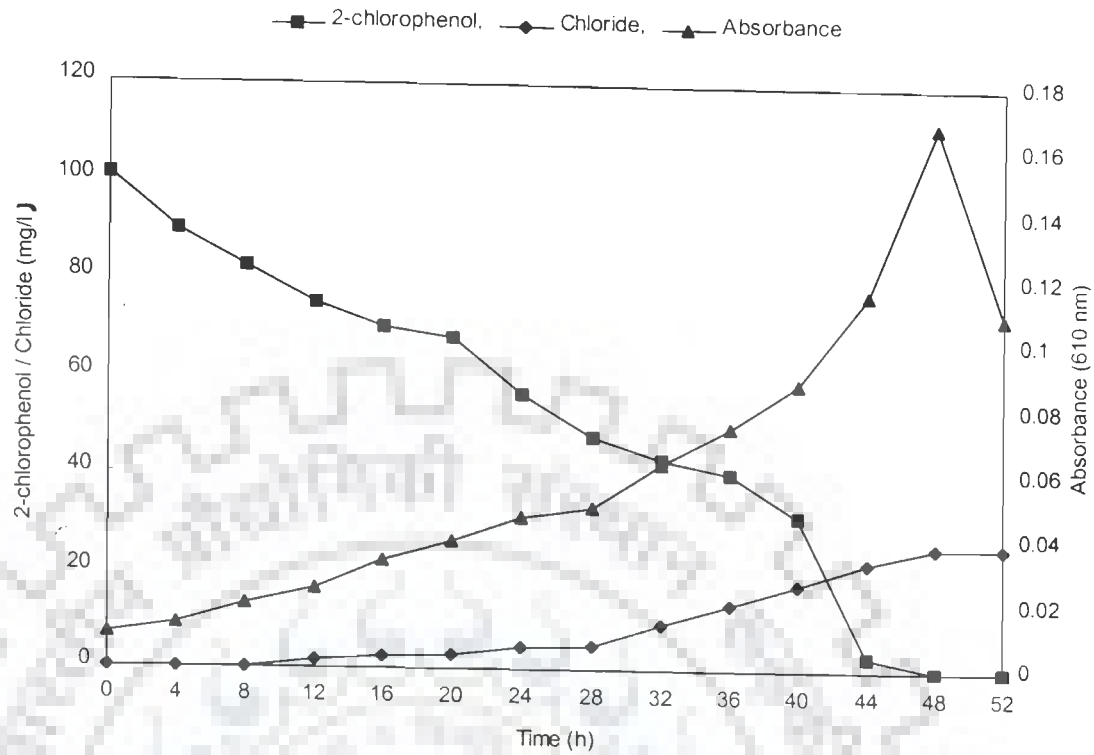


Figure 29

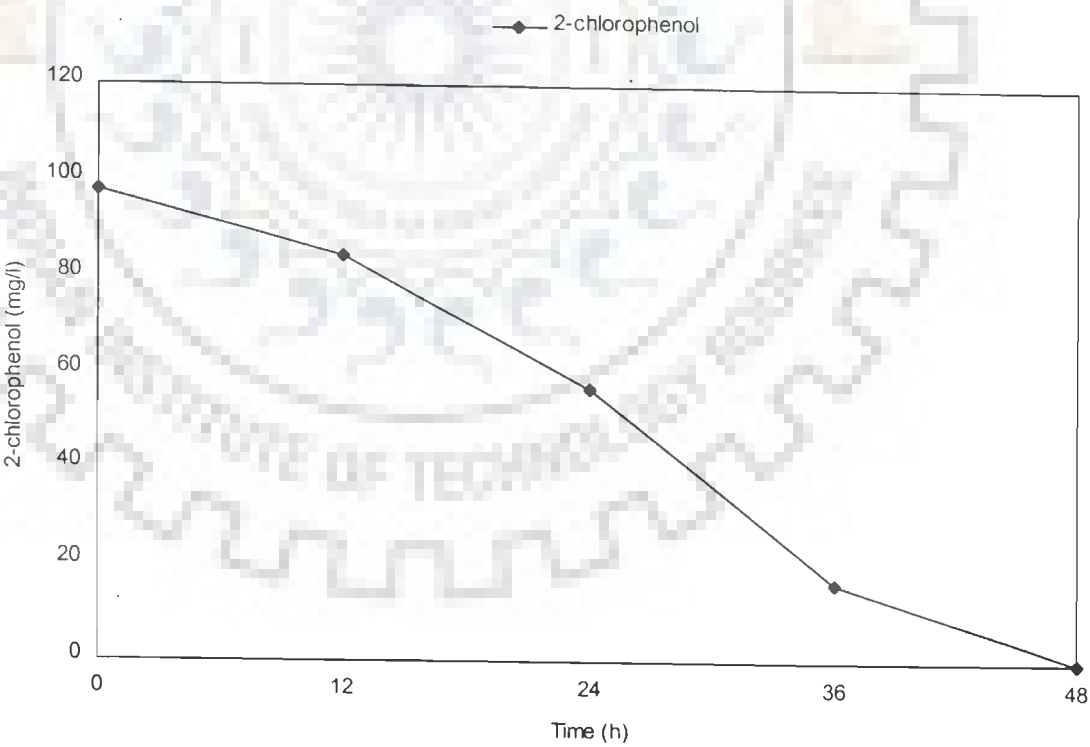


Figure 30

4.9.2 Degradation of phenol by mixed cultures of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1

Phenol (100 mg l^{-1}) was shown to be degraded by *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 in 24 h (Figure 31) and 30 h (Figure 32) and showed specific growth rate of 0.146 h^{-1} and 0.123 h^{-1}, respectively (Table 37). Phenol at similar concentration was observed to be removed in lesser time period of 20 h when treated by the mixed culture of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 (Figure 33). Hence, the rate of degradation of phenol by mixed cultures was higher as compared to degradation rate by pure cultures of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 (Table 36).

Colony count for both the cultures as CFU/ml was evaluated at 4 h interval (Table 34). The initial count of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 was observed to be $4.6x10^3$ CFU/ml and $4.4x10^3$ CFU /ml respectively. Colony count of both the populations showed a steady increase up to 8 h, after which colony count of *Rhodococcus* sp. M1 increased as compared to *Pseudomonas fluorescens* P1. Total counts of *Rhodococcus* sp. M1 reached a maximum of $1.43x10^7$ CFU/ml as compared to $2.8x10^5$ CFU/ml as detected for *Pseudomonas fluorescens* P1 after an incubation period of 20 h wherein the substrate was completely degraded. *Rhodococcus* sp. M1 was thus observed to undergo a rapid growth than *Pseudomonas fluorescens* P1 with phenol as the substrate and hence considered as the dominant population.

Table 34. Growth behaviour of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 as mixed culture in presence of phenol as the substrate

Time (h)	Total colony count (CFU/ml)		Colony count of *Rhodococcus* sp. M1 (CFU/ml)		Colony count of *Pseudomonas fluorescens* P1 (CFU/ml)	
	Range	Average	Range	Average	Range	Average
0	$6.0x10^3 - 1.05x10^4$	$9.0x10^3$	$3.0x10^3 - 7.5x10^3$	$4.6x10^3$	$3.0x10^3 - 5.0x10^3$	$4.4x10^3$
4	$1.2x10^4 - 1.8x10^4$	$1.38x10^4$	$6.8x10^3 - 8.5x10^3$	$7.1x10^3$	$4.0.x10^3 - 8.5x10^3$	$6.7x10^3$
8	$1.8x10^4 - 8.0x10^4$	$7.2x10^4$	$1.5x10^4 - 5.8x10^4$	$5.5x10^4$	$7.0x10^3 - 2.0x10^4$	$1.7x10^4$
12	$1.4x10^5 - 2.0x10^5$	$1.8x10^5$	$1.05x10^5$-$1.58x10^5$	$1.43x10^5$	$1.5x10^4 - 4.5x10^4$	$3.7x10^4$
16	$1.1x10^6$ - $2.25x10^6$	$1.75x10^6$	$9.5x10^5 - 1.8x10^6$	$1.7x10^6$	$1.3x10^4 - 6.8x10^4$	$5.5x10^4$
20	$1.1x10^7 - 3.0x10^7$	$1.45x10^7$	$9.5x10^6 - 1.6x10^7$	$1.43x10^7$	$1.3x10^5 - 4.8x10^5$	$2.8x10^5$

Initial concentration of the substrate was 100 mg l^{-1}
Values of viable plate count for *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 are average of five sets of experiment

Figure 31. DEGRADATION OF PHENOL BY *Rhodococcus* sp. M1

The study was carried out in modified M3 medium containing phenol (100 mg l^{-1}) at 32°C under shaking condition (120rpm). The cells grown on DSM-65 medium was acclimatized to phenol before using it for the present study. Phenol and growth was estimated as described in methods.

Figure 32. DEGRADATION OF PHENOL BY *Pseudomonas fluorescens* P1

The study was carried out in modified M3 medium containing phenol (100 mg l^{-1}) at 32°C under shaking condition (120 rpm). Inoculum was prepared by growing the culture in nutrient broth. The culture was then acclimatized to phenol before using it for the present study. Phenol and growth was estimated as described in methods

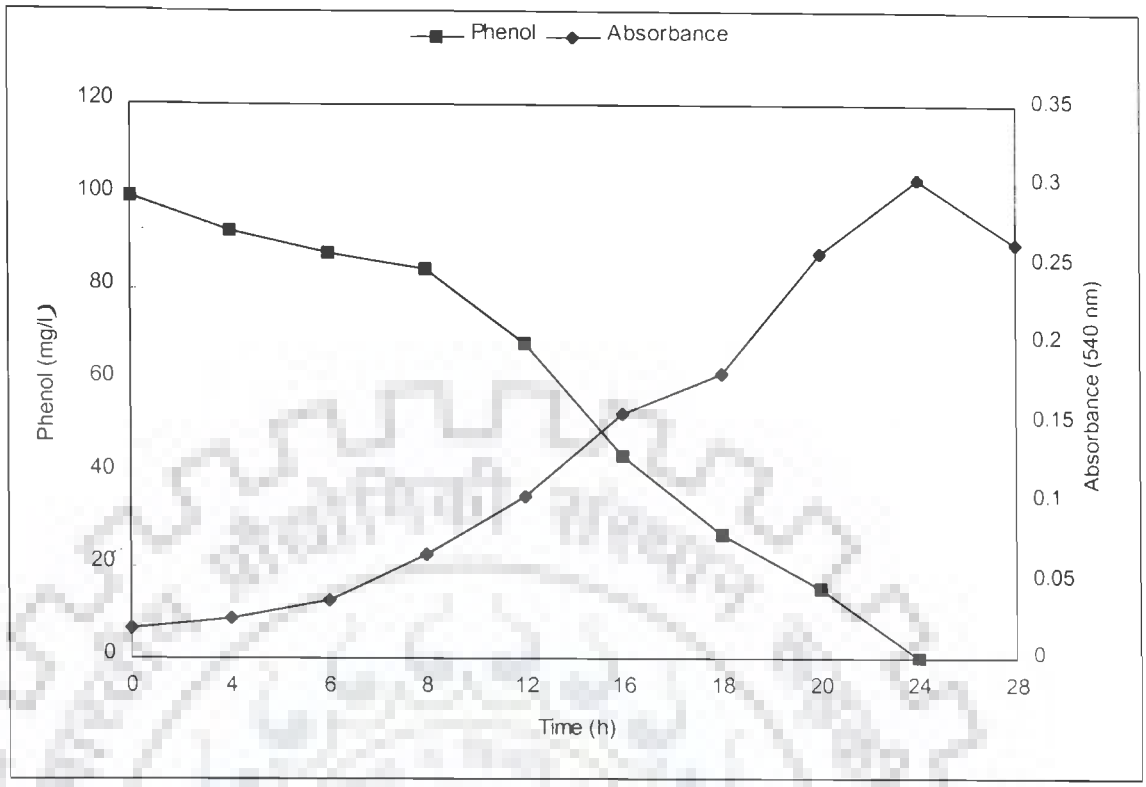


Figure 31

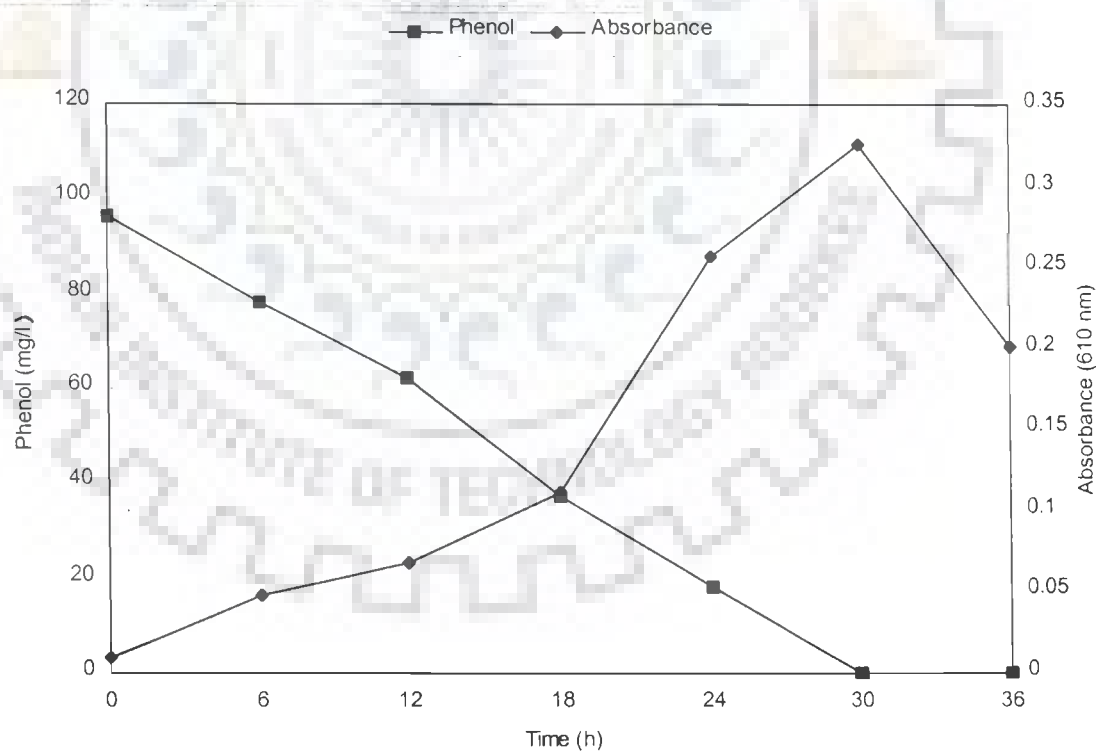


Figure 32

Figure 33. PHENOL DEGRADATION BY THE MIXED POPULATION OF *Rhodococcus* sp. M1 AND *Pseudomonas fluorescens* P1

The study was carried out in modified M3 medium containing phenol (100 mg l^{-1}) at 32°C under shaking condition (120 rpm). *Rhodococcus* sp. M1 and *Psedomonas fluorescens* P1 were acclimatized to phenol and was used as the inoculum. Appropriate inoculum of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 was added to the modified M3 medium so as to give initially identical CFU/ml (Colony Forming Unit) for both the populations. Substrate concentration was estimated as described in methods.

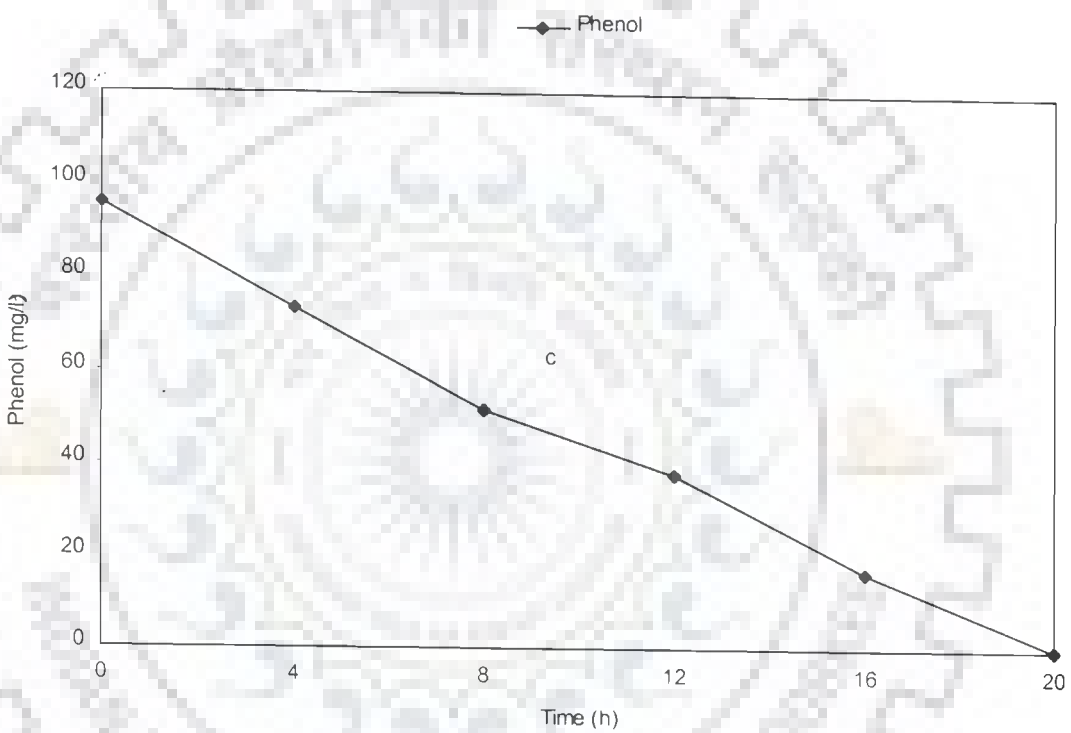


Figure 33

4.9.3 Degradation of p-cresol by mixed cultures of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1

Degradation of p-cresol at an initial concentration of 100 mg l^{-1} was observed by *Rhodococcus* sp. M1 in 20 h (Figure 34) and by *Pseudomonas fluorescens* P1 in 24 h (Figure 35). Almost similar specific growth rate was observed on p-cresol (100 mg l^{-1}) for both the cultures (Table 37). However, removal of p-cresol by mixed population of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 resulted into quicker (16 h) degradation of the substrate (Figure 36). Rate of degradation of p-cresol (100 mg l^{-1}) by mixed culture was considerably higher as compared to the degradation rate by the pure culture (Table 36). Total count for both these cultures degrading p-cresol was evaluated at 4 h interval and is presented in Table 35. Initial counts for *Rhodococcus* sp. M1 and for *Pseudomonas fluorescens* P1 respectively were $4.6x10^3$ CFU/ml and $4.4x10^3$ CFU/ml. Simultaneous growth of both the populations was observed. Both the cell populations showed a steady increase in the growth resulting into about similar cell counts. Total count of *Rhodococcus* sp. M1 reaches a maximum value of $6.6x10^6$ after an incubation period of 16 h in which the substrate is completely degraded, whereas total count for *Pseudomonas fluorescens* P1 after similar period of incubation was estimated to be $5.6x10^6$ CFU/ml. Thus both the populations appear to be coexisting under the conditions when p-cresol was added as the substrate.

Table 35. Growth behaviour of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 as mixed culture in presence of p-cresoll as the substrate

Time (h)	Total colony count (CFU/ml)		Colony count of *Rhodococcus* sp. M1 (CFU/ml)		Colony count of *Pseudomonas fluorescens* P1 (CFU/ml)	
	Range	Average	Range	Average	Range	Average
0	$5.5x10^3 - 1.1x10^4$	$9.0x10^3$	$3.0x10^3 - 5.0x10^3$	$4.6x10^3$	$2.8x10^3 - 5.2x10^3$	$4.4x10^3$
4	$7.0x10^3 - 1.1x10^4$	$9.5x10^3$	$5.8x10^3 - 7.5x10^3$	$6.5x10^3$	$1.5x10^3 - 4.0x10^3$	$3.0x10^3$
8	$3.2x10^4 - 7.0x10^4$	$5.7x10^4$	$2.7x10^4 - 4.0x10^4$	$3.5x10^4$	$1.8x10^4 - 2.8x10^4$	$2.2x10^4$
12	$7.5x10^5 - 9.0x10^5$	$8.7x10^5$	$4.0x10^5 - 5.0x10^5$	$4.5x10^5$	$3.5x10^5 - 4.5x10^5$	$4.2x10^5$
16	$1.1x10^7 - 2.0x10^7$	$1.22x10^7$	$6.5x10^6 - 8.0x10^6$	$6.6x10^6$	$5.2x10^6 - 6.0x10^6$	$5.6x10^6$

Initial concentration of the substrate was 100 mg l^{-1}
Values of viable plate count for *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 are average of five sets of experiment

Figure 34. DEGRADATION OF P-CRESOL BY *Rhodococcus* sp. M1

The study was carried out in modified M3 medium containing p-cresol (100 mg l^{-1}) at 32°C under shaking condition (120rpm). The cells grown on DSM-65 medium was acclimatized to p-cresol before using it for the present study. Substrate and growth was estimated as described in methods.

Figure 35. DEGRADATION OF P-CRESOL BY *Pseudomonas fluorescens* P1

The study was carried out in modified M3 medium containing p-cresol (100 mg l^{-1}) at 32°C under shaking condition (120 rpm). Inoculum was prepared by growing the culture in nutrient broth. The culture was then acclimatized to p-cresol before using it for the present study. Substrate and growth was estimated as described in methods

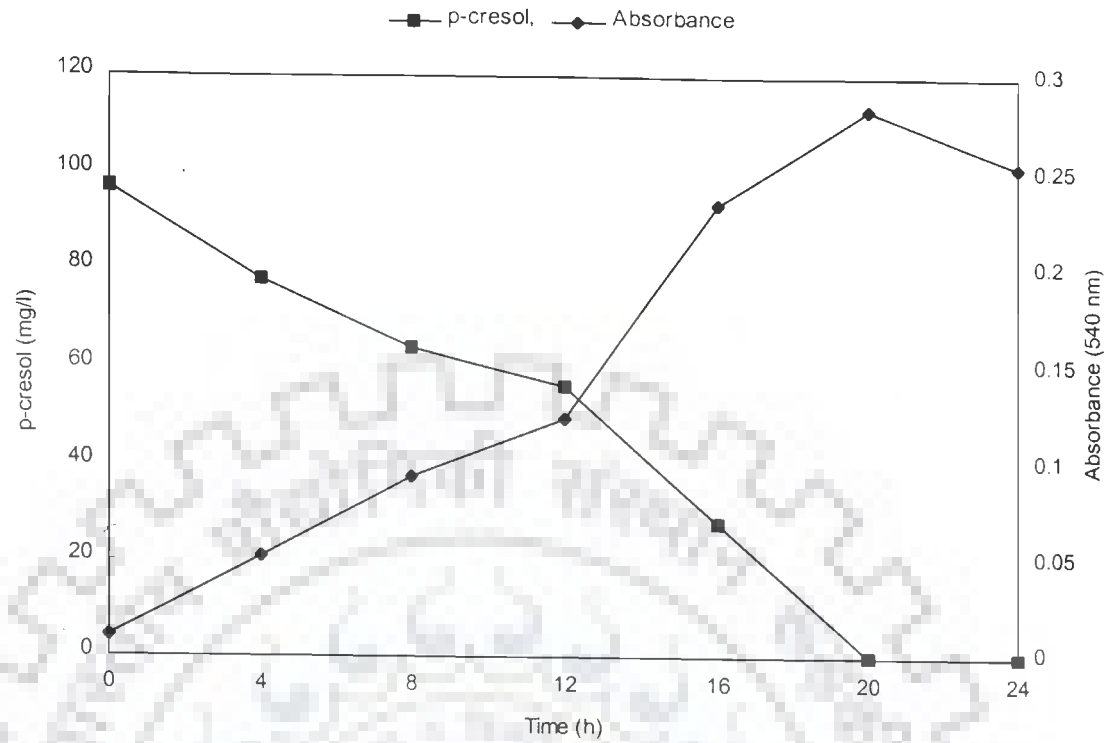


Figure 34

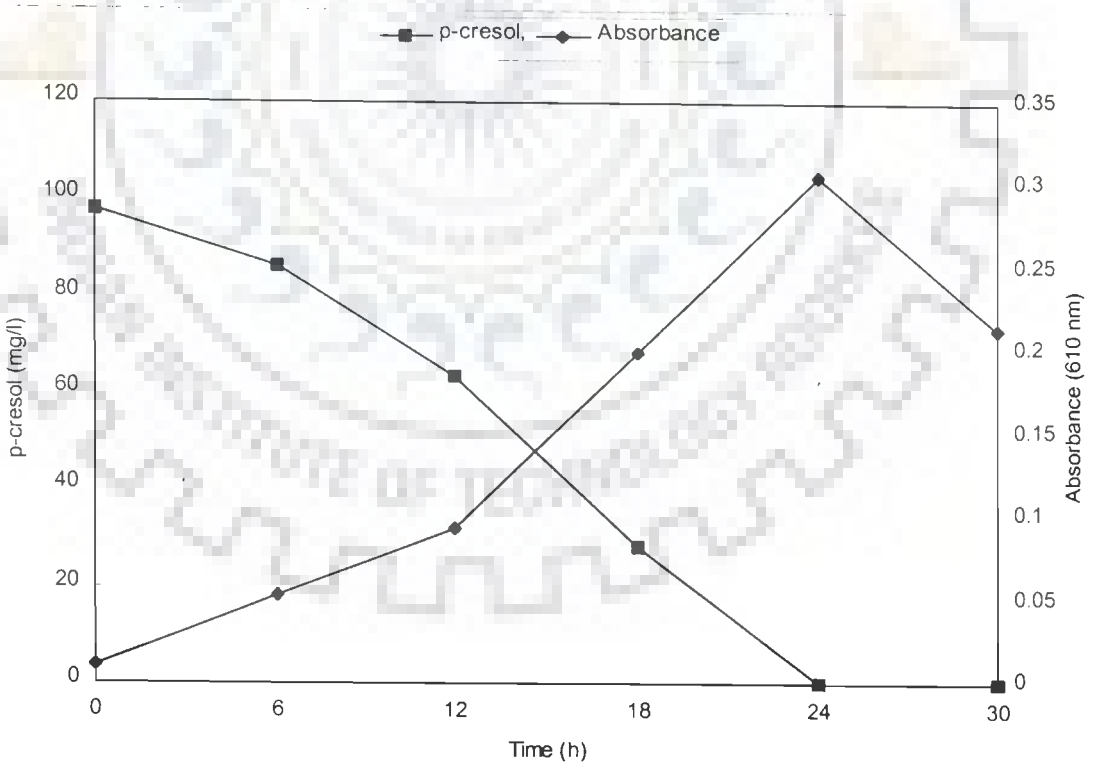


Figure 35

Figure 36. DEGRADATION OF P-CRESOL BY THE MIXED POPULATION OF *Rhodococcus* sp. M1 AND *Pseudomonas fluorescens* P1

The study was carried out in modified M3 medium containing phenol (100 mg l^{-1}) at 32°C under shaking condition (120 rpm). *Rhodococcus* sp. M1 and *Psedomonas fluorescens* P1 were acclimatized to p-cresol and was used as the inoculum. Appropriate inoculum of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 was added to the modified M3 medium so as to give initially identical CFU/ml (Colony Forming Unit) for both the populations. Substrate concentration was estimated as described in methods.

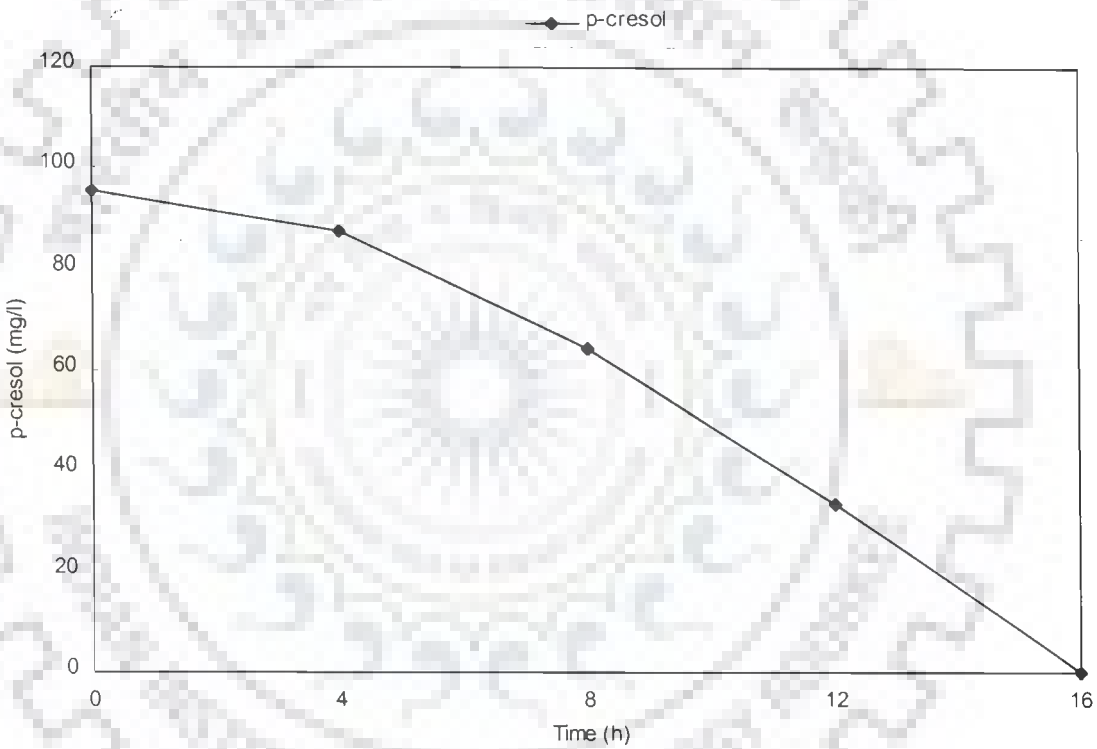


Figure 36

Table 36. Rate of degradation of 2-chlorophenol, phenol and p-cresol by pure culture of *Rhodococcus* sp. M1, *Pseudomonas fluorescens* P1 and mixed culture

Sl. No.	Substrates (100 mg l^{-1})	Strain	Rate of degradation (mg l^{-1} h^{-1})
1.	2-chlorophenol	a. *Rhodococcus* sp. M1	9.8
		b. *Pseudomonas fluorescens* P1	1.92
		c. Mixed Culture	1.73
2.	Phenol	***a.*** *Rhodococcus* sp. M1	4.5
		b. *Pseudomonas fluorescens* P1	3.5
		c. Mixed Culture	4.7
3.	p-cresol	***a.*** *Rhodococcus* sp. M1	4.5
		b. *Pseudomonas fluorescens* P1	4.1
		c. Mixed Culture	6.6

Table 37. Specific growth rate of pure cultures of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 on 2-chlorophenol, phenol and p-cresol

Sl. No.	Substrates (100 mg l^{-1})	Strain	μ (h^{-1})
1.	2-chlorophenol	*a. Rhodococcus* sp. M1	0.211
		b. Pseudomonas fluorescens P1	0.05
2.	Phenol	*a. Rhodococcus* sp. M1	0.146
		b. Pseudomonas fluorescens P1	0.123
3.	p-cresol	*c. Rhodococcus* sp. M1	0.14
		d. Pseudomonas fluorescens P1	0.135

μ, Specific growth rate of the culture

4.10 Biological treatment of 2-chlorophenol and 4-chlorophenol by a trickling filter reactor

Based on the batch studies conducted in the laboratory for the degradation of monochlorophenol (2-chlorophenol and 4-chlorophenol), attempts were made to find the feasibility of using a trickling filter reactor for the degradation of these substrates by conducting bench scale studies. Accordingly, a laboratory model of trickling filter was fabricated (Plate 4) and was filled with gravel of size 3 to 4 cm. The culture of *Rhodococcus* sp. M1 used in the batch scale study for the degradation of 2-chlorophenol and 4-chlorophenol was seeded on the gravel medium. *Rhodococcus* sp. M1 was grown in DSM-56 medium to get substantial amount of the culture for seeding in the reactor. The *Rhodococcus* sp. M1 inoculum was then uniformly seeded over the gravel medium. Phenol at a concentration of 200 mg l^{-1} was fed in the reactor for a period of two weeks to promote the growth of the culture that had been seeded. The reactor was then fed with 2-cholorophenpol (100 mg l^{-1}) for stabilization. 2-chlorophenol concentration, chloride and pH of the effluent were monitored. Following a period of one month the reactor was stabilized for the removal of 2-chlorophenol. Experiments were then carried out to find out the optimal organic and hydraulic loading for effective removal of the monochlorophenolic compounds. Subsequently the performance of the reactor was evaluated for treatment of 2-chlorophenol and 4-chlorophenol at the optimal organic and hydraulic loading.

Plate 4: A laboratory model of the trickling filter reactor used to study biological treatment of chlorophenols by whole cell immobilization of *Rhodococcus* sp. M1 on the gravel medium.


Trickling
Filter Unit
Peristaltic
Pump
Effluent
Influent

4.10.1 Optimization of the organic loading rate for 2-chlorophenol and 4-chlorophenol degradation

Optimization of organic loading rate for 2-chlorophenol in the range of 0.0518 to 1.037 g COD/l-day was carried at constant hydraulic flow rate of 30 ml/min. The concentration range of the substrate for optimizing the organic loading rate was based on the results obtained from batch scale study of 2-chlorophenol degradation. After each change in organic loading, the reactor was allowed to reach a steady state operation. Percent reduction in COD was computed and is presented in Figure 37. Results indicate that the organic loading rate for 2-chlorophenol up to 0.259 g COD/l-day showed COD reduction of 98.3% after which the efficiency of the reactor was observed to decline. Reduction in COD was not observed when organic loading rate for 2-chlorophenol was increased to 1.037 g COD/l-day. The results obtained indicated that the organic loading rate of 0.259 g COD/l-day of 2-chlorophenol was optimum for the efficient removal of the substrate.

Organic loading rate for 4-chlorophenol was studied for the range 0.0518 g to 0.778 g COD/l-day (Figure 37). Reduction in COD of 94% was observed for organic loading rate of 0.129 g COD/l-day after which the substrate removal was shown to decline. Removal of substrate was not observed at the organic loading rate of 0.778 g COD/l-day. Thus the study indicated an organic loading rate of 0.129 g COD/l-day for 4-chlorophenol as optimal for effective removal of 4-chlorophenol.

Figure 37. OPTIMIZATION OF ORGANIC LOADING FOR THE TREATMENT OF 2-CHLOROPHENOL AND 4-CHLOROPHENOL BY TRICKLING FILTER REACTOR

Synthetic waste containing monochlorophenolic isomer (2-chlorophenol/ 4-chlorophenol) was fed in the reactor. *Rhodococcus* sp. M1 acclimatized to respective chlorophenols was seeded on the gravel medium. Organic loading of 2-chlorophenol and 4-chlorophenol for the range 0.0518 to 1.037 g COD/l-day and 0.0518 to 1.037 g COD/l-day respectively was studied. Grab samples were collected at periodic intervals of 6 h. Chlorophenol concentration was estimated as described in methods.

4.10.2 Optimization of hydraulic loading for the degradation of 2-chlorophenol and 4-chlorophenol

The hydraulic flow rate was optimized for the trickling filter reactor at flow rates ranging from 20 to 60 ml/min. Feed flow rate was increased in a stepwise manner maintaining a constant organic loading rate of 0.259 g COD/l-day for 2-chlorophenol. At each step the trickling filter reactor was allowed to reach a steady state operation. Percent reduction in COD was evaluated (Figure 38). Complete removal of the substrate was observed for hydraulic loading rate up to 30 ml/min. The efficiency of the reactor was shown to decrease with further increase in the flow rate. Removal of substrate by the reactor was not observed at hydraulic loading rate of 60 ml/min. The study thus showed that the hydraulic loading rate of 30 ml/min was optimal for the efficient removal of 2-chlorophenol by the reactor.

Optimization of hydraulic loading rate was carried out for the treatment of 4-chlorophenol by the trickling filter reactor for flow rate in the range of 20 ml/min to 60 ml/min at optimum organic loading rate of 0.129 g COD/l-day (Figure 38). Result indicate a similar trend as that of 2-chlorophenol showing the optimum flow rate to be 30 ml/min. Complete removal of substrate was observed for flow rate up to 30 ml/min after which substrate degradation efficiency of the reactor declines. Removal of 4-chlorophenol was not observed at the flow rate 60 ml/min.

Figure 38. OPTIMIZATION OF HYDRAULIC LOADING FOR THE TREATMENT OF 2-CHLOROPHENOL AND 4-CHLOROPHENOL BY TRICKLING FILTER REACTOR

Composition of the waste is as described in methods, the seed culture used and sampling interval is as described in Figure 37. Treatment of chlorophenols (2-chlorophenol/ 4-chlorophenol) was studied for the flow rate in the range, 20 ml/min to 60 ml/min. Chlorophenol concentration was estimated as described in methods.

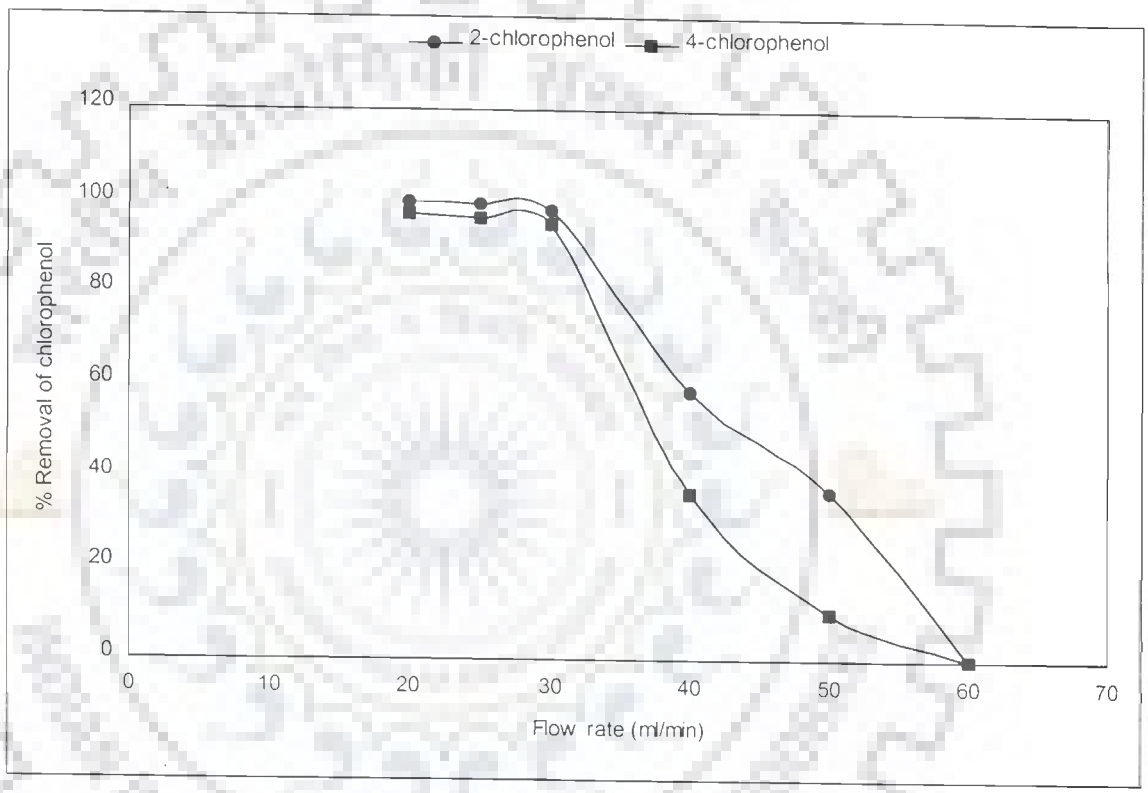

2-chlorophenol
4-chlorophenol
% Removal of chlorophenol
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
Flow rate (ml/min)

Figure 38

4.10.3 Operation of the trickling filter reactor at optimal organic and hydraulic loading for the treatment of 2-chlorophenol and 4-chlorophenol

The removal of 2-chlorophenol and 4-chlorophenol was studied separately, operating the trickling filter reactor at optimal organic and hydraulic loading for a period of one month. The results of the study are summarized in Table 38 and Table 39 for 2-chlorophenol and 4-chlorophenol respectively. Substrate concentration, pH, suspended solids, chloride and COD were monitored in the influent and the effluent to evaluate the performance of the reactor. The effluent samples were collected at one hour interval and composited for six hours before subjecting it to analysis. The value of the physicochemical parameter studied and presented (Table 38 and 39) are average of 30 days, for which the study was carried out. Efficient removal of 2-chlorophenol was supported by 84.6% reduction in COD and release of 13.26 mg/l of chloride, which is equivalent to the theoretical value. Similarly, degradation of 4-chlorophenol in the reactor showed 77.5% reduction in COD value and release of stoichiometric amount of chloride. Negligible decrease in pH was observed for both these substrates.

Table 38. Degradation of 2-chlorophenol in trickling filter reactor at optimal organic loading and hydraulic loading

Parameter	Influent (mg/l)	Effluent (mg/l)	% Reduction
2-chlorophenol	50 ± 3.0	2.2 ± 1.0	95.6 ± 1.0
COD	70.62 ± 5.0	10.83 ± 3.0	84.6 ± 1.0
Chloride	1.83 ± 0.5	13.26 ± 2.0	-
Suspended solids	Nil	10 ± 2	-
pH	7.3 ± 0.0	7.25 ± 0.0	-

Organic loading rate- 0.259 g COD/l-day; Flow rate 30 ml/min

Table 39. Degradation of 4-chlorophenol in trickling filter reactor at optimal organic loading and hydraulic loading

Parameter	Influent	Effluent	% Reduction
4-chlorophenol	25 ± 2.0	1.5 ± 1.0	94.0 ± 1.0
COD	73.52 ± 5.0	16.5 ± 3.0	77.5 ± 1.0
Chloride	2.8 ± 0.5	6.26 ± 2.0	-
Suspended solids	Nil	12.0 ± 2.0	-
pH	7.28 ± 0.0	7.26 ± 0.0	-

Organic loading rate- 0.129 g COD/l-day; Flow rate 30 ml/min

CHAPTER-5

5.0 *Discussion*

Anthropogenic introduction of chlorophenols into the environment due to industrial and agricultural uses is great cause of concern because of their toxic nature, carcinogenicity and persistence in the environment (Knackmuss and Hellwig, 1978). Biological approach for the removal of chlorophenols has been reported to be gaining advantage over physicochemical methods of treatment as it does not produce toxic and undesirable intermediates and is less expensive (Kim and Maier, 1986; Autenrieth and Bonner, 1991). Biological degradation of chlorophenols by few bacterial species have been reported by some researchers (Karns *et al.*, 1983; Edgehill and Finn, 1983; Saber and Crawford, 1985). Among the bacterial species, degradation of chlorophenols by *Rhodococcus* sp. have been reported by few (Apajalahati *et al.*, 1986; Apajalahati and Salkinoja-Salonen, 1986; Allard *et al*, 1987; Haggblom *et al,* 1989; Zaitsev *et al*, 1995). *Rhodococci* are being increasingly used in bioremediation sites and waste water treatment systems for the removal of toxic and recalcitrant compounds because of their metabolic versatility and robustness in natural environment (Finnerty, 1992; Warhurst and Fewson, 1994). The organisms show high substrate affinity and persistence in the environment even under adverse conditions, show lack of catabolite repression and flocculating growth which favors their high settleability in biological treatment systems (Salkinoja-Salonen *et al.*, 1991).

The present study thus focuses on the isolation of a *Rhodococcus* sp capable of degrading chlorophenols with increased efficiency and also to evaluate the kinetics of chlorophenols degradation. Further, the chlorophenol degradation has also been analysed when present in mixture with other contaminants as normally reported to occur in environment. In addition, microorganisms are seldom reported to occur as single species in natural condition, hence the degradation of chlorophenol and related phenolics by mixed cultures with *Rhodococcus* as one of the culture component was investigated.

5.1 Enrichment, isolation and identification of *Rhodococcus* sp.

Rhodococcus sp. was isolated from the habitat for this organism specified in literature. *Rhodococci* have frequently been isolated from soil, freshwater and from soil contaminated with petroleum compounds (Dobrovolskaya and Tretyakova, 1991; Maeda *et al.*, 1995). In the present study isolation of *Rhodococcus* was carried out from garden soil and oil contaminated soil. Samples from oxidation pond and compost were also used for enrichment and isolation as they are generally found to be a rich source of microbial populations. M3 medium described in Prokaryotes (Starr *et al.*,1981) was taken up for the selective isolation of *Rhodococcus* sp. Growth in the M3 medium was noted by increase in turbidity and was shown to be positive for all the sources of enrichment, except for the oil contaminated soil from gas station (Table 13).

Enrichment samples showing positive growth in the specific medium for *Rhodococcus* (M3 medium) was processed for monochlorophenol (2-chlorophenol) degradation by acclimatization procedure. Several researchers have reported

degradation of chlorophenol in the order of ortho>meta>para (Boyd *et al.*, 1983). 2-chlorophenol being the ortho isomer of monochlorophenol was thus chosen for initial acclimatization. Sodium propionate in the M3 medium, which is the carbon source, was gradually removed and replaced by 2-chlorophenol supplementation. Similar process of acclimatization was followed for the culture WR1306 where benzene was used as the substrate for enrichment and was replaced stepwise by chlorobenzene. As soon as chlorobenzene was used as the sole growth substrate, cells were plated on nutrient agar for isolation of pure culture (Reineke and Knackmuss, 1984).

Enrichment samples showing growth in M3 medium containing 2-chlorophenol as the only carbon source was used for the isolation of pure culture by plating on M3 medium. Five pure cultures were isolated from different sources (Table 14). The pure cultures were then identified on the basis of colony, cell morphological, physiological and biochemical characteristics. The isolates were characterized by pigmented colonies, pigmentation varying from orange, pink, red and buff (Table 15 and Plate 1). Another significant feature of the colonies was dense and elevated center and flattened margin. Margin and surface properties were observed to change with age. Colony and cell morphological characteristics (Table 16) of the isolates was found to be similar to that of *Rhodococcus* described in Bergey's manual of Systematic Bacteriology (Goodfellow, 1989) leading to a possibility of the cultures to be *Rhodococcus*.

Rhodococci as described in literature are gram positive, non-motile with pigmented colonies. *Rhodococci* are characterized by typical growth cycle varying from coccus to rod. Rhodococcal cells were reported to show a tendency to clump together (Prokaryotes 1981; Bergey's Manual of Systematic Bacteriology, 1989).

Physiological and biochemical characterization of the isolates was carried out for the confirmation of the identity of the isolates to be *Rhodococcus* sp. The biochemical characteristics of the isolates (Table 17) show these to be catalase and urease positive. Starch hydrolysis was observed for all the isolates (except for isolate M3), but was unable to utilize casein. These were shown to utilize citrate and acetamide (except for M3) and also to reduce nitrate, showed negative reaction for oxidase and gelatin liquefaction and were characterized to be strictly aerobic as they were capable of only oxidizing the glucose when grown on glucose oxidation-fermentation medium. All the isolates were shown to produce acid from maltose, fructose, sucrose, dextrose, trehalose (Table 18). Biochemical characteristics and sugar utilization pattern of the isolates when compared to that described in Bergey's Manual of Systematic Bacteriology (1989) confirms the isolates to be *Rhodococus* sp.

5.2 Screening of the *Rhodococcus* isolates for phenolics and chlorophenols degradation

The five isolates of *Rhodococcus* sp. obtained after enrichment and isolation procedure and designated as M1, M2, M3, M4 and M5 were screened with the objective of getting potential culture(s) having capability to degrade chlorophenols. Chlorophenols in most of the cases were reported to be present along with phenol and cresols in the industrial effluent (Gu and Korus, 1995). Hence the feasibility of application of the culture for effective treatment of chlorophenolic wastes desires that the culture have the ability to degrade phenol and cresols. Isolates therefore, were screened for their capability to degrade phenols and cresols along with chlorophenols.

The biodegradation potential of phenols, cresols (ortho, meta and para) and chlorophenols (2-chlorophenol, 4-chlorophenol. 2.4-dichlorophenol and 2,4,6-trichlorophenol) by the isolates are presented in Tables 19 and 21 respectively. Substrate degradation, if not observed to support growth, does not imply complete mineralization of the substrate. Hence in order to ensure the ability of the isolates to completely utilize the substrate, growth of the isolates on phenolics and chlorophenols was measured and the results are presented in Tables 20 and 22 respectively. The results had indicated that the *Rhodococcus* isolate M1 as the most potential culture since it had maximum ability for degradation for wider range of phenolics as compared to other isolates. *Rhodococcus* sp M1 isolated from garden soil could grow on phenol, cresols (ortho, meta and para) and chlorophenols (2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol).

The other *Rhodococcus* isolate M5 from compost was observed to degrade phenol and cresols but was unable to grow on all the chlorophenols. It was found to degrade 2-chlorophenol but was unable to grow on 4-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol. Isolate M4 from oil contaminated site of garage could grow on all the chlorophenols and phenol but was unable to degrade cresols. *Rhodococcus* sp M1 was thus identified as the most potential culture showing maximum extent of degradation for wider range of substrates as compared to other isolates and hence was selected for further studies.

5.3 Induction of *Rhodococcus* sp for degradation of 2-chlorophenol

Rhodococus isolate M1 selected on the basis of screening, had maximum extent of degradation for 2-chlorophenol out of the four chlorophenols (2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol) analysed. Degradation of 2-chlorophenol at an initial concentration of 100 mgl^{-1} commenced after a prolonged lag period of 5days. Complete degradation of 2-chlorophenol was achieved after a period of 45 days (Figure 3). With an aim to reduce the lag period and subsequently the time taken for 2-chlorophenol removal, the *Rhodococcus* isolate M1 was subjected to induction using different aromatic compounds. Toluene enriched culture from activated sludge showed rapid removal of chlorophenols in a study by Ryding *et al* (1994). The enzyme 1,2-pyrocatechase necessary for the ring cleavage was shown to be induced in cells of *Alcaligenes* strain A175 when grown on toluene (Schraa *et al.*, 1986) Thus, toluene was selected as one of the inducers. Haggblom *et al* (1989) had shown transformation of chlorophenols by *Rhodococcus rhodochrous* and *Rhodococus* sp. strain P1 AN117 that were capable of degrading phenol. Hence phenol was identified as another possible inducer. Further, there are catabolic pathways in which homologous reactions are carried out on substrates which are similar but not identical in structure (Gibson, 1984). Benzoic acid catabolism in *Pseudomonas putida* (Clarke, 1984) and phenol degradation pathway (Autenrieth and Bonner, 1984) had shown similarity with that of monochlorophenol metabolism. Hence, these two compounds were also selected.

Growth ability of the Rhodococcus isolate M1 was studied for toluene, benzoic acid, phenol and catechol (Figure 4). Cultured cells of the isolate M1, enriched on the

above mentioned compounds were used in their logarithmic phase of growth to study the degradation of 2-chlorophenol at an initial concentration of 100 mgl^{-1}. Complete removal of the chlorophenol was noted for toluene (Figure 5), and benzoic acid (Figure 6) enriched cells in 30 days and 48 h respectively, whereas Phenol (Figure 7) and catechol (Figure 8) induced cells showed 2-chlorophenol removal in 35 days and 45 days respectively. Benzoic acid induced cells of *Rhodococcus* sp. M1 showed complete removal of 2-chlorophenol in least time period as compared to toluene, phenol and catechol enriched cells. The benzoic acid induced culture of *Rhodococcus* sp. M1 was thus used for optimization of temperature, pH and for kinetic studies.

Spain *et al.* (1989) had shown monohydroxylation of several mono and dichlorophenols by a dioxygenase of a toluene grown *Pseudomonas putida* F1 that though not conclusive but appeared to be involved in degradation of chlorophenol. Phenol and catechol showed similarity in catabolic pathway with that of chlorophenol. However, phenol enriched cells of *Rhodococcus* sp. was shown to transform and not completely degrade the chlorophenol as detected by Knackmuss and Hellwig (1978). Benzoate grown cells of *Pseudomonas* B13 showed the presence of the enzyme, catechol 1,2 dioxygenase (Knackmuss and Hellwig, 1978). Catechol dioxygenase is the enzyme that acts on chlorocatechol to break the ring structure. Even though the catechol 1,2 dioxygenase, essential for ring cleavage, has also been detected in toluene grown cells of *Alcaligenes* strain A175 (Schraa *et al.* 1986) and phenol grown cells of *Pseudomonas* sp. B13 (Knackmuss and Hellwig, 1978), however an increased level of this enzyme to a greater extent may account for higher degradative capability for 2-chlorophenol by the benzoate induced cells as observed in the present study. However

Further work is required to investigate the changes at cellular and molecular level leading to increased levels of degradation by benzoic acid induced cells.

5.4 Optimization of temperature and pH for chlorophenol degradation by *Rhodococcus* sp. M1

Benzoic acid enriched cells of *Rhodococcus* sp. M1, acclimatized to 2-chlorophenol was used to study the optimum temperature and pH for maximum degradation of chlorophenols. *Rhodococcus* sp. M1 was shown to grow on 2-chlorophenol in the temperature range of 10°C to 40°C (Figure 9), however, the optimum temperature for degradation of 2-chlorophenol was found to be 32 - 35°C. Temperature was observed to had profound effect on the growth of *Alcaligenes* sp. with chlorobenzene as the substrate, maximum degradation occurred at 29°C (Schraa *et al.*, 1986).

The optimum pH for the growth of *Rhodococcus* sp. M1 on 2-chlorophenol was observed to be in the range of pH 7.0 to 8.0 (Figure 10). Growth of the organism on 2-chlorophenol was reported to be negligible for pH value lower than 6.5. However, *Rhodococcus* sp. M1 was observed to grow in alkaline pH, up to pH of 11.5. Strains of *Ochrobacterium anthropi* also displayed alkaliphilic properties with notable 4-chlorophenol and 2,4-dichlorophenol degradation at the pH 8.5 - 9.5, and the catabolic activity was observed up to pH of 11 (Muller *et al.*, 1998). *Rhodococcus chlorophenolicus* and *Flavobacteria* led to PCP degradation at an alkaline pH of about 8 and 8 - 8.5 respectively (Martinson *et al.*, 1986). In the acid environment bacterial metabolism is slower and chlorophenols are more toxic than at higher pH (Valo *et al.*,

1990). This is because the toxicity of chlorophenols is usually attributed to the acid (undissociated form) condition. Hence, the toxicity of a chlorophenol decreases as pH increases (Stanlake and Finn, 1982; Kishino and Kobayashi, 1995). Increase in the duration of lag phase with decreasing pH for strain NC degrading pentachlorophenol further substantiates this theory (Vandenberg and Olsen, 1981). Thus chlorophenols should be less toxic to microorganisms at elevated pH values, which may allow bacteria to utilize these at high concentrations (Maltseva and Oriel, 1997). The ability of *Rhodococcus* sp. M1 to grow at high pH values hence increases the possibility of degradation of higher concentration of chlorophenols by this culture.

5.5 Kinetics of degradation of different chlorophenols by *Rhodococcus* sp M1

Several strains of bacteria had been reported to degrade chlorophenols under aerobic conditions (Stanlake and Finn, 1982; Karns *et al.*, 1983; Saber and Crawford, 1985; Maltseva and Oriel, 1997; Valenzuela *et al.*, 1997; Aranda *et al.*, 1999; Wang *et al.*, 2000). Degradation of chlorophenols by pure cultures of *Rhodococcus* has been reported by very few researchers. *Rhodococcus chlorophenolicus, Rhodococcus* sp. strain CP-2 and *Rhodococcus* sp. strain CG-1 degrade series of chlorinated phenols ranging from dichlorophenols to pentachlorophenol (Apajalahati *et al* 1986; Apajalahati and Salkinoja-Salonen, 1986; Haggblom *et al.*, 1989; Allard *et al.*, 1987; Gorlatov and Golovleva, 1992; Finkelshtein *et al.*, 2000). *R. erythropolis* 1CP was shown to grow on 4-chlorophenol and 2,4-dichlorophenol at concentration of 0.1 g/l and 0.05 g/l respectively (Gorlatov *et al.*, 1989; Gorlatov and Golovleva, 1992). *Rhodococcus opacus* was shown to grow on 2-chlorophenol, 3-chlorophenol and 4-chlorophenol (Zaitsev *et al.*, 1995). *Rhodococcus rhodochrous* and *Rhodococcus* sp.

strain P1 and AN117 were reported to transform 3-chlorophenol, 4-chlorophenol, 2,3-dichlorophenol and 3,4-dichlorophenol (Haggblom *et* al., 1989). Thus in the degradation studies reported so far employing different bacteria and *Rhodococcus* strains, degradation of chlorophenol was achieved for low concentration of the substrate in considerably longer time period. In many cases only dechlorination was observed and no degradation was reported. The present study with *Rhodococcus* sp. M1 was thus carried out to attempt complete degradation of high concentration of chlorophenols. The total concentrations of chlorophenols in contaminated waters have been reported to be several tens of mg/l (World Health Organization, 1989), therefore, higher concentrations of chlorophenols were selected for the present study to represent a worst case treatment scenario. Also the present study using the culture *Rhodococcus* sp. M1 was aimed at reducing the lag period and subsequently the time taken for degradation of chlorophenols.

Benzoic acid induced cells of *Rhodococcus* sp. M1 acclimatized to respective chlorophenols was used to study the kinetics of degradation. 2-chlorophenol was observed to be degraded up to a concentration of 300 mgl^{-1} (Figure 11a - h). Complete degradation of 2-chlorphenol up to a concentration of 250 mgl^{-1} was evident by the release of more than 90% of the theoretical amount of chloride and also no metabolites were detected through spectroscopic and gas chromatographic analyses. Detectable residual soluble COD was not observed after the degradation of 2-chlorophenol up to a concentration of 250 mgl^{-1}. The initial concentration appeared to affect the extent of degradation and the degradation rate increased with increasing substrate concentration. Maximum rate of degradation was observed for 2-chlorophenol concentration of 100

mgl^{-1}, after which rate of degradation decreases (Table 23). Increase in degradation rate with substrate concentration that subsequently decreased after reaching a maximum was also observed for the degradation of BTEX (benzene, toluene, ethylbenzene and xylene) by a coculture of *Pseudomonas putida* and *Pseudomonas fluorescens* (Shim and Yang, 1999). Complete degradation of 2-chlorophenol at a concentration of 300 mgl^{-1} was not achieved as indicated by the release of chloride up to 82.65% of the theoretical amount. Residual soluble COD of 60.8 mg/l further authenticates the incomplete degradation of 2-chlorophenol at a substrate concentration of 300 mgl^{-1}. Removal of 2-chlorophenol up to 48% was observed at a concentration of 350 mgl^{-1} (Figure 11 i), however, negligible release of chloride and increase in cell growth at this concentration possibly indicates the transformation and not the degradation of the substrate. Formation of brown colouration of the medium further supports this possibility. The brown colour in the medium could probably be due to the formation of quinonic catechol radicals caused by autopolymerizing reactions that limited further catechol metabolism. Such autopolymerizing reactions have been reported for chlorocatechols by Haller and Finn (1979).

4-chlorophenol was observed to be degraded up to a concentration of 100 mgl^{-1} (Figure 12 a - e). Release of more than 90% of the theoretical amount of chloride and no detectable residual soluble COD indicates complete mineralization of the substrate up to concentration of 75 mgl^{-1}. Residual soluble COD of 16.4 mgl^{-1} was observed after the degradation of 100 mgl^{-1} of 4-chlorophenol, suggesting incomplete removal of the substrate. Only 43.27% degradation was observed when 4-chlorophenol concentration was increased to 150 mgl^{-1} (Figure 12 f). However, negligible increase in cell growth

and insignificant amount of chloride released at 4-chlorophenol concentration of 150 mgl^{-1} suggests that the degradation observed was due to transformation of the substrate. No growth was observed when the chlorophenol was added at higher concentration (200 mgl^{-1}) (Figure 12 g).

The organism when grown on 4-chlorophenol showed prolonged lag phase and increased period of degradation as compared to 2-chlorophenol. In addition, the culture showed negligible growth on 4-chlorophenol at the concentrations higher than 100 mgl^{-1}. This is possibly because of the substrate induced toxicity of the compound at concentrations higher than 100 mgl^{-1}. However, the growth of *Rhodococcus* sp. M1 on 2-chlorophenol was observed up to concentration of 300 mgl^{-1}. 4-chlorophenol thus appeared toxic at much lower concentration (100 mgl^{-1}) as compared to 2-chlorophenol (300 mgl^{-1}). The position of the chlorine substituents in phenol ring was observed to profoundly affect the chlorophenol toxicity as reported by Liu *et al.*, (1982). Para substituted phenols were shown to be more toxic than the ortho isomer in the degradation by *Bacillus* sp. strain TL81. Degradation of chlorophenol in the order of ortho> meta > para has been reported by several researchers (Boyd et al, 1983; Genthner *et al.*, 1989; Wood *et al.*, 1989, Connor and Young, 1996; Jin and Bhattacharya, 1997) thus further supporting the finding by Liu et al (1982). The ability of *Rhodococcus* sp. M1 to degrade higher concentration of 2-chlorophenol as compared to 4-chlorophenol in the present study could be in line with these findings. However in the case of *Rhodococcus opacus* 1CP the rate of growth decreased from 4-chlorophenol to 3-chlorophenol and then to 2-chlorophenol (Finkelshtein *et al.*, 2000).

The toxicity of chlorophenol to microorganism was reported to increase with the degree of chlorination in the phenol nucleus (Liu *et al.*, 1982). Similar observations were obtained for the present study with 2,4-dichlorophenol and 2,4,6-trichlorophenol. Degradation of 2,4-dichlorophenol and 2,4,6-trichlorophenol was observed for lower concentration of these substrates as compared to the monochlorophenolic isomers (2-chlorophenol and 4-chlorophenol). 2,4-dichlorophenol and 2,4,6-trichlorophenol was degraded only up to a concentration of 50 mgl^{-1} and 25 mgl^{-1} respectively, after a prolonged lag period and requires significantly longer period for degradation (Figure 13 a - e and Figure 14 a - c). Complete mineralization of 2,4-dichlorophenol was achieved up to 25 mgl^{-1} of the substrate as evident from no residual soluble COD. Complete degradation of 2,4,6-trichlorophenol was not achieved even at a low concentration of 10 mgl^{-1}. This is evident from release of chloride only up to 79.77% of the theoretical value (Figure 14a).

5.5.1 Evaluation of kinetic parameters for the degradation of different chlorophenols by *Rhodococcus* sp. M1

Although studies on degradation of chlorophenols have been extensively reported, relatively few studies have been carried out to evaluate the basic parameters, which describe the kinetics of chlorophenol utilization. Determination of kinetic parameters for chlorophenolic (2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol) degradation provides a foundation for design and development of operational strategies of biological treatment systems for treating such wastes and hence is essential. Kinetic parameters for the degradation of

monochlorophenol (2-chlorophenol, 4-chlorophenol), dichlorophenol (2,4-dichlorophenol) and trichlorophenol (2,4.6-trichlorophenol) were studied for *Rhodococcus* sp. M1. For all the chlorophenolic compounds studied, specific growth rate was observed to decrease with increase in substrate concentration. Similar trend was observed for *Alcaligenes eutrophus* by Hill *et al.* (1996) using phenol as the sole carbon source. Specific growth rate was observed to drop linearly with phenol concentration. Such trend could not accurately fit with Haldane substrate inhibition kinetics. However by regressing a straight line through the data (ie. substrate concentration against specific growth rate)the following model equation was developed:

$$\mu = \mu_{max} . (1-S_1 / K_{i1})$$

Where $[S_1]$ refers to the concentration of the substrate. The given equation represents a linear case of the general empirical model proposed by Luong (1987) for substrate inhibition. The intercept of the line obtained by plotting specific growth rate with initial substrate concentration gives a maximum specific growth rate (μ_{max}). The slope gives an inhibition parameter (K_{i1}). Value of K_{i1} gives the maximum phenol concentration beyond which no growth should be observed in batch culture (Hill *et al.*, 1996).

Value for μ for *Rhodococcus* sp. M1 when grown on 2-chlorophenol at a concentration of 10 mgl^{-1} was computed to be 0.221 h^{-1} (Table 24). Specific growth rate showed a linear drop with increasing substrate concentration and gives a value of 0.043 h^{-1} at 2-chlorophenol concentration of 300 mgl^{-1}. Lower growth rate with increase in substrate concentration could be as a consequence of substrate inhibition.

Decrease in specific growth rate with increase in substrate concentration was also observed for *Artrobacter* sp. degrading pentachlorophenol (Stanlake and Finn, 1982). Increase in growth rate with substrate concentration was observed for *Ralstonia* sp. strain RKI degrading 2,6-dichlorophenol. The maximal growth rate (0.082 h^{-1}) was reached when 2,6-dichlorophenol concentration was 300 μM after which the growth rate decreased rapidly with substrate concentration (Steinle *et al.*, 1998). Value of μ for *Rhodococcus opacus* GM-14 on 50 mgl^{-1} of 2-chlorophenol was 0.15 h^{-1} which is lower than *Rhodococcus* sp. M1 giving a value of 0.224 h^{-1}. Specific growth rate however showed lower value for *Rhodococcus* sp. M1 as compared to *Rhodococcus opacus* GM-14 for substrate concentration greater than 50 mgl^{-1}. However the lag period for degradation of 2-chlorophenol by *Rhodococcus opacus* GM-14 was significantly high as compared to *Rhodococcus* sp. M1. Values of μ was in the range of 0.0481 to 0.024 h^{-1} for 4-chlorophenol concentration varying from 10 to 100 mgl^{-1} (Table 25). Growth rate of *Pseudomonas* sp. B13 on 4-chlorophenol reported by Knackmuss and Hellwig (1978) showed a higher value of 0.4 h^{-1} as compared to the M1 strain as reported in this present study. However, 4-chlorophenol was degraded only up to a concentration of 20 mgl^{-1}. In the present study, the specific growth rate for 2,4-dichlorophenol was observed to be 0.0221 h^{-1} at a concentration of 10 mgl^{-1} (Table 26). The value of μ declined with increasing concentration of 2,4-dichlorophenol and was 0.01 h^{-1} at higher substrate concentration (50 mgl^{-1})). Tyler and Finn (1974) reported the specific growth rate of 0.12 h^{-1} on 2,4-dichlorophenol for a *Pseudomonas* sp. upto a substrate concentration of 25 mgl^{-1} beyond which it inhibited growth.

Maximum specific growth rate (μ_{max}) of *Rhodococcus* sp. M1 on 2-chlorophenol was computed to be 0.248 h^{-1} (Figure 15) that remarkably dropped to 0.0493 h^{-1} for 4-chlorophenol (Figure 16). Further decrease in the μ_{max} value to 0.244 h^{-1} was observed for 2,4-dichlorophenol (Figure 17). Doubling time of *Rhodococcus* sp. M1 for non-inhibitory concentration of 2-chlorophenol was found to be 3.13 h (Table 27). Similar doubling time of 3 h was reported for *Rhodococcus opacus* GM-14 on 2-chlorophenol (Zaitsev *et al.*, 1995). However with *Azotobacter chroococcum* MSB1, higher doubling time of 4 h was observed on 2-chlorophenol (Balajee and Mahadevan, 1990). Doubling time of the organism was found to increase significantly in case of 4-chlorophenol giving a value of 16.6 h (Table 27). Doubling time of *Rhodococcus* sp. M1 in 2,4-dichlorophenol was observed to be remarkably high and was found to be 37.41 h.

Growth yield of *Rhodococcus* sp. M1 on 2-chlorophenol, 4-chlorophenol and 2,4-dichlorophenol calculated on the basis of substrate utilized decreases with increase in initial substrate concentration possibly because of substrate induced toxicity. Growth yield was in the range of 0.087 to 0.026 g mol^{-1} for 2-chlorophenol up to a concentration of 300 mgl^{-1} (Table 24). On 4-chlorophenol growth yield was lower compared to 2-chlorophenol and was in the range of 0.065 to 0.033 g mol^{-1} for substrate concentration up to 100 mgl^{-1} (Table 25). Growth yield for 2,4-dichlorophenol concentration up to 50 mgl^{-1} ranged from 0.065 to 0.045 g mol^{-1} (Table 26). Low growth yield of *Rhodococcus* sp. M1 on 2-chlorophenol, 4-chlorophenol and 2,4-dichlorophenol is an advantage for wastewater treatment systems as biomass disposal problem will be less severe (Oh *et al.*, 1994)

Substrate uptake rate had shown the similar decreasing trend for 2-chlorophenol, 4-chlorophenol and 2,4-dichlorophenol. Substrate uptake rate varied in the range 0.267 to 0.59 mg/mg.h for 2-chlorophenol (Table 24). Considerable decrease in the substrate uptake rate was observed when the concentration of 2-chlorophenol was increased from 100 to 150 mgl^{-1}. Substantial decrease in the substrate uptake rate was observed for 4-chlorophenol and was in the range 0.136 to 0.059 mg/mg.h (Table 25). Substrate uptake rate further declined for 2,4-dichlorophenol and varied from 0.082 to 0.024 mg/mg.h (Table 26).

Inhibition parameter (K_{il}) i.e. the concentration of the substrate beyond which no growth is observed was computed for 2-chlorophenol, 4-chlorophenol and 2,4-dichlorophenol. Value of K_{il} for the chlorophenolic compounds in the present study agreed with the experimental value obtained from shake flask study. K_{il} value for 2-chlorophenol was 354.28 mgl^{-1} (Figure 15). No growth was observed for 2-chlorophenol beyond the concentration 350 mgl^{-1}. Similarly the K_{il} value for 4-chlorophenol was observed to be 164.33 mgl^{-1} (Figure 16). Batch study showed inhibition of growth by 4-chlorophenol beyond 150 mgl^{-1}. Hence the K_{il} value for 4-chlorophenol was supported by the result obtained in batch study. 2,4-dichlorophenol gives a K_{il} value of 81.3 mgl^{-1} (Figure 17), which agrees with the experimental value where no growth was observed beyond 50 mgl^{-1}. Anaerobic propionate fed enrichment culture showed significantly lower K_{il} value of 68 mgl^{-1}, 59 mgl^{-1} and 35 mgl^{-1} for 2-chlorophenol, 4-chlorophenol and 2,4-dichlorophenol respectively (Jin and Bhattacharya, 1997) as compared to *Rhocdococcus* sp. M1. Higher the K_{il} value, less sensitive is the culture to substrate inhibition (Shim and Yang, 1999). Hence in the

present study low K_{ii} for 2,4-dichlorophenol as compared to 2-chlorophenol and 4-chlorophenol indicated that 2,4-dichlorophenol was more toxic than the monochlorophenolic isomers.

5.6 Degradation of mixed substrate by *Rhodococcus* sp. M1 using 2-chlorophenol, phenol and p-cresol

In majority of the industrial effluents and bioremediation sites organic pollutants are found as mixtures, making it important to understand the growth behavior and degradation pattern of a microbial species on mixture of organic substrates. Industrial synthesis of chlorophenolic compounds involves the chlorination of phenol, hence the wastewater will often contain phenol along with chlorinated phenols (Gu and Korus, 1995; Hill *et al.*, 1996). Effluent from the herbicide manufacturing plant was reported to contain phenol and cresols along with chlorophenol (Bond and Straub, 1973, Hinteregger *et al.*, 1992; Menke and Rehm, 1992). Efficient removal of the waste, containing mixture of phenol and cresol along with chlorophenols demands the study of the degradation pattern of the mixed substrate by *Rhodococcus* sp. M1.

Degradation of chlorophenol in mixed substrate form have been investigated by other workers and showed preferential or diauxie as well as a simultaneous pattern of utilization. Preferential utilization was observed for the mixture of chlorophenols consisting of 2-chlorophenol, 4-chlorophenol; 2-chlorophenol and phenol by *Alcaligenes* sp. A7-2 (Menke and Rehm, 1992) and also for the mixture of pentachlorophenol and phenol by enrichment culture (Klecka and Maier, 1988). In the

first case 4-chlorophenol was the preferred substrate whereas phenol was completely exhausted before 2-chlorophenol in the second case. In the mixture of pentachlorophenol and phenol, pentachlorophenol was utilized as the preferred substrate. Simultaneous utilization pattern was reported for a mixture of chlorophenols having dichlorophenol, trichlorophenol and pentachlorophenol by a *Flavobacterium* sp. (Gu and Korus, 1995) and for the mixture of pentachlorophenol and 2,4,5-trichlorophenol by enrichment culture (Klecka and Maier, 1988).

The present study showed a unique trend of preferential but simultaneous utilization of the substrates. In sequential or diauxic growth, adaptation to the less preferred carbon source is completely prevented in the presence of the preferred compound. However in the degradation of binary mixture of 2-chlorophenol (100 mgl^{-1}) and phenol (50 mgl^{-1}), though phenol was the preferred substrate but 2-chlorophenol was observed to be simultaneously removed (Figure 19 and 20). Phenol was removed in the first 48 h and 2-chlorophenol was simultaneously removed and showed concentration of 53.2 mgl^{-1}after 48 h. However, chloride released (5.4 mgl^{-1}) was significantly lower than the theoretical value expected for the amount of 2-chlorophenol removed (Figure 20). Lower chloride released and high residual COD of 139.2 mg l^{-1} after 48 h (Figure 21) indicate 2-chlorophenol to be presumably undergoing transformation or cometabolized along with phenol. Cometabolic removal of 2,4,6-trichlorophenol with phenol as the growth substrate was reported with *Pseudomonas* sp. (Wang *et al.* 2000). Cometabolism of 4-chlorophenol with phenol as the growth substrate was reported for *Alcaligenes eutrophus* (Hill *et al.* 1996). Transformation of 4-chlorophenol was also observed by resting, phenol induced

Acinetobacter culture (Hao *et al.*, 2002). Although in first 48 h, though 2-chlorophenol was simultaneously utilized but phenol was presumed to be the primary substrate. Shift of the substrate from phenol to 2-chlorophenol when the former was completely utilized could have possibly resulted in an intermediate lag. Hence diauxic growth pattern as observed reflect preferential utilization of phenol over 2-chlorophenol (Figure 20).

Similar pattern of degradation was observed with the mixture of 2-chlorophenol (100 mgl^{-1}) and p-cresol (50 mgl^{-1}) where p-cresol was the favoured substrate (Figure 23 and 24). Complete removal of p-cresol was observed in 36 h. 2-chlorophenol was simultaneously degraded to show removal of 58% in 36 h. Chloride value of 11.5 mgl^{-1} and residual soluble COD of 100.92 mg l^{-1} (Figure 25) in this case indicated transformation or cometabolization of 2-chlorophenol along with p-cresol. Growth curve however in this case is not biphasic as it gives a singular peak after 48 h when both the substrates are utilized (Figure 24). Gradual increase in growth was observed up to 42 h after which there was a sharp increase to give a pronounced peak at 48 h.

In the multiple substrate utilization of 2-chlorophenol, phenol and p-cresol, phenol was the preferred substrate to be removed first (Figure 26 and 27). Complete removal of phenol after 72 h was followed by the degradation of p-cresol and 2-chlorophenol in 96 h and 120 h respectively. Delayed degradation of 2-chlorophenol compared to phenol and p-cresol could be due to the electron withdrawing effect of the chlorosubstituent, which deactivates electrophilic substitution (Menke and Rehm, 1992). In this case also 2-chlorophenol showed simultaneous removal along with phenol and p-cresol. The degradation of 2-chlorophenol however was not supported by

release of chloride. Release of chloride was observed only after 72 h of growth. High value of residual soluble COD of 145.25 mg l^{-1} (Figure 28) and no release of chloride after 72 h when phenol was completely removed again suggested transformation or cometabolism of 2-chlorophenol and not complete removal. Residual soluble COD was reported to be negligible after the complete removal of 2-chlorophenol in 120 h. In this case also *Rhodococcus* sp. M1 showed a biphasic growth pattern giving the first peak after 96 h when both phenol and p-cresol were completely utilized after which the culture had a decreased growth followed by an increase in growth resulting into another peak after 120 h (Figure 27). In this case shift in substrate to 2-chlorophenol after complete utilization of phenol and p-cresol resulted in negative growth.

Growth on binary substrate mixtures of 2-chlorophenol with phenol and 2-chlorophenol with p-cresol started with no apparent lag phase. However, growth on the substrate mixtures consisting of 2-chlorophenol, phenol and p-cresol was initiated after a lag of 12 h presumably because of substrate toxicity. Specific growth rate of *Rhodococcus* sp. M1 showed a decrease when grown on binary or tertiary mixture of 2-chlorophenol, phenol and p-cresol as compared to their growth rates when these substrates were used singly (Table 29).

Further lower rate of degradation of the substrates (2-chlorophenol, phenol and p-cresol) was observed when utilized as mixed substrate as compared to their degradation as single substrate (Table 28). Similar observation was made with mixture of benzene and toluene where the rate of degradation of either benzene or toluene in the presence of the other was slower than the degradation rate of either substrate alone (Chang *et* al., 1993). In another study by Deeb. and Alvarez-Cohen. (!999), substrate

degradation patterns by a microbial consortium over a range of BTEX (benzene, toluene, ethylbenzene and xylene) concentrations for individual aromatics were found to differ significantly from patterns for aromatics in mixture. In this case, presence of toluene, benzene or ethylbenzene had a negative effect on xylene degradation rate. Substrate interaction in the present case thus can be considered inhibitory to each other as degradation rate of the individual substrates decreases. Rate of degradation of 2-chlorophenol was less in tertiary mixture with phenol and p-cresol (0.33 mg l^{-1} h^{-1}) and in binary mixture with phenol (1.04 mg l^{-1} h^{-1}) as compared to binary mixture with p-cresol (2.08 mg l^{-1} h^{-1}). Thus 2-chlorophenol in tertiary mixture with phenol and p-cresol and binary mixture with phenol was presumed to be more toxic to the organism as compared to the mixture with p-cresol. A number of studies have demonstrated that closely related chemical compounds are more likely to interfere with each other's metabolism when present as mixed substrate (Halter *et al.* 1978). In the present study, it can be presumed from the decreased degradation rates that 2-chlorophenol, phenol and p-cresol interfere with each other's metabolism when present in mixed substrate form causing significant enhancement in time needed for degradation and consequently lower rate of degradation of these substrates.

5.7 Degradation of phenolics and 2-chlorophenol by mixed culture of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1

Rhodococci in bioremediation sites or when used in waste water treatment systems are expected to coexist with other microorganism and compete with other microbial populations having similar degradation capability. Hence the virtual

application of the *Rhodococcal* culture for chlorophenol and phenolics degradation in contaminated sites and wastewater treatment demands a mixed culture study with another bacterial culture. This is to evaluate the eventual fate of the *Rhodococcal* population in presence of another competing bacterial culture having similar degradative ability. *Pseudomonas* being the most widely present in the environment may dominate in the biological treatment systems, hence the same was considered suitable and selected for the present study. A *Pseudomonas* sp. was isolated from soil and was designated as P1. It was identified as *Pseudomonas fluorescens* based as per the criteria as described in Bergeys Manual of Systematic Bacteriology (1989) that was based on colony characteristics, cellular morphology (Table 30), biochemical characteristics (Table 31), sugar utilization (Table 32) and mol% G+C of the DNA. Interaction of the *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 populations were studied when grown on 2-chlorophenol, phenol and p-cresol. The individual populations were enumerated by plate count method. *Rhodococcus* sp M1 showing positive growth on acetamide was enumerated by plating on acetamide agar. *Pseudomonas fluorescens* P1 showing negative growth on acetamide (Bergey's manual of systematic bacteriology, 1989) was evaluated by deducting the *Rhodococcal* count from total count on nutrient agar plate.

Degradation of chlorophenol and phenolics by mixed microbial community has been reported by several groups (Flyubjerg et al. 1993; Puhakka et al. 1995; Caldeira et al. 1999; Farrell and Quilty 1999). Coculture studies with *Rhodococcus* sp. and *Pseudomonas* sp. has been reported for growth on crude oil (Hamme and Ward 2001). However the interactive behaviour between the constituent microbial species during

coculture has been scarcely reported. Types of the interactions among the microbial populations may be categorized as neutral, positive or negative (Stanier et al. 1986). In the present study the interaction between the *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 when grown on 2-chlorophenol and phenol could be considered as of negative - competitive type, since in both the cases, population of one organism dominates over the other. However, growth of the mixed culture on p-cresol showed a neutral type of interaction since the growth behaviour of both the cultures was almost similar.

Specific growth rate of *Pseudomonas fluorescens* P1 on 2-chlorophenol was lower (0.05 h^{-1}) as compared to *Rhodococcus* sp. M1 (0.211h^{-1}) (Table 37). However, in coculture state, with 2-chlorophenol as the substrate *Pseudomonas fluorescens* P1 tends to overtake *Rhodococcus* sp. M1. The heterotrophic bacteria *Klebsiella pneumoniae* though showed growth rate twice that of *Pseudomonas aeruginosa,* was shown to coexist stably in laboratory biofilm due to spatial distribution (Stewart *et al.* 1997). The degradation rate of 2-chlorophenol also decreased significantly from 9.8 mg l^{-1} h^{-1} when degraded by pure culture of *Rhodococcus* sp. M1 to 1.73 mg l^{-1} h^{-1} by mixed cultures (Table 36). Degradation rate of 2-chlorophenol by mixed cultures and *Pseudomonas fluorescens* P1 almost showed a similar value of 1.73 mg l^{-1} h^{-1} and 1.92 mg l^{-1} h^{-1} respectively (Table 36). *Rhodococcus* sp. M1 at the end of 48 h, when 2-chlorophenol was completely utilized although showed a lower value of $5x10^5$ CFU/ml as compared to *Pseudomonas fluorescens* P1 ($1.1x10^7$ CFU/ml), but was not completely eliminated by *Pseudomonas* overgrowth (Table 33).

Phenol at 100 mg l^{-1} was degraded in 24 h by *Rhodococcus* sp. M1 (Figure 31) whereas similar concentration of phenol was degraded in 30 h by *Pseudomonas fluorescens* P1 (Figure 32). A coculture of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 degraded the same substrate in lesser time period as compared to degradation by individual culture (Figure 33). Rate of degradation of phenol by mixed cultures was higher as compared to degradation rate by pure cultures of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 (Table 36).

Growth on phenol showed *Rhodococcus* sp. M1 to be the dominant species. Simultaneous increase in both the populations was observed at the initial stage of substrate utilization (Table 34). This resulted in the degradation of 50% of the substrate in 8 h and the total count of both the cultures when computed was found to be similar at this stage. This however was followed by gradual increase in the *Rhodococcal* population as compared to *Pseudomonas* and subsequently became the dominant population. *Rhodococcus* sp. M1 showed a higher count of $1.43x10^7$ CFU/ml as compared to *Pseudomonas fluorescens* P1 showing a count of $2.8x10^5$ CFU/ml when the substrate is completely degraded after 20 h. Slightly higher specific growth rate of 0.146 h^{-1} for pure culture of *Rhodococcus* sp. M1on phenol as compared to growth rate of 0.123 h^{-1} for *Pseudomonas fluorescens* P1 further supported these observations (Table 37).

Rhodococcus sp. M1 and *Pseudomonas fluorescens* P1 was shown to degrade 100 mg l^{-1} of p-cresol in 20 h (Figure 34) and 24 h (Figure 35) respectively. Degradation of similar concentration of p-cresol was however achieved in 16 h when mixed culture of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 was used

(Figure 36). The decreased period of degradation could possibly result due to similar growth rates of 0.14 h^{-1} for *Rhodococcus* sp. M1 and 0.135 h^{-1} for *Pseudomonas fluorescens* P1 on p-cresol thus indicating neutral type of interaction between these cultures (Table 37). Similar total count of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 was observed on p-cresol after 16 h of incubation (Table 35).

5.8 Biological treatment of monochlorophenols in a trickling filter reactor

Studies were carried out by immobilizing the microorganisms in the form of a fixed film on gravel medium in a trickling filter reactor, with the aim of developing a biological system for treatment of industrial wastes containing chlorophenolic compounds. Degradation of monochlorophenols (2-chlorophenol and 4-chlorophenol) was studied in the laboratory model of a trickling filter reactor (Plate 4). The seed culture for the reactor i.e. *Rhodococcus* sp. M1 used in the present study is characterized by flocculating growth and hence showed a high settleability. Considering the high settleability of the seed cultures, suspended growth system can be considered as to be more suitable option than fixed film reactor. However, high aeration in the aerobic suspended growth reactor causes stripping of the monochlorophenols due to the volatile nature of the compound. Hence, fixed film reactor was selected for the treatment of monochlorophenol. Monochlorophenolic isomers (2-chlorophenol and 4-chlorophenol) were specifically selected for the present study as they showed a high extent of degradation as compared to 2,4-dichlorophenol and 2,4,6-trichlorophenol in the batch culture studies.

The organic loading rate and hydraulic loading rate of the trickling filter reactor was optimized for the effective treatment of the monochlorophenolic synthetic waste. The reactor was shown to treat 2-chlorophenol for higher organic loading rate of 0.259 g COD/l-day as compared to 4-chlorphenol of 0.129 g COD/l-day (Figure 37) at an optimum flow rate of 30 ml/min (Figure 38). The results of bench scale studies is thus in confirmation with batch studies where 4-chlorophenol was degraded for lower concentration of substrate as compared to 2-chlorophenol. The performance of the reactor was then evaluated for both 2-chlorophenol (Table 38) and 4-chlorophenol (Table 39) individually at optimum organic loading and hydraulic loading rate and showed more then 90% removal of the substrates. The high rate of substrate removal is also supported by 84.6% reduction in COD for 2-chlorophenol (Table 38) and 77.5% reduction in COD for 4-chlorophenol (Table 39). Release of more than 90% of theoretical amount of chloride for both 2-chlorophenol and 4-chlorophenol further supports complete removal of these substrates. High suspended solids in the effluent are one of the primary disadvantages of trickling filter system. However, in the present study, suspended solids in the effluent of both 2-chlorophenol and 4-chlorophenol is 10 $mg\ l^{-1}$.

Treatment of chlorophenolic waste by fixed film system has been reported by very few researchers. However, most of the studies are with higher chlorophenols such as pentachlorophenol. A study by Westmeier and Rehm (1987) showed treatment of municipal water contaminated with 4-chlorophenol in a packed bed fermentor using lecaton as the support material. Decontamination rate was 300 µmol/l/h using *Alcaligenes* sp. A7-2 as the seed culture.

SUMMARY AND CONCLUSION

Isolation of microorganisms belonging to the *Rhodococcus* sp. was carried out from varied sources in M3 medium specified for *Rhodococcus*. Sources identified for the isolation of *Rhodococcus* sp. were garden soil, oil-contaminated soil, compost and pond sediment. Enrichment cultures in M3 medium were subjected to 2-chlorophenol acclimatization by gradual replacement of sodium propionate with 2-chlorophenol. Five isolates were obtained from 2-chlorophenol acclimatized enrichment sample from the sources selected and were subjected to identification. The isolates were identified as *Rhodococcus* sp. on the basis of colony, cell morphological, physiological and biochemical characterization.

The five isolates identified as *Rhodococcus* sp. were designated as M1, M2, M3, M4, M5 and screened for their ability to degrade different chlorophenols (2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol), phenol and cresols (ortho, meta and para). Of the five isolates screened, the culture M1 isolated from garden soil showed maximum extent of degradation for wider range of phenolics (phenol, cresols) and chlorophenols (2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol). Thus the isolate M1 denoted as *Rhodococcus* sp. M1 was taken up for further studies.

Degradation of 2-chlorophenol by *Rhodococcus* sp. M1 was observed to commence after a lag period of 5 days and is completely removed after 45 days at an initial substrate concentration of 100 mg l^{-1}. To reduce the lag period for the

degradation of 2-chlorophenol, the cells of *Rhodococcus* sp. M1 were grown on different inducers such as toluene, benzoic acid, phenol and catechol. Benzoic acid induced cells degraded 2-chlorophenol (100 mg l^{-1}) completely in 48 h, whereas toluene induced cells took 30 days and phenol and catechol induced cells took more than 30 days for the degradation of similar concentration of 2-chlorophenol. Benzoic acid induced culture of *Rhodococcus* sp. M1 acclimatized to 2-chlorophenol was thus used for the studies on optimization of pH and temperature as well as degradation kinetics of chlorophenols.

Rhodococcus sp. M1 was able to grow and degrade 2-chlorophenol in the temperature range of 10 to 40°C however, the optimum temperature was observed to fall between 32 to 35°C. Study of the pH revealed the ability of *Rhodococcus* sp. M1 to grow up to pH 11.5. In the acidic range, growth and degradation was not observed below pH of 6.5. Optimum pH for *Rhodococcus* sp. M1 for 2-chlorophenol degradation was observed to be in the range of pH 7.0 to 8.0.

Rhodococcus sp. M1 was studied for the degradation of 2-chlorophenol (10 to 400 mg l^{-1}), 4-chlorophenol (10 to 200 mg l^{-1}), 2,4-dichlorophenol (10 to 100 mg l^{-1}) and 2,4,6-trichlorophenol (10 to 50 mg l^{-1}). 2-chlorophenol could be degraded up to a concentration of 300 mg/l. However, maximum rate of degradation was observed at 100 mg l^{-1} and no growth was observed at 400 mg l^{-1}. Degradation of 4-chlorophenol was observed up to 150 mg l^{-1} with prolonged lag phase and increased period of degradation. With 2,4-chlorophenol and 2,4,6-trichlorophenol degradation was achieved up to 50 mg l^{-1} and 25 mg l^{-1} respectively.

With the increase in initial substrate concentration, the specific growth rate and substrate uptake rate was observed to decrease for all the chlorophenols used in the study. Specific growth rate (μ) was in the range of 0.221 to 0.043 h^{-1} for 2-chlorophenol and maximum specific growth rate was evaluated to be 0.248 h^{-1}. Specific growth rate showed a lower value for 4-chlorophenol as compared to 2-chlorophenol and was observed to be in the range 0.0481 to 0.024 h^{-1} and μ_{max} value was calculated to be 0.0493 h^{-1}. Specific growth rate value shows further decline for 2,4-dichlorophenol and falls in the range of 0.022 to 0.01 h^{-1} with a μ_{max} value of 0.0244 h^{-1}. Substrate uptake rate (SUR) for 2-chlorophenol was computed to be in the range of 0.267 to 0.059 mg/mg.h. The SUR for 4-chlorophenol, was observed to be in the range of 0.1361 to 0.059 mg/mg.h. Substrate uptake rate further decreased for 2,4-dichloropohenol and was observed to be in the range 0.082 to 0.024 mg/mg.h.

Degradation of 2-chlorophenol was studied in mixture with phenol and cresol by *Rhodococcus* sp. M1. Degradation of the binary mixture of 2-chlorophenol at an initial concentration of 100 mg l^{-1} and phenol at a concentration of 50 mg l^{-1} showed simultaneous utilization of the substrates. However, phenol was preferentially removed in 48 h. Gradual reduction in the concentration of 2-chlorophenol was observed and was completely removed after 96 h. Degradation of the mixture of 2-chlorophenol (100 mg l^{-1}) and p-cresol (50 mg l^{-1}) showed similar degradation pattern. Both the substrates were simultaneously utilized but p-cresol was preferentially utilized in first 36 h followed by removal of chlorophenol in 48 h. Degradation of mixture of 2-chlorophenol, phenol and p-cresol at an initial concentration of 50 mg l^{-1} for each substrate was studied. Rate of degradation for all the substrates was observed to

increase compared to their degradation, when present as binary substrate mixture or in pure substrate form. Phenol, p-cresol and 2-chlorophenol were observed to be simultaneously reduced. However, phenol was observed to be the most preferred substrate as it was completely utilized in 76 h followed by the degradation of p-cresol after 96 h. Complete removal of 2-chlorophenol was observed after 120 h.

Degradation of 2-chlorophenol, phenol and p-cresol at an initial concentration of 100 mg l^{-1} for each of the substrates was investigated by *Rhodococcus* sp. M1 in coculture. For this study, a *Pseudomonas* sp. was isolated, identified as *Pseudomonas fluorescens* and was designated as *Pseudomonas fluorescens* P1. A coculture of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 was used as inoculum for the degradation of 2-chlorophenol, phenol and p-cresol. Complete degradation of 2-chlorophenol by mixed culture, showed higher cell count for *Pseudomonas fluorescens* P1 ($1.1x10^7$) as compared to *Rhodococcus* sp. M1 ($5x10^5$) thus indicating *Pseudomonas fluorescens* P1 to be the dominant population. The trend was observed to be the reverse for the degradation of phenol by the mixed culture. Complete removal of phenol showed a higher cell count ($1.43x10^7$) for *Rhodococcus* sp. M1 compared to *Pseudomonas fluorescens* P1 ($2.8x10^5$) in the mixed culture. Complete degradation of p-cresol showed similar CFU/ml value for both the populations. *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 showed a cell count of $6.6x10^6$ and $5.6x10^6$ for the degradation of p-cresol thus indicating neutral interaction between both the populations.

Degradation of monochlrophenolic isomers (2-chlorophenol / 4-chlorophenol) was studied in a fixed film system by immobilizing the chlorophenol acclimatized

Rhodococcus sp. M1 on the gravel medium in a trickling filter reactor. Synthetic wastewater fed in a trickling filter reactor was composed of monochlorophenols (2-chlorophenol / 4-chlorophenol), phosphate and nitrate. Initially experiments were carried out with 2-chlorophenol in the waste water which was followed by 4-chlorophenol. Organic loading and hydraulic loading was varied for both 2-chlorophenol and 4-chlorophenol in order to achieve the optimum. Results indicated that the optimal organic loading for 2-chlorophenol and 4-chlorophenol was 0.259 g COD/l-day and 0.129 g COD/l-day respectively. Optimization of hydraulic loading showed an optimum flow rate of 30 ml/min for efficient removal of the chlorophenols treated. The trickling filter reactor was then run at optimum organic and hydraulic loading conditions for both 2-chlorophenol and 4-chlorophenol individually and physicochemical parameters such as suspended solids, chloride, chemical oxygen demand (COD) and pH in the effluent was monitored along with substrate concentration. Efficient removal of both 2-chlorophenol and 4-chlorophenol was supported by release of chloride and reduction in COD.

The significant findings of the present study could be briefly summarized as follows:

(i) A potent naturally occurring strain of *Rhodococcus* sp. M1 was isolated from garden soil with the capability of degrading a wide range of phenolics (phenol, o-cresol, m-cresol and p-cresol) and chlorophenols (2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol). The culture was shown to grow in high pH and is thus suitable to be used for alkaline waste.

(ii) To improve the degradation of 2-chlorophenol and subsequently other chlorophenols, the culture was induced with toluene, benzoic acid, phenol and catechol. Benzoic acid induced cells showed the maximum extent of degradation in remarkably less time period.

(iii) Microrganisms for degradation of chlorophenols so far reported showed long lag periods and low degradation rates. Also chlorophenols in most of the cases is not completely degraded but only dechlorinated. In the present study, the benzoate induced culture of *Rhodococcus* sp. M1 showed complete degradation of monochlorophenols (2-chlorophenol and 4-chlorophenol), 2,4-dichlorophenol and 2,4,6-trichlorophenol in significantly reduced time with negligible lag.

(iv) Evaluation of kinetic parameters for the degradation of a pollutant is essential for the design of a biological treatment system. Scanty reports are available on the kinetics of chlorophenol degradation. In the present study, the kinetic parameters for the degradation of monochlorophenol (2-chlorophenol, 4-chlorophenol) and 2,4-dichlorophenol were evaluated. *Rhodococcus* sp. M1 demonstrated inhibitory kinetics for 2-chlorophenol, 4-chlorophenol and 2,4-dichlorophenol degradation.

(v) Chlorophenol in wastewater is generally present along with phenol and cresols. Hence a degradation study of the mixed substrate consisting of 2-chlorophenol, phenol and p-cresol was carried out to observe the degradation pattern. Simultaneous but preferential pattern of utilization of the substrates was observed. Competitive inhibition pattern of substrate utilization was evident in

the present case from the decrease in the degradation rate for all the substrates in mixture compared to their degradation as pure substrates.

(vi) For the application of the *Rhodococcus* sp. M1 in biological treatment system, the interaction of the culture with a fast growing bacterial species like *Pseudomonas* sp. having similar degradative ability was studied. Interaction between the populations of *Rhodococcus* sp. M1 and *Pseudomonas fluorescens* P1 was studied for the degradation of 2-chlorophenol, phenol and p-cresol. Competitive type of interaction between the cultures was observed when grown on 2-chlorophenol and phenol. Growth on p-cresol showed neutral type of interactions.

(vii) Biological degradation study of monochlorophenols using a trickling filter reactor revealed that such a fixed film system for the treatment of industrial waste containing chlorophenols can be developed and successfully operated using *Rhodococcus* sp. M1 as an initial seed for developing microbial film on the gravel medium in the trickling filter reactor.

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*Due to non-availability of literature and some patented information, various review articles were referred to seek the information and used as cross references.

RESEARCH PAPERS ACCEPTED/COMMUNICATED AND RESEARCH ABSTARCT PRESENTED IN CONFERENCES

1. **M. Goswami**, N. Shivaraman, R. P. Singh (2002). Kinetics of chlorophenol degradation by benzoate-induced culture of *Rhodococcus erythropolis* M1. *World Journ of Microbiol and Biotechnol.* (Accepted)

2. **M. Goswami,** N. Shivaraman, R. P. Singh (2002). Microbial degradation of 2-chlorophenol, phenol and p-cresol as mixed substrate by *Rhodococcus erythropolis* M1. *Water Res.* (Communicated)

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