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STUDIES ON MICROBIAL CONSORTIA DEGRADING PHENOL IN AQUEOUS PHASE

THESIS SUBMITTED
TO THE UNIVERSITY OF KERALA
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY IN MICROBIOLOGY

BY

AMBUJOM S.

BIOCHEMICAL PROCESSING & WASTEWATER TECHNOLOGY
REGIONAL RESEARCH LABORATORY, CSIR
THIRUVANANTHAPURAM-695 019
INDIA

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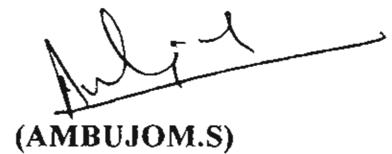
CERTIFICATE

Certified that the work presented in this thesis entitled **"Studies on Microbial Consortia Degrading Phenol in Aqueous Phase"** submitted herewtih by **Ms. Ambujom.S** for the award of **Doctor of Philosophy in Microbiology** of the University of Kerala is an authentic scientific record of the research work carried out by her under my guidance and supervision, and no part thereof has been submitted for any degree or award.

(Dr. V.B. Manilal)
Supervising Guide

DECLARATION

I hereby declare that the matter inculded in this thesis is the result of the investigations carried out by me in the Biochemical Processing & Wastewater Technology Division of Regional Research Laboratory, Trivandrum, under the guidance of Dr .V.B. Manilal and the same has not been submitted elsewhere for a degree or diploma.



(AMBUJOM.S)

Dedicated to Appa, Amma and Radha

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AMBUJOM.S

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ABSTRACT

Microbial consortia have been identified as potent tools for waste treatment including discharges of toxic chemicals. Activated sludge is the most widely used microbial consortium in the wastewater treatment. Knowledge of the factors that determine the growth, stability and performance of the consortium are essential for achieving optimum results on wastewater purification. The present research was focused on gaining more insight on the structure and stability of a phenol degrading consortium which was developed and maintained in a fed-batch reactor by feeding 500 mg phenol/l/day.

Analysis of the consortium had shown that it was consisted of 8 phenol degrading bacteria and 2 non-phenol bacteria which belonged to the genera of *Bacillus*, *Streptomyces*, *Pseudomonas* and *Rhodococcus* with varied phenol degradation and growth in phenol or its derivatives. The best phenol degrading isolates (AS_5) could remove 400 mg phenol during 24 hours, while the slow phenol degrader (AS_7) of the consortium could remove only 120 mg phenol from the above concentration.

The performance of a consortium would, naturally be guided by the functional characteristics of the constituent members. The attempt to study the population of phenol degrading consortium indicated that the individual population were under continuous shift and was not in the same scale. Despite the varied growth characteristics of individual members in the consortium stable performance was exhibited by the consortium.

Significantly higher degradation rate of phenol was observed with the consortium than the individual isolate at varying concentration of phenol from 400 mg/l to 700 mg/l. Compared to pure culture, the consortium was better equipped for degradation of phenol in pure or mixture and under stressed conditions.

The phenol degrading consortium in this study was constituted of a highly heterogeneous population in terms of their morphology (size, shape, pigmentation and gram reaction), biochemistry (presence of ortho and meta pathway for cleavage of phenol), physiology (ability to utilise variety of phenols, cresols, benzenes, catechol and resorcinol) and growth rate. The presence of two non-phenol bacteria in the consortium added more heterogeneity to the system. The stability and degradation rate of the consortium were superior to those of mixed cultures made from less heterogeneic members.

These observations made us to conclude that the stability of a microbial consortium is found to be conformed through the diverse characteristics of its constituent members.

A completely functional and stable consortium could be established slowly in 3 months period by reconstituting all isolates of the consortium. Before it reached its final stage of stability a lot of changes was noticed in the system. However, the exact reason for the delay in attaining the stability was not clear from the reconstitution studies.

CHAPTER 1

INTRODUCTION

The commonly encountered organic pollutants in the environment are benzene, toluene, ethyl benzene, xylene, polycyclic aromatic hydrocarbons, aromatic sulfonates, aromatic amines, azoaromatics, nitroaromatics, phenol and its derivatives, polychlorinated biphenyl's etc. These compounds are mainly originated from industrial operations.

Phenol (Fig. 1.1) and its derivatives are aromatic molecules containing hydroxyl, methyl, amide or sulphonic groups attached to the benzoid ring structure. The origin of phenols in the environment is by both anthropogenic as well as xenobiotic sources. The former are from run-offs of urban areas and from decaying lignocellulosic materials. The xenobiotic sources are industrial wastes derived from petrochemical-oil refining, chemical manufacturing process such as phenol manufacturing plants, polymeric resin producing industry, pharmaceutical industry, wood processing industry, pesticide manufacturing plants, explosive manufacturing, textile and colour industries. The annual world production of phenol alone is around 1.25×10^9 kg (Bechard *et.al.* 1990).

Major sources of phenols and related aromatics from the industrial sources are given in Table 1.

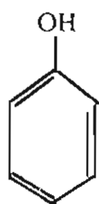


Fig.1.1 Phenol

Table 1.1 Sources of phenols and related aromatic compounds in wastewater (Kumaran and Paruchuri, 1997)

Source	Siginificant organic compounds	Form
Petroleum refining	Hydrocarbons (alkanes, cycloalkanes, polyaromatic hydrocarbons), benzenes, substituted benzenes, toluene, n-octanes, n-decanes, naphthalenes, biphenyls and phenol	Solid, liquid & particulate
Petrochemicals	Naphthalene, heptanes, benzenes, butadiene, phenol and resorcinol	Aqueous
Basic organic chemical manufacture	M-aminophenol, resorcinol, dintrophenol, p-nitrophenol, 3-nitrophenol, benzene, sulphonic acids, anniline, chlorobenzenes and toluene	Soild & particulate
Coal refining	Phenol, catechol, o-,m-, p-cresols, resorcinol, hydroquinone, pyrogallol, polyaromatic hydrocarbons, pyridines, pycolines, lutidines, xylenes and toluenes	Liquid & aqueous suspension
Pharmaceuticals	Toluenes, benzyl alcohols, phenyl acetic acid, chlorinated products of benzene, chloroform, ether and ethyl alcohol	Liquid
Tannery	Tannin, catechin, phenol, chlorophenol and nitrophenol	Aqueous
Pulp and paper mills	Lignin, vanillic acid, dehydrodivanillin, ferulic acid, cinnamic acid, synringic acid, vieratric acid, protocatechuric acid, gentistic acid, benzoic acid, guadiachols, catechol, coniferylalcohol, dehydro-diconiferyl alcohol, phenylpropionic acid, phenols and chlorophenols	Liquid & aqueous suspension

Phenol, also known as carbolic acid or hydroxy benzene and cresols are antiseptic agents used in surgery in mid 1800's. Later studies revealed that phenol is a toxic and malodorous substance having a wide range of adverse health effects on higher organisms. Hence, phenol and its derivatives are classified as priority pollutants by the U.S. Environmental Protection Agency and were designated as poison B.

Phenol poisons through inhalation or skin adsorption and ingestion. Continuous ingestion of phenol for a prolonged time causes mouth sore, diarrhoea, excretion of dark urine and impaired vision at concentration levels ranging between 10 and 240 mg/l (Barker *et.al.* 1978). Lethal blood concentration for phenol is of the order of 4.7 to 130 mg/100ml. Phenols are toxic to several biochemical functions (Kumaran, 1986). Even the low phenol concentration of 5 μg per litre can impart typical smell upon chlorination. Hence, W.H.O. has prescribed a concentration of 1μg/l as the permissible limit of phenol for drinking water. Elimination of phenol, thus is a necessity to preserve the environmental quality.

Legal restrictions and high levels of wastewater discharge are among the driving forces behind the development and application of industrial wastewater treatment. Economic considerations frequently dictate the treatment systems to be chosen, although phenol and its derivatives can be treated both by physico-chemical methods and biological processes.

a) Physico-chemical removal of phenol and its derivatives from water

The treatment of phenol containing wastewater to attain a harmless level of 0.5 mg/l is difficult by many physical and chemical methods, because of its high solubility and stability (Zhang , 1980).

Physical treatment of phenol wastewater is done mainly through adsorption, while the chemical processes include oxidation reactions using ozone, ozone/ UV, Ozone/hydrogen peroxide, photolysis, heterogeneous photocatalysis using hydrogen peroxide/UV, wet air oxidation, supercritical water oxidation and electrochemical oxidation.

Sorption method is effectively used to remove phenol and its derivatives from aqueous systems. Sorption of phenol or its chlorinated derivatives on various solid media such as, activated carbon (Streat *et. al.* 1995 and Furya *et. al.* 1996) ,granular and fibrous activated carbon (Brasquet *et. al.* 1996, Mollah and Robinson *et. al.* 1996 (a, b), Juang *et. al.* 1996), graphite carbon black (Kiraly *et.al.* 1996), anion-exchange resin (Lee and Ku, 1996) and spent oil shale (Darwish *et. al.* 1996) have been reported.

Ozonation was found to be successful in destroying phenol and substituted phenols in oil shale wastewater. Ozonation of 2-chlorophenol (2-CP) , 4-chlorophenol (4-CP), 4-chlororesorcinol, 6-chloro-m-cresol and 2,4-dichlorophenol results in the production of quinones, and inorganic chloride was shown by Trapido *et.al.* (1995). End products of the above reaction was found to be toxic to biological organisms (*Daphina magna*). Individual and total phenolic compounds oxidation coefficient was evaluated with a simulated film absorber and an experimental column for the degradation of phenol and its derivatives from oil shale wastewater was demonstrated by Kallas *et. al.* (1995). The ozone/hydrogen peroxide treatment in the degradation of chlorobenzene was resulted in the production of 2- and 4-chlorophenol as first oxidation products (Cortes *et. al.* 1996).

There are many reports on the removal of phenol and its derivatives by photolysis (Castrantas *et.al.* 1990 and Lipcynska-kochany, 1992). Baum and Oppenlander (1995) developed a vacuum UV photoreactor for photomineralization of 2,4-dichlorophenol and other chlorinated organics. Enhancement of photooxidation of methoxy phenol due to naturally occurring organic matter was reported by Canonica and Hogine (1995).

One of the chemical methods for the treatment of phenol and its derivatives in wastewater is by heterogeneous photocatalysis (Okamoto *et.al.* 1985 (a, b), Muneer *et.al.* 1992, D'Oliveria *et.al.* 1993. and Pelizzetti *et.al.*1993). Complete oxidation of phenol and TOC using a photocatalytic membrane, in presence of hydrogen peroxide was studied in both laboratory and pilot scales. The major claim was the advantage of using colloidal TiO_2 suspension for the treatment (Barni *et.al.* 1995 a, b). Normally, degradation of 3-chlorophenols by heterogeneous photocatalysis leads to para-hydroxylated products and chloroquinone. According to Dong *et.al.* (1995) the chlorocatechols formed from 3-chlorophenol by heterogeneous photocatalysis in presence of hydrogen peroxide is less toxic to biological organisms. A small difference in the degradation rates between the isomers during photocatalytic oxidation of chlorophenol was reported by Wang *et.al.* (1995). Photocatalytic degradation of 2,4-dichlorophenol (2,4-DCP) was found to be depended on the pH of the system (Tang and Huang, 1995).

Use of TiO_2 coated fiber-optic reactor for the complete oxidation of 4-chlorophenol (4-CP), pentachlorophenol (PCP), dichloroacetate and oxalate was reported by Peil and Hoffmann, (1995, 1996). Formation and subsequent destruction of polyhydroxylated PCBs during dechlorination of dichlorophenol through photocatalytic treatment were observed by Minero *et.al.* (1995).

Advanced oxidation processes involve the use of photocatalytic TiO_2 to destroy a variety of organic contaminants. Particulate TiO_2 suspensions irradiated with UV light shorter than 385 nm was catalysed autooxidation of 4-CP, by the addition of oxyanion oxidants, for example, chlorite (Martin *et.al.* 1995). Oxidation of four chlorinated phenols, pentachlorophenol (PCP), 2,4-dichlorophenol (2,4-DCP),

3,5-dichlorophenol (3,5-DCP) and 2,3,5-trichlorophenol (2,3,5-TCP) using an aqueous TiO_2 with UV irradiation was resulted in the production of toxic intermediates to *E. coli* in the case of PCP and 2,3,5-TCP (Jardim *et .al.* 1997).

The decomposition of 2-chlorophenol (2-CP) in aqueous solution by UV/TiO_2 has been studied under various pH, light intensities and types of TiO_2. Few hours of illumination in the acidic solution resulted in complete disappearance of the compound (Ku *et.al.* 1996). Haarastick *et.al.* (1996) reported that bicarbonate and chloride were acted as photoinhibitors in the photocatalytic oxidation of 4-chlorophenol and p-toluenesulfonic acid in a fluidized-bed reactor. UV induced peroxidation of phenol to 1,2-benzoquinone and its ring cleavage products (Scheck and Frimmel, 1995) was explained with a model by Shen *et. al.* (1995). Phenolic wastewater treatment with wet air oxidation at low temperature and pressure was observed by the addition of hydrogen peroxide (Lin and Wu, 1996).

Gopalan and Savage (1995) developed a model for the chemical oxidation of phenol through dimerization in super critical water oxidation. In the super critical oxidation of phenol, carbon dioxide yield was found to be enhanced by the addition of metal catalyst (Ding *et. al.* 1995). More than 96% of phenol removal at residence time of 3.38 seconds through supercritical oxidation was reported by Kranjc and Levec (1996). They also observed that removal rate by oxidation was mainly depended on phenol concentration and oxygen availability.

Electrochemical oxidation was able to remove 99 % of initial COD from a phenolic wastewater. Complete removal of phenol was observed at the graphite anode with maximum current efficiency (Kannan *et.al.* 1995). Complete oxidative degradation of 4-chlorophenol (4-CP), 3,4-dichloroaniline and 2,4,6-trinitrotoluene

with intense shockwave (160 mm) and ultraviolet radiation in a electrohydraulic discharge process was reported by Willberg *et. al.* (1996).

Adsorption of phenol on granular activated carbon column using an electric current showed that the adsorptive capacity of GAC decreased after both cathodic and anodic treatments. While, under anoxic conditions adsorptive capacity decreased only after anodic treatments (Mehta and Flora, 1997), study of Petrier and Francony (1997) showed that the treatment of both atrazine and pentachlorophenol was more efficient at 500 than at 20 KHz ultrasonic waves.

Horseradish peroxidase was effectively used to catalyse phenol degradation from foundry wastewater (Cooper and Nicell, 1996). This enzymatic method could remove 99 % of 3.5 mM phenol in water.

Though several of the above mentioned methods have been tried for the clean up of phenol and phenolic derivatives in environment, they were found to be expensive and difficult in practising because of the incomplete removal and generation of toxic intermediates (Jardim *et. al.* 1997). Therefore development and design of a simple, less expensive and effective procedure such as biological systems received much attention.

(b) Biological treatment of phenol and phenolic derivatives

Objective of any biological treatment is to remove soluble, or colloidal or suspended biologically transformable organics in water. This is achieved by bringing active micro-organisms in contact with the wastewater. Biological treatments are of two kinds; one is aerobic which works in presence of oxygen while the other is anaerobic treatment in which hydrogen or carbon dioxide act as the electron acceptor. Based on the type of growth pattern aerobic treatment is classified as (1) ***suspended***

growth aerobic treatment i.e. activated sludge, aerated lagoon, etc. and (2) *attached growth aerobic treatment* i.e. trickling filter, rotating biological contactors, aerobic filter etc.

Activated sludge process (ASP) is one of the most widely accepted biological systems for the treatment of phenolic wastes. Activated sludge is a suspension of micro-organisms maintained in a relatively homogenous state by mixing and turbulence through aeration whereby soluble, colloidal and suspended organics are oxidised by the organisms. Micro-organisms make use of organic pollutants for their growth and functioning of cellular process by electron transport mechanisms (Fig.1..2).

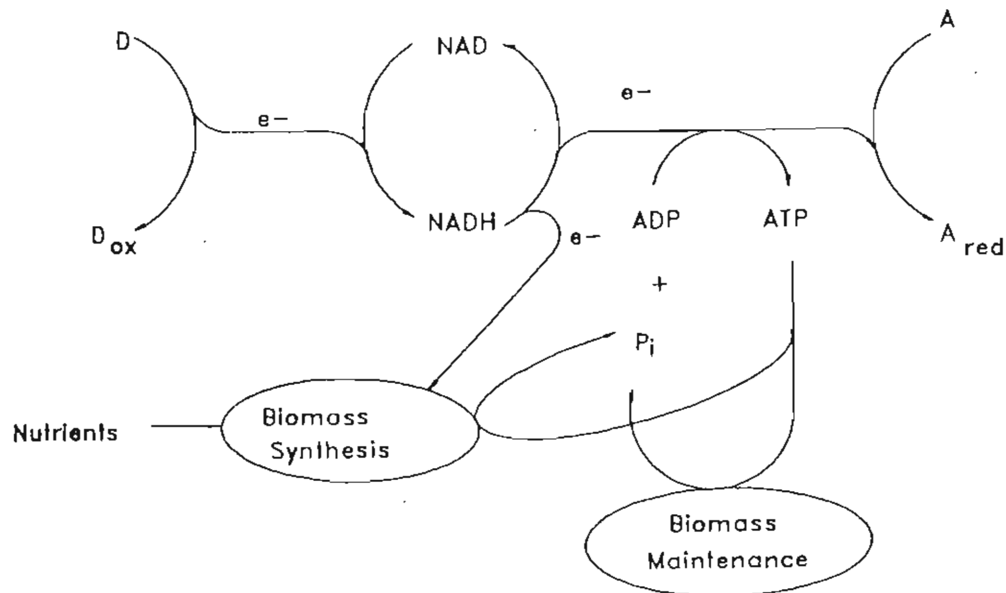
(i) Aerobic degradation of phenol by monoculture

Reports on degradation of aromatic compounds in nature by aerobic process has been documented from the beginning of 20th century. Micro-organisms that degrade phenol were isolated as early as 1908 (Evans, 1947) and biodetoxification of phenol was proven as a viable method for remediating phenol containing wastewater.

Many studies have shown that phenol oxidised completely to carbon dioxide and water by bacteria, fungi, yeast, algae, and water-plants. Aromatic compounds being rich in carbon content once the rings are cleaved by the organisms, the products (organic acids) formed enter into the energy cycle (Fig.1.2)

Concentration of the toxic substrates is one of the major factors affecting the bioremediation. So attempts have been made to isolate and characterise the individual isolates capable of degrading different concentration of phenol and its derivatives. Phenol degradation by pure culture was extensively studied using groups of bacteria belonging to *Pseudomonas, Alcaligenes and Acinetobacter species.* (Bayly and Wigmore, 1973, Piper 1989, Fewson, 1991 and Kiyohara *et.al.* 1992). Among the

a) Energy and electrons flows for the primary substrates



b) Information Flow

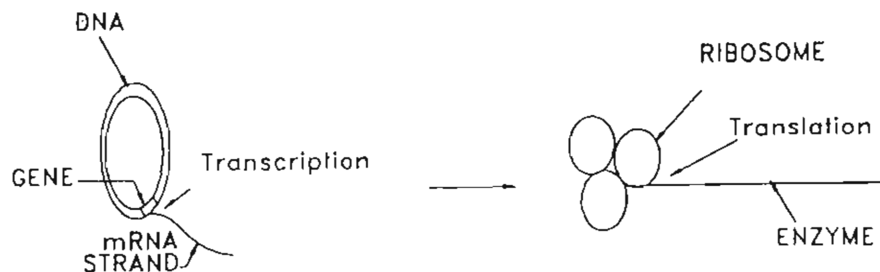


Fig.1.2 Simple representations of electron and energy flows(a) and information flow (b) in a bacterial cell. D- electron donor, D_{ox}-oxidized product from the donor, A - electron acceptor, A_{red} - reduced product the acceptor, e^- - electron, NAD- nicotinamide adenine dinucleotide, NADH - reduced NAD, ADP - adenosine-diphosphate, ATP- adenosine triphosphate, and P_i - inorganic phosphate (Rittmann,1992).

Pseudomonas species, *P. putida* was found to be the most potent to degrade phenol. A psychrotrophic strain *P. putida* QS was able to degrade 200 mg/l of phenol within 59.5 hours at 10° C, while at 30° C the above strain showed less growth and degradation (Kotturi *et. al.* 1991). Continuous culture study of *P. putida* ATCC 17484 revealed that it was able to degrade 3.0 g/l of phenol having dilution rates ranging from 0.074 to 0.085 h^{-1} (Allsop *et. al.* 1993).

Phenol degradation by *Pseudomonas putida* NCIMB 10015 from a biofilter with a maximum load of 133.2 $gm/m^3/h$ of phenol in waste gases was able to degrade 90.8 % of fed phenol (Zilli *et al.* 1993) . In the studies of Ahamad *et. al.* (1996) it is reported that 4 isolates of Pseudomonads, *Pseudomonas* sp. CPC1, *P. aeruginosa* COPC3, *P.aeruginosa* COPC4 and *Pseudomonas sp.* SOPC5 degraded 1000 mg/l of phenol each during 138 hours of incubation, while one of the Pseudomonas species *P.stutzeri* SPC2 could degrade only 500 mg/l of phenol in 138 hours. A phenol adapted Pseudomonas sp. strain CP4 was capable of degrading 470 mg/l of phenol during 12 hours of incubation which was half of the time required by the unadapted cells to degrade the same amount of phenol (Babu *et.al.* 1995).

Semicontinuous and continuous degradation of phenol by *Pseudomonas putida* P8, immobilized on activated carbon was reported by Ehrhardt and Rehm (1989). The above strain was able to degrade 360 mg/l/h. Collins and Daugulis (1997) have reported the removal of high initial concentrations of phenol (4, 10, 28 g/l) in a two-phase organic/aqueous partitioning fermenter inoculated with *Pseudomonas putida* ATCC 11172 in batch and fed-batch conditions. Capacity to hold high rate of phenol (4g/l, 10g/l and 28 g/l) without substrate inhibition is the main advantage of this system. Although polymer entrapped *Pseudomonas* cells were

capable of degrading 3 g/l of phenol as reported by Bettman and Rehm (1985), free cells of *Pseudomonas* were able to degrade only 1 g/l of phenol in 35 hours of incubation. According to Sarnaik and Kanekar (1995), of the four Pseudomonas , degrading methyl violet and phenol from a dye industry effluent , two strains *P. alcaligens*, and *P.putida. biovar B* completely degraded 344 mg/l of phenol within 48 hours of incubation. While, the strain *P.mendocina*, and *P. stutzeri* degraded only 61 and 70% of phenol during 24 and 48 hours respectively. In a biofilm system operated in continuos mode having different ratios of surface, *Pseudomonas putida* ATCC 1172 could degrade phenol at the rate of 0.23 g/l/hr (Molin and Nilsson, 1985).

Another well-studied group of bacteria capable of degrading phenol was *Acinetobacter* sp. Phenol tolerance of *Acinetobacter* species ranging from a concentration of 0.2 to 1.5 g/l was reported by Beveridge and Tall (1969) and D' Aquino *et.al.* (1988).

Boopathy 's work (1995) indicated that *Desulfovibrio* species were able to remove 0.047 mg/l of phenol within 60 hours of incubation.

(i) Degradation of phenol by yeast

Phenol degradation capacity of the yeast species *Rhodotorula sp.* strain was reported by Katayama-Hirayama *et.al.* (1991) and was found to degrade phenol from a concentration of 50 to 1000 mg/l but the growth was decelerated above 250 mg/l of phenol .

(ii) Phenol degradation by fungi

Metabolism of phenol by fungal strains has also received much attention. Jones *et al.* (1995) showed that the degradation of 0.188 mg/l of phenol within 12 hours of incubation using by *Aspergillus fumigatus*. Biodegradation of phenolic

compounds in vinasse using two fungal species *Aspergillus terreus* and *Geotrichum candidum* was reported by Gracia *et. al.* (1997). Approximately 66 % of total phenols and 94 % of ortho phenol were removed by *A. terreus*, while the strain *G. candidum* could correspondingly remove 70 % and 91 % respectively.

(iii) Phenol degradation kinetics

Knowledge of the kinetics of degradation is essential to derive the process variables for the treatment of toxic compounds. Biodegradation kinetics of mixtures containing phenol as the primary substrate and an inhibitory co-metabolite (4-chlorophenol) was studied using *P. putida* PPG4 (ATCC 17453) which could utilise both phenol and 4-chlorophenol simultaneously (Saez and Rittman, 1993). Effect of carbon to nitrogen ratio on the kinetics of phenol degradation by *Acinetobacter johnsonii* was studied by Hoyle *et. al.* (1995). Phenol disappearance and population were studied at two different CN ratios (CN 1.5 and CN 5.60); in this dual-substrate model, a linear growth on phenol was observed during the period of nitrogen availability, and first order metabolism of phenol without growth during the depletion of nitrogen. Influence of superficial gas flow rate on the biofilter performance inoculated with *Pseudomonas putida* was studied using first order substrate elimination kinetics (Zilli *et. al.* 1996). The elimination capacity was strongly depended on the organic load of the filter bed. This biofilter system possessed removal efficiencies (0.86 to 0.90) over wide ranges of phenol concentration upto 750 mg m^{-3} with a superficial gas flow rates ranging between 30 and 92 m^3/h^{-1}. Inhibition kinetics on phenol degradation of *Pseudomonas cepacia* strain was investigated under unstable steady state conditions by Schroder *et. al.* (1997). By controlling a continuously operated bioreactor with an inhibitory substrate (Phenol) at the unstable steady state

in the inhibitory branch (both substrate limitation and under high substrate concentrations) the kinetic inhibition parameters could be estimated under steady state conditions. This was helpful to validate inhibition mechanisms and avoid any discrepancies caused by batch-generated data and their application to continuous culture. Phenol degradation kinetics of *Acinetobacter calcoaceticus* and *Pseudomonas fluroscens* 2218 using Monod's and Haldane equations using steady and unsteady state operations were shown by Kumaran and Paruchuri (1997). Competition for the common substrate as well as non-competitive inhibition by secondary inhibitors were reported as the possible reasons for lower growth. Aerobic degradation kinetics of phenol in biofilm inoculated with *Pseudomonas putida* NCIMB 10015 having thickness ranging from 9 to 90 µm showed that at low flow rates, biomass grew so abundantly and outer active layers were sufficient to remove all the substrate available in the system. While, at high flow rates biomass was unable to withstand substrate overloading and most of the phenol left was undegraded (Converti *et. al.* 1997). Kinetics of phenol biodegradation in the presence of glucose showed that substitutable substrates which are dissimilar in origin and molecular structure may involve in an uncompetitive cross-inhibitory interaction when they are simultaneously removed. When easily degradable substrates may not enhance the per-unit amount of biomass, the compounds were classified as toxic ones (Wang *et. al.* 1996).

(iv) Phenol metabolism

There are many reports on micro-organisms degrading phenol through ortho and meta cleavage pathways. The enzymes involved in both pathways were reported as different in nature and activity. Paller *et.al.* (1995) reported the degradation of phenol using catechol 1,2-dioxygenase in *Acinetobacter calcoaceticus* NCIB 8250 G. An

intradiol ring fission, leading to the dead end product 2-methyl muconolactone during the degradation of meta and ortho cresol metabolism in *Bacillus pumilus* precultured in phenol was reported by Gunther *et. al.* (1995).

Phenol hydroxylase (E.C.14.1.3.7.) catalysed the initial step of microbial phenol mineralization in presence of catechol as source of oxygen. Phenol hydroxylase of the yeast *Trichosporon cutaneum* was purified and its kinetics has been studied in detail (Neujahar and Gaal, 1973). The above was also detected in *Candida tropicalis* and *Candida maltosa* (Neujahar *et. al.* 1974, Krug and Straube, 1986 and Hofmann and Kruger, 1985). Isolation of NADH and NADPH-dependent phenol hydroxylases was partially characterized from a cell free extract of phenol degrading *Brevibacterium fuscum* (Nakagawa and Takeda, 1962), thermotolerant *Bacillus stearothermophilus* (Gurujayalekshmi and Oriel, 1989), soil actinomycete *Streptomyces steonii* (Antai and Crawford, 1983), *Rhodococcus* sp. (Golovle and Eroshina, 1982). The plasmid-encoded phenol hydroxylase from *Pseudomonas* sp. strain CF 600 (Powlowski and shingler, 1990) is a multicomponent enzyme that is generally similar to other multicomponent mono and dioxygenases, whereas cloned enzyme from *Pseudomonas picketti* PK01 is similar to the phenol hydroxylase of *Trichosporon cutaneum* (Kukor and Olsen, 1992).

Both catechol 1,2-dioxygenase and catechol 2, 3-dioxygenase indicating ortho and meta pathways respectively were found to be present in the fungi *Aspergillus fumigatus* degrading phenol (Jones *et. al.* 1995). Reduction of phenol content and toxicity in olive oil mill effluent by ligninolytic white rot fungus *Pleurotus ostreatus* producing phenol oxidases has been reported by Martirani *et.al.* (1996). A fungal peroxidase that degrades phenol has been reported in batch, continuous and

discontinuous semi batch reactors of the fungus *Coprinus macrohizus* (Al Kassim *et.al.* 1994). Degradation of phenol by enzymes similar to phenolic oxidase originated from the root cells of aquatic weed *Eichhorina crassipes* in presence of trace metals, enabled upto 74 % of 200 mg/l of phenol during 24 hours (Nor, 1994). Phenol degradation by an algae *Ochromonas danica* through meta pathway by virtue of the presence of meta cleaving enzymes such as phenol mono-oxygenase, catechol 2,3-dioxygenase, 2-hydroxy (alkyl) muconate semialdehyde dehydrogenase was reported by Semple and Cain (1996).

(v) Genetic approach on phenol degradation

Reports on the genetic component present in the microbial communities responsible for phenol degradation are very limited. Characterisation of a phenol hydroxylase gene *phe*A form *Bacillus stearothermophilus* BR 219 has been reported by Kim and Oriel (1995). Selvaratnam *et. al.* (1995) studied the *in situ* expression of the catabolic gene *dmp* N gene of phenol degrading *Pseudomonas putida* ATCC 11172 in a sequencing batch reactor where the expression of *dmp* N gene, decreased with decrease in the phenol concentration. It was also found that the rate of synthesis of *dmp*N *m*RNA is induced by phenol.

Taylor *et.al.* (1997) reported the distribution of phenol degraders in a biofilm using radio labelled DNA probes (toluene-3-mono-oxygnease (*tbu*) or toluene dioxygnease (*tod*)). Phenol degrading strains in the biofilm found to possess *tod* pathway than the bacteria which is not capable of degrading phenol.

(c) Biodegradation of derivatives of phenol

(i) Halogenated phenols

Among halogenated phenols that cause environmental pollution the chlorophenols rank first. They are produced in a massive scale as pesticides, wood preservatives and as disinfectants (Alexander, 1981 and Renike and Knackmus, 1984).

Fava *et al.* (1995) have reported the aerobic dechlorination and degradation of 2-chlorophenol (2-CP), 3-chlorophenol (3-CP) and 4-chlorophenol (4-CP) by a *Pseudomonas picketti* strain. This strain was capable of dechlorination and degradation of 2-CP, 3-CP and, 4-CP at initial concentrations of 1.51 mmol $^{-1}$, 0.57 mmol $^{-1}$ and 0.75 mmol $^{-1}$ during 30, 30, and 40 hours of incubation respectively. Catabolic effect of vitamins on the aerobic degradation and dechlorination of 2-CP and 4-CP by a *Pseudomonas picketti* strain LD1 and 4-chlorobiphenyl by *Pseudomonas* sp. CPE1 at an initial concentration of 150 mg/l was studied by Kafkewitz *et. al.* (1996). A solution containing biotin, folic acid, pyridoxine hydrochloride, riboflavin, thiamine hydrochloride, niacin, pantothenic acid, cyanocobalamin, p-amino benzoic acid and thioctic acid at a concentration of 600 ppb increased the degradation of the target compound (7.16 %). Kramer and Kory (1987) have isolated two 4-chlorophenol degrading bacteria belonging to *Arthrobacter* sp capable of degrading 100 mg/l of 4-chlorophenol during 24 hours of incubation, at a temperature of 25 ^{0} C and pH below 8.3. A substrate inhibition was observed with this strains at 37 ^{0} C as well as below 25 ^{0} C and at pH lower than 7 or greater than 9. Menke and Rehm (1992) has studied the degradation of mixtures of monochlorophenols and phenol as substrates using free and immobilised cells of *Alcaligenes sp.* Total degradation of 50 mg/l of

4-chloro-2 methyl phenol, 2,4-dichlorophenol and 4-chlorophenol by an activated sludge isolate within 25 hours has been studied by Lechner *et. al.* (1995).

Bae (1996) reported the biodegradation of 4-chlorophenol by a new isolate, *Arthrobacter ureafaciens* CPR706, through an unreported hydroquinone pathway. This culture was able to degrade 4-chlorophenol at a faster rate and to have a much higher substrate tolerance than organisms that degrade 4-chlorophenol through chlorocatechol pathway. An isolate from a wastewater treatment plant, *Pencillium simplicissimum* SK9117, metabolized 3-chlorophenol, 4-chlorophenol, and 4-bromophenol when unsubstituted phenol also was supplied. It was also discussed that the culture could not grow when monochloro- or monobromo-phenols was supplied as the sole carbon and energy source, and there was no release of chloride and bromide ions. The auxenic culture was able to grow and release fluoride ions during the complete mineralization of 3- and 4-flurophenol (Robert *et. al.* 1996).

Kramer and Kory (1992) isolated a para-chlorophenol (p-CP) degrading 2 gram positive *Coryneform* bacteria from a continuous culture system. Each isolate was capable of degrading 100 mg/l of p-CP within 32 and 48 hours of incubation. Valenzuela *et al.* (1997) have reported the complete degradation of 400 mg/l of 2,4-dichlorophenoxyacetic acid and 40-100 mg/l of 2,4,6-trichlorophenol (2,4,6-TCP) by *Alcaligenes eutrophus* JMP134 (pJP_4) isolated from bleached Kraft mill effluent. This strain could degrade higher concentration of 2,4,6-TCP alone and in mixtures containing 2,4-dichlorophenoxyacetate , 4-CP, and 2,4,5-TCP.

A number of bacterial isolates with the ability to degrade PCP and other chlorinated compounds have been isolated from a variety of environments, usually with a history of PCP exposure. Nohynek *et. al.* (1995) described the taxonomy of four

pentachlorophenol (PCP) degrading bacterial strains such as *Arthrobacter sp.* ATCC 33790, *Flavobacterium sp.* ATCC 39723, *Pseudomonas sp.* RA2 and *Sphingomonas chlorophenolica* sp. nov. Resnick and Chapman (1994) have reported in their study that complete degradation of 100 mg/l of pentachlorophenol, 2,3,4-trichlorophenol, 2,4,6-trichlorophenol, 2,3,5,6-tetrachlorophenol and 2,4-dichlorophenol, 2,5-dichlorophenol and 2,6-dichlorophenol using a *Pseudomonas* sp. polyurethane foam has shown enhanced PCP degradation in liquid culture (Briglia *et. al.* 1990). *R.. chlorophenolicus* PCP-1 could also degrade PCP anaerobically in presence of iodosobenzene at a rate similar to aerobic degradation of PCP (Uotila *et. al.* 1992).

Topp *et. al.* (1988) found that 50 mg/l PCP was degraded only after a lag phase of 90 hours when it was introduced as the sole carbon source. *Rhodococcus (Mycobacterium) cholrophenolics* PCP-1 maintained higher viability in soil than in liquid culture and consequently was more effective at mineralising PCP in soil (Middeldrop *et. al.* 1990 and Brigila *et. al.* 1994). *Arthobacter* sp. ATCC 33790 mineralised an influent of 525 mg/l PCP in a chemostat reactor. (Stanlake and Finn, 1982 and Edgehill, 1994). This strain could also remove 85 % of PCP at concentrations between 150 and 200 mg PCP/kg of soil (Edgehill and Finn, 1983). A *Pseudomonas sp.* isolated by Trevors (1982) grew rapidly at 10 mg/l PCP, but was completely inhibited the growth at 75 mg/l. Radehaus and Schmidt (1992) found cells of *Pseudomonas (Sphingomonas) sp.* RA2 to exhibit decreased viability at 200 mg PCP/l. The growth rate was 0.09/h at 40 mg/l, it was reduced to 0.05/h at 150 mg/l.

Aerobic metabolism of pentachlorophenol by spent saw dust culture of shiitake mushroom (*Lentinus edodes*) in soil was reported by Okeke *et.al.*(1993). Utilization

of halogenated benzenes, phenols and benzoates by *Rhodococcus opacus* GM - 14 was reported by Zatisev *et .al.* (1995). This strain had specific growth rates of 0.27 and 0.29 h^{-1} on phenol and 3-chlorophenols respectively. The cell free extracts of a *Flavobacterium sp.* degrading pentachlorophenol has also degraded 3-chlorophenol, 3-bromophenol and 3-iodophenol. The extracts from the PCP induced cells were able to release iodine from 3-iodophenol at the expense of NADPH and oxygen (Xun and Orser 1991).

(ii) Nitrophenols

Nitroaromatic compounds are found in wastewaters of industries manufacturing dyes, explosives, and other chemicals etc. The compound, 2,4-dinitrophenol (2,4-DNP) is an especially interesting one as it has been shown to be mineralised by some bacteria in pure cultures (Lenke *et .al.* 1992, Hess *et. al.* 1993 and Lenke and Knackmuss, 1996). Two *Rhodococcus erthyropolis* strains from soil and river water were shown to use 92 mg/l of 2,4-DNP as sole carbon and energy source (Lenke *et .al.* 1992). A nitrophenol degrading sulfate reducer was isolated from swine manure by Boopathy (1995). This strain utilised both phenol and p-chlorophenol, but failed to utilise the phenolic compounds with two or more chlorine atoms as it was lacking enzymes to dechlorinate the poly-chlorophenols. The concentration upto 150 mg/l of para nitrophenol (PNP) was degraded by *Corneybacterium* sp. strain 8/3 isolated from soil as reported by Horakov and Kotouckova (1996). This strain required to be pre-incubated at 50 mg PNP for 72 hours to achieve growth which was partially suppressed at high concentration of PNP. Phenol and glucose addition did not show any effect on the degradation of PNP by a *Corynebacterium* strain Z4. While addition of 100 mg/ml of glucose inhibited PNP

degradation by *Pseudomonas* MS, and phenol addition enhanced the PNP degradation by *Pseudomonas strain* GR in waste water (Zaidi and Mehta, 1995).

Major limitations of pure culture systems in biodegradation are the incomplete degradation of toxic compounds, difficulty in the maintenance of sterility and change in expressions due to mutation. So the use of mixed cultures degrading toxic compounds have become practical and easiest in waste treatment applications.

(d) Mixed Culutre / Consortia

Complete mineralization of a toxic compound often does by the sequential metabolism of two or more organisms where individual population alone fails to transform the compound. Mixed culture or consortia possesses genetic complement to code for the entire biodegradation enzymes by virtue of presence of a group of organisms with varied genetic capabilities (Slater and Godwin, 1980). The problem of toxic intermediates produced during the xenobiotic degradation by pure cultures could also be overcome by mixed cultures or microbial consortia that have a wider spectrum of metabolic properties. Microbial consortia possess many factors which enhance metabolism of toxic compound, such as production of growth factors, removal of toxic intermediates and recalcitrants removal by co-metabolism etc (Singleton, 1994). In nature, usually microbial communities exist as either loose or tight associations depending upon the conditions of the environment in which they live. The communities that have tight associations exhibit proper ties and characteristics that may not otherwise be present in any other combination of micro-organisms or in their individual populations.

Although the degradation ability of a microbial consortium to degrade toxic compounds is now fairly well-documented, there are still questions about their

biological performance with respect to structure and stability. Answers to these questions will extend the applicability of microbial consortium in the bioremediation of phenol and other toxic chemicals.

(i) Definition of microbial consortium

A microbial consortium can be defined as the tight associations existing among members of microbial communities enabling them to survive and perform more productively in a particular environmental condition (Whittenbury, 1978). Two characteristics of microbial consortium as pointed out by Slater and Lovatt (1981) are their combined metabolic activity and higher interacting properties. Consortium can be regarded as well balanced micro-ecosystems and include all bacterial species necessary for the degradation of organic pollutants present in the wastewater to which it is exposed. Microbial consortium consists of mechanically stable, separate entities without any regular shape .

In contrast to other types of immbolized biomass, microbial consortium has no inert carrier material that neither plays an essential role in the transformation, nor is an important factor of its stability. However, constitution of microbial consortium is based on the principle of autoimmbolization, due to the presence of particular active biomass. Table 1.2 lists the most important characteristics of microbial consortium.

(ii) Microbial consortium characterization

The quality of a microbial consortium involved in biodegradation mainly depends on the biological characteristics of the whole system. Availability of nutrients, primary substrates, proper microbial populations containing coded information etc. are the major factors that determine the extent of biodegradation. Through adequate and accurate consortium characterization, appropriate predictions about the

functioning of consortium type can be made. However, so far, very few reports are available on the characterization of consortium involved in the degradation of pollutants.

Table 1.2 Consortium characteristics important to the functioning of aerobic wastewater treatment systems.

Characteristics	
Development	The development of microbial consortium is a continuous process of autoimmbolization of individual isolates. In most of the cases consortium is formed and not developed.
Biological activity	Microbial consortium degrades / mineralizes the polluting compounds completely. The inhibitory metabolites formed during degradation may be used as carbon source for secondary substrate utilizing members (Singleton, 1994).
Mechanical strength	The activity of constitutent members in the consortium will hold-up the system, and provides mechanical strength.

Phenol degradation performance of mixed cultures or consortia has been studied mainly with immobilised microbial systems. Dwyer *et. al.* (1986) have claimed the successful degradation of phenol by immobilising a consortium consisting 3 physiological groups of organisms, phenol oxidising bacterium, a *Methanothrix* like bacterium and a hydrogen utilising methanogen in agar. The degradation of toxic compounds by mixed cultures immobilized on activated carbon has been reported as a combination of physical adsorption and biological degradation. This combined effect assumes an intrinsic relationship between the activated carbon and bacteria in the same environment, thus regenerating the surface of the adsorbent/carrier as diffusion and degradation proceeds (Abu-Salah *et. al.* 1996). The adsorbed material desorbes and diffuses out of the carbon and is metabolized by the bacteria. Cultures adsorbed onto activated carbon, have shown as more effective in the studies of a 2 member

consortium consisted of bacteria *Pseudomonas putida P8* and yeast *Cryptococcus elinovii H1* immobilised on activated carbon (Ehrhardt and Rehm, 1985 and Morsen and Rehm, 1985). However, these consortium failed to degrade in the same rate when they were immobilised on sintered glass system (Morsen and Rehm, 1990).

A syntrophic consortium consisting of a short and long rod shaped bacteria and a strain of *Desulfo vibrio* was grown with a methanogen, which was capable of degrading 10 mM phenol within 25 hours of incubation (Knoll and Winter, 1989). Competition between two microbial populations *Pseudomonas putida* ATCC 17514 and *P. resinovorans* ATCC 14235 for phenol degradation were observed in a sequencing fed-batch reactor by Dikshitulu *et.al.*(1993). This study has indicated the coexistence of two pure competitors, which was stable even at a varied flow rate. Khoury *et. al.* (1992) has reported the aerobic degradation of phenol to an extent of 376.64 mg/l phenol with 20 hour of growth by a 3 member consortium consisting of *Pseudomonas fluorescens* III, an oxidase negative bacterium and *Acinetobacter johnsonii*, a spindle shaped organism.

Difference in phenol feeding patterns that could alter the structure of a phenol degrading microbial community capable of co-metabolizing trichloroethylene was studied by Shih *et al.* (1996). Enhanced degradation of phenol has been reported by the addition of 4 types of soils such as loam, sandy loam, silty loam and silty clay loam with mixed activated sludge culture. The silty loam and silty clay loam enhanced the phenol degradation from 30 to 55%, presumably due to the formation of bacterial colony on the surface of sand which perhaps, develop resistance to the toxicity of chemicals (Lo and Hung, 1993).

(iii) Derivatives of Phenols

Degradation of derivatives of phenol using microbial consortium has been limitedly reported. Parker *et. al.* (1994) investigated a pilot scale activated sludge treatment for 2-chlrophenol (2-CP) and 2,4,6-trichlorophenol (2,4,6-TCP) and pentachlorophenol (PCP). Of these PCP was absorbed to a great extent and biodegraded than 2,4,6-TCP and 2-CP respectively. An aerobic 3 membered mixed culture consisting of one *Pseudomonas sacharophila* and two strains of *Pseudomonas sp.* in a continuous flow fluidized bed reactor, was capable of degrading a mixture of 2,4,6-TCP, 2,3,4,6-tetrachlorophenol (TeCP) and PCP (Puhakka *et. al.* 1995). Enhancement of pentachlorophenol degradation due to the addition of peptone and glucose was observed with a 3 member consortium consisted of *Flavobacterium glueum, Agrobacterium radiobacter* and *Pseudomonas* sp. (Yu and Ward, 1994) .

Indications are there for improving biodegradation by applying methods to enhance physical contact of cells with compounds. Silverstein *et. al.* (1994) reported an enhanced degradation of 2,4-dinitrophenol, when a three member consortium consisted of *Rhodococcus sp.* was subjected to mechanical shearing and incubation in EDTA to improve dispersion.

An activated sludge capable of degrading p-nitrophenol at varying concentrations such as 0.05, 0.2, 0.5 and 2.0 mM were operated in batch and continuous mode. In continuous mode, system was stable to exposure of inhibitive level of PNP whereas batch mode showed much shift towards varying PNP concentrations (Matusi *et. al.* 1994).

Simultaneous degradation of 3-chlorobenzene, phenol, o-, m- and p-cresol with a 2 member consortium consisted of *Pseudomonas aeruginosa* and *Pseudomonas*

strain was reported by Babu *et.al.* (1995). This consortium could degrade both phenol and chlorobenzoates upto 10 mM within 84 hours of incubation (Babu *et. al.* 1995). Another aerobic consortium degrading 2 g/l of monoaromatic compounds was isolated from olive oil mill effluent by Zouari and Ellouz (1996).

A simplified monod kinetic model for aerobic degradation of two important phenolic consistutents, tyrosol and caffeic acid of olive oil mill waste water was reported with a heterogeneous microbial culture (Borja *et. al.* 1995).

Most of the above studies were focused on the degradation ability, degradation kinetics, effect of other nutrients on degradation and it is fairly established that microbial consortia are capable of degrading phenol and its derivatives under various environmental conditions. However, information is lacking about the members and their specific interactive roles of a defined consortium involved in the degradation of phenol or any other toxic compounds.

(e) Scope of present work

Major biodegradation benefits are; (1) complete transformation of organic compounds into harmless form, (2) easy and cheap removal, (3) restoration of ecosystem imbalance and (4) release of sustainable energy which can be made useful for other functions. Removal of organic compounds using microorganisms is presently an accepted system of bioremediation of soil, water and gases. Biodegradation of compounds are achieved mainly through enzymatic action of microorganisms/microcosms present in a particular community. A microbial consortium offers sequential transformations of chemicals in an orderly manner and it is tolerable to a wide range of environmental conditions. The consortium possesses diverse metabolic capabilities acquired through the members of its community. It is

being evolved by the association of microbial strains. A stable consortium is being continually maintained without much changes.

Acquiring knowledge of the factors affecting the quality and growth of mixed cultures involved in the biodegradation becomes increasingly important, especially because currently, almost all waste treatment reactors are running with mixed cultures / consortia. **The present work was to gain more knowledge on the behaviour of a mixed culture about its constitution, stability and performance in phenol degradation, which could be successfully applied to the biodegradation of many other organic pollutants also.**

This thesis comprises of 8 chapters, in which the first chapter covers a brief review of physico-chemical and biological treatment of phenol containing wastewater. The advantages of using mixed culture in the bioremediation of toxic compounds are also discussed.

Materials and methods followed in the study are presented in Chapter 2. Chapter 3 deals with the isolation and characterisation of phenol degrading organisms from the microbial consortium developed for the present studies. Chapter 4 describes the population dynamics of microbial consortium, factors affecting and controlling the distribution of microbial population and its performance in an activated sludge reactor during 2 years of operation.

In Chapter 5 studies on phenol degradation stability of a microbial consortium and its best phenol degrading isolate are presented under various stress conditions like, different initial concentrations of phenol and presence of mixed substrate.

Chapter 6 of the thesis deals with the heterogeneity of the microbial consortium with respect to degradation ability and stability of the system. Chapter 7 of the thesis

describes reconstitution of microbial consortium from the isolated individual strains and Chapter 8 is the concluding chapter in which summary of the results and a detailed bibliography are presented.

CHAPTER 2

MATERIALS AND METHODS

The phenol degrading microbial consortium was developed and maintained in two fed-batch activated sludge reactor systems as per the following .

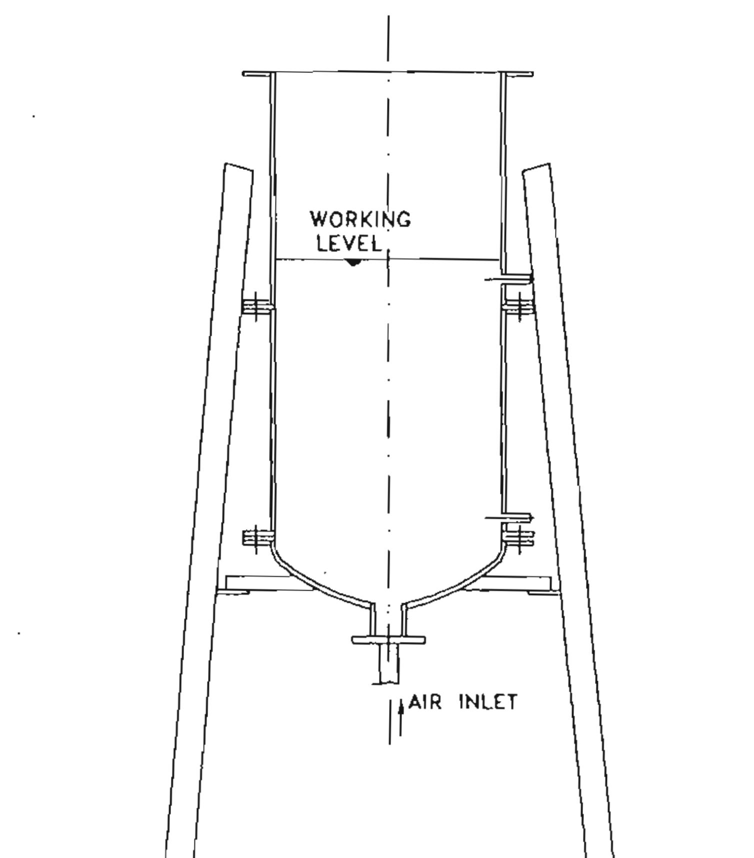


Fig. 2.1 Schematic diagram of fed-batch reactor (Type-1)

2.1 Reactor-1

Material of construction	: Stainless Steel
Capacity	: 14 litres
Height of reactor	: 510 mm
Internal diameter	: 248 mm
Rate of air supply	: 4 litres per minutes
Hydraulic retention time	: 14 days
Mode of operation	: Fed- batch

2.2 *Reactor-2*

Material of construction	: Borosil Glass
Capacity	: 1 litre
Height of reactor	: 210 mm
Internal diameter	: 90 mm
Rate of air supply	: 4 litres per minute
Hydraulic retention time	: 0.5 days
Mode of operation	: Fed- batch

2.3 *Batch Studies*

0.25 litre and 0.5 litre shaken flasks containing 100 ml media were used to assess the individual phenol degradation activity.

2.4 *Media*

Composition of the basal media used for the studies is presented in the Table 2.1. All chemicals used are of reagent grade quality. Chlorine free water was used for all media preparation. The media used for non-phenol degraders are given in Table 2.2.

Table 2.1 Basal salt medium composition.

Ingredients	quantity
K_2HPO_4	0.5 g/l
$MgSO_4 . 7H_2O$	0.2 g/l
$CaCl_2 . 2H_2O$	0.01 g/l
NH_4NO_3	3 g/l
$FeSO_4 . 7H_2O$	0.01 g/l
$MnSO_4 . H_2O$*	0.5 mg/l
$ZnSO_4$ *	0.5 mg/l
$CuSO_4 . 5H_2O$*	0.5 µg/l
$CoCl_2 . 6H_2O$ *	0.5 µg/l

* Trace elements

Phenol at a concentration of 0.5 g /l was added as sole carbon source. The pH of the medium was 7.2. The media were solidified with 1.5 % agar .

Table 2. 2 Media composition for isolation of non- phenol degraders

(a) *Nutrient Broth*

Ingredients	g/l
Peptone	5.0
NaCl	5.0
Beef extract	1.5
Yeast extract	1.5
Water	1 litre

(b) *Nutrient Agar*

Ingredients	g/l
Peptone	10.0
NaCl	5.0
Beef extract	5.0
Agar	15.0
Water	1 litre

(c) *Yeast Extract Agar*

Ingredients	g/l
Peptone	10.0
NaCl	5.0
Beef extract	5.0
Yeast extract	5.0
Agar	15.0
Water	1 litre

(d) *Lactose Extract Agar*

Ingredients	g/l
Peptone	10.0
NaCl	5.0
Beef extract	5.0
Lactose	5.0
Agar	15.0
Water	1 litre

2.5 Analysis of Sample

(a) Measurement of pH and DO

Dissolved oxygen (DO) and pH were routinely monitored with a DO probe (Jet-way POM 7) and a pH electrode (Thoshniwal cat. no. CL54).

(b) Phenol estimation

Phenol was analysed periodically in the supernatant of cell free medium using spectrophotometric method (ASTM, 1979) and direct measurement method. The spectrophotometric method is based on the reaction between phenol and 4-aminoantipyrene in the presence of ferricyanide under alkaline condition to give a

red coloured product. The absorbance of the resulting product was measured in a double beam spectrophotometer at a wavelength of 510 nm against blank.

Direct measurement of phenol was done with the cell free extract of sample absorbance at 270 nm. Absorbance was found to be linearly dependent on concentration upto 80 mg/l phenol.

(c) *Biomass concentrations*

Concentration of biomass was done by centrifuging a known volume of culture sample at 10,000 rpm for 15 minutes at 4 ^{0}C. After washing with buffer solution the pellet was transferred and dried to constant mass at 105 ^{0}C in a preweighed test tube. The difference between the initial and final weight was calculated as dry weight of biomass. Turbidity measurements at 600 nm was also followed to determine the biomass concentration in the samples.

(d) *Total count and viable count*

Sample containing the bacteria is serially diluted and each dilution were placed on phenol agar media and nutrient agar media. Colonies developed were taken as viable counts.

(e) *Mixed liquor suspended solids (MLSS)*

MLSS was measured (APHA, 1985) by filtering 50 ml of activated sludge through preweighed whatman filter paper no.42. The filter paper was dried to constant mass at 103± 2 °C for 1 hour.

MLSS was calculated using the formula,

$$\text{MLSS in mg/l} = \frac{(A-B) \times 1000}{\text{Sample volume (ml)}}$$

where, A= weight of filter paper + dried residue (mg),

B= weight of filter paper (mg).

(f) *Total chemical oxygen demand (TCOD)*

TCOD of the sample was analysed using the following principle. The organic matter gets oxidized completely by potassium dichromate in the presence of concentrated sulphuric acid to produce carbondioxide and water. The excess potassium dichromate is titrated with standard ferrous ammonium sulphate. The volume of dichromate consumed is proportional to the oxygen required for the oxidation of the organic matter.

(g) *Total organic carbon*

Total organic content in the sample were analysed using TOC- 5000 A analyser (Shimadzu). Dissolved organic carbon (DOC) and inorganic carbon (IC) of the culture were determined after its centrifugation for 20 minutes at 10,000 rpm. The difference of total carbon (TC) and inorganic carbon (IC) gives DOC/TOC.

(h) *Gram staining*

It was performed by following the method as described by Collins and Lyne (1984).

(i) *Microscopy*

Microscopical examination of the bacterial isolates and the consortium was carried out using Nikon light microscope .

(j) *Scanning electron microscopy*

The harvested cells were washed with phosphate buffer having the pH 7.0 and fixed in 5 % glutaraldehyde for 3 hours. The cells were again washed with phosphate buffer and dehydrated by washing with increasing concentration of acetone and finally suspended in absolute acetone. The cells in acetone were frozen, etched for 10 min at

60 °C and coated with gold and viewed in the scanning electron microscope (Hitachi S 2400).

(k) *Thin -layer chromatography*

Culture filtrates were adjusted to pH 2, with con. HCl and extracted twice with ethyl acetate. The organic extracts were combined and dried over anhydrous sodium sulfate. Products in the extract were separated using thin layer chromatographic plates coated with silica gel in a solvent mixture of chloroform, ethanol and 0.1N sodium hydroxide in the ratio of 100:5:1.

(l) *Storage of isolates*

The phenol degrading bacterial isolates were grown on phenol agar slant (500 mg/l) for 72 hours and they were transferred to incubator at 37 °C. The non-phenol degraders were maintained on nutrient agar slants and stored at 4 °C. Cultures were subcultured in every 3 months.

CHAPTER 3

ISOLATION, IDENTIFICATION AND CHARACTERISATION OF THE MEMBERS OF PHENOL DEGRADING MICROBIAL CONSORTIUM

3.1 *Introduction*

Phenol or phenolic derivatives are the major constituents of some chemical, pharmaceutical, petrochemical, textile and coffee industrial effluents, which are usually treated by microbiological methods such as activated sludge, trickling filters etc. (Chudoba *et. al.* 1989, Hess *et. al.* 1990)

Most of the laboratory studies reported on phenol degradation have been tried with pure strains (Sokol, 1988, Katayama-Hirayama *et. al.* 1991, Zilli *et. al.* 1993). The following reports are among the few biodegradation studies of phenolic compounds employing microbial consortia. Zache and Rehm (1989) have reported phenol degradation by a 2 member consortium consisted of the bacteria, *Pseudomonas putida* P8 and the yeast *Cryptococcus eliniovi* H1. This consortium was able to degrade 1200 mg/l of phenol in 80 hrs of incubation. Similarly a 3 member aerobic consortium degrading phenol consisted of *Pseudomonas fluorescens III* strain, *Acinetobacter* and a spiral shaped strain has been reported by Khoury *et al.* (1992), which could degrade 376.44 mg/l of phenol during 20 hours of incubation. Knoll and Winter (1989) have reported a four member anaerobic consortium capable of degrading 188 mg/l of phenol in 6 hours duration. These studies have mainly focused on phenol metabolism and degradation capability of the cultures.

In order to describe any microbial system, isolation and characterisation of the members in the community, is a perquisite. The present chapter describes the isolation, identification, characterisation and phenol degradation properties of microorganisms of the activated sludge developed in a fed-batch reactor.

3.2 *Materials and Methods*

(a) *Inoculum*

About 200 ml of sewage sample collected from a sewage pumping station in Trivandrum city, India, and soil suspension of domestic waste discharge site were used as inoculum.

(b) *Type I fed-batch reactor*

The phenol degrading activated sludge was developed from the sewage seed in a fed-batch reactor as follows. In the first step concentration was slowly raised to 800 mg/l which took about 3 months. After that the phenol concentration of activated sludge was reduced and maintained at 500 mg/l (Table 3.1). The reactor was daily fed with enrichment media in a litre of chlorine free water and 500 mg/l of phenol. The other reactor conditions were the following: working volume 14 litre, stirring with air flow greater than 4 lpm, temperature 30±3 °C, dissolved oxygen 1.4 mg/l, pH 6-8, hydraulic retention time 14 days, dilution factor 0.042 h^{-1}, influent chemical oxygen demand 1487.3 mg/l, effluent chemical oxygen demand 296.80 mg/l and biomass 2900-3400 mg/l. The fed-batch reactor was maintained for 5 years under laboratory conditions.

(c) *Isolation of individual members of consortium*

Isolation of bacteria was done after 8 months of inoculation of the fed-batch reactor. Sample of 1ml from the reactor was subjected to sonication for a minute using Vibronics (ultrasonic processor, capacity 120 v, India) to separate the cells from aggregates. The samples were diluted in phosphate buffered saline solution (NaCl, 8.0 g/l, K_2HPO_4, 1.21 g/l and KH_2PO_4, 0.3 g/l) having the pH 7.3. Ten fold dilutions were prepared in a total volume of 10 ml.

Table 3.1 Scheme of reactor feeding and microbial isolation

Day of feed	Seed	Feed concentraion used	Isolation
0	Sewage seed	14ml of EM+ 1 mg/l of peptone and 400 mg/l of Phenol	-
2	-	1 litre enrichment media + 1 mg/l peptone and 100 mg/l of phenol	-
3-4	-	No feed	-
5	soil suspension of domestic waste	No feed	-
6-10	-	No feed	-
11-12	-	1 litre EM +100 mg/l peptone and 100 mg/l phenol	-
13-16	-	1 litre EM+100 mg/l peptone and 150 mg/l phenol	-
17-21	-	1 litre EM + 100 mg/l peptone and 200 mg/l phenol	-
22-24	-	1 litre EM + 100 mg/l peptone and 250 mg/l phenol	-
25-32	-	1 litre EM +100 mg/l peptone and 300 mg/l phenol	-
33-36	-	1litre EM+100 mg/l peptone and 400 mg/l phenol	-
37-44	-	1litre EM + 100 mg/l peptone and 500 mg/l phenol	-
45-66	-	1litre EM + 100 mg/l peptone and 600 mg/l phenol	-
67-74	-	1litre EM + 100 mg/l peptone and 600 mg/l phenol	-
75-78	-	1litre EM + 100 mg/l peptone and 600 mg/l phenol	-
79-83	-	1litre EM + 100 mg/l peptone and 700 mg/l phenol	-
84-87	-	1litre EM + 100 mg/l peptone and 800 mg/l phenol	-
88-101	-	1 litre EM + 100 mg/l peptone and 800 mg/l phenol	-
102-104	-	No feed	-
105-108	-	1 litre EM + 100 mg/l peptone and 400 mg/l phenol	-
109-265	-	1 litre EM + 100 mg/l peptone and 500 mg/l phenol	The strain AS_1 to AS_4 isolated without sonication
266-1800	-	1litre EM without peptone	The strain AS_5 , AS_6 , AS_7 AS_8 , were isolated after sonication for 1 minute

EM Enrichment media

The samples were vortexed for 5 minutes before dilution. The samples were also vortexed briefly at each dilution step before transfer. The dilution were plated on phenol agar medium and incubated at 37°C for 3 to 5 days. Colonies, differentiated on the basis of colony morphologies/colour, were counted, and transferred to phenol agar slants. To ensure the purity of each isolate, the colonies were repeatedly streaked on agar plates and picked up separated colonies. Phenol degraders were identified by their ability to grow on phenol. Non-phenol degraders were isolated by plating the serially diluted samples on yeast extract agar, nutrient agar, and lactose agar. Colonies of different morphologies were counted, picked and transferred into phenol broth for 6 hours. The cultures from the phenol broth were streaked on nutrient agar medium. Each isolated colonies were plated on agar to ensure the purity of the isolates.

(d) *Screening of Individual isolates for phenol degradation activity*

Phenol degradation pattern of individual strains was studied by inoculating them into 500 ml shaken flasks containing 100 ml of mineral salts solution, (composition as given in Chapter 2) with 500 mg/l of phenol. Inoculum of each isolate was used with almost the same cell quantity as that of the consortium in the fed-batch reactor . Inoculated flasks were placed on orbital shaker adjusted to 200 rpm at 30 ±3°C. The samples were drawn and analysed periodically.

(e) Identification of isolates of the consortium

Morphology of colonies was identified based on their growth on phenol and nutrient agar media. Gram staining property of each isolate was tested and microscopically examined. Individual isolates of the consortium were identified based on their growth pattern at 0, 25, 37, 40, 50 ^{0}C, and on biochemical tests of urease, catalase, methyl red, H_2S production, Voges-Porskauer indole and nitrase tests. The

identification of the cultures were confirmed by Microbial Technology and Culture Collection, Institute of Microbial Technology, Chandigarh, India.

3.3 Results

(a) Isolates of consortium

A stable bacterial consortium was formed in the fed-batch reactor by feeding 500 mg/l of phenol every day. Repeated isolation of cultures on phenol and non-phenol media confirmed the presence of 10 bacterial strains, evenly numbered as AS_1 to AS_8 as phenol degraders and those failed to grow on phenol were designated as non-phenol degraders (AS_9 and AS_{10}). All the 8 phenol degraders were able to grow on catechol, cresols and chlorophenol media also. The non-phenol bacterial members of the consortium were able to grow on glucose, lactose, yeast extract, formate medium.

Table 3.2 and 3.3 show the characteristics of the isolates where majority of them belonged to *Bacillus* (AS_2, AS_3, AS_5, AS_9 and AS_{10}) and the rest of the strains were *Pseudomonas* sp (AS_4 and AS_7), *Rhodococcus rhodococcus* (AS_6) and *Streptomyces* (AS_1). The isolate AS_8 could not be identified.

(b) Phenol degradation

The results of phenol degradation by the consortium at different time intervals show that more than 96 % of the fed phenol (500 mg/l) was degraded in the first half of incubation and the remaining 4 % of phenol was degraded in the second half (Fig.3.1).

The phenol degradation rate of individual isolates of the consortium were presented in Table 3.4. Among the individual isolates the *Bacillus* strain (AS_5) was the best phenol degrader which could degrade 72 % (360 mg) of fed phenol (500 mg/l) during 24 hours, while the AS_7 *Pseudomonas* sp was the slowest phenol degrader.

Although the non-phenol degrading bacteria (AS_9 and AS_{10}) of the consortium were unable to degrade the phenol, they could tolerate upto 700 mg/l of phenol. Table 3.5 shows the cell concentration of each bacterial isolates in the consortium. A wide variation of the cell counts was observed among the strains, as low as 10^4 /ml and as high as 10^{14} / ml during one cycle of feeding.

Table 3.2 Cultural characteristics of bacterial isolates of phenol degrading consortium

Strains	Size	Pigmentation	Shape	Margin	Elevation	Surface	Optical properties
AS_1	Regular	Dark yellow	Circular	Entire	Flat	Smooth	Opaque
AS_2	Irregular	White	Rhizoid	Filamentous	Flat	Smooth	Translucent
AS_3	Small	Cream	Circular	Entire	Flat	Smooth	Opaque
AS_4	Small	Yellow	Circular	Entire	Flat	Smooth	Opaque
AS_5	Small	Blue	Rhizoid	Filamentous	Flat	Smooth	Opaque
AS_6	Small	Brown	Rhizoid	Filamentous	Flat	Smooth	Transparent
AS_7	Irregular	Blue outside	Circular	Entire	Rised	Smooth	Opaque
AS_8	Circular	Rose	Circular	Entire	Rised	Smooth	Opaque
AS_9	Irregular	Brown	Irregular	Lobate	Flat	Smooth	Translucent
AS_{10}	Irregular	Brown	Irregular	Scrate	Flat	Smooth	Opaque

Media : Basal salt and agar with 500 mg/l phenol for $AS_1$to AS_8 and Nutrient agar for AS_9 and AS_{10}

Incubation Temperature : 37 °C, Age : 72 hours

3.4 Discussion

Results of the present study indicate that the phenol degradation by the consortium was complete and faster than the individual isolates.

There are reports on phenol degradation by a consortia consisting of one yeast and one bacteria degrading 1200 mg/l in 80 hours (Zache and Rehm, 1989) and a 3 member bacterial consortium degrading 376 mg/l of phenol in 20 hours (Khoury *et. al* .1992).

Table 3.3 Morphological and biochemical characteristics of bacterial isolates of the consortium

	Microbial isolates of AS									
Test	AS_1	AS_2	AS_3	AS_4	AS_5	AS_6	AS_7	AS_8	AS_9	AS_{10}
Colony morphology	Circular	Rhizoid	Circular	Circular	Rhizoid	Rhizoid	Irregular	Circular	Irregular	Irregular
Colony colour	Dark yellow	White	Cream	Light yellow	Blue	Pale	Bluish rose	Rose	Brown	Brownish rose
Gram reaction	-	+	+	-	+	+	-	-	+	+
Cell morphology	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Cell length (µm)	1.25	0.45	3.3	1.6	1.6	0.8	1.7	3.3	1.2	2.9
Cell diameter (µm)	0.63	0.13	0.67	0 66	0.6	0.39	1	0.67	0.38	0.38
Urease	+	-	+	-	-	+	-	-	+	-
Catalase	+	+	+	+	-	+	+	+	+	+
Nitrase	+	-	+	+	+	+	+	+	+	-
Methyl Red	-	-	-	-	-	-	-	-	-	-
H_2S	+	+	+	+	+	+	+	+	-	-
Voges-Porskauer	-	-	-	-	-	-	-	-	-	-
Indole	+	-	-	-	+	+	+	+	-	-
Growth										
0°C	-	-	-	-	-	-	-	-	-	-
25°C	+	+	+	+	+	+	+	-	-	-
37°C	+	+	+	+	+	+	+	+	+	+
40°C	+	+	+	+	+	+	+	+	+	+
50°C	-	-	-	-	-	-	-	+	+	+
Presumptive* identification	*Streptomyces sp*	*Bacillus sp.1*	*Bacillus sp.2*	*Pseudomonas sp.1*	*Bacillus sp.3*	*Rhodococcus rhodococcus*	*Pseudomanas sp.2*	*Not detected*	*Bacillus sp.4*	*Bacillus sp.5*

* The identification was confirmed by MTCC, IMT , Chandiagarh, India

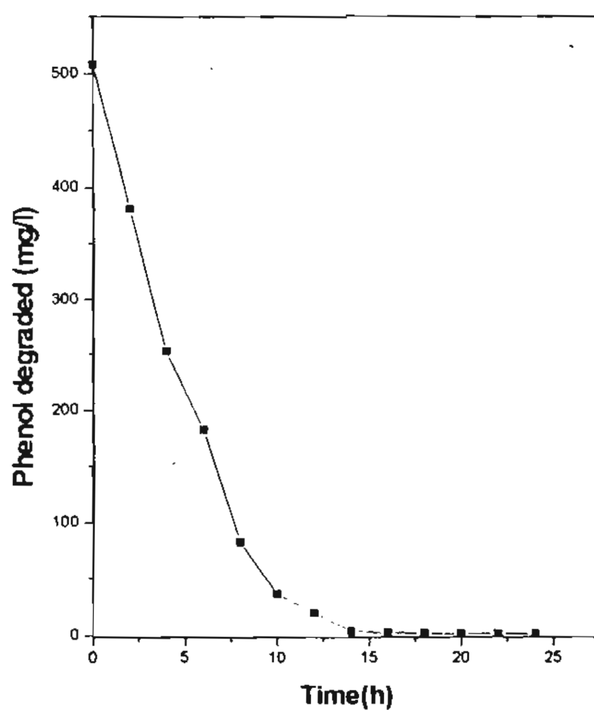


Fig.3.1 Phenol degradation by consortium in fed-batch reactor during 24 hours

Table 3.4 Bacterial isolates and their respective phenol degradation rate at 500 mg/l

Bacterial strains	% of phenol degraded at		
	24h	48h	72h
AS_1	32	64	86
AS_2	60	86	100
AS_3	34	87	98
AS_4	54	86	99
AS_5	72	87	100
AS_6	40	70	97
AS_7	24	51	86
AS_8	26	49	99
AS_9	0	0	0
AS_{10}	0	0	0

Table 3.5 Individual population of fed-batch activated sludge reactor maintained at 500 mg/l phenol

Strains	Cells/ml present in Fed-batch reactor*		
	Lower range	Upper range	variation
AS_1	$2X10^4$	$1X10^6$	± 1
AS_2	$2X10^6$	$1X10^9$	± 2
AS_3	$6X10^6$	$6X10^{10}$	± 1
AS_4	$2X10^6$	$3X10^{11}$	± 1
AS_5	$4X10^6$	$4X10^{10}$	± 1
AS_6	$7X10^6$	$2X10^{12}$	± 2
AS_7	$2X10^6$	$1X10^{11}$	± 1
AS_8	$2X10^8$	$2X10^{11}$	± 1
AS_9	$1X10^8$	$1X10^{10}$	± 1
AS_{10}	$1X10^{10}$	$2X10^{11}$	± 1

*Counts taken after 24 hours of feeding from 20 experiments

Unlike the above, the consortium developed in this study had a higher specific phenol degradation as evidenced by the result of degrading 96 % of fed phenol (500 mg/l) during 12 hours.

Information is lacking how larger membered consortia performs better. Consortium of the present study had as many as 8 members of phenol and two non-phenol bacteria, and which performed significantly better than in the earlier studies.

Although the specific involvement of individual members of the consortia are not clear from the results, directly or indirectly involved in the degradation of phenol, they possessed versatile features in their degradation pattern, and other characteristics. Most interestingly, such a heterogeneous group co-existed in the system performed stable degradation. Perhaps, their heterogeneous attributions of the isolates may have played greater roles in keeping the system coherent and stable.

Obviously, the non-phenol degraders were phenol tolerant upto 700 mg/l of phenol and their role in the degradation may be indirect through the utilisation of metabolites which would even inhibitory to the system itself.

In conclusion, this chapter describes the isolation and identification of phenol degrading consortium which consisted of eight phenol degrading and two non-phenol degrading bacteria, despite the limitations of individual members in the degradation of phenol.

CHAPTER 4

POPULATION DYNAMICS IN MICROBIAL CONSORTIUM DEGRADING PHENOL

4.1 Introduction

Microbial consortium is being utilised in wastewater treatment for more than a century. Being the active agent responsible for biodegradation, biomass of activated sludge has been a subject of major research and it receives utmost importance and focus even now.

Currently, the activated sludge processes are mainly characterised and evaluated by (1) measuring biomass quantity, (2) sludge activity, and (3) enzyme activity (Wanner, 1994).

In microbial consortium, the relationships between individuals are controlled by certain factors which can alter the functioning of the system through changes in the population dynamics. Hence, it is important to study the biological structure of microbial consortium (e.g. activated sludge) and their performance.

Attempts have been made to describe the microbial population degrading 2,4-dinitrophenol (Schmidt and Gier, 1989), 2,4-dichlorophenol (Chudoba *et .al.* 1989) and p-nitrophenol (Matsui *et.al.* 1994). These studies have mainly focused to quantify organisms (total biomass) involved in toxic compound degradation as well as its influence on the total population. Information on the dynamics of members of a consortium is lacking in the literature, which will help to know more about the function of a consortium as a community.

The objectives of the present work are (1) to study the variation of individual as well as total population in the activated sludge during its operational period, (2) to

study the factors controlling the population dynamics and (3) to analyse the stability of the activated sludge with respect to their constitution.

4.2 Materials and Methods

(a) Fed-batch sludge reactor

The phenol degrading activated sludge was developed and maintained in a fed-batch reactor for this study as presented in Chapter .2.

(b) Analysis

Phenol, cell number and biomass were estimated by following the method described in Chapter 2. Settled sludge volume of the fed-batch reactor was monitored in a 1 litre graduated container at varying time intervals (APHA, 1985).

(c) Studies on individual population of activated sludge

Variation in the viable count of activated sludge was analysed on hourly, daily, weekly and monthly basis by surface plating on phenol and nutrient agar medium. Results presented are the average of 25 experiments.

4.3 Results

(a) Treatment performance

A fed-batch reactor of activated sludge was operated by feeding 500 mg phenol/l/day and kept the hydraulic retention time of 14 days. The variation of typical operational parameters of the reactor viz. MLSS, pH, dissolved oxygen and individual bacterial count were estimated periodically.

The total biomass content (MLSS) of the fed-batch reactor was in the range of 1.0 g/l to 3.4 g/l during the year 1994 and 1995 (Fig.4.1). Except for few months, phenol degradation activity of the reactor was above 99 % (Fig. 4.2).

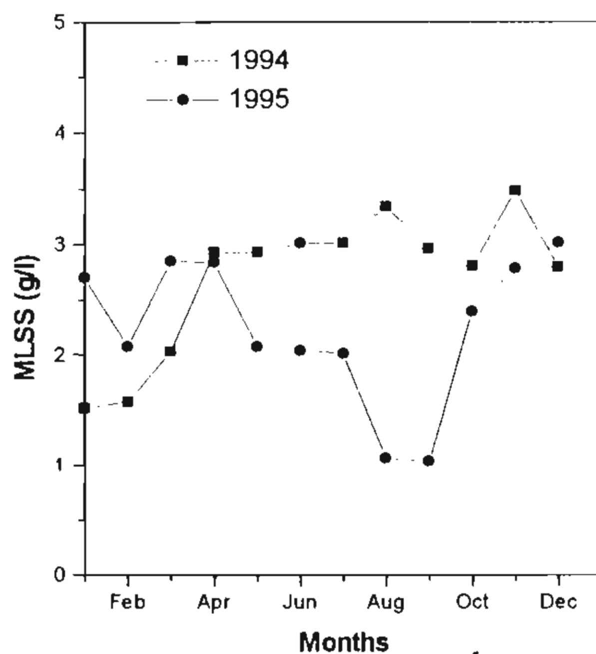


Fig.4.1 Monthly variation of MLSS of fed-batch reactor

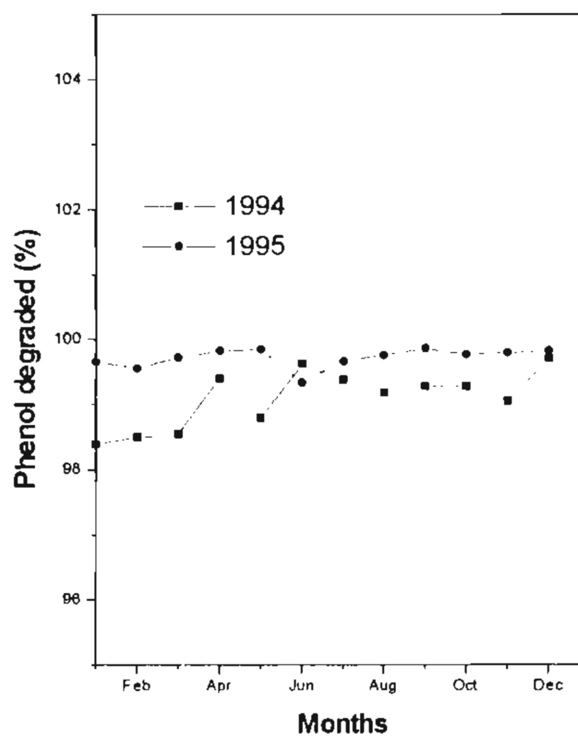


Fig.4.2 Phenol degradation in fed-batch activated sludge reactor

Measurements of pH and phenol degradation at every 2 hours in a week are presented in Table. 4.1. The variation of pH in a day was slightly higher (6.3 to 8.0) than the pH measurements on different days (7.0 to 8.0). The temporary decline of pH due to phenol feeding used to be elevated with the progress of phenol degradation.

(b) Fluctuation of individual members of phenol degrading microbial consortium

Estimation of individual bacteria in every alternate hour showed their specific pattern of growth, for example, the AS_5 exhibited fastest growth rate and both AS_7 and AS_8 had the slowest growth rate (Fig. 4.3a to d).

Individual population had undergone continuous shift in the consortium, though the rate of shift was different for each one. Comparatively, less shift was noticed with the slowest phenol degrader AS_7 and with non-phenol degraders (AS_9 and AS_{10}) (Fig. 4.4, 4.5, 4.6, and 4.7). However, the total bacterial count was almost steadily maintained on daily, weekly and monthly estimation (Fig. 4.8).

Individual fluctuation was found to be maximum for *Bacillus* sp. 1 (AS_2) (Fig. 4.3a, 4.4a, 4.5a, 4.6a and 4.7a). However, the slow phenol degrader *Pseudomonas* sp. 2 (AS_7) showed only less variation throughout the period of operation (Fig. 4.4c, 4.5c, 4.6c and 4.7c).

The changes in the population of the members of consortium could not be correlated with the variation in the pH.

4.4 Discussion

Population dynamics of a defined phenol degrading microbial consortium maintained in controlled conditions were studied for 2 years consecutively, to

Table 4.1 Daily variation of pH and phenol degradation in fed-batch activated sludge reactor

	Monday		Tuesday		Wednesday		Thursday		Friday		Saturday		Sunday	
Time (h)	pH	Phenol removal (%)	pH	Phenol removal (%)	pH	Phenol removal (%)	pH	Phenol removal (%)	pH	Phenol removal (%)	pH	Phenol removal (%)	pH	Phenol removal (%)
0	7.6	0	7.3	0	7.1	0	6.9	0	6.6	0	6.6	0	6.6	0
2	7.1	26.7	6.9	24.7	6.8	25.4	6.5	27	6.3	28.2	6	30.3	6	31.9
4	7	54	7	38.1	6.8	38.6	6.4	53.5	6	49.4	5.7	55	5.2	48.3
6	7.3	77	7	68	7.3	58.1	6.7	74.1	6.2	75.4	6	76	5.5	82.5
8	7.4	96.2	7.1	92	7.5	88.6	6.9	88.1	6.7	95.1	6.5	96	6.3	93.8
10	7.4	96.7	7.3	97	7.5	96.9	7	96.4	6.7	97	6.5	97.1	6.2	96.9
12	7.4	96.9	7.4	97.2	7.5	97.2	7	97.9	6.8	98	6.6	98.2	6.6	97.9
14	7.5	99.2	7.5	98.3	7.6	98.5	7.1	98.3	6.8	99	6.7	99.1	6.7	98
16	7.5	99.5	7.5	99	7.6	99	7.1	99	6.9	99	6.7	99.3	6.8	99
18	7.6	99.5	7.5	99.4	7.7	99.4	7.1	99.4	7	99.2	6.8	99.5	6.8	99.4
20	7.7	99.5	7.6	99.5	7.5	99.5	7.1	99.5	7	99.2	6.8	99.6	6.8	99.5
22	7.8	99.5	7.7	99.5	7.5	99.5	7.2	99.6	7	99.3	6.9	99.6	6.9	99.5
24	8	99.5	7.8	99.6	7.5	99.7	7.5	99.7	7	99.4	6.9	99.7	7	99.6

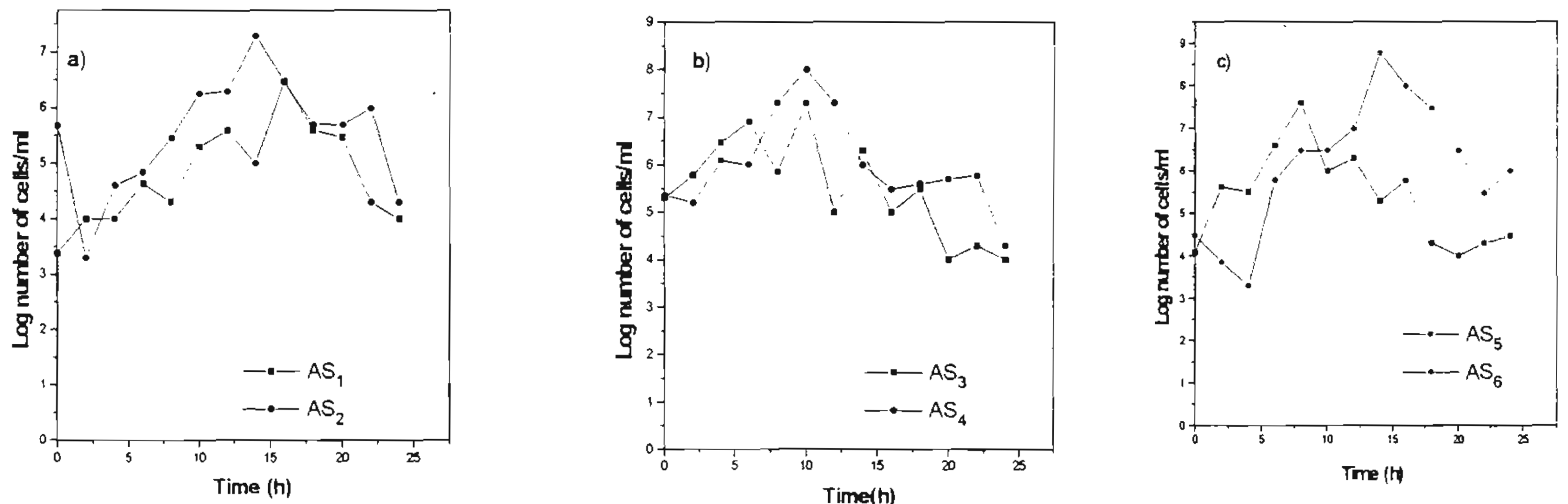
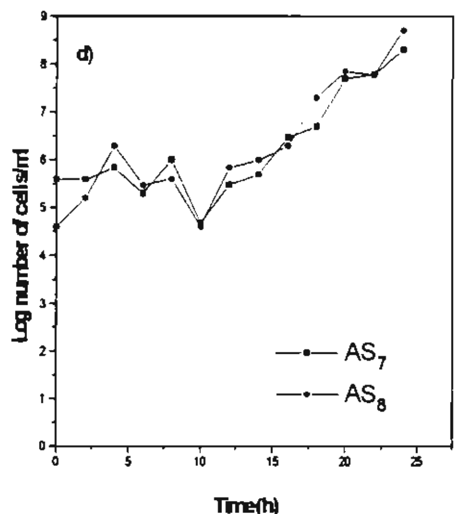


Fig. 4.3 Growth profile of bacterial members in consortium at 500 mg/l of phenol concentration (a) AS_1 & AS_2, (b) AS_3 & AS_4 and (c) AS_5 and AS_6



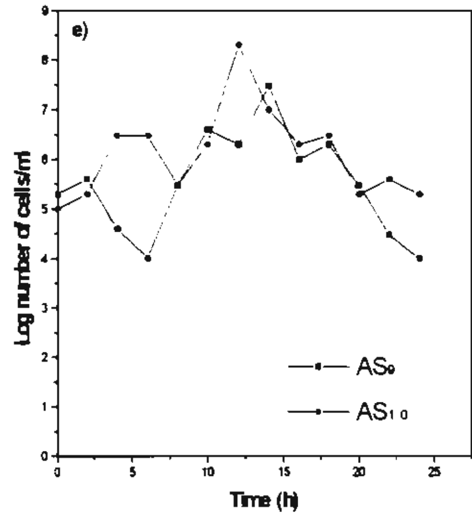
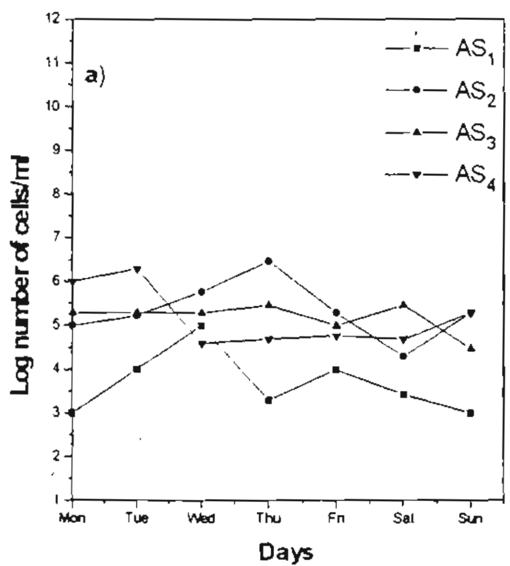
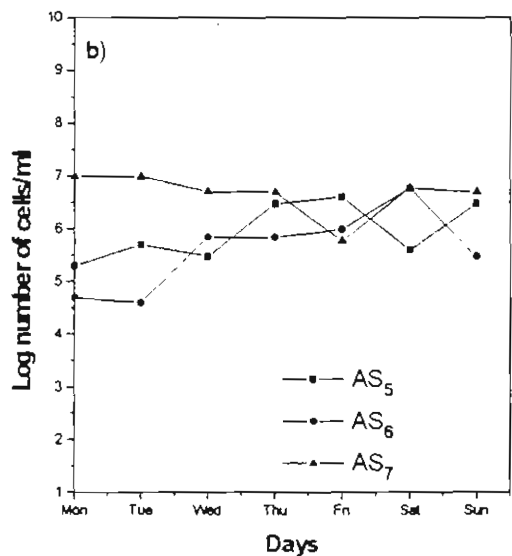


Fig.4. 3 Growth profile of bacterial members in consortium at 500 mg/l of phenol concentration (d) AS_7 & AS_8 and (e) AS_9 & AS_{10}





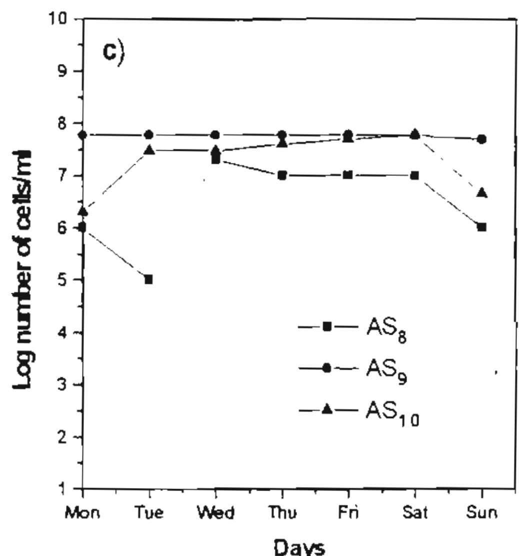
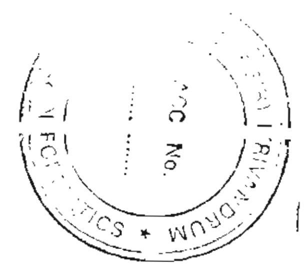


Fig. 4.4a to c Daily variation of individual bacteria in consortium



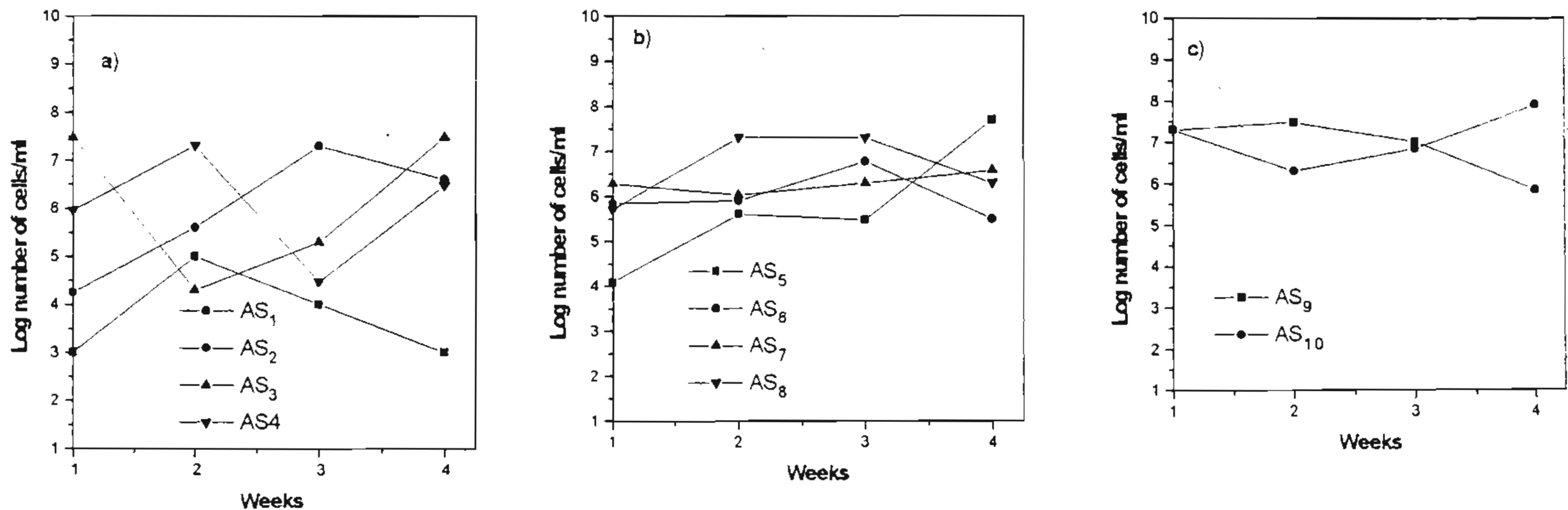


Fig. 4.5a to c Weekly variation of individual bacteria in consortium

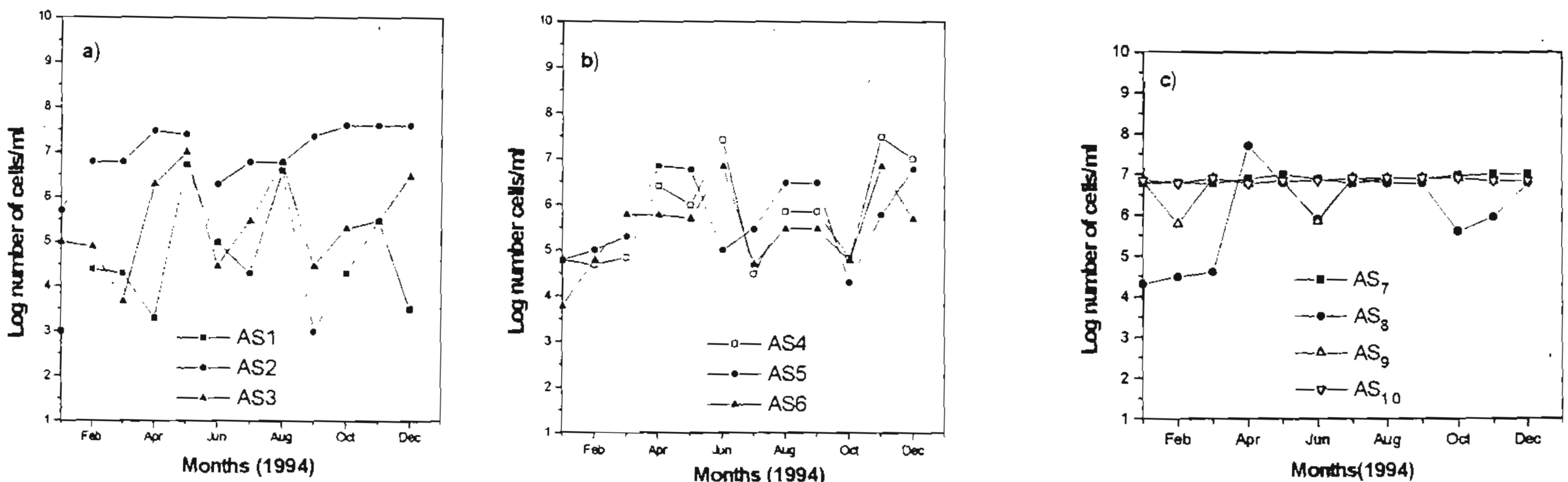


Fig.4.6a to c Monthly variation of individual bacteria in consortium

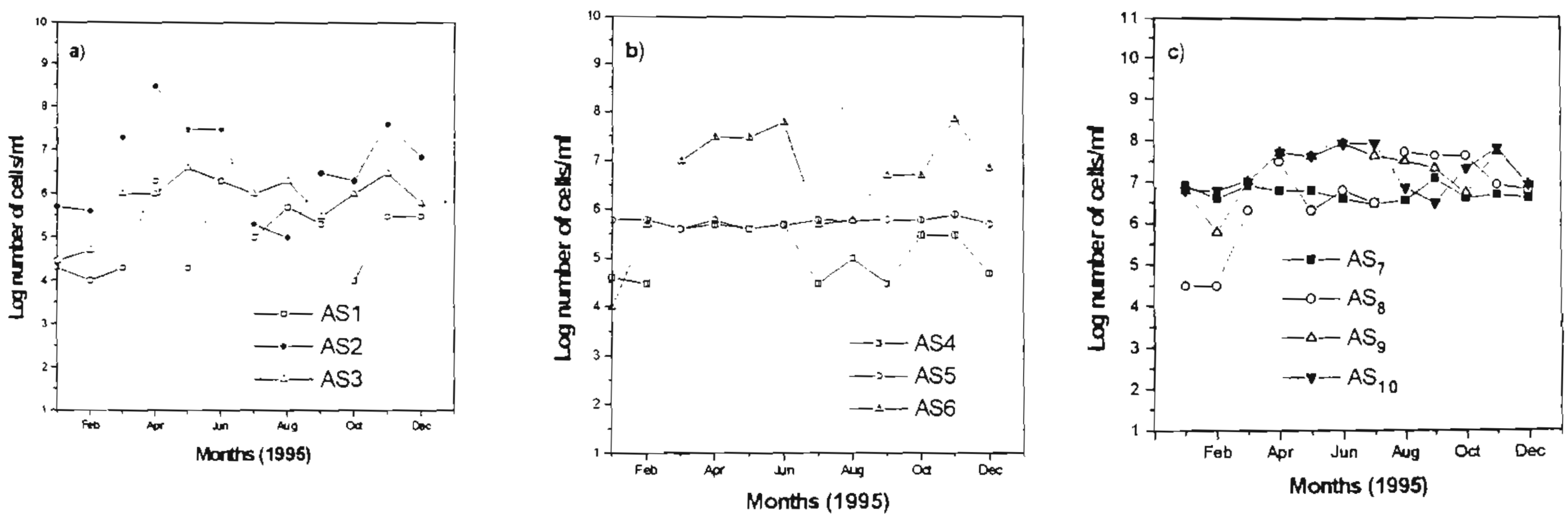


Fig.4.7a to c Monthly variation of individual bacteria in consortium

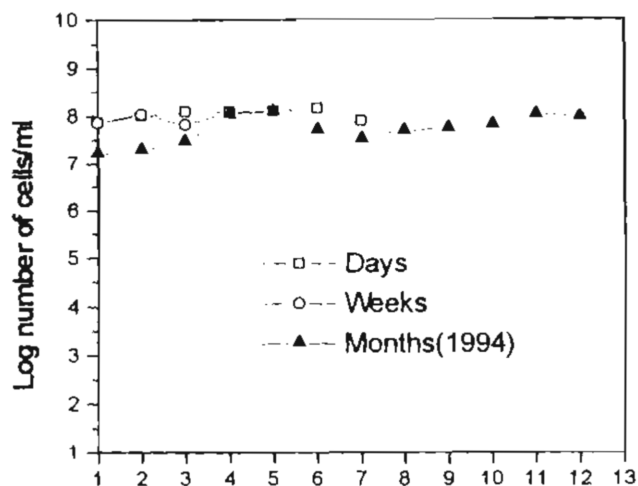


Fig.4.8 Total bacterial count of consortium taken daily, weekly and monthly

determine the behaviour of individual members in the consortium involved in degradation.

In the present system both continuously shifting and steady populations were present, while the total population found to be almost steady throughout the operational period. Bacterial fluctuations of the consortium indicate that a balanced interaction existed between the members of phenol degrading population, and the individual population engaged in this type of interaction were not shifting independently. As a result of this dynamics the stability would have brought out in the system to perform complete degradation.

In the present consortium, non-phenol degraders were remained as passive members of thecommunity with respect to direct degradation of phenol. Each members of consortium had a different growth pattern. These inherent growth characteristics of the individual members presented them suitable places in the

consortium growth and activity. The level of phenol utilisation was also found different with each individual. This would play a major role not only in growth pattern of members, but also on the degradation efficacy.

MLSS of phenol degrading activated sludge indicated that it had a slightly higher biomass concentration than that of conventionally operating activated sludge reactor where, MLSS varied between 1 to 2 g/l (Zhang and Yamamoto, 1996). However, the present activated sludge had relatively slow setting rate than the conventional one.

The present microbial consortium was stable with respect to biodegradation of phenol and constitutional aspects, at the same time the members within it were at continuous variation in their viable count. Even during these perturbance, elimination of a particular population was not observed.

In conclusion, the phenol degrading microbial consortium possessed highly dynamic members subjected to continuous changes in their numbers without altering much on their total population and function. It works as a microcommunity capable of mutually sustaining, constantly utilising and disparting energy with less interdependency.

CHAPTER 5

STABILITY OF A MICROBIAL CONSORTIUM AND ITS BACTERIAL ISOLATE ON PHENOL DEGRADATION UNDER STRESS CONDITIONS

5.1 Introduction

Phenol and its derivatives are common environmental pollutants discharged through wastewater's from fossil fuel refining processes, phenol manufacturing plants, pharmaceutical and a variety of other industries. Concentration of phenol and its substitutes in these industrial effluents may present in varying levels. For instance, Coke industry waste may contain high concentration of phenol upto 6000 mg/l (Ehrhardt and Rehm 1989) whereas petroleum refinery effluent contains a phenol concentration of 61 mg/l (Viraraghavan and Robbins 1994).

Most of the studies on biodegradation of phenol have been carried out with pure culture, where the factors such as changes in concentration (Katayama *et.al.* 1991, Paller *et.al.* 1995, Zaidi and Imam 1996), presence of its derivatives or other easily degradable compounds (Schmidt *et.al.* 1987, Zaidi and Mehta 1995, Zaidi *et.al.* 1996), anoxic conditions, etc. have been observed as limitation in the degradation.

Mixed populations have been known for some of their advantages in the degradation of phenolic compounds (Hess *et. al.* 1990, Khoury *et.al.* 1992, Bisaillon *et.al.* 1993, Gallert and Winter, 1993, Silverstein *et. al.* 1994, Ambujom and Manilal 1995 and Puhakka *et. al.* 1995). But information on their degradation stability with respect to changed concentrations and mixed substrates is insufficient to conceive its application as the sole method for decontamination of wastewater.

The present chapter deals with the stability of a phenol degrading microbial consortium and its best phenol degrading isolates under different stress conditions, such as varying feed concentrations of phenol and mixing phenol with glucose and

2,4-dichlorophenol. It also examines the factors controlling the stability of a system with respect to biodegradation of phenol.

5.2 Materials and Methods

(a) *Isolation*

The phenol degrading microbial consortium was developed by enrichment culture technique in a mineral salt medium containing 500 mg/l of phenol as sole carbon source as already described in Chapter 2.

(b) *Phenol degradation in varying concentrations*

The phenol degradation studies using consortium and the isolate AS_5 were performed in 500 ml shaken flasks containing 100 ml enrichment media and 400, 450, 500, 550, 600, 650 and 700 mg/l of phenol were added after autoclaving the medium at 121 ^{0} C for 20 minutes. The flasks were then inoculated with 0.27% (w/v) of AS_5. Same inoculum density was used from the phenol degrading fed-batch reactor as bacterial consortium. Inoculated flasks were then placed in an orbital shaker adjusted to 200 rpm at 30 ± 3 ^{0}C for 24 hours and samples drawn at an interval of 2 hours for analysis.

(c) *Phenol degradation in mixture of compounds*

The effect of glucose and 2,4-dichlorophenol on the degradation of phenol by the consortium and AS_5 were studied by inoculating them to shaken flasks containing 100 ml of medium with 500 mg/l of phenol and 160 mg /l of glucose and 500 mg/l of phenol with 50 mg/l of 2,4-dichlorophenol. Control experiment was done with 500 mg/l of phenol in 100 ml enrichment medium. Biomass concentration used in each test were 0.8 % (w/v). Inoculated flasks were placed on orbital shaker adjusted to 200 rpm at 30 ± 3 ^{0}C.

d) *Analytical methods*

The concentration of phenol in samples were analysed colourimetrically using 4-aminoantipyrene method. Viable counts were determined by serial dilution technique. Growth of individual culture was monitored by measuring absorbance at 600 nm. Dry weight of the cells was determined gravimetrically after drying the samples for 90 °C for constant weight. Mixed liquor suspended solids (MLSS) were determined as described in Chapter 2 .

5.3 Results

(a) Degradation of phenol by microbial consortium and AS_5 at varying initial concentration

The degradation of phenol ranging from 400 to 700 mg/l was studied by employing microbial consortium developed on 500 mg/l of phenol and one of its best isolates AS_5 degrading phenol. During 24 hours, consortium could degrade 99.8 % (399 mg/l) of fed phenol (400 mg/l) and the amount of phenol degradation was increased steadily with the increase of phenol concentration as it was observed as 633 mg at 700 mg/l concentration (Fig.5.1).

The degradation of phenol by AS_5 was significantly lower than the consortium as it was observed as 320 mg/l of the fed 400 mg/l phenol. Unlike in the consortium the degradation of phenol declined drastically beyond the concentration of 600 mg/l (Fig.5.1).

(b) Degradation of phenol by microbial consortium and AS_5 in presence of glucose or 2,4-dichlorophenol

Addition of 160 mg/l of glucose with 500 mg/l phenol feed resulted in an increased degradation of phenol by both consortium and pure culture during the early hours of incubation (Fig.5.2a and b).

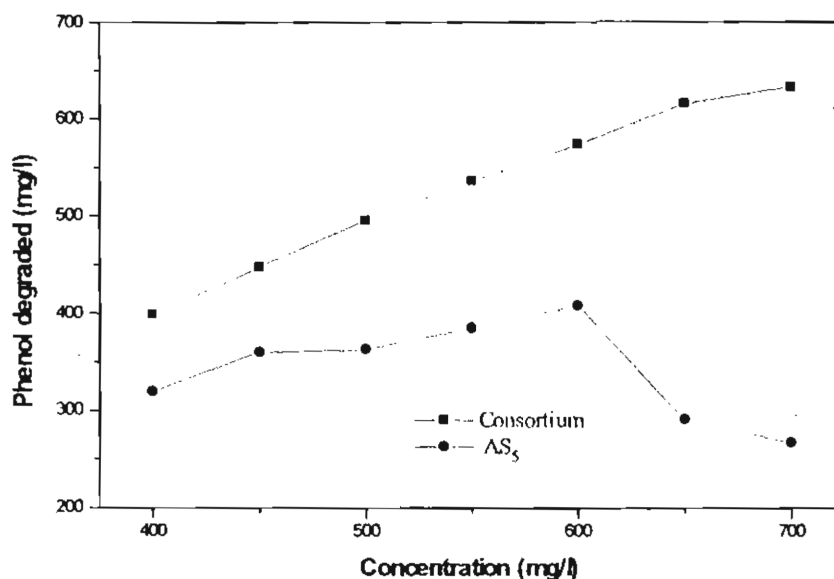


Fig.5.1 Amount of phenol removed by consortium and AS_5 at different phenol concentrations during 24 hours of shaking

It was also observed that the glucose degradation was higher in the case of consortium (89 %) than the pure culture (46 %) during 24 hours (Fig.5.3). The degradation of phenols by consortium from a mixture of 50 mg/l 2,4-dichlorophenol and 500 mg/l phenol was 527 mg/l (Fig.5.2 a), which is comparable to that of 536 mg/l from 550 mg/l of phenol, feed alone (Fig.5.4).

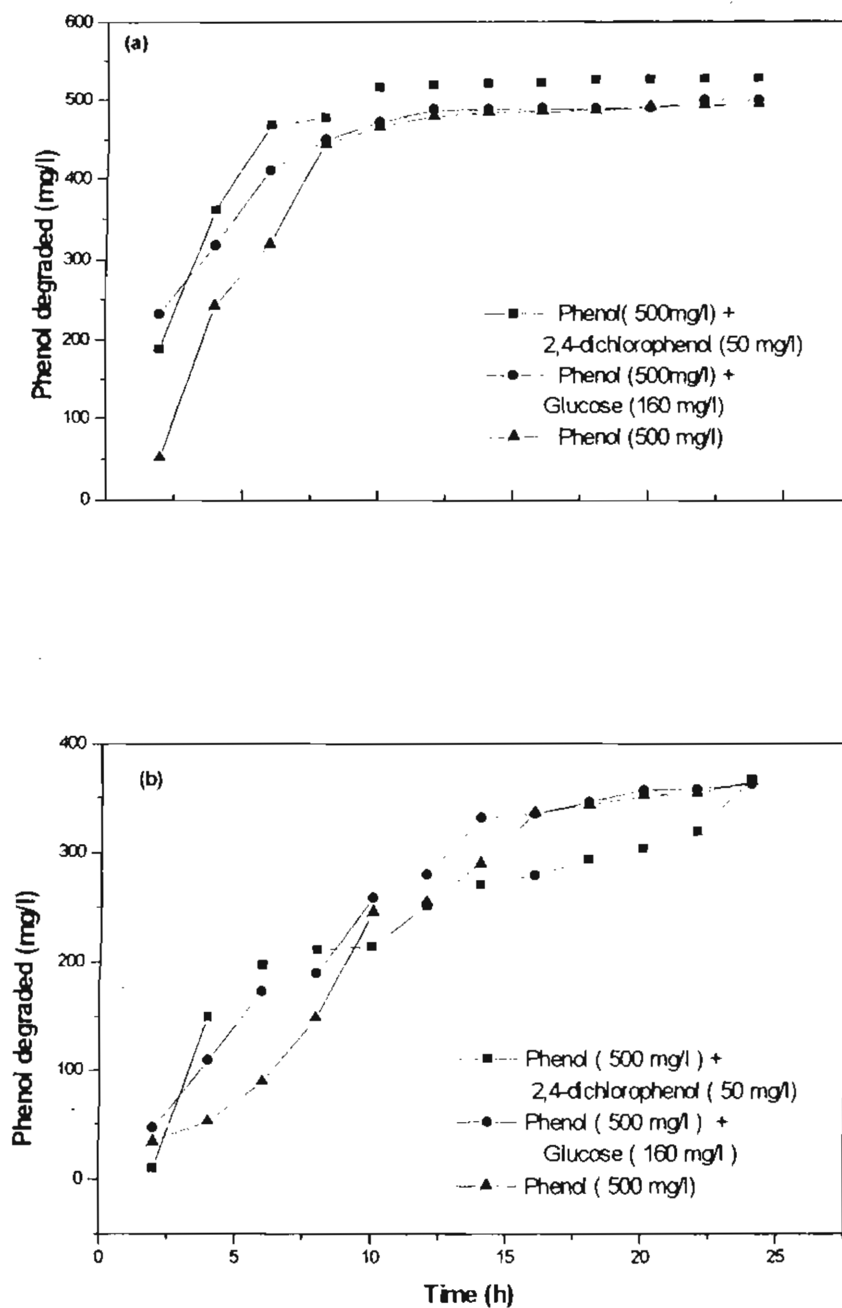


Fig.5.2 Phenol degraded by the consortium (a) and pure culture AS_6 (b) with or without other carbon sources during 24 hours

However, pure culture could degrade only 363 mg of phenol from the mixture of 50 mg/l of 2,4-dichlorophenol and 550 mg/l phenol, which is same as that of phenol degradation (363 mg) by AS_5 in 500 mg/l phenol feed alone (Fig.5.1).

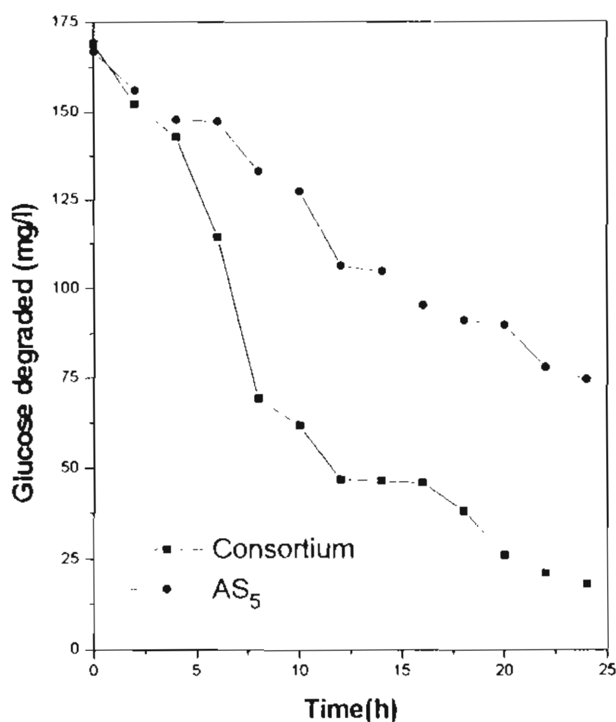


Fig.5.3 Amount of glucose degraded by consortium and pure culture during 24 hours of shaking

5.4 Discussion

Microorganisms are capable of degrading and utilising organic pollutants as sole carbon and energy source in pure cultures, but their performance may alter when they are introduced into the natural environment. The changes in concentrations, presence of other organic compounds and unavailability of oxygen are the few major factors affecting the performance of the bacteria under natural conditions.

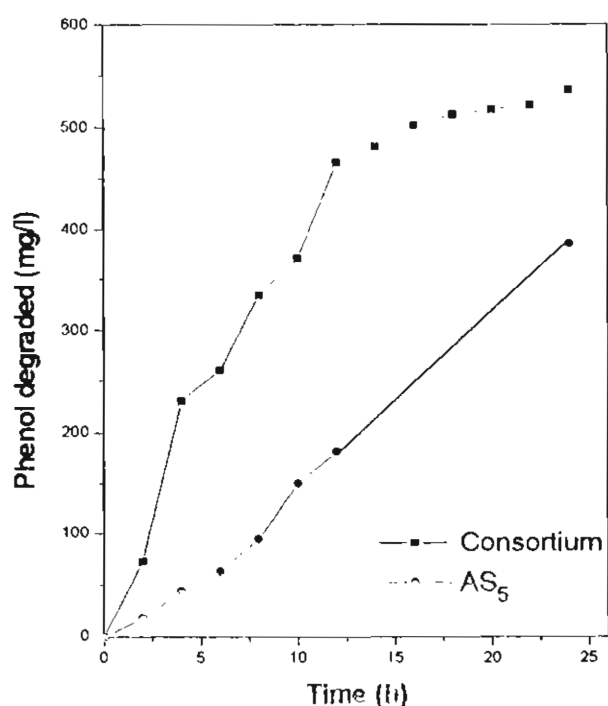


Fig.5.4 Phenol degraded by the consortium and pure culture AS_5 at 550 mg/l phenol during 24 hours

Results of the phenol degradation studies by microbial consortium showed that the phenol degradation was enhanced with the increased feed in the range of concentrations tried (400, 450, 500, 550, 600, 650 and 700 mg/l) 700 mg/l (Fig.5.1). But the pure culture could not obtain higher degradation rate beyond 600 mg/l phenol. This indicates the better stability of consortium under varying feed concentrations.

In the study of Khoury et al (1992), an inhibition of phenol degradation has been observed at a concentration of 376.44 mg/l by a 3 member aerobic consortium consisted of *Pseudomonas fluroscens III, Acinetobacter johnsonii* and a spiral shaped oxidase positive organism. Similarly, an inhibition of 4-chlorophenol by a 3 member mixed culture consisted of *Pseudomonas, Flavobacterium* and *Alcaligenes* at the concentration of 400 mg/l has been reported by Buriton *et. al.* (1994). Importantly, in

the present study, the consortium could easily degrade 633 mg phenol in 700 mg/l concentration without inhibition. One speciality of this consortium is that it consists of 10 members including 2 non-phenol degraders. It is assumed that the presence of such a large community with varied metabolic capabilities have helped to degrade phenol at a faster rate, and thus helped to reduce the concentration effect, and toxicity stress. The high removal of phenol by the consortium may be also due to greater absorption capability of certain members or because of their special uptake of phenol from a lower concentration. Possible removal of inhibitory metabolites in a consortium, which is reported in many earlier studies, may have contributed positively in this case also.

Presence of more than one organic compounds in the system may affect the biodegradative performance of microorganisms (Zaidi and Mehta, 1996). Rozich and Colvin (1986) have reported the inhibition of phenol by glucose addition to a heterogeneous population previously acclimated to phenol. But in the present case, an enhanced degradation of phenol was observed by the addition of glucose at least in the early hours. The consortium could degrade 527 mg of phenol from the mixture of 50 mg/l of 2,4-dichlorophenol and 500 mg/l phenol, which shows that the system was capable of degrading both phenol and 2, 4-dichlorophenol. Such a trend was not seen from the pure culture as it could take only 363 mg from the same mixture (Fig.5.2 b). Simultaneous degradation of both glucose and phenol indicates that, substitutable substrates which are dissimilar in origin and molecular structure may be involved in the non *inhibitory interaction.. Thus, results of this study show the unique potential of* phenol *microbial consortium.*

In the present study, it was found that consortium structure was maintained same under varied experimental conditions and long duration of operation. The stable degradative activity of the consortium was also maintained stable despite of the stresses such as change in concentrations and mixing of other substrate other than phenol.

CHAPTER 6

IMPORTANCE OF HETEROGENEITY IN THE MICROBIAL CONSORTIUM FOR STABLE PHENOL DEGRADATION

6.1 Introduction

Microbial consortium or mixed culture offers advantages over pure culture of having increased metabolic capabilities which enable them to overcome the difficulty in the complete degradation of a particular substrate (Davison *et. al.* 1994). This phenomenon is evidently expressed in the degradation of a large spectrum of environmental pollutants. It was also not exceptional to the biodegradation of phenol (Khoury *et. al.* 1992, Bisaillon et. *al.* 1993, Gallert and Winter, 1993 and Ambujom and Manilal, 1995), chlorinated phenols (Puhakka *et. al.* 1995) and nitrophenols (Hess *et. al.* 1990, Silverstein *et. al.* 1994).

The structure of a microbial community degrading organics depends on its constituent members, and their ability of degradation or mineralization. Variations among the constituent members of a consortium have been reported in terms of their morphology and physiology. Davison *et. al.* (1994) have isolated a nine membered aerobic bacterial consortium degrading poly chlorinated biphenyls, in which they differ in morphological and physiological attributes. Juteau *et.al.* (1995) have found a methanogenic consortium degrading pentachlorophenol consisted of seven morphologically different bacteria.

Degradation of herbicide dicamba with a consortium and its isolates showed that the rate of degradation is high in consortium than that of pure culture. This is probably due to the presence of different species and with varied physiological nature (Fogarty and Tuovinen, 1995).

Phenol and its derivatives are metabolised via two different pathways initiated either by ortho or meta cleavage (Dagley, 1985 (Fig.6.1), Muller and Babel, 1994). Aerobically phenol is first converted to catechol, a reaction which is catalysed by a monoxygenase. Subsequently, the catechol is degraded via an ortho- or meta-fission to intermediates of the central metabolism. The initial ring fission is catalysed either by an ortho-cleaving enzyme, catechol 1,2-dioxygenase or by a meta cleaving enzyme catechol 2,3-dioxygenase, where the product of the ring cleavage is the cis-cis muconic acid for the former and 2-hydroxy muconic semialdehyde for the later (Ahamd and Kunhi, 1996). Metabolic diversity of pure culture degrading through both pathways is also documented. Many *Pseudomonas* species for example, *P. putida* (Morsen and Rehm,1990) and *P.aeruginosa* (Ribbons, 1970) degrade phenol through meta-pathway. There are only few reports on bacteria utilizing phenol through ortho-pathway which include *Alcaligenes* sp. A72, a genetically modified strain (Menke and Rehm, 1992), *Pseudomonas cepacia* CMA1 (Stockinger *et.al.* 1992) *Pseudomonas stutzeri* (Palleroni, 1984) etc. Phenol and cresol metabolism through ortho cleavage pathway has been reported in *Bacillus pumilus* by Gunther *et.al.* (1995). It is found that most of the yeast species degrade phenol through ortho-pathway; for example , *Cryptococcus elinovii H1* (Morsen and Rehm, 1990) and *Rhodotorula* (Katayama-Hirayama *et.al.* 1991). Evidence of operating both pathways for the metabolism of phenol was reported in the fungi *Aspergillus fumigatus* (Jones *et.al.* 1995). The meta cleavage pathway for aromatic ring degradation was reported in a eukaryotic alga *Ochromonas danica* (Simple and Cain, 1996).

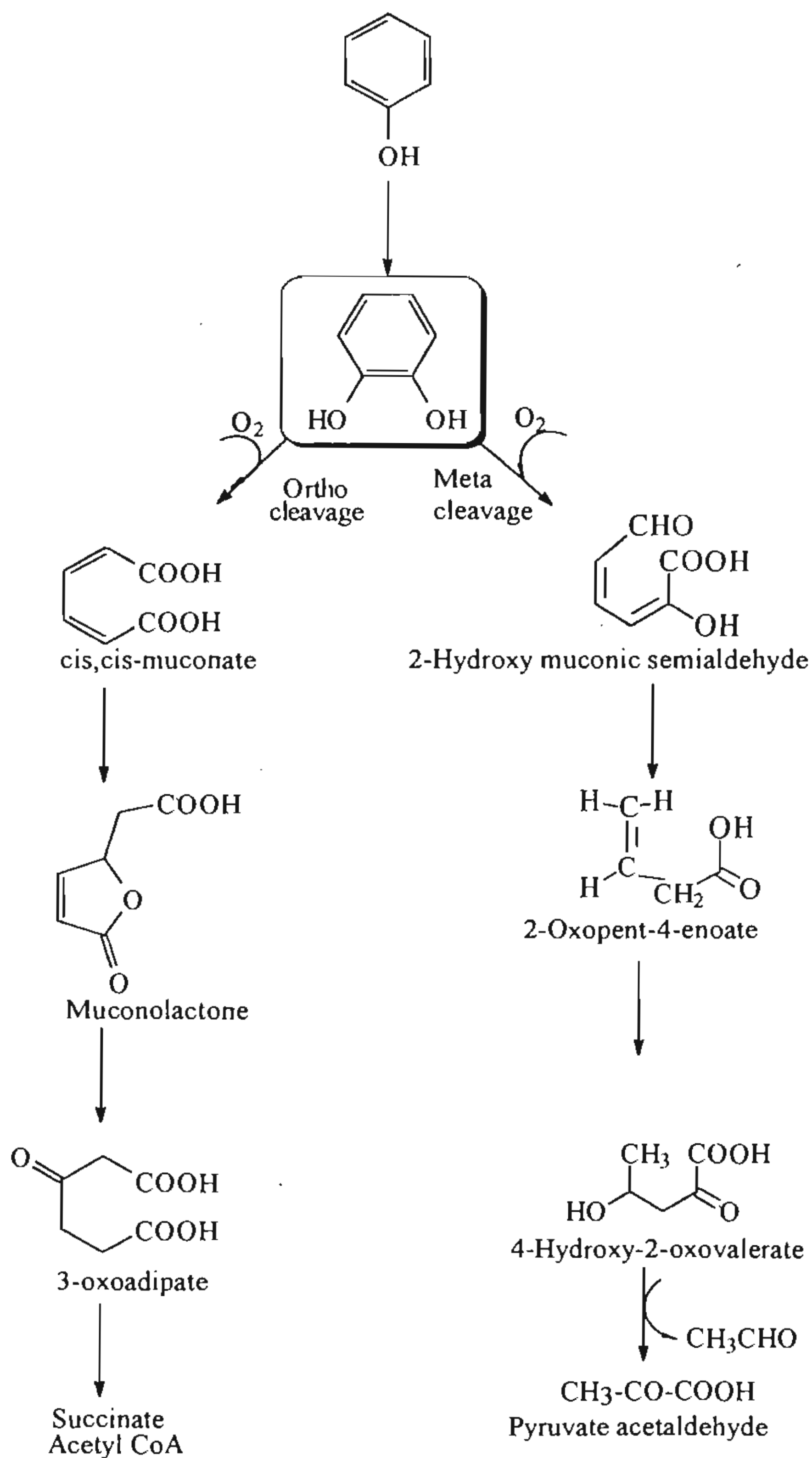


Fig.6.1 Aerobic degradation pathway of phenol

It has not been clearly understood how the metabolic diversity of the members of a consortium degrading toxic compound harmoniously works to achieve their complete degradation. However, the strong thinking is that metabolic diversity may play a significant role in the biomineralization of organic pollutants in the environment.

This chapter of the thesis deals with the importance of heterogeneity of microbial population of consortium in their morphological, biochemical and physiological attributes related to biodegradation of phenol and its constitutional stability.

6.2 Materials and Methods

(a) Morphological studies

Morphology was studied by (i) plating the consortium isolates on phenol agar and on nutrient agar, (ii) observing the culture under light microscope, (iii) examining after fixing and staining and (iv) scanning electron microscopy of the isolates as in the Chapter 2.

(b) Biochemical studies

(i) Identification of cleavage pathways for phenol degradation

Production of the yellow product α-hydroxy muconic semialdehyde (α HMS) or β-ketoadipate from catechol was tested for detecting meta or ortho ring fission pathways. (Bergey's manual, 1986). A suspension of 10 ml of 72 hour phenol grown bacteria was concentrated to 2 ml by centrifugation at 10,000 g for 15 minutes at 4°C. From the concentrate 0.5 ml was resuspended in 2 ml of 0.2 M Tris buffer (pH 8.0) added with 0.5 ml of toluene to solubilize the cell membrane, which was shaken with 0.2 ml of 1.0 M catechol solution. Appearance of the yellow colour within a few minutes was the indication of meta cleavage activity. To 2.5 ml of cell suspension, 1g of $(NH_4)_2SO_4$ was added and incubated for 1 hour at 30°C. The

sample pH was adjusted to ~10 with 0.5 ml ammonia (5N) and a drop of 1 % sodium nitroprusside was added to the mixture. Appearance of a deep purple colour was observed for ortho cleavage activity.

(ii) Degradation studies with ortho/meta cleavagers

Phenol degradation pattern of all phenol degraders were studied individually by inoculating them into 500 ml Erlenmeyer's flask containing 100 ml enrichment media and 500 mg/L of phenol as sole carbon source. The flasks were inoculated with 2 ml of 72 hour grown bacterial suspension having an optical density of 0.6 at 600 nm, and placed it on orbital shaker adjusted to 200 rpm for 72 hours at 30 ± 3°C.

All phenol degrading members (ortho/meta) of the consortium were inoculated individually or in mixtures into 500 ml Erlenmeyer's flasks containing 100 ml enrichment media and 500 mg/l of phenol. The concentration of the inoculum used in each flask was 0.62 % (w/v) for ortho cleavagers, and 0.34 % (w/v) for meta cleaving organisms. The flasks were incubated on an orbital shaker adjusted to 200 rpm at 30 ± 3 °C and samples were drawn for analyses at an interval of 2 hours.

The impact of varying concentrations of phenol on degradation by ortho and meta cleavager was studied by inoculating 0.4 % (w/v) of cells into 500 ml Erlenmeyer's flasks having 100 ml enrichment media supplemented with phenol at the concentrations 400, 450, 500, 550, 600, 650 & 700 mg/l. The flasks were placed on an orbital shaker adjusted to 200 rpm at 30 ± 3°C. Samples were drawn at an interval of 2 hours for analysis.

(iii) Degradation studies with ortho and meta with or without non-phenol degraders

Meta and ortho cleavagers were studied in combination for their phenol degradation by inoculating *1 member* (AS_5) , 2 members (*1* ortho + *1* meta), 4

members (2 ortho + 2 meta) and 6 members (3 ortho + 3 meta) into 500 ml Erlenmeyer's flasks containing 100 ml enrichment media and 400, 500, 700 mg/l of phenol. The concentration of cells used for inoculation was 0.33 % (w/v) .

Phenol degradation of 8 member consortium (3 ortho + 5 meta) was studied by inoculating 0.3 % (w/v) into 500 ml Erlenmeyer's flasks containing 100 ml enrichment media with 500 mg/l of phenol.

Effect of the presence of non phenol degraders (AS_9 & AS_{10}) with ortho and meta cleavagers in the degradation of phenol was studied by inoculating them into 500 mg/l of phenol at a cell concentration of 0.56 % (w/v). Presence of non-phenol degrader (AS9 + AS_{10}) with all meta and ortho cleaving strains in their degradation of 400, 500, 700 mg/l of phenol was studied by inoculating them at a cell concentration of 0.37 % (w/v) in 500 ml Erlenmeyer's flasks containing 100 ml media and respective phenol concentration. Inoculated flasks were placed on an orbital shaker adjusted to 200 rpm at $30 \pm 3\,^{0}C$.

In all above the studies biomass concentration used was same as that of control system.

(c) Physiological studies

Physiological characteristics of individual members of the consortium were studied by inoculating them into 400, 500, 700, 900, 1000 mg/l of phenol and 100 mg/l of 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, o-, m-, and p-cresol, nitrobenzene, 2-chlorobenzene, 4-hydroxybenzoic acid, resorcinol, catechol and salicylic acid slants.

The ability of non-phenol degrading members of consortium to utilise various carbon sources were examined by inoculating them into 100 mg/l of glucose, lactose, ammonium formate and catechol media.

6.3 Results

Diversity of the members of phenol degrading microbial consortium were studied in their morphological, biochemical and physiological attributes.

(a) Morphological studies

Both gram positive and gram negative rods were found in the consortium, with the size varied from 0.45 to 3.3 μm cell length and 0.13 to 1 μm cell diameter (Fig.6.2 a to j photomicrograph).

(b) Biochemical studies

Biochemical characteristics of individual members of the consortium were examined by identifying their cleavage patterns of phenolic ring fission. It was identified that AS_1, AS_2, AS_4, AS_5 and AS_8 as meta cleavagers and AS_3, AS_6 and AS_7 as ortho degraders.

(i) Degradation of phenol by ortho/meta cleavagers

The results of phenol degradation studies employing individual isolates are shown in Table 6.1 and Fig. 6.3. The isolate AS_5 showed the highest level (360 mg) of degradation from 500 mg/l of phenol during 24 hours, whereas the AS_7 exhibited the lowest level. In general, the bacteria degrading through ortho pathway had slightly lower rate of degradation compared to meta cleavagers. The phenol degrading members of consortium except AS_1, AS_4, AS_7 and AS_8 maintained near neutral pH conditions in the system (Table 6.2).

Table 6.1 Bacterial isolates and their respective phenol degradation rate at 500 mg/l

Bacterial strains	mg of phenol degraded during		
	24h	48h	72h
AS_1	160	320	430
AS_2	300	430	500
AS_3*	170	435	495
AS_4	270	430	495
AS_5	360	435	500
AS_6*	200	350	485
AS_7*	120	255	430
AS_8	130	245	495

* Ortho cleavagers

The phenol degradation profile of bacterial isolates at varying concentrations of phenol ranging from 400 to 700 mg/l is given in Fig.7.3. The best phenol degrading isolate (AS_5) showed an increase in degradation upto 600 mg/l, whereas the remaining did not show much degradation beyond 550 mg/l or 500 mg/l of phenol.

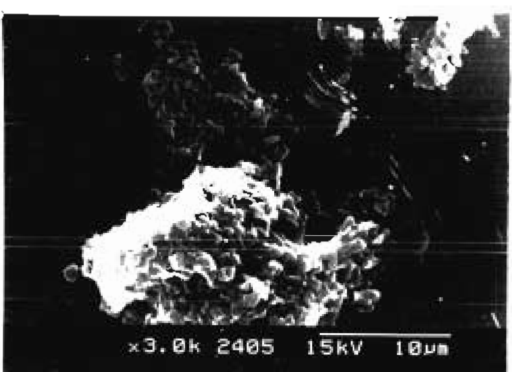
Table 6.2. Variation of pH in the culture medium of phenol degrading members of consortium at 500 mg/l

Strains	0h	2h	4h	6h	8h	10h	12h	14h	16h	18h	20h	22h	24h
AS_1	6.7	6.7	6.6	6.6	6.5	6.6	6.5	6.5	6.4	6.3	6	5.8	5.6
AS_2	6.7	6.8	6.7	6.6	6.6	6.7	6.6	6.6	6.6	6.5	6.4	6.2	6
AS_3	6.8	6.8	6.7	6.6	6.6	6.7	6.6	6.6	6.6	6.6	6.3	6.2	6
AS_4	6.4	6.7	6.6	6.6	6.5	6.8	6.7	6.7	6.7	6.7	5.8	5.5	4.3
AS_5	6.7	6.7	6.6	6.6	6.6	6.6	6.6	6.5	6.5	6.4	6.3	6.2	6
AS_6	6.8	7	6.7	6.7	6.6	6.7	6.6	6.6	6.6	6.4	6.3	6.2	6.1
AS_7	6.8	6.8	6.7	6.7	6.7	6.7	6.6	6.5	6.5	6.3	5.4	4.6	4.3
AS_8	6.8	6.8	6.7	6.8	6.7	6.7	6.7	6.7	6.5	6.4	6.2	5.6	5.2

In general, the phenol degradation by ortho cleaving members of consortium was lower than that of meta cleavagers.

Fig. 6.2 Morphological diversity characteristics of members of consortium

(a) AS_1, (b) AS_2, (c) AS_3, (d) AS_4, (e) AS_5, (f) AS_6, (g) AS_7, (h) AS_8, (i) AS_9 and (j) AS_{10}



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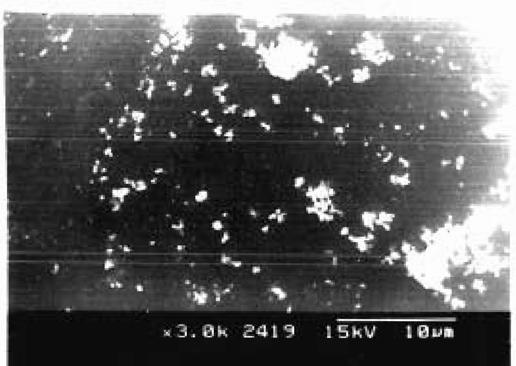

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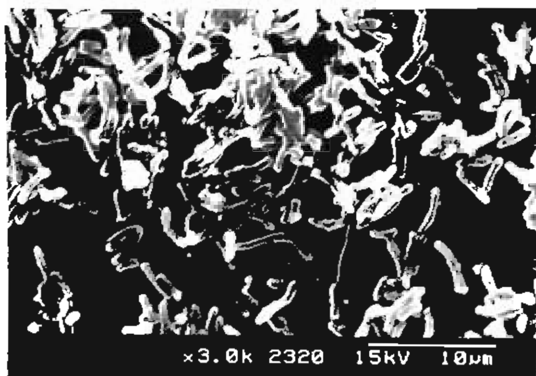

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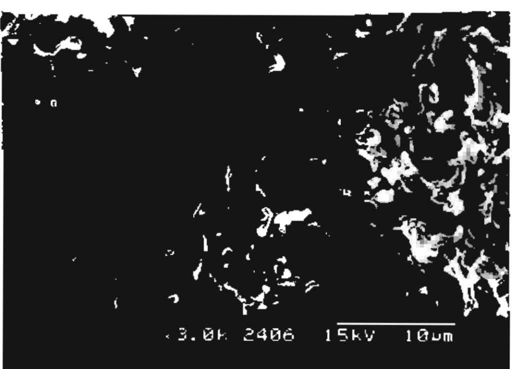

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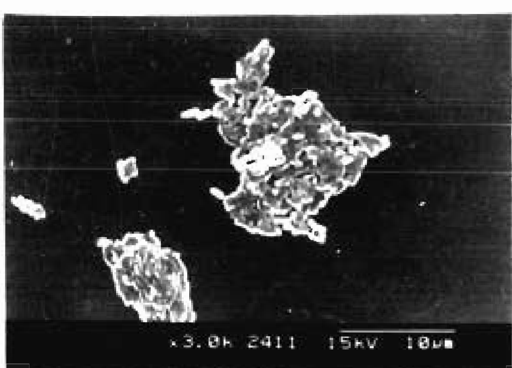

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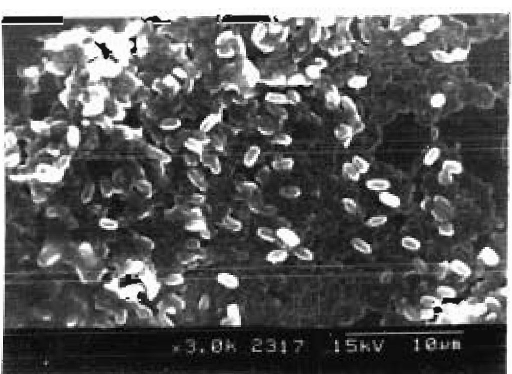

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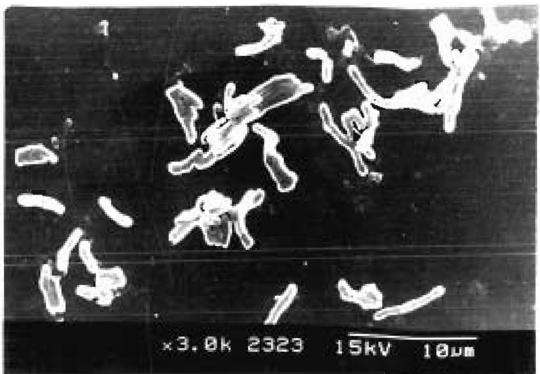

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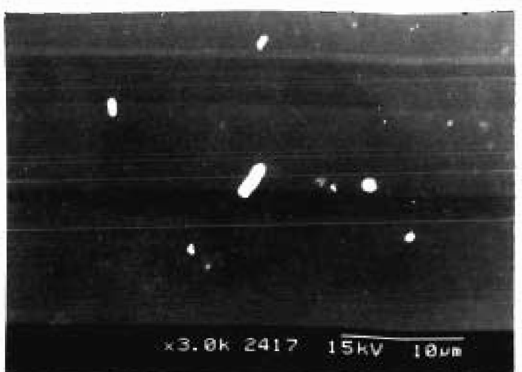

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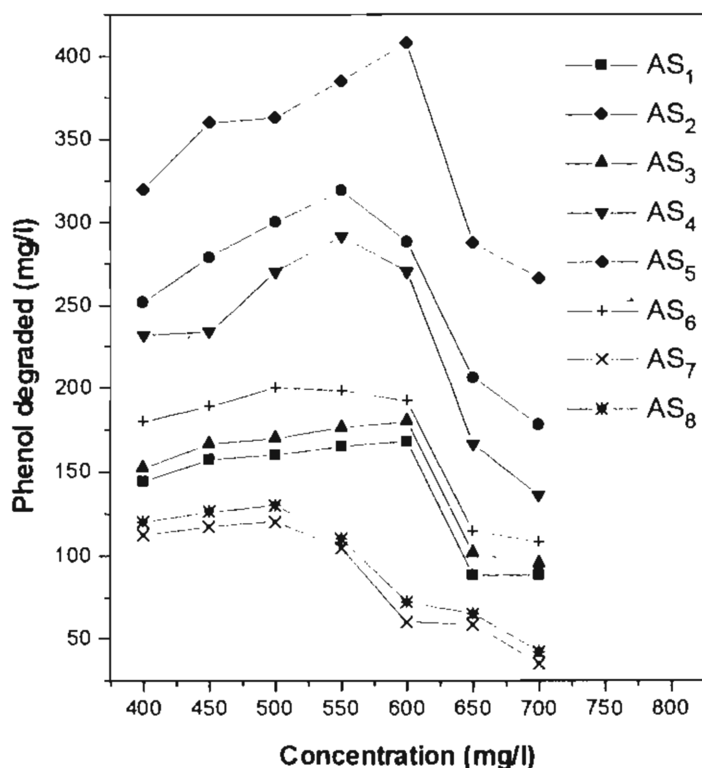

Fig.6.3 Phenol degradation by the isolates of consortium during 24 hours

The studies with 1 meta (AS_5), 2 meta (AS_5 + AS_8), 3 meta (AS_5 + AS_1 + AS_2), 4 meta (AS_5 + AS_1 + AS_4 + AS_8) and 5 meta (AS_5 + AS_1 + AS_2 + AS_4 + AS_8) showed increased phenol degradation with increase in members of the culture as evidenced from Fig.6.4a.

The pH was maintained close to neutral range (pH 6.69 to 6.12) in the case of 1 meta and 3 meta cleavagers added system, but in the cultures of 2 meta, 4 meta and 5 meta cleaving mixtures the pH was dropped from 6.7 to 4.0. (Fig.6.4b).

The results of degradation by the ortho cleavagers indicated that highest rate of phenol degradation with the mixture of 3 ortho (AS_3 + AS_6 + AS_7). The rate of degradation was declined with lesser members (Fig.6.5).

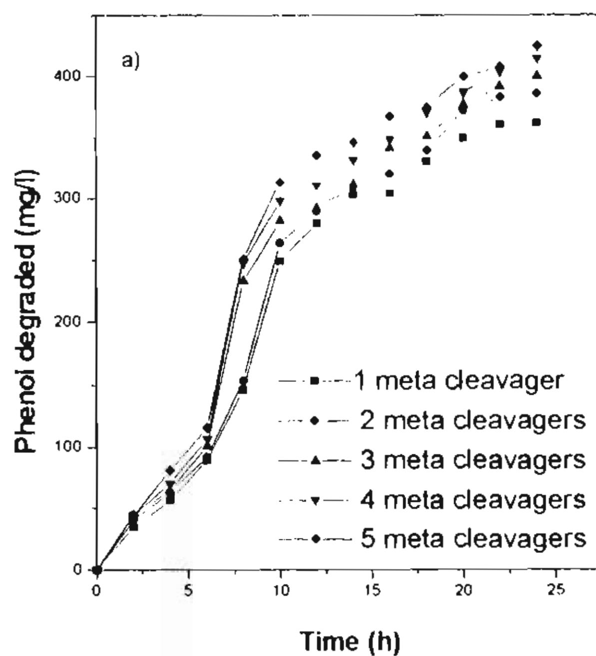

Fig.6.4a Pattern of phenol degradation at 500 mg/l by one or more meta cleavagers

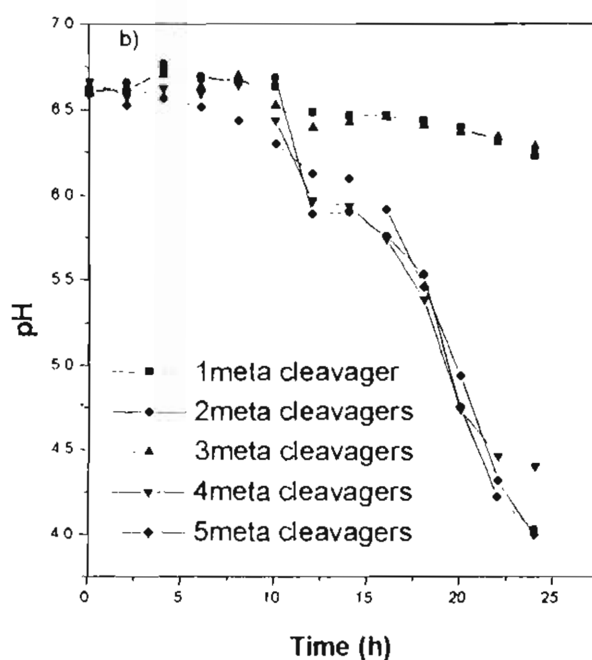

Fig.6.4b pH variation during degradation of phenol (500 mg/l) by meta cleavagers

Degradation of phenol was affected above the acclimated level (500 mg/l) of phenol in both the group of meta and ortho cleavagers. But the degradation of ortho cleavagers was significantly affected at the highest concentration (700 mg/l) tried (Fig.6.6a).

The meta cleavagers maintained their pH in the neutral range (Fig.6.6b). But, the pH was dropped significantly i.e. from 6.79 to 3.43 with ortho clevagers during increasing concentration (Fig.6.6c).

(ii) Mixed culturing of ortho and meta with or without non-phenol degraders

Results of the studies on degradation of phenol at the concentrations of 400, 500 and 700 mg/l employing 1 member (AS_5), 2 member [one meta (AS_5) + one ortho (AS_7)] , 4 member [2 meta (AS_1 + AS_4) + 2 ortho (AS_3 + AS_6)], 6 member [3 meta (AS_2 + AS_5 + AS_8) + 3 ortho (AS_3 + AS_6 + AS_7)] and 8 member [5 meta (AS_1 + AS_2 + AS_4 + AS_5 + AS_8) + 3 ortho (AS_3 + AS_6 + AS_7)] mixed system are presented in Fig.6.7, where the degradation of 1 and 2 member system the degradation was affected at 700 mg/l of phenol concentration. On the contrary, a significant increase in degradation was observed with the mixtures of 4,6 and 8 member mixtures as the phenol concentration was increased.

The amount of phenol degradation achieved at different concentrations by the 1, 2, 4, 6 and 8 member consortium is presented in the Fig.6.8 which clearly, shows the poor performance of 1 and 2 member system at 700 mg/l of phenol. The degradation rate of phenol was significantly increased with number of members in 700 mg/l of phenol .

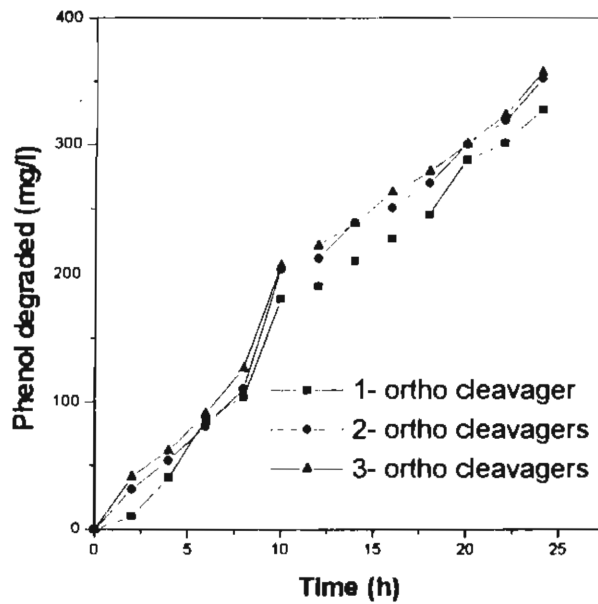

Fig.6.5 Phenol degradation at 500 mg/l by ortho cleaving organisms

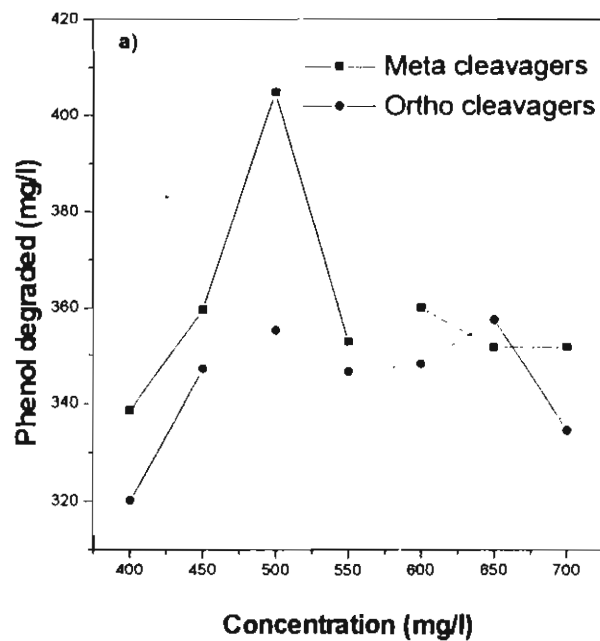

Fig.6.6a Phenol degradation by 3 member meta/ ortho cleavaging organisms during 24 hours at varying initial concentration of phenol

In the consortium including 8 phenol degraders and 2 non-phenol degraders the pH was maintained at neutral range unlike, the ortho cleavagers mixed systems where

wide variations of pH was noticed. The biodegradation of phenol by the ortho/meta cleavagers was influenced by the additions of non-phenol degraders (Fig.6.9a, b). When the meta and ortho separately cultured with non-phenol degraders the pH was reduced from 6.6 to 5.0 (Fig.6.10a, b).

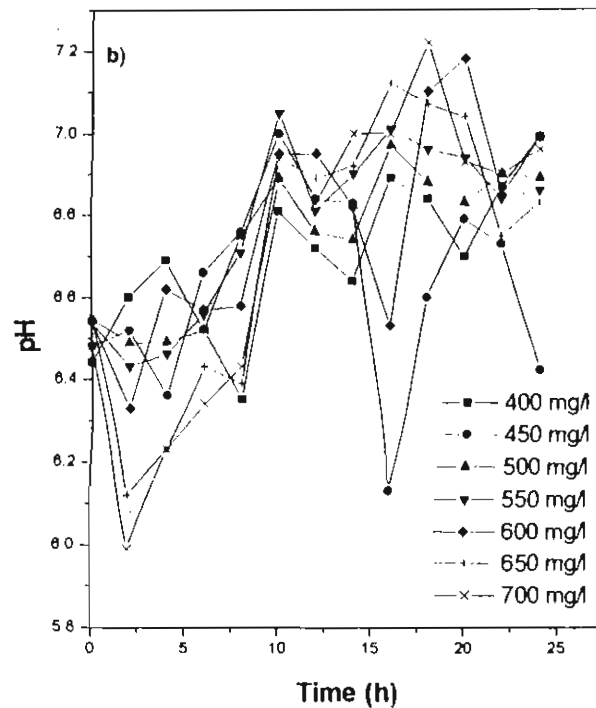

Fig. 6.6b pH variation in 3 meta cleavager consortium fed with different phenol concentrations

The presence of non-phenol degraders with 8 phenol degraders (5 meta + 3 ortho) showed the maximum degradation of 399.9 mg from 400 mg/l of phenol and 621.3 mg from 700 mg/l of phenol during 24 hours (Fig.6.8).

There was not much effect by increase in the biomass concentration from 0.5 to 1.0 % of AS_7 (slow phenol degrading ortho cleavager) with AS_5 (best phenol

degrading ortho cleavager) as evidenced by the marginally increased degradation of 84 to 86 % at 500 mg/l concentration (Fig.6.11).

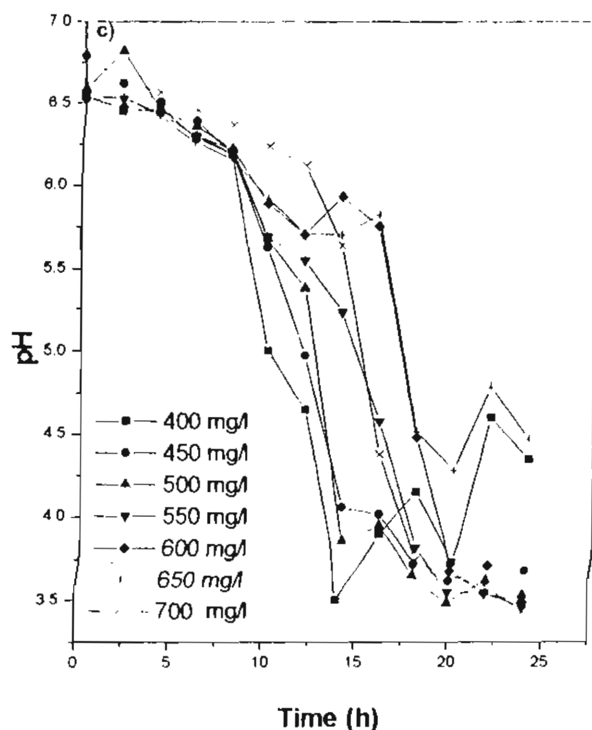

Fig.6.6c pH variation in 3 ortho cleavager consortium fed with different phenol concentrations

Physiological studies:

Pattern of phenol degradation by the members of consortium in the utilisation of various carbon sources is given in Table 6.3. All phenol degrading members except AS_7 and AS_8 of the consortium were able to degrade phenol upto the maximum concentration of 1000 mg/l phenol tried. The strains AS_7 and AS_8 failed to grow beyond 700 mg/l phenol concentration.

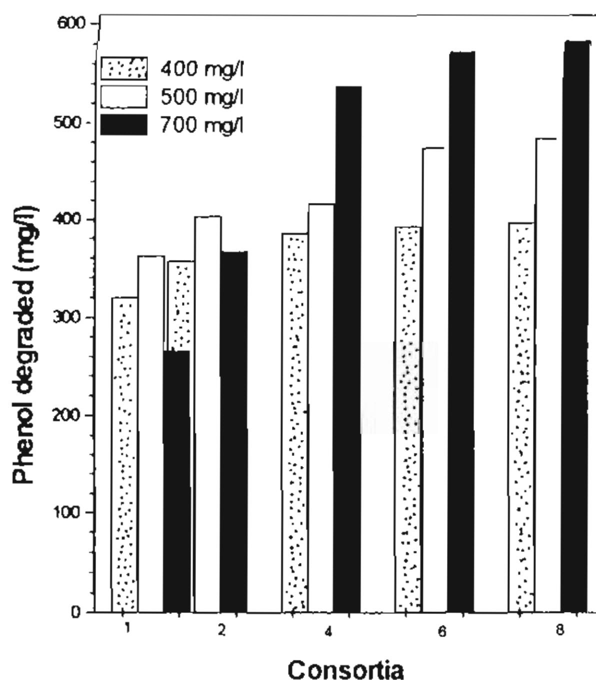

Fig.6.7 Phenol degraded by different consortia fed with varied phenol concentration during 24 hours

Phenol degrading members of consortium such as AS_1, AS_2, AS_3, AS_5, AS_7 and AS_8 were able to grow on 100 mg/l of chloro- derivatives of phenol, cresols, catechol, salicylic acid, 4-hydroxybenzoic acid, 2-chlorobenzoic acid, nitrobenzene and resorcinol. However, the strain AS_4 failed to grow on salicylic acid, 2-chlorobenzoic acid, 4-hydroxybenzoic acid , nitrobenzene and resorcinols. The strain AS_6 could not grow on all the above compounds except resorcinol (Table 6.3).

The non-phenol degrading members of the consortium were unable to utilize any of the aromatic compounds tried as their carbon sources, but they were able to grow on ammonium formate agar medium.

Table.6.3 Physiological characteristics of the members of consortium (AS)

Compounds	AS_1	AS_2	AS_3	AS_4	AS_5	AS_6	AS_7	AS_8	AS_9	AS_{10}
Phenol 400mg/l	+	+	+	+	+	+	+	+	-	-
Phenol 500 mg/l	+	+	+	+	+	+	+	+	-	-
Phenol 700 mg/l	+	+	+	+	+	+	+	+	-	-
Phenol 900 mg/l	+	+	+	+	+	+	-	-	-	-
Phenol 1000 mg/l	+	+	+	+	+	+	-	-	-	-
2-chlorophenol*	+	+	+	+	+	+	+	+	-	-
3-chlorophenol*	+	+	+	+	+	+	+	+	-	-
4-chlorophenol*	+	+	+	+	+	+	+	+	-	-
2,4-dichlorophenol*	+	+	+	+	+	+	+	+	-	-
p-cresol*	+	+	+	+	+	+	+	+	-	-
m-cresol*	+	+	+	+	+	+	+	+	-	-
o-cresol*	+	+	+	+	+	+	+	+	-	-
Catechol*	+	+	+	+	+	+	+	+	-	-
Salicylic acid*	+	+	+	-	+	-	+	+	-	-
4-hydroxybenzoic acid*	+	+	+	-	+	-	+	+	-	-
2-chlorobenzoic acid*	+	+	+	-	+	-	+	+	-	-
Nitrobenzene*	+	+	+	-	+	-	+	+	-	-
Resorcinol*	+	+	+	-	+	+	+	+	-	-

* Concentration 100 mg/l

6.4 Discussion

The results of this study demonstrated the significance of diverse characteristics of the members of the consortium for its stable phenol degradative performance.

It was observed that a heterogeneous group of microorganisms was worked together as a consortium to degrade the phenol. Although the bacteria were all rods, they possessed *different size (Photomicrograph 6.2a to j) and physiology in terms of* specific growth and degradation rate (Table 6.3).

In the degradation of phenol, bacteria of the consortium operated both meta cleavage and ortho cleavage pathways which were found to be advantageous for maintaining a stable system. The role of two bacteria AS_9 and AS_{10} was not definitely

involved in the degradation of phenol directly due to their incapability to degrade phenol, but was of help in the group indirectly. It is clear from the results that this consortium could degrade phenol at a higher rate than individual isolates.

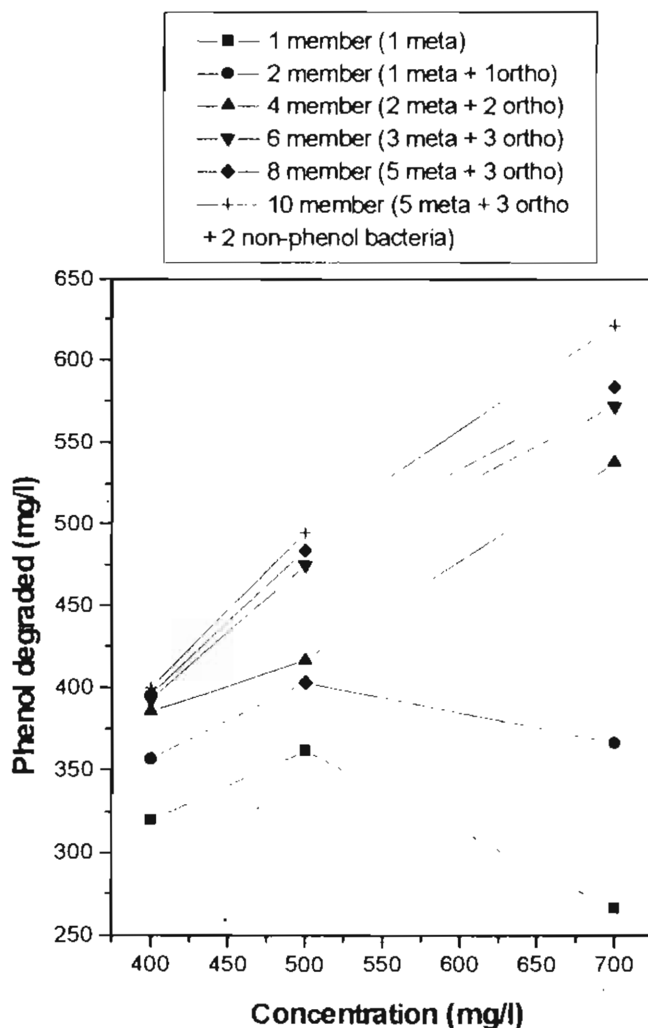

Fig.6.8 Amount of phenol degraded by constituted consortia at varying concentration of phenol during 24 hours

Metabolites formed during the two pathways are of different kinds. Ortho cleavage pathway is chromosomally encoded one (Holloway and Morgan, 1986) and meta cleavage pathway is plasmid encoded one (Harayama *et.al.* 1987).

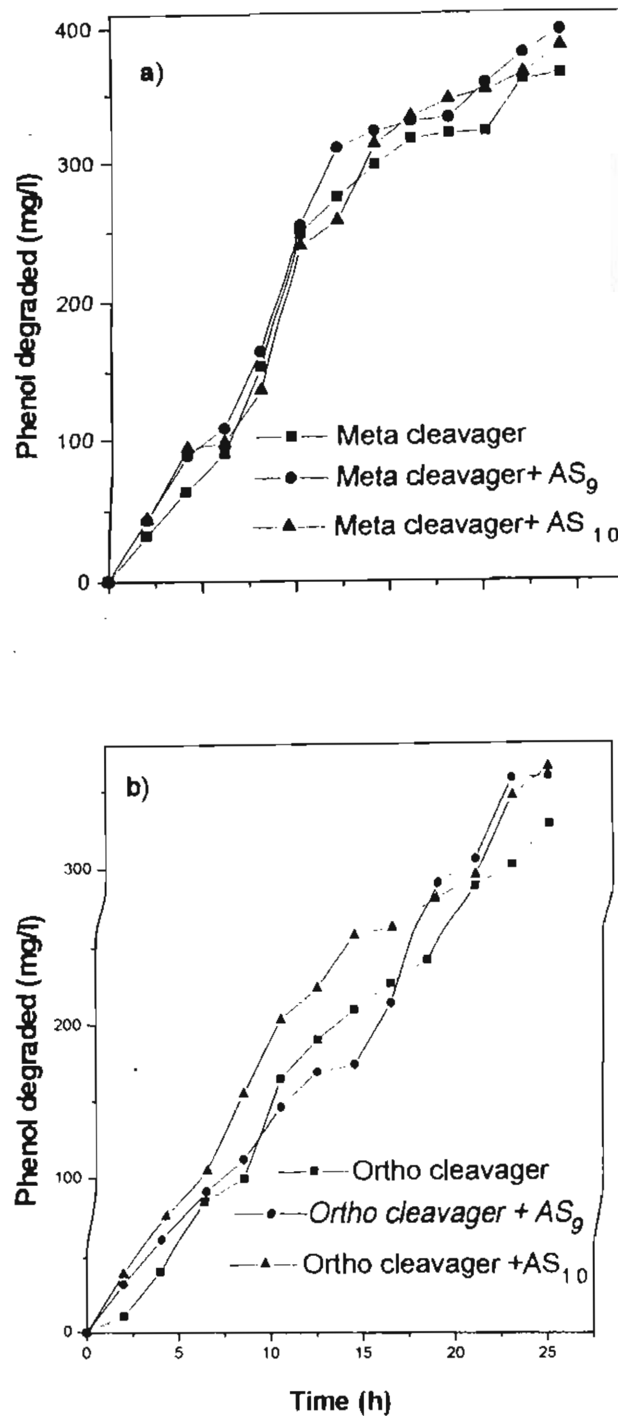

Fig.6.9 Phenol degradation by meta (a) and ortho (b) cleavagers with or without non-phenol bacteria AS_9 and AS_{10} at 500 mg/l

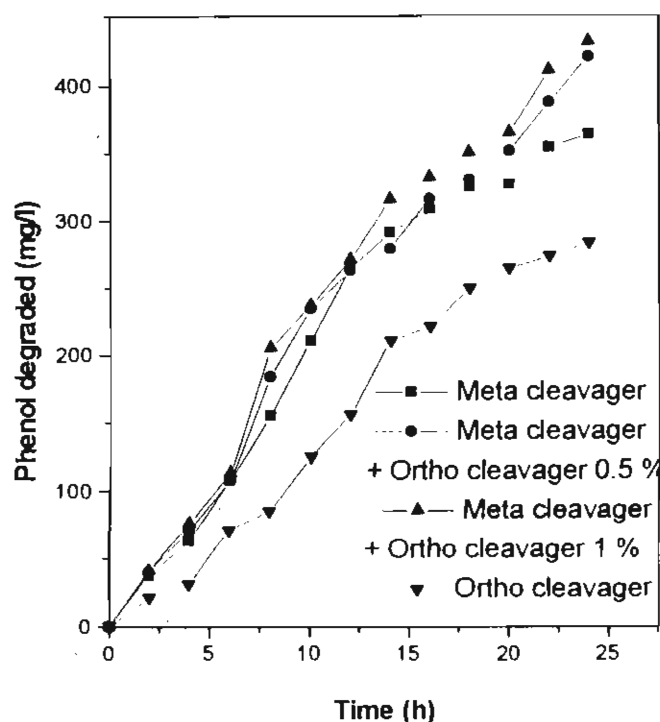

Fig.6.11 Effect of biomass concentration on phenol degradation at 500 mg/l

Due to such differences, the possible inhibition of phenol degradation may be reduced in the consortium considerably. Presumably, the consortium were able to perform stable degradation because of its diverse biochemical pathways and degradation potential.

Physiological attributes of individual members of consortium indicated that the members possess differences in their utilisation of carbon sources in terms of quality and nature. This again shows their physiological diversity which ultimately helped to improve the degradation capability of the consortium. Bell and Tremaine (1995), have expressed their findings that diverse bacterial community will be utilising a large

number of carbon sources because of the increased metabolic capabilities of the members likely to acquired by the community.

It was shown from the above observations that the present phenol degrading consortium was constituted of heterogeneous microorganisms possessing diverse characteristics and qualities. It was also found the heterogeneic community performed better than homogeneic as it evident from the results (Fig. 6.7 and 6.8).

The heterogeneic characteristics of associate members of consortium seems to play a significant role in maintaining structure and functional stability of the consortium.

CHAPTER 7

RECONSTITUTION OF MICROBIAL CONSORTIUM FOR PHENOL DEGRADATION

7.1 Introduction

Wastewater containing phenol and its derivatives have traditionally being treated by physico-chemical methods. Recently, biodegradation have gained importance and many microbial strains have been studied, obviously to develop a suitable system. A large number of bacteria, few yeasts and fungi have been experimented and found as powerful agents for biodegradation. Meanwhile, microbial consortia in the form of activated sludge were recognised for their application in phenolic wastewater treatments. The distinct advantage is their higher level of degradation and stable performance.

The consortia are not developed rather formed naturally. Thorough understanding of consortia in terms of constitution and role of constituent members in function and stability is advantageous for developing suitable and active consortia faster.

Storage of microbial consortium as such for reuse is not possible due to limitations in maintaining cultural properties of constituent members. While, long term preservation of individual isolates of consortium is comparatively simple and easy with desired cell densities and physiological status in proper cultural media. But the task ahead is the reconstitution of the consortium from the isolate to obtain same activity.

Few reports are available on the reconstitution of bacterial consortia, though not very successful for degrading toxic compounds. Reconstitution of microbial consortium from the isolated strains degrading β -O-4 lignin- related dimeric

compound resulted in the loss of metabolic capability of individual strains (Cespedes *et.al.* 1992). The isolates degrading aromatic compounds from olive oil mill effluent have showed lesser activity after reconstitution (Zouari and Ellouz, 1996). The present study of consortium reconstitution was to evaluate individual specificity with respect to its stability and function.

This chapter of thesis deals with the reconstitution of a phenol degrading consortium from isolated individual members. The fluctuations of individual members, performance of phenol degradation, and factors influencing the stability of reconstituted consortium were also studied.

7.2 *Materials and Methods*

Phenol degrading microbial consortium was developed using enrichment techniques as already described in Chapter 2.

Individual strains from the consortium were isolated by plating in appropriate dilutions on phenol agar medium and nutrient agar medium. Isolates were identified as per the procedure given in Chapter 3.

(a) Preparation of bacterial inoculum for reconstitution

In order to prepare the inoculum, all eight phenol degrading bacterial isolates were separately cultured for 72 hours in the liquid medium containing 500 mg/l of phenol. The non-phenol degraders were cultured in nutrient broth for 72 hour at 37 °C. After incubation the cells were harvested by centrifugation at 10,000g for 15 min. at 4 °C. The harvested cells were washed twice with buffered phosphate saline solution. Consortium was reconstituted (Fig.7.1) by mixing the washed cells of all the 10 members at a biomass concentration (2.7 g/l) similar to that of fed-batch reactor (Chapter 2).

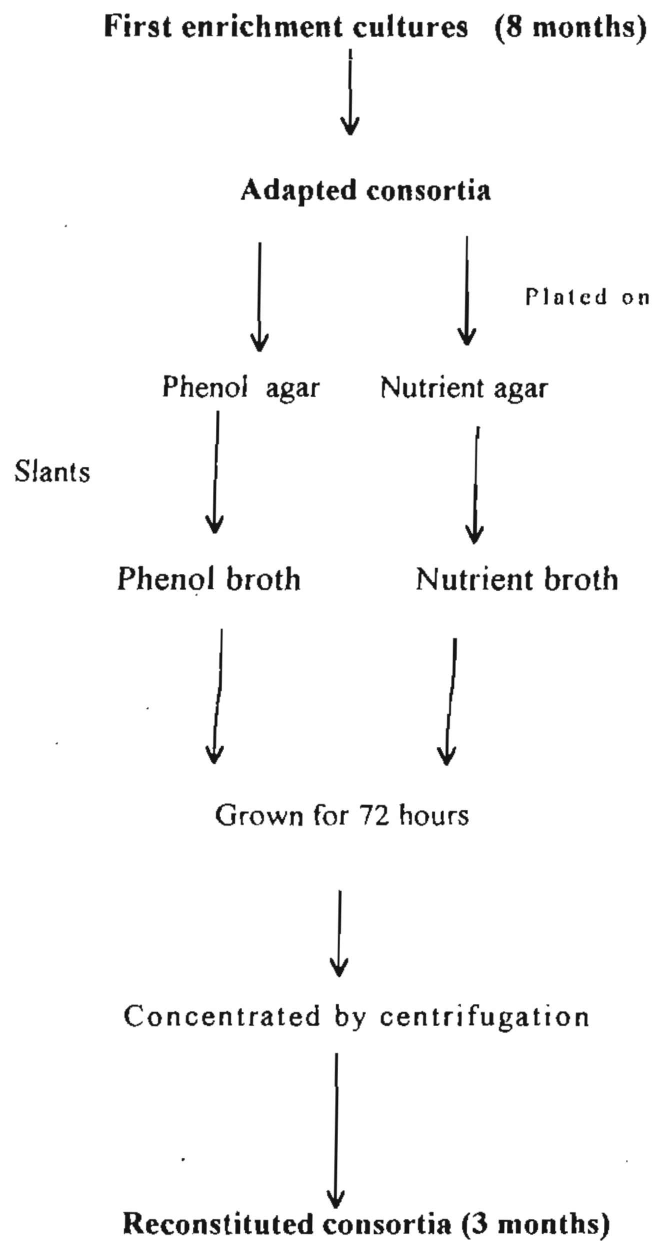

Fig.7.1 Steps in the reconstitution of phenol degrading microbial consortium

(b) Activity *of reconstituted consortia*

Activity of the reconstituted consortia was monitored by estimating phenol degradation, MLSS and viable cell count on phenol agar and nutrient agar. Sample pH was measured with the help of an electrode.

7.3. *Results*

The colour of active consortium was muddy brown throughout the period of operation. But from day 0 to 9 of reconstitution consortium showed a change of colour from white to dark brown and during which the biomass content, phenol degradation activity and pH also decreased (Fig 7.2, 7.3, and 7.4.) However, after 2 months, the dark brown colour of consortium disappeared and original colour (muddy brown) reappeared. These changes indicate that a disturbance in phenol degradation have contributed to biomass fluctuations or biomass fluctuations affected the degradation of phenol in the system.

The total biomass as well as individual population were declined to half of the initial concentration during initial 3 weeks time of reconstitution (Table 7.1). Further, the system re-established to the original concentration within 2 weeks time. Except the changes in the individual population numbers, there was no elimination of strains in the consortium. However, both the individual population and MLSS were maintained almost constant after reaching to the maximum activity in terms of phenol degradation (Fig.7.3).

(a) Activity of reconstituted consortium

The phenol degradation profile of reconstituted consortium showed a significant reduction during the period of affected growth as indicated from the degradation of 450 mg/l of phenol to 175 mg/l per day (Fig.7.3).

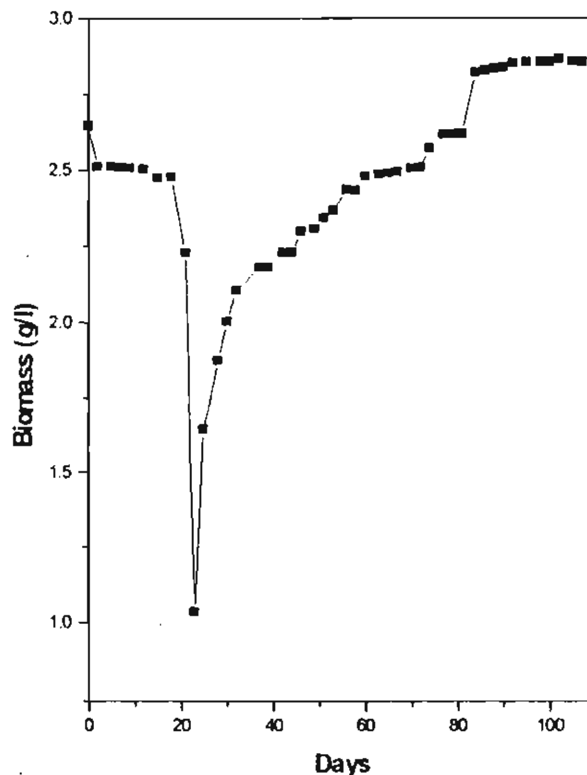

Fig.7.2 MLSS in the 500 mg/l phenol fed-batch reactor inoculated with reconstituted consortium

However, along with the promoted growth of the reconstituted consortium, the phenol degradation also increased and reached to a removal of 99. 8% of 500 mg/l fed phenol after stabilisation in 15 weeks.

pH of the system showed a significant decrease from initial value of 7.35 to 3.2 during the period of reconstitution . By regaining activity, the consortium pH was again shifted to neutral side which was maintained during the further studies (Fig.7.4).

7.4 Discussion

Reconstitution of bacterial mixture was aimed to reproduce phenol degrading bacterial population having the same constitution and function as that of original consortium.

Table 7.1 Variation of individual population in reconstituted consortium (AS)

Strains	0 day	12th day	23rd day	37th day	46th day	60th day	72nd day	84th day	95th day
AS_1	1.1×10^{6}	6×10^{6}	1.1×10^{4}	1×10^{6}	1×10^{8}	1×10^{8}	1×10^{7}	3×10^{7}	4×10^{7}
AS_2	1.3×10^{6}	4×10^{5}	1×10^{4}	1×10^{5}	2×10^{7}	1×10^{7}	2×10^{7}	4×10^{6}	5×10^{6}
AS_3	1.4×10^{6}	4×10^{8}	1×10^{4}	2×10^{8}	1×10^{6}	3×10^{6}	2×10^{6}	2×10^{5}	3×10^{5}
AS_4	3×10^{5}	3×10^{7}	1×10^{4}	4×10^{6}	4×10^{5}	1×10^{6}	5×10^{6}	1×10^{6}	5×10^{6}
AS_5	1×10^{6}	5.2×10^{5}	1×10^{4}	4×10^{7}	4×10^{7}	4×10^{6}	2×10^{6}	2×10^{6}	6×10^{6}
AS_6	7×10^{6}	3×10^{5}	1.6×10^{4}	1×10^{7}	1×10^{8}	2×10^{7}	2×10^{7}	2×10^{7}	7×10^{6}
AS_7	4×10^{6}	3×10^{6}	1×10^{4}	2×10^{7}	1×10^{6}	2×10^{7}	1×10^{6}	2×10^{8}	6×10^{8}
AS_8	1×10^{7}	2×10^{8}	5×10^{5}	2×10^{8}	2×10^{7}	2×10^{7}	2×10^{7}	1×10^{7}	4×10^{7}
AS_9	5.9×10^{8}	3×10^{7}	3×10^{5}	2×10^{7}	1×10^{8}	1×10^{7}	2×10^{7}	1×10^{7}	4×10^{7}
AS_{10}	1.2×10^{8}	2×10^{6}	3×10^{7}	1×10^{7}	1×10^{8}	2×10^{7}	2×10^{7}	1×10^{7}	5×10^{7}
Total viable count	7.4×10^{8}	6.7×10^{8}	3×10^{7}	5×10^{8}	4.8×10^{8}	2×10^{8}	1.2×10^{8}	2.8×10^{8}	7.9×10^{8}
MLSS (g/l)	2.7	2.5	1	2.2	2.3	2.5	2.5	2.8	2.9
% phenol degraded from 500 mg/l	90.8	85.9	35.6	74.8	78.8	84.7	86	97	98

According to the earlier works (Cespedes *et. al.* 1992 and Zouari and Ellouz, 1996) the reconstitution of microbial consortium involved in the biodegradation from the isolated strains was found to be a difficult one, mainly because of the change of metabolic properties of isolated strains.

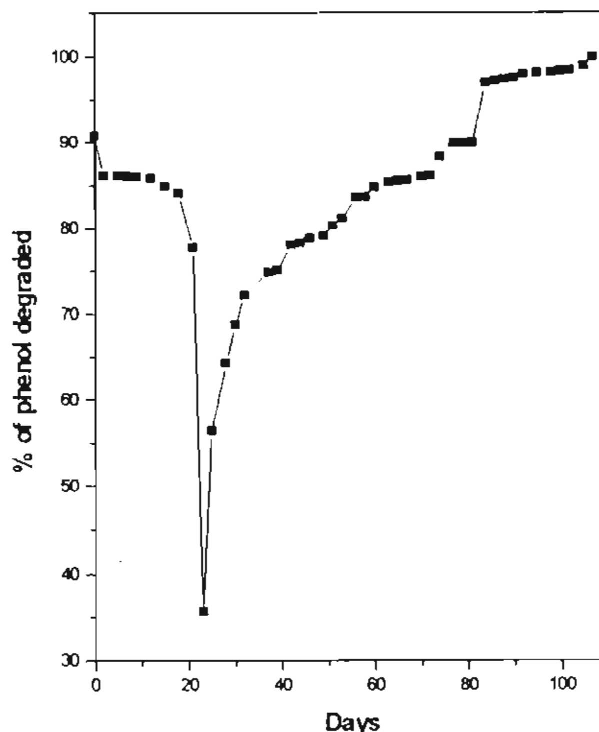

Fig.7.3 Phenol degradation profile of reconstituted consortium at 500mg/l

While, in the present study, the phenol degrading microbial consortium was reconstituted successfully from the isolated strains, and it required around 3 months duration to attain stability and performance as that of parent consortium.

In the study of Shih *et. al.* (1996) a difference in colour of system from white to yellow and then back to white was observed in a phenol degrading microbial community capable of co-metabolizing trichloroethylene. This colour change of system is due to the accumulation of α -HMS a yellow intermediate of phenol degradation resulted from the biomass fluctuations.

In the present system also, a colour change from white to dark brown and back to original colour was observed during the period of less activity and biomass concentration. This result indicates that there was a biomass fluctuation in the consortium and it was regained after 3 months of operation.

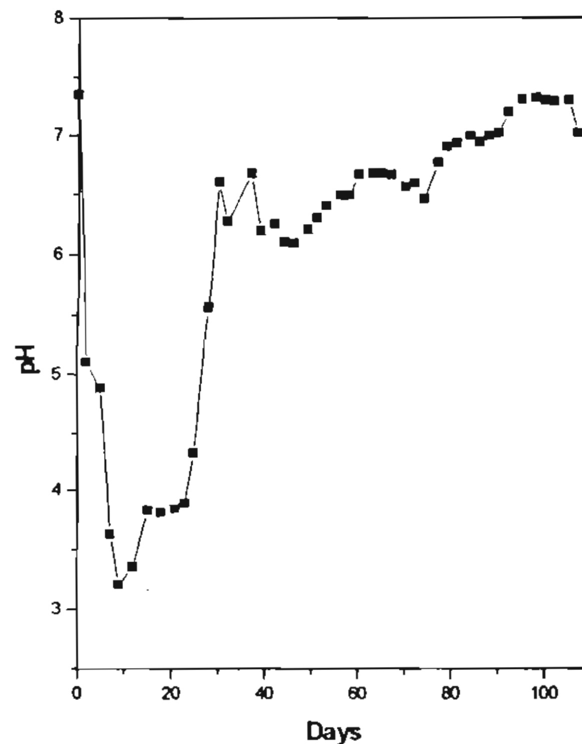

Fig.7.4 pH variation in the reconstituted consortium fed with 500 mg/l phenol

It is evident from the results that stable constitution and function of the consortium could not be attained soon after mixing of all the isolates of the consortium. Although individual members were mixed and grown under same conditions as it was optimised for activated sludge. The establishment of consortium was a slow process. The exact reason for the delay in reconstituting consortium by this method is not clear from the studies. Probably, certain characteristics of bacterial strains helped to interact and co-exist in a consortium might be temporaily lost during partition and pure culturing and they could regain such qualities slowly when they put together again.

CHAPTER 8

SUMMARY

In the biological treatment of organic pollutants, microbial consortium is widely used as an active agent because of its distinct advantages which include complete transformation of pollutants, higher metabolic capabilities, prevention of invasion of contaminants and tolerance to environmental stress.

Microbial consortium is a *'black box'* in which effective decontamination process occurs through a series of complex mechanisms. In this study of microbial consortium an attempt was made to open the *'black box'* for gathering information on their function, complexity, diversity and stability.

(i) Microbial consortium

Microbial consortium has been defined as any tight association of microbial communities which enable them to survive and perform better in a particular environmental condition than existing separately.

The consortia are formed by natural selection under specified conditions suitable for the existence and growth of the community. The components of a consortium are the individual microbial members joining the association.

A phenol degrading activated sludge (AS) termed as *"Consortium"* was developed and maintained in a fed-batch reactor to remove 0.5g phenol/l/day. The consortium was constituted of two non-phenol bacteria and eight phenol degraders of which 4 were gram negative rods and the rest were gram positive rods. The microbial strains of the consortium were identified as *Bacillus*, *Streptomyces*, *Pseudomonas* and *Rhodococcus* (Chapter 3).

Study on the phenol degradation ability of individual isolates indicated that everyone possessed varied capability. The best phenol degrading isolates AS_5 (*Bacillus* sp.3) of the consortium could degrade 400 mg phenol in a day and the corresponding value for the slow phenol degrader AS_7 (*Pseudomonas* sp.2) was 120 mg phenol.

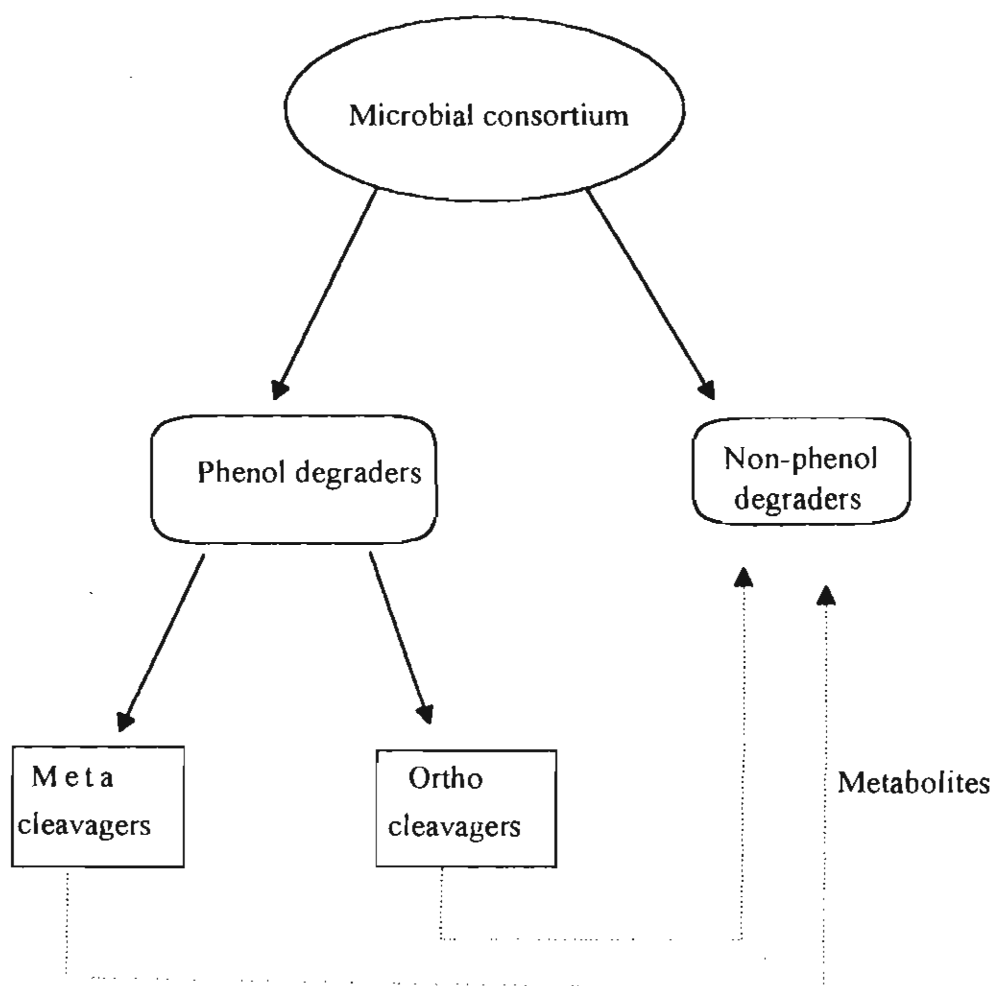

Fig. 8.1 Schematic representation of phenol degrading consortium

The phenol degradation rates of individual members were (in the increasing order); *Pseudomonas* sp.2 < unidentified strain < *Streptomyces* sp.< *Bacillus* sp.2 < *Rhodococcus rhodococcus* < *Pseudomonas* sp.1 < *Bacillus* sp.1 < *Bacillus* sp.3.

However, the consortium constituted of the above slow, medium and fast phenol degraders were shown a steady degradation of 621 mg phenol per day.

(ii) Population dynamics

Individual population in a community has undergone continuous changes, presumably due to the influences of biotic and abiotic factors (Zhang and Yamamoto, 1996). Similarly, a continuous shift in the individual population was observed in the present study. But the level of shift was varied with individual members; for instance, maximum fluctuation in population was evident in the case of AS_2 (*Bacillus* sp. 1) and the minimum fluctuation was observed in the population of AS_7 (*Pseudomonas* sp. 2) and non-phenol degraders (*Bacillus* sp. 4 and *Bacillus* sp. 5) .

Inspite of this continuous shift in the individual population, the total cell count was found to be constant and performed stable degradation throughout the operation period. Growth of individual bacteria was found to be comparable to its rate of degradation (Chapter 4).

(iii) Stability of degradation

As indicated in Chapter 5 of this thesis, a positive correlation was observed between activity of microbial consortium and the concentration of phenol in the range of 400 mg/l to 700 mg/l. The individual isolates could not have such an increasing rate of phenol degradation by the increase of concentration from 400 mg/l to 700 mg/l.

A distinct phenol degradative performance was exhibited by the consortium in the presence of related compound such as 2,4-dichlorophenol and unrelated compound such as glucose. The degradation pattern appeared to be of uncompetitive

non-inhibitory type (Chapter 5).

(iv) Heterogeneity of consortium

One of the remarkable characteristics of the microbial consortium was its diverse nature attributed from its constituent members. The members were heterogeneous in terms of morphology (pigmentation, size, shape and gram reaction), biochemistry, physiology, phenol degradation and growth pattern.

Colony morphology of the members of the consortium was circular, rhizoid, irregular with a colour variation of yellow, white, cream, blue, bluish rose, rose, brown and brownish rose. All of them were rods, with a size variation from 0.45 μm to 3.3 μm cell length and 0.13 to 1 μm cell diameter.

The consortium possessed bacterial members operating ortho and meta cleavage pathways for phenol degradation. Many physiological differences were observed with the microbial members of this stable consortium that worked for the study period of four years without loosing its attributes, stability and function (Chapter 6)

(v) Reconstitution

An attempt was made to study the possibility of reconstituting microbial consortium with the isolated members. It was found to be a slow process as it took 3 months to obtain a stable consortium. Significantly, longer period of 8 months was required to develop the original consortium degrading phenol. The exact reason for the delay in achieving the stability of consortium by reconstitution was not evident from the study. Perhaps, certain qualities required to be maintained in a consortium by the members could have altered and affected which might have resulted in their

compatibility temporarily (Chapter 7).

This concludes the " *Studies on microbial consortia degrading phenol in aqueous phase*" which brought out many important observations stated as above.

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