STUDIES ON BIODEGRADATION OF NITROPHENOL ISOMERS BY MIXED BACTERIAL CULTURES

A thesis submitted to the
UNIVERSITY OF MYSORE
for the Degree of
DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

By
SHABANA BASHEER

Fermentation Technology & Bioengineering Department
Central Food Technological Research Institute
Mysore-570 013, India
October - 2003

DECLARATION

I hereby declare that the thesis entitled "***Studies on biodegradation of nitrophenol isomers by mixed bacterial cultures*** " submitted for the degree of Doctor of Philosophy in Biotechnology to the University of Mysore is the result of work carried out by me under the guidance of Dr. S. Divakar in the Department of Fermentation Technology and Bioengineering, Central Food Technological Research Institute, Mysore during the period November 1998 to October 2003.

I further declare that the results of the work have not been submitted for the award of any degree, diploma or fellowship.

Date : 29-10-2003 Shabana Basheer

Mysore

Dr S. DIVAKAR
Scientist
Fermentation Technology & Bioengineering Dept.

29th October 2003

CERTIFICATE

I hereby declare that the thesis entitled "***Studies on biodegradation of nitrophenol isomers by mixed bacterial cultures***" submitted by Ms. Shabana Basheer for the degree of Doctor of Philosophy in Biotechnology of the University of Mysore is the result of research work carried out by her at the Department of Fermentation Technology and Bioengineering, CFTRI, Mysore under my guidance during the period from November 1998 to October 2003

Dr. S. Divakar
Guide

To my sister…

Rahmathunnisa S.A.

ACKNOWLEDGEMENTS

I think if anyone honestly reflects on who we are, how we got there, what we think we might do well, and so forth, we discover a debt to others. This page is specifically designed to note my appreciation of those people who stand out most notably in my mind as to contributing towards my thesis.

Words are inadequate to express my gratitude to **Dr. Divakar S., Scientist, Fermentation Technology and Bioengineering, CFTRI**, my guide and mentor, for his rock solid support and constructive criticism. I can only hope that a small part of his chemical intuition, breadth of knowledge and depth of understanding has rubbed off on me

I thank **Dr. Kunhi A.A.M., Scientist** (Retd), **Food Microbiolgy, CFTRI**, my erstwhile supervisor, for taking me as a Research Assistant and kindling interest and leading me towards Doctorate studies

I wish to sincerely thank **Dr. Prakash V., Director, CFTRI**, for providing infrastructure, lending moral support and for always being there to see me through my difficulties

I thank **Dr. N.G. Karanth, Head, FTBE**, for his support and encouragement

I am indebted to **Dr. M.C. Mishra, Scientist, FTBE,** for the inumerable favours, foremost being free access to the lab, his moral support and affection

I owe a debt of gratitude to the staff at **Dept. of FTBE- Mr. N.P. Ghildyal, Dr. S.G. Prafulla, Dr.M.S. Thakur, Dr. Avinash S., Mr. Eugene Raj, Mr. Varadaraj** and others who made my Doctorate studies possible with their overwhelming and unforgettable support

I specially thank **Dr.M.K. Gowthaman, Scientist, CLRI,** for just being himself and keeping me sane and positive in my moments of madness

I thank **Dr. M.C. Varadaraj, Head, HRD,** for characterizing the bacterial cultures and for being so kind

I appreciate **Mr. M.R. Radhakantha, COA,** for his encouragement to move ahead

I thank **Mr. Akmal Pasha** and staff, **FICP**, for their help

I have been fortunate to have some of the best and most supporting **friends at FTBE.** The cheerfulness and happiness brought in by them shall always be cherished

I thank the **Staff** and my **colleagues at FM**, for their co-operation

I deeply appreciate the technical and research assistance accorded by the **Central Intruments Facility & Services, FOSTIS, CFTRI**, and **Sophisticated Intruments Facilty, Indian Institute of Science,** Bangalore

I thank **Mr. Abdul Khayoum,** for all his help in formatting the thesis.

I am thankful to the **Security Staff,** CFTRI for providing me security

I wish to thank the **Council of Scientific and Industrial Research,** Govt. of India for awarding me a Senior Research Fellowship

I gratefully thank my **Dee, family and friends,** who stood by me with love, forgiveness and lots of patience

I also appreciate **many others** whom I have never met but whose published work inspired me

I have saved the best for the last and the best compliments are for my parents, **Mr. S.A. Basheer** and **Mrs. Tajunnisa,** whom I feel God sent to make me strong and took them away to make me stronger

I close by thanking **Almighty God** for these people and my thesis. Whatever good may come of this work, the credit belongs to Him. After all, He already knows how microbes work

Shabana Basheer

CONTENTS

CONTENTS

LIST OF FIGURES

CHAPTER 3

3.1 Biomass estimation during degradation of subtoxic levels of ONP, MNP, PNP and their mixture

3.2 Degradation pattern of subtoxic concentrations of ONP, MNP, PNP and Mix NPs

3.3 Degradation pattern of subtoxic concentrations of ONP, MNP, PNP. Residual substrate analysis by HPLC

3.3a HPLC profile during degradation of subtoxic concentration of ONP

3.3b HPLC profile during degradation of subtoxic concentration of MNP

3.3c HPLC profile during degradation of subtoxic concentration of PNP

3.3d HPLC profile during degradation of subtoxic concentration of a mixture of mononitrophenol isomers

3.4 Progress curve of nitrite release during degradation of subtoxic concentrations individual mononitrophenol isomers and their mixture by the consortium

3.5 Progress curve of ammonia production during MNP degradation by the nitrophenol degrading consortium

3.6 Degradation pattern of a mixture of ONP, MNP and PNP. Residual substrate analysis by HPLC

3.7 Degradation of 0.1mM mononitrophenols by the consortium induced with 0.5% sodium acetate

3.8 Degradation of 0.1mM mononitrophenol isomers by the consortium induced with o-cresol

3.9 Degradation of 0.1mM mononitrophenol isomers by the

(SNP-4)

4.5 Percent removal of 0.1mM substrate by *Pseudomonas* sp.(SNP-5)

4.6 Percent removal of 0.1mM substrate by *Pseudomonas alcaligenes* (SNP-6)

4.7 Percent removal of 0.1mM substrate by *Psuedomonas* sp. (SNP-7)

4.8 Percent removal of 0.1mM substrate by *Sarcina maxima* (SNP-8)

4.9 Degradation pattern of a mixture of mononitrophenol isomers by SNP-1

4.10 Degradation pattern of a mixture of mononitrophenol isomers by SNP-2

4.11 Degradation pattern of a mixture of mononitrophenol isomers by SNP-3

4.12 Degradation pattern of a mixture of mononitrophenol isomers by SNP-4

4.13 Degradation pattern of a mixture of mononitrophenol isomers by SNP-5

4.14 Degradation pattern of a mixture of mononitrophenol isomers by SNP-6

4.15 Degradation pattern of a mixture of mononitrophenol isomers by SNP-7

4.16 Degradation pattern of a mixture of mononitrophenol isomers by SNP-8

4.17 Degradation of 0.1mM substrate by well induced cells of *Sarcina maxima*

4.18 Percentage of ONP degradation and nitrite release by *Sarcina*

microbial consortium

6.2a 500MHz ^{1}H NMR spectrum showing the region for 6.0-8.5ppm of the reaction mixture obtained by the degradation of MNP by the microbial consortium

6.2b 500MHz ^{1}H NMR spectrum showing the region for 5.5-9.0ppm of the reaction mixture obtained by the degradation of MNP by the microbial consortium

6.3a 500MHz ^{1}H NMR spectrum showing the region for 0.5-5.5ppm of the reaction mixture obtained by the degradation of PNP by the microbial consortium

6.3b 500MHz ^{1}H NMR spectrum showing the region for 5.5-11ppm of the reaction mixture obtained by the degradation of PNP by the microbial consortium

6.4a 500MHz ^{1}H NMR spectrum showing the region for 0-2.9ppm of the reaction mixture obtained by the degradation of ONP by *Sarcina maxima*

6.4b 500MHz ^{1}H NMR spectrum showing the region for 3.0-6.0ppm of the reaction mixture obtained by the degradation of ONP by *Sarcina maxima*

6.4c 500MHz ^{1}H NMR spectrum showing the region for 6.0-8.0ppm of the reaction mixture obtained by the degradation of ONP by *Sarcina maxima*

6.5a 500MHz ^{1}H NMR spectrum showing the region for 6.85-7.80ppm of he reaction mixture obtained by the degradation of MNP by *Sarcina maxima*

6.5b 500MHz ^{1}H NMR spectrum showing the region for 2.0-5.0ppm of the reaction mixture obtained by the degradation of MNP by *Sarcina maxima*

SCHEMES

LIST OF TABLES

ABBREVIATIONS

AC - aminocatechol

ADNT - α-amino-4,6-dinitrotoluene

ATA - anaerobic toxicity assay

AU - activity units

BHI - brain heat infusion

BMP - biochemical methane potential

BQ - benzoquinone

BT - benzenetriol

C - catechol

CFU - colony forming units

d - doublet

dd - doublet of a doublet

DAHAT - 2,4- diamino-6-hydroxyl aminotoluene

DANT - 2,4- diamino-6- dinitrotoluene

2D HMQCT - Two-Dimensional Heteronuclear Multiple Quantum Coherence Transfer

Dinoseb - 2-sec-butyl-4,6-dinitrophenol

DMSO - dimethyl sulphoxide

DNOC - dinitro-o-cresol

DNP - dinitrophenol

DNT - dinitrotoluene

Fenitrothion - o,o-dimethyl-o- (3-methyl-4-nitrophenyl) thiophosphate

GC - gas chromatography

^{1}H - proton

HAB - hydroxylaminobenzene

γ-HMA - **γ-**hydroxyl maleylacetate

HMUAL - hydroxy muconicsemialdehyde

HMX - octahydro-1,3,5,7- tetranitro-1,3,5,7- tetracozine

HPLC - high pressure liquid chromatography

HQ - hydroquinone

β-KA - β-ketoadipic acid

m - multiplet

MA - maleyl acetate

Mix NPs - mixture of the three mononitrophenol isomers-ONP, MNP and PNP

MNC - 4-methyl-5- nitrocatechol

MNP - m-Nitrophenol

MP - methyl parathion

MR-VP - methyl red- Voges Proskauer

MUA - cis,cis- muconic acid

MUL - muconolactone

NAD - nicotinamide adenine dinucleotide

NADPH - nicotinamide adenine dinucleotide phosphate

NC - nitrocatechol

NHQ - nitrohydroquinone

NMR - nuclear magnetic resonance

OD - optical density

ONP - o-Nitrophenol

PCP - pentachlorophenol

Picloram - o,o-dimethyl-o-4-nitro-m-tolyl phosphorothioate

PNP - p-Nitrophenol

RDX - hexahydro-1,3,5-trinitro-1,3,5- triazine

s - singlet

TCA - tricarboxylic acid cycle

TLC - thin layer chromatography

TNT - 2,4,6- trinitrotoluene

UV - ultraviolet

CHAPTER 1

INTRODUCTION

Biotechnology encompasses an important science, **Bioremediation**, which significantly deals with biotic transformations of consequential pollutants/contaminants. It offers various options for combating the menace of disturbing ecosystems arising due to irate xenobiotics. Today we talk in terms of not only pollutant/xenobiotic residues but also their conjugates and bound forms. Hence both biotic and abiotic transformations of parent xenobiotics and their fate and consequence in soil, water and air have generated immense interest. Soil is a major reservoir of microorganisms that plays an important role in maintaining its fertility. Xenobiotic compounds introduced into soil present daunting challenges to the soil microflora.

1.1 NITROAROMATIC COMPOUNDS

Nitroaromatic compounds form an important class of xenobiotic compounds. A vast majority of these compounds detected in the environment are anthropogenic in nature and nitrosubstituted aromatic compounds are important building blocks for the large scale synthesis of pesticides, pharmaceuticals, plastics, azo dyes and explosives and also serve as precursors for the production of aminoaromatic derivatives (Kearney and Kaufmann, 1976; McCormick et al., 1976; Schwarzenbach et al., 1988). As a consequence, nitroaromatic compounds have become pollutants in rivers, wastewaters, groundwater, pesticide treated soils and the urban atmosphere (Golab et al., 1979; Grosjean, 1985, Keith and Telliard, 1979; Piet and Smeenk, 1985; Zoeteman et al., 1980). Nitroaromatics are also present in combustion emissions and airborne particulate matter (Meijers and vander Lur, 1976; Pitts, 1982; Schuetzle, 1983; Tokiwa and Ohnishi, 1986). Nitrobenzenes, nitrotoluenes, nitrophenols and nitrobenzoates are used in the manufacture of pesticides, dyes, explosives, polyurethane foams, elastomers and industrial solvents. Nitrobiphenyls are important plasticizers and wood preservatives

(Masse et al., 1985). Chloramphenicol and nitrozepam are example of drugs. HMX, RDX and TNT have been extensively used as explosives and pose, currently, the most visible environmental problem (Hartter, 1985). The discharge of nitroaromatic compounds in wastewater and application as pesticides (Parathion, Dinoseb, Fenitrothion) have broadened their environmental impact and called for solutions for redemption of these toxic compounds. Some are highly mutagenic and toxic. Ortho-nitrophenol (ONP), 2,4-dinitrophenol and para-nitrophenol (PNP) are listed as priority pollutants by the U.S Environmental Protection Agency (Callahan et al., 1979; Keith and Telliard, 1979). As the demand for agricultural produce increases, so inevitably does the need for pesticides. Currently, organophosphate compounds are one of the most widely used class of pesticides in industrialized countries. High level exposure to these neurotoxins results in acetylcholine accumulation, which interferes with muscular responses, leading to the possibility of death. Repeated or prolonged exposure can cause delayed cholinergic toxicity and neurotoxicity (Tuovinen et al., 1994). Parathion and Methyl parathion are two popular organophosphate pesticides used for agricultural crop protection (Kumar et al., 1996). PNP is not only used extensively in manufacturing processes but is also a major metabolite in the hydrolysis of parathion and methyl parathion. As a result it can build up in the soil. These compounds may enter industrial waste streams, where they cause deleterious consequences for the following reasons: (i) the majority of nitroaromatic compounds are highly toxic to microorganism and may destabilize the continuos treatment systems by inhibition of growth; (ii) nitro groups and chloro substituents, reduce the electron density of the aromatic ring and thus impede electrophilic attack of oxygenases and oxidative degradation of nitroaromatic compounds; (iii) because of their electrophilic character they are also subject to reduction of the nitro groups which generate biologically inert azo, azoxy- and polymeric compounds (McCormick et al., 1976, 1978). Biologically, nitroaromatic compounds are either simply transformed to dead end products, by several microorganisms, which many a time prove to be more toxic than the parent compound or they may actually utilize the nitroaromatic compounds as a carbon and/or nitrogen sources.

1.2 REVIEW OF LITERATURE

1.2.1 Biodegradation of nitroaromatic compounds

Considerable amount of work has been done on development of treatment systems by biodegradation. It has been observed that microorganisms have capacity to convert nitroaromatic compounds into intermediates that can serve as growth substrates. Populations of microbes able to degrade nitroaromatics or any other compounds can arise by different means. If the chemical in question is a close analog to an ubiquitous microbial substrate, native soil microflora may degrade the molecule. Degradative populations could still arise through natural selection in contaminated environments. In the former case, biodegradation by *in situ* microorganisms should always be possible, while in the latter it might occur only at older spill sites. Because of natural selection process, it is commonly assumed that a bacterial population in older, more heavily contaminated spill sites will be more adapted to degradation of the contaminant. Such organisms may be suitable candidates for use in bioremediation (Crawford, 1995; Kaake et al., 1994; Marvin-Sikkema and de Bont, 1994). Despite the toxicity of nitroaromatic compounds, many microorganisms are able to transform or degrade them (Table 1.1).

Table 1.1 Representative bacteria reported to degrade nitroaromatic compounds

	Organism	Nitroaromatic compound	Reference
1.	*Arthrobacter eutrophus* JMP134	2,6-DNP (2,6-dinitrophenol)	Ecker et al., 1992
2.	*Arthrobacter aurescens* TW 17	4-nitrophenol	Hanne et al., 1993
3.	*Bacteroides fragilis*	1-nitropyrene	Kinouchi and Ohnishi, 1983
4.	*Clostridium acetobutylicum*	Chloramphenicol, 2-/3-nitrophenol , 2-/3-/4-nitrobenzoate, 2-nitrobenzaldehyde	O'Brien and Morris (1971).
5.	*Clostridium pasteurianum, Eschericia coli, Viellonella alkalescens*.	trinitrophenol, 40 nitro compounds, including nitrophenols, nitrobenzoates, nitrotoluenes	McCormick et al. (1976).
6.	*Desulfotomaculum orientis, Desulfococcus multivorons.*	4-nitrophenol	Gorontzy et al., 1993
7.	*Flavobacterium*	2-nitrobenzoic acid	Cain 1966 ; Ke et al., 1959.
8.	*Haloanaerobium praevalens, Sphorohalobacter marismoruti*	nitrobenzene, 2-/3-/4-nitrophenol, 2-/3-/4-nitroaniline, 2-4-dinitrophenol, 2,4-dinitroaniline	Oren et al, 1991

9.	*Methanobaterium formicicum*	3-nitrophenol, 4-nitrophenol, 2,4-dinitrophenol, 4-nitrobenzoic acid, 4-nitroaniline	Gorontzy et al., 1993
10.	*Methanosarcina barkeri*	3-nitrophenol, 4-nitrophenol, 2,4-dinitrophenol, 4-nitrobenzoic acid, 4-nitroaniline	Gorontzy et al., 1993
11.	*Moraxella sp.*	4-nitrophenol	Spain et al., 1997
12.	*Nocardia sp*.strainTW12	4-nitrophenol	Hanne et al., 1993
13.	*Nocardia alba*	2,4-dinitrophenol	Germanier and Wuhrman , 1963.
14.	*Pseudomonas putida* B2	3-nitrophenol	Zeyer et al., 1986
15.	*Pseudomonas sp.* strain HBX	trinitrophenol	Traxler et al., 1974
16.	*Rhodobacter capsulatus* EIFI	2,4-dinitrophenol, 2-nitrophenol, 3-nitrophenol, 4-nitrophenol	Blasco and Castillo, 1993
17.	*Sporohalobacter marismoruti* ATCC 35420	2-nitrophenol, 3-nitrophenol, 4-nitrophenol, Nitrobenzene,	Oren et al., 1991

		nitroanilines, 2,4-dinitrophenol, 2,4-dinitroaniline	
18.	*Rhodococcus erythropolis* HL 24-2	2,4-dinitrophenol, Picric acid	Lenke and Knackmuss, 1992.
19.	*Pseudomonas pseudoalcaligenes*	nitrobenzene	Nishino and Spain, 1993
20.	*Ralstonia eutropha* JMP 134	3-nitrophenol	Schenzle et al., 1997
21.	*Comamonas* sp. strain JS 765	nitrobenzene	Nishino and Spain, 1995
22.	*Pseudomonas putida* 2NP8	2-nitrophenol, 3-nitrophenol, nitrobenzene	Zhao et al., 2000
23.	*Veillonella alcalescens*	Trinitrophenol and related compounds	McCormick et al., 1976

The mechanisms of the reactions, their regulation and the nature of enzymes will provide fertile areas for research. Understanding the molecular basis for the catabolic sequence will allow their capabilities to be enhanced and exploited for practical purposes. Significant progress has recently been made in studies of aerobic and

anaerobic biodegradation of nitroaromatic compounds, making **bioremediation**, a feasible method for restoration of sites contaminated with these compounds. Relatively expensive physical (e.g. incineration) or chemical (e.g. solvent extractions) treatments may be replaced in the future by effective and cost-saving bioremediation technologies (Marvin-Sikkema and de Bont, 1994).

Several barriers must be overcome before biodegradation can provide an efficient treatment strategy for nitroaromatic compounds:

(a) The toxicity of nitroaromatics to microorganisms.
(b) Low bioavailability due to insolubility or sorption of the contaminant.
(c) Complications caused by mixture of nitroaromatic contaminants.
(d) Lack of catabolic systems able to degrade these compounds in the microbial community.

Microorganisms may treat these chemicals as sources of energy, carbon or nitrogen bringing about extensive degradation in the process or as a cometabolite (Alexander, 1967). This phenomenon becomes important especially during the metabolism of any chemical compound by a microbial community or a consortium. Availability of a number of factors such as temperature, salinity, pH, redox potential, microbial biomass, prior exposure can affect the degradation rate and thus the fate of a toxicant.

Various reports exist regarding the utilization of nitroaromatic compounds as carbon, nitrogen or energy sources or all of these *Pseudomonas putida* B2 grew on ONP (o-Nitrophenol) as sole C- and N-source (Folsom et al., 1993). A microbial culture isolated from a pesticide contaminated soil utilized PNP as sole C- and N-sources at 30°C with shaking. Around eight bacterial strains were isolated from different polluted sites in Bulgaria and USA. From these, three strains could use PNP as a sole N- and C-source while four strains used PNP as a N-source only. Another strain identified as *Ochromobacter anthropi* B_3 used 2,4-dinitrophenol as a nitrogen source (Petrova and

Laha, 1995). *Pseudomonas cepacia* RKJ 200 isolated by selective enrichment utilized PNP as sole C-, N- and energy source (Prakash et al., 1996). *Arthrobacter protophormiae* RKJ 100 was able to utilize PNP or 4-nitrocatechol as its sole C-, N- and energy sources producing p-benzoquinone (BQ) and hydroquinone (HQ) via the □-ketoadipate pathway (Chauhan et al., 2000). Bacterial culture *Ralstonia* sp. SJ 98, *Arthrobacter protophormiae* RKJ 100 and *Burkholderia cepacia* RKJ 200 were reported by Bhushan et al. (2000) to be using PNP as sole C-, N- and energy source. A PNP adapted microbial population (from an activated sludge) retained in porous particles utilized PNP as a sole C-source and degraded PNP releasing nitrite without significant accumulation of intermediate metabolites (Xing et al., 1999). Ramanathan and Lalithakumari (1999) observed that *Pseudomonas* sp. A3, isolated from soil, shown to degrade methyl parathion (MP) and other pesticides used the aromatic portion (PNP) as a C- and energy source during hydrolysis of MP. Three *Arthrobacter* sp. isolated from parathion contaminated soil could use PNP as C-source (Hanne et al., 1991).

Additional carbon sources and inorganic nutrients have been shown to have a profound effect on the degradation of nitrophenols. Mohammed et al.,
(1992) isolated from industrial sludge, a strain of *Pseudomonas cepacia* capable of using either PNP, DNP, DNOC or NB as its sole N-source but utilized succinic acid as a primary C-source.. Addition of citrate as a secondary C-source could not improve bacterial growth of *Pseudomonas putida* 2NP8 on nitrobenzene but the strain was able to use ONP and MNP (m-Nitrophenol) as growth substrates (Zhao and Ward, 2000). A mixed culture comprising of *Comomonas testosteronii* and *Acidovorax delafieldie* showed no increase in rate of growth and degradation of 20mg/l PNP with the addition of 1% yeast extract (Zhao and Ward, 1999). Zaidi and Mehta (1992) observed that the addition of glucose, sodium citrate and sodium acetate enhanced the degradation of PNP by inoculated bacteria. Growth on a second compound may substantially alter the kinetics of mineralization of low concentration of organic chemicals in loamy soil (Scow et al., 1989). At a concentration of 10μg/g soil, phenol slowed the initial rate of mineralization but increased the final extent of mineralization of 5mg of radio labelled PNP/g soil, whereas glucose and glutamate had no effect. Glucose stimulated PNP

mineralization by *Corynebacterium* sp., in samples from Beebe Lake and Cayuga Lake (Zaidi et al., 1989). An acclimated sludge was able to digest ONP in low concentration and addition of glucose promoted the anaerobic digestion of nitrophenols as well as upgrade the toxicity tolerance of the sludge. The reaction rate constant increased along with an increasing nitrophenol concentration (Tseng et al., 1996). Addition of 100μg/ml of glucose as a second substrate did not enhance the degradation of 20μg/ml of PNP in lake water by *Corynebacterium* sp. Z4 whereas glucose used at the same concentration inhibited degradation of 20μg PNP in wastewater by *Pseudomonas* sp. MS. While phenol and glucose increased the extent of PNP degradation by *Pseudomonas* sp. GR, phenol had no effect on PNP degradation of PNP by *Corynebacterium* sp. Z4 (Zaidi and Mehta, 1995). Acclimation time for 2 μg/l PNP degradation increased from 6-12 days in the presence of 10 mg/l phenol, but lower phenol levels were observed to increase the acclimation period to 8 days (Wiggins and Alexander, 1988). Mineralization of phenol or PNP was rapid and *Corynebacterium* grew extensively in solutions of 5mM and 10mM phosphate whereas growth was reduced in medium containing high iron concentrations. Calcium at 5mM but not at 0.5mM inhibited PNP mineralization by *Corynebacterium* sp. at a phosphate concentration of 0.2-0.5mM (Robertson and Alexander, 1991). Addition of phosphate, nitrate or sulfate (at 10mM) decreased the acclimation period for mineralization of low concentrations of PNP (2mg-2μg/ml) in lake water (Jones and Alexander, 1988a).

Other factors like inoculum size, substrate concentration, adaptation, varying pH and temperature conditions have been reported to have profound effect on the rate of degradation and extent of mineralization of nitrophenols. *Pseudomonas putida* PNP1 aerobically cultured in a strongly buffered mineral medium at pH 7 and 30ºC was used for purification of wastewater containing PNP in a continuously working aerobic solid bed reactor. An optimal load of 270 mg/l/hr was almost completely degraded whereas loads upto 500 mg/l/hr could be degraded only with an increase in aeration rate (Ray et al., 1999). A PNP degrading strain PNP1 in ammonium containing mineral medium grew optimally at pH 7 and at a temperature between 30-35°C and showed stoichiometric nitrite release. In ammonium free medium the maximum specific growth

rate was reduced. Weak inhibition was observed below 40 mg/l whereas acute toxicity occurred at 600 mg/l (Loeser et al., 1998). Zaidi and Imam (1995) suggested that bacteria capable of degrading high concentration of toxic chemicals could be isolated from sites contaminated with high concentration of toxic chemicals. They found that *Pseudomonas putida* isolated from a heavily contaminated petrochemical plant in Gyanilla PR rapidly degraded only high concentration (1-100μg/ml) of PNP, but not low concentrations (1-10μg/ml). Dramatic detoxification of mononitrophenols occurred at subtoxic levels by granular sludge in an upflow anaerobic sludge blanket digester (Donlon et al., 1996). Transformation rate of PNP by pentachlorophenol degrading *Sphingomonas* sp. UG30 and *Sphingomonas chloramphenolica* strains RA2 and ATCC 39723 in mineral salts-glucose medium was dependent on the initial concentration with the optimum rate at 310μM and inhibition occurring at 1,100 μM or more. An initial lag was eliminated on pre-exposure of UG30 to PNP (Leung et al., 1997). An indigenously isolated bacteria isolated from pesticide amended soil utilized PNP as sole C- or N-sources with the optimal concentration of PNP in the medium being 30 mg/l and a concentration 60 mg/l being inhibitory (Javanjal and Deopurkar, 1994).

An acclimated sludge was able to anaerobically digest ONP in low concentration and addition of glucose promoted the digestion of nitrophenols as well as upgraded the toxicity tolerance of the sludge (Tseng et al., 1996). In an anaerobic biological fluidized bed used to treat synthetic wastewater containing three types of nitrophenols, PNP was found to be most toxic of the nitrophenols to methane producing bacteria followed by MNP and ONP (Tseng and Yang, 1995). Zaidi and Mehta (1994) suggested that the inoculum size may be important in the success of inoculation to enhance biodegradation at low concentrations based on their observation that when 10,000 cells/ml of *Corynebacterium* species were added to ground water containing 26mg of PNP/ml, it degraded only 5% in 48h but degraded 70% when inoculum size was increased to $1x10^5$ cells/ml. Nishino and Spain (1993) observed a lag period when *Pseudomonas putida* JS444 was treated with 300μg/l PNP. The length of the lag was inversely proportional to the cell density but was not affected by PNP concentrations over a range of 15-5000μg/l. *Pseudomonas cepacia* at a concentration of 330 cells/ml did not

mineralize 1.0μg of PNP/ml (lake water) but 80% of PNP was mineralized when the cell concentration was increased to 33,000 to 360,000 *P.cepacia* cells/ml (Ramadan et al., 1990). Similarly higher biomass allowed methanogenic cultures to be less impacted by nitrophenols (Uberoi and Bhattacharya, 1997). Increased inoculum size from 300,000 to 500,000 cells/ml shortened acclimation period and increased the rate and extent of mineralization in case of *Corynebacterium* sp Z-4 mutant whereas a reverse reaction was observed in case of *Pseudomonas putida* (Zaidi and Imam, 1996). Most mononitrophenol degradation studies have been carried out at near room temperature and around neutral pH conditions. A mixed culture consisting of *Comamonas testesteronii* and *Acidovorax delafieldii* were tested to degrade both nitrophenols and nitrobenzene in 250ml Erlenmeyer flasks incubated at room temperature with agitation at 200 rpm (Zhao and Ward, 1993).

Dimkov and Topalova (1994) studied the degradation of phenol, ONP, MNP and PNP at an optimum pH and temperature of 7.2 and 28^{o}C respectively using 55 culture isolates from polluted soil. *Corynebacterium* sp.8/3 grown at 26^{o}C aerobically in mineral medium at pH 7.2 converted 50 mg/l PNP to 4-nitrocatechol. This conversion was affected by the pH of the medium in case of encapsulated cells of strain 8/3 (Kotouchkova et al., 1997). A PNP degrading strain PNP1 grew optimally at pH. 7 and at a temperature between 30-35^{o}C and showed stoichiometric release of nitrite. (Loeser et al., 1998). The optimum conditions for the biodegradation of nitrobiphenyls to nitrobenzoic acid and nitrophenol and subsequent degradation of nitrophenol with release of nitrite were at pH 7.5, 30^{o}C and cell density of 1.5 OD at 590nm (Ali Sadat and Walia, 1996). An optimum temperature of 25^{o}C and pH 8 were observed by Horakova and Kotouchkova (1996) for PNP degradation by growing as well as resting cells of C*orynebacterium* sp. 8/3.

Effect of acclimation, induction, release of nitrite and CO_2, behavioural changes in degrading organisms, capability of enzymes in degrading related compounds led several workers to look into the genetic aspects of nitrophenol degradation. *Pseudomonas* isolates used compounds such as glucose and fructose as sole C-source

as well as methyl parathion and PNP. The degradation of these compounds by the *Pseudomonas* isolates was found to be plasmid-encoded (Cortez et al., 1989). The PNP degrading bacterium harboured a plasmid approximately 36kb in size, while the methyl parathion-degrading bacterium contained many plasmids. Five soil *Actinomycetes* capable of degrading PNP contained large plasmids. Spontaneously cured variants of one isolate simultaneously lost the ability to degrade PNP. Conjugal transfer of PNP back into the used strain restored its ability to degrade PNP indicating that the degradation genes for that isolate were plasmid encoded (Hanne et al., 1991). A 50-kb plasmid carried the PNP degradation genes in the strain *Pseudomonas cepacia* RKJ 2000 which also encoded resistance to inorganic zinc ions (Prakash et al., 1996).

Chauhan et al. (2000) conducted studies on a PNP-derivative and a PNP + transconjugant which demonstrated that the genes for the 4-nitrocatechol pathway reside on the plasmid present in *Pseudomonas cepacia* RKJ200 (now *Burkholderia cepacia*). Since both PNP and 4-nitrocatechol are degraded via hydroquinone (HQ) formation and it was likely that the same set of genes encode further metabolism of HQ in nitrocatechol (NC) and PNP degradation. Similar studies conducted using *Arthrobacter protophormiae* RKJ100 indicated that same genes were probably involved in the degradation of PNP and NC and investigations revealed a 65,000 bp plasmid containing genes for the degradation of PNP and NC which has potential applications in bioremediation and soil decontamination (Chauhan et al., 2000)

1.2.2 Microbial mineralization of Nitroaromatic compounds

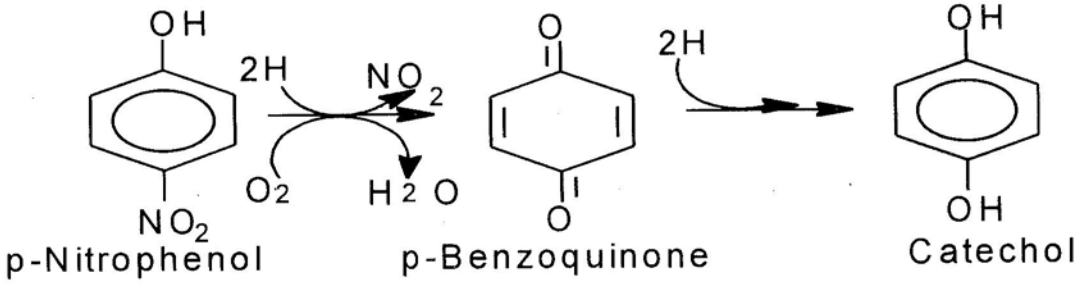
Several microorganisms have been isolated, which degrade nitroaromatic compounds. Degradation could occur under both aerobic and anaerobic conditions with or without enzymes. Presently four mechanisms of microbial mineralization of nitroaromatic compounds are known.

1.2.2.1 An initial oxygenation reaction yielding nitrite

Oxidative removal of the nitro group from the aromatic nucleus yielding nitrite has been demonstrated in various bacteria **(Fig 1.1).** Some bacteria mineralize these compounds

completely but use them as a nitrogen source by oxygenolytic removal of the nitro group (Bruhn et al., 1987; Dickel and Kanckmuss, 1991). The enzymes responsible for the removal of the nitro group have been identified. Zeyer and Kochar (1988) isolated and purified nitrophenol oxygenase from *Pseudomonas putida* B2 which stoichiometrically converted ONP to catechol and nitrite. Raymond and Alexander (1971) proposed a conversion wherein a *Flavobacterium* converts nitroaromatics to nitrocatechols before removing the nitro groups as nitrite.

Fig. 1.1 Initial oxygenolytic reaction yielding nitrite

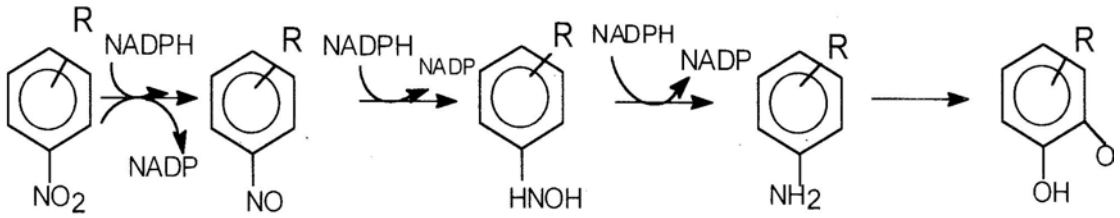


1.2.2.2 Reductive transformation reaction

Several microorganisms degrade nitroaromatic compounds by initially reducing the nitro-substituent to an amino group, which may subsequently be released as ammonia (Zeyer and Kearney, 1984) **(Fig 1.2).** The action of nitroreductases has been demonstrated in cell free systems under both aerobic and anaerobic conditions (Kinouchi and Ohnishi, 1983; McCormick et al., 1976; Villanaueva, 1964). Schenzle et al. (1997) found that *Ralstonia eutropha* JMP134 (Pemberton et al., 1979) converted MNP using it as its sole source of nitrogen, carbon and energy. The reduction proceeds via a nitroso and a hydroxylamino group. Theaminoaromatic product is further degraded in the presence of oxygen by aniline oxygenases to ammonium and catechol which is further mineralized by ring cleaving enzymes. This pathway is involved in the degradation of nitrobenzoates, nitrotoluenes and nitrophenols. Some bacteria are not

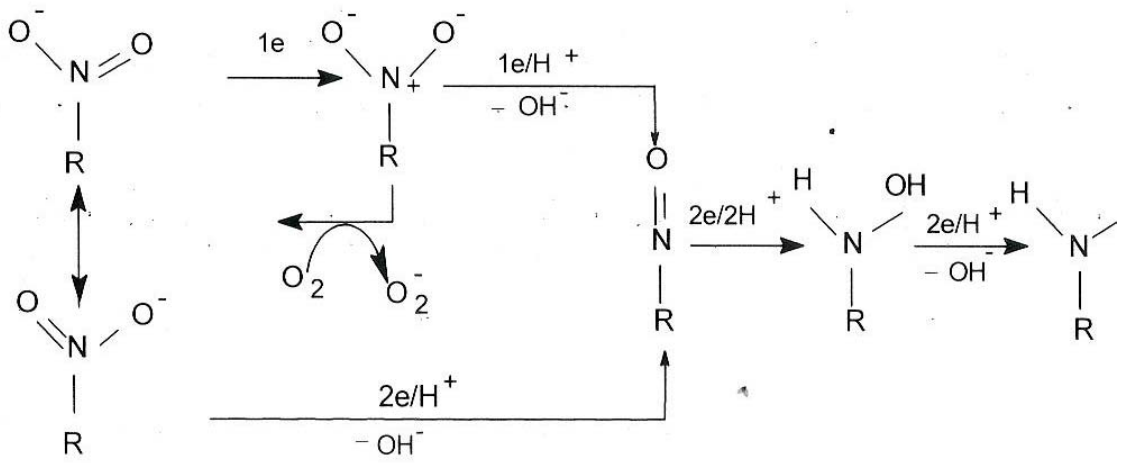
capable of mineralizing nitroaromatics completely after reduction of the nitro group but use the liberated ammonia as a nitrogen source (Preuss et al., 1993; Boopathy and Kulpa 1993; Boopathy et al.,1993).

Fig. 1.2 Pathway of the removal of the nitro group from nitroaromatics by initial reduction reactions



The nitro group exists as a resonance hybrid **(Fig. 1.3)**. Because the oxygen atoms are more electronegative than the nitrogen atom, the polarization of the nitrogen-oxygen bond causes the nitrogen atom to carry a partial positive charge and to serve as an electrophile. Therefore, the common reaction of the nitro group in biological systems is reduction, which can proceed either by one electron or two-electron mechanism. In addition, iron (II) and other metal ions and reduced sulfate compounds (Dunnivant et al., 1992; Gorontzy et al., 1993; Heijmann et al., 1993; Preuss et al., 1993) can serve as reductants for the non-enzymatic reduction of nitroaromatic compounds. Both the nitro group and the amino group are relatively stable. However sequence of reactions involved in reduction of the nitrogroup to the amine produces highly reactive intermediates. The nitroso and hydroxylation groups are electrophiles that can interact with bio-molecules to cause toxic, carcinogenic and mutagenic effects (Beland et al., 1985; Hlavica, 1982; Weisberger, 1978).

Fig.1.3 Reduction of nitro groups by one electron and two electron mechansims [Spain (ed.)1995]



The one-electron reduction of the nitro group produces a nitro radical anion, which can be oxidized to the starting material by molecular oxygen with the concomitant production of superoxide. The cycle leads to the designation of enzymes that catalyse one-electron reduction of the nitrogroup as “oxygen sensitive” (Bryant and De Luca, 1991). Enzymes from a variety of sources catalyze one electron reduction of the nitro group. These include anaerobic bacteria such as *Clostridium* sp. (Angermaier and Simon,1983), facultative bacteria such as *Eschericia coli* (Peterson et al., 1979) and *Enterobacter* sp. as well as plants and animals (Bryant and De Luca, 1991).

Reduction of the nitro group by the sequential addition of pairs of electrons is “oxygen insensitive” because no radicals are produced (Bryant and De Luca, 1991). Nitroreductases of this type convert nitro groups to either hydroxylamines or amines by the addition of electron pairs donated by reduced pyridine nucleotides. The electron pathway goes through the formation of nitroso derivatives, which are difficult to detect due to their reactivity. Both nitroso and hydroxylated intermediates can react readily

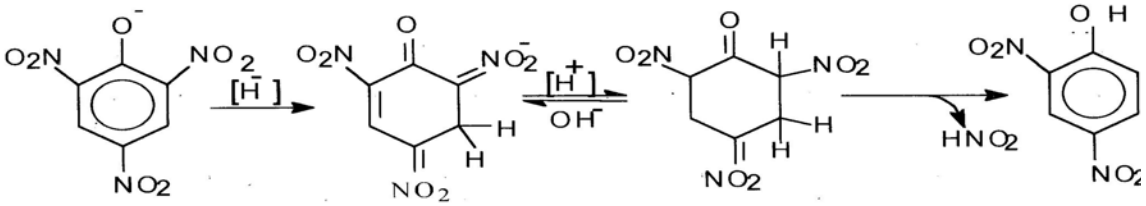
with a variety of biological materials including condensation reactions, for example, non-enzymatic production of azoxy compounds in the presence of oxygen (McCormick et al., 1976).

The ease of reduction of the aromatic nitro group depends on the nature of other substituents on the ring and on the reducing potential of the environment. Electron withdrawing groups activate the molecules for reduction of the nitro group, whereas electron donating groups make the ring more susceptible to electrophilic attack. In the case of nitrotoluene, the number of nitro groups increases the probability of reduction and the probability of electrophilic attack decreases. Therefore, reduction of one nitro group of TNT is very rapid under a variety a conditions, including those prevalent in growing cultures of aerobic bacteria. In contrast, reduction of α-amino-4, 6-dinitrotoluene (ADNT) requires a lower redox potential, and reduction of 2, 4-diamino-6-dinitrotolune (DANT) requires a redox potential below 200mv (Funk et al., 1993), because the electron-donating properties of the amino groups lower the electron deficiency of the molecule.

1.2.2.3 Complete reductive removal of the nitro group by the formation of a hydride-Meisenheimer complex

This pathway is characterized by the complete reductive removal of the nitro group as nitrite and the formation of a hydride-Meisenheimer complex as one of the metabolites indicating an initiation of nucleophilic attack on the aromatic ring **(Fig 1.4)** by the hydride ion. Lenke and Knackmuss (1992) used *Rhodococcus erythropolis* to utilize picric acid which was metabolized to form a orange-red hydride-Meisenheimer complex and was further converted to 2,4,6-trinitro-cyclohexane with concomitant liberation of nitrite.

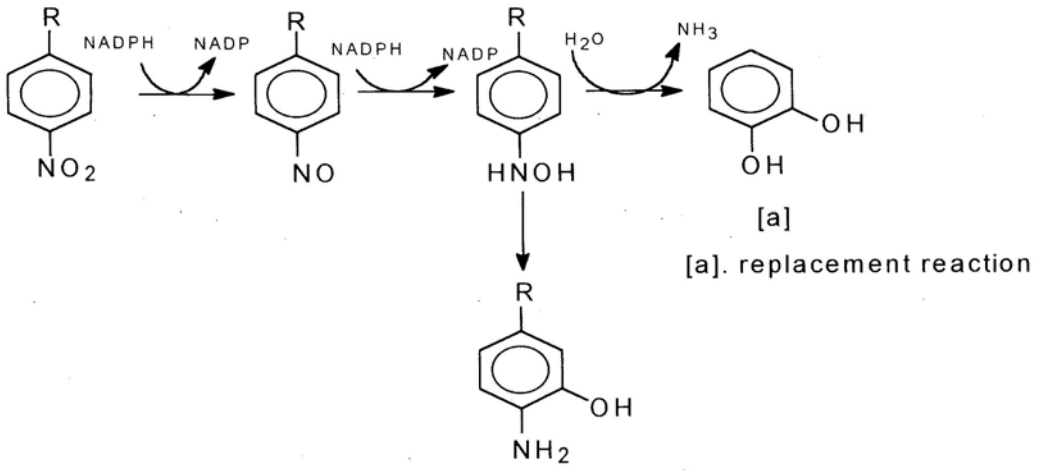
Fig.1.4 Complete reductive removal of nitro group from nitroaomatic compounds



1.2.2.4 Degradation of nitroaromatics via partial reduction and replacement reactions

Non-polar nitroaromatic compounds are considered resistant to microbial attack (Fewson, 1981). This is due in part to the reduction of electron density in the aromatic ring by the nitro group hindering electrophilic attack by oxygenases and thus preventing aerobic degradation. The accumulation of ammonia but not nitrite in media in nitrobenzene grown culture of *Pseudomonas alcaligenes* JS45 suggested that initial attack on the nitro group was reductive rather than oxidative leading to formation of hydroxyl aminobenzene (HAB) requiring 2 mol of NADPH. The HAB undergoes catalyzed rearrangement analogous to Bamberger rearrangement to form aminophenol (Nishino and Spain, 1993). This intramolecular Bamberger rearrangement reaction **(Fig 1.5),** (Bamberger, 1894, Shine, 1967; Sone et al., 1981) resulted in release of ammonia via ring fission of aminophenol. Implication of this type of rearrangement has been extensively described by Corbett and Corbett (1995).

Fig. 1.5 Partial reduction and replacement reaction



[b]. Bamberger-like rearrangement

1.2.3 Anaerobic degradation of nitroaromatic compounds

The reactions of nitroaromatic compounds in anaerobic systems almost exclusively involve the reduction of the nitrogroup. McCormick et al. (1976) clearly demonstrated that *Viellonella alkalescens* could reduce TNT and also identified some of the enzymes involved. Subsequently, a variety of other bacteria have been shown to reduce aromatic nitro compounds under anaerobic conditions (Angermaier and Simon, 1983; McCormick et al., 1976, Oren et al., 1991; Rafii et al., 1991 and Schackmann and Müller, 1991). Boopathy and Kulpa (1993) conducted studies on *Desulflovibro* sp. strain B that uses TNT and a variety of other nitroaromatic compounds as the source of nitrogen for growth and also as the terminal electron acceptor. The nitro compounds are reduced to the corresponding amines and proposed that the amino groups are removed from the aromatic ring by a reduction deamination mechanism analogous to that

described by Schnell and Schink (1991). Preuss et al. (1993) isolated a strain of *Desulfovibrio* by selective enrichment with pyruvate as carbon source, sulfate as the

terminal electron acceptor, and TNT as the source of nitrogen. The strain fixes atmospheric nitrogen and can also use ammonia as its nitrogen source. Several strains of *Clostridia* have been studied because of their ability to reduce nitroaromatic compounds (McCormick et al., 1976; Preuss et al., 1993; Rafii et al., 1991). Angermaier and Simon (1983) provided evidence that hydrogenase and ferrodoxin in *Clostridium kluyveri* are responsible for a one-electron reduction of nitroaromatic compounds. Rafii et al. (1991) characterized oxygen sensitive enzymes from several strains of *Clostridium* isolated from human faecal matter. The enzymes reduced 4-nitrobenzoate and several nitropyrenes to the corresponding amines. Hydrogenase from *Clostridium pasteurianum* and carbon monoxide dehydrogenase from *Clostridium thermoacticum* reduce DANT to DAHAT when ferrodoxin is included in the reaction mixture (Preuss et al., 1993). The reduction also took place with reduced ferrodoxin or methyl viologen in the absence of enzymes suggesting that the enzymes reduce ferrodoxin and not nitroaromatic compounds. Kaake et al. (1992, 1994) used an anaerobic mixed culture for the biodegradation of Dinoseb (2-sec-butyl-4,6-dinitrophenol) under methanogenic conditions with starch as the electron donor. Similar enrichment cultures degraded RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) and TNT to non-detectable levels in contaminated soil (Funk et al., 1993). Culture of *Clostridium bifermentans* isolated from other enrichments (Crawford, 1995) degraded both RDX and TNT.

O'Connor et al. (1989) studied the toxicity and anaerobic biodegradability of substituted phenols under methanogenic conditions using two anaerobic bioassays - the biochemical methane potential (BMP) and the anaerobic toxicity assay (ATA) to evaluate the stoichiometric conversion of added substrate carbon to CO_2 and CH_4. It was observed that ONP and PNP were completely mineralized. Significant transformation occurred and aminophenols were detected for higher concentrations. Mononitrophenols were degraded (Blasco and Castillo, 1992) to a lesser extent than 2,4-DNP under light anaerobiosis with the exception of ONP which was considerably metabolized in the absence of O_2 probably because of anaerobic reduction of the nitro group. Weak growth and no nitrite excretion were also observed in the presence of toxic levels of mononitrophenols (0.5mM), thus suggesting the elimination of nitro

groups under anaerobiosis. Resting cells of *Ralstonia eutropha* JMP134 (Schenzle et al., 1997) metabolized MNP to N-acetylaminohydroquinone under anaerobic conditions.

1.2.4 Biodegradation by fungi

The non specific lignolytic system produced by white rot fungus, *Phanerochaete chrysosporium* consisting of a complex system of extracellular peroxidases, small organic molecules and hydrogen peroxide is capable of degrading a wide range of synthetic chemicals including nitroaromatic compounds. Several groups (Bumpus and Tatarko, 1994; Fernando et al., 1990; Michels and Gottschalk, 1995; Spiker et al., 1992; Stahl and Aust, 1993; Valli et al., 1992) have reported degradation and even mineralization of nitroaromatic compounds by *P. chrysosporium*. Most fungi can catalyse the reduction of at least one nitro group of TNT (Parrish, 1977). Mycelia of *P. chrysosporium* reduce TNT to a mixture of 2-amino-4.6-dinitrotoluene (Stahl and Aust, 1993). Under lignolytic conditions, the amino compounds disappear and mineralization can be fairly extensive. In contrast, Valli et al. (1992), suggested that 2,4-dinitrotoluene is reduced predominantly to 2-amino-4-nitrotoluene by intracellular enzymes. Catalyzed by manganese peroxidase, it is further converted to 4-nitro-1,2-benzoquinone which is reduced to 4-nitrocatechol but could provide no strong evidence and suggested that 4-nitrocatechol could serve as a substrate for oxidative removal of the nitro group. Michels and Gottschalk (1995) provided strong evidence that under nonlignolytic condition TNT is reduced to 4-amino-2,6-dinitrotoluene via the corresponding hydroxylamino intermediate. Hoffrichter et al. (1993) showed cometabolic degradation of ONP, MNP and PNP by *Penicillium* sp. B 7/2 growing at the expense of glucose.

1.2.5 Aerobic Biodegradation

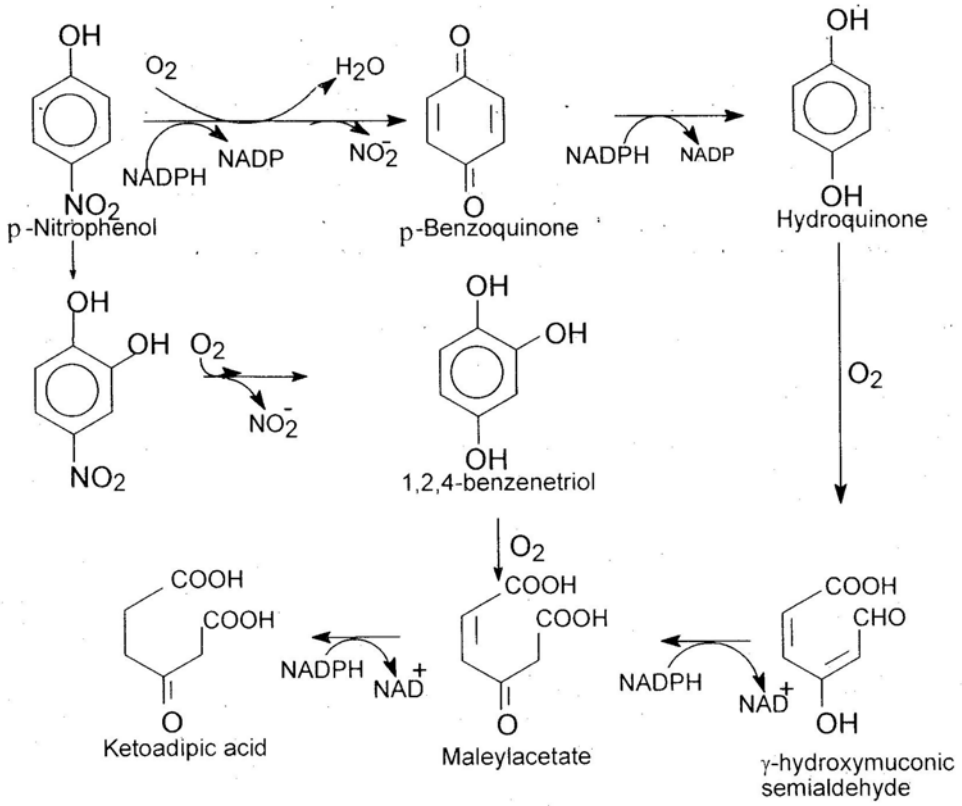
Bacteria can often derive carbon, nitrogen and energy from degradation of nitroaromatic substrates. Ability of bacteria to degrade nitrophenols and nitrobenzoates was reported many years ago. The earliest studies regarding degradation of mononitrophenol isomers by Baumann and Herter (1877-78) and Meyer (1905) had shown quantitatively

in rabbits that o-, m- and p-nitrophenols conjugated *in vivo* with glucoronic and sulphuric acid. Meyer was able to detect the reduction of m-and p-isomers. Oettingen (1949) found PNP to be more toxic than its isomer. Robinson et al. (1951) conducted studies on the extent of reduction of mononitrophenols in rabbits and found complete conjugation with glucoronic and sulphuric acids in doses of 0.2 to 0.3g/kg. A number of bacteria recently have been reported to degrade, aerobically, a wide range of polar and non-polar nitroaromatic compounds. Such bacteria use a variety of strategies for the removal of/or transformation of the nitro group aerobically. These include (a) monooxygenase-catalyzed elimination of the nitro group as nitrite (b) dioxygenase-catalyzed insertion of two hydroxyl groups with subsequent elimination of the nitro group as nitrite (c) partial reduction of the nitro group to a hydroxylamine which is the substrate for rearrangement or hydrolytic reactions and elimination of ammonia and (d) partial reduction of the aromatic ring to form a Meisenheimer complex and subsequent elimination of the nitro group as nitrite.

1.2.5.1 Monooxygenase-catalyzed initial reaction

Some of the earliest reports on the biodegradation of nitroaromatic compounds involved studies of bacteria that can grow on nitrophenols (Simpson and Evans, 1953). They provided preliminary evidence in 1953 that a strain of *Pseudomonas* could convert PNP to hydroquinone. Studies with a partially purified enzyme (Spain et al., 1979) revealed that a strain of *Moraxella* degrades PNP by initial oxygenase attack that results in the release of nitrite and accumulation of hydroquinone requiring 2 moles of NADPH to oxidize each mole of PNP **(Fig 1.6)** corresponding to nitro and hydroxyl group.

Fig 1.6 Monooxygenase catalyzed initial reaction of nitroaromatic compounds



Experiments with $^{18}O_2$ provided rigorous evidence that the mechanism of the reaction is a monohydroxylation (Spain et al., 1979) and preliminary evidence suggested that the enzyme was a flavoprotein monooxygenase. The stoichiometry and hydroquinone accumulation as the first detectable intermediate suggests that the initial product of the reaction is 1,4-benzoquinone. However an inducible quinone reductase could not be easily separated from the membrane bound oxygenase that catalyzed the initial reaction. The hydroquinone produced served as the substrate for ring fission reaction catalyzed by a ferrous iron-dependent dioxygenase yielding γ-hydroxy muconicsemialdehyde which was oxidized to maleyl acetic acid. Catalytic amounts of NAD^+ were required for the conversion of the ring fission product to β-ketodipate via

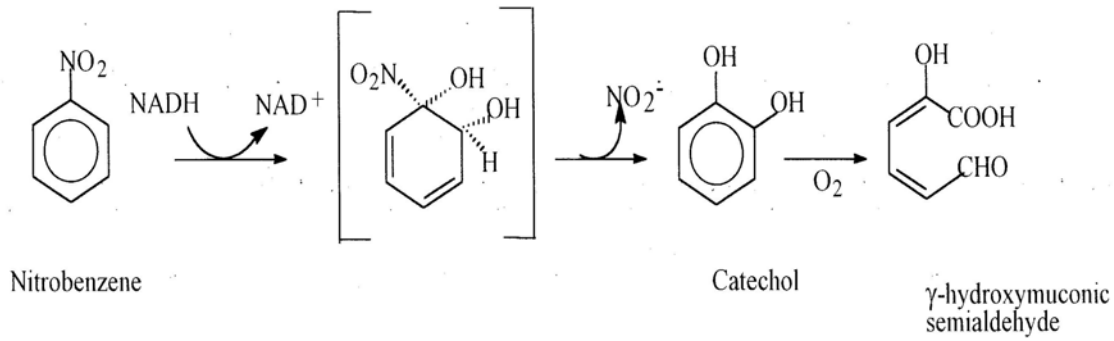
reduction of maleyl acetate in cell extracts because the two reactions recycle the cofactor. Hanne et al. (1993) proposed a similar pathway using *Arthrobacter* and a *Nocardia* sp. which converted PNP to hydroquinone. Cell extracts grown on PNP also contained enzymes which converted it to 1,2,4-benzentriol. In contrast Jain et al. (1994) isolated an *Arthrobacter* sp. which seemed to degrade PNP to 1,2,4-benzentriol via 4-nitrocatechol by a monooxygenase catalyzed hydroxylation at the ortho position. This was suggested by Raymond and Alexander (1951) who confirmed the conversion of PNP to 4-nitrocatechol (UV, Visible, IR, TLC and GC) by resting cells of a soil bacterium which was able to use PNP a source of carbon and energy and released stoichiometric amounts of nitrite. Jain et al. (1994) observed that 1,2,4-benzenetriol was further oxidized by an ortho ring fission to maleyl acetic acid but the enzyme responsible could not be detected. However an enzyme that converts PNP to 4-nitrocatechol has been purified from a strain of *Nocardia* sp. grown on PNP (Mitra and Vaidyanathan, 1984) and a similar enzyme activity has been demonstrated in another strain of *Nocardia* after growth on phenols (Hanne et al., 1993).

Similarly, monooxygenase catalyzed conversions of ONP were reported by various researchers. Zeyer and Kearney (1984) isolated and purified an NADPH dependent monooxygenase that catalyzed the conversion of ONP to catechol with the concomitant release of nitrite and oxidation of 2 moles of NADPH. Catechol was subsequently oxidized by 1,2-dioxygenase and was degraded further giving cis, cis-muconic acid and muconolactone through an ortho cleavage pathway (Zeyer and Kocher, 1988). Spain et al. (1979) proposed that the enzymatic reaction produces 1,2-benzoquinone by a mechanism analogous to the reaction catalyzed by PNP oxygenases. The activity of key enzymes of the pathway, nitrophenol oxygenase, catechol 1,2-dioxygenase and lactonizing enzymes in cell extracts and catechol 2, 3-dioxygenase (key enzyme of meta cleavage pathway) were detected thus confirming previous reports (Zeyer and Kearney, 1984). The ortho-nitrophenol monooxygenase is unusual among monooxygenases that catalyse the removal of aromatic nitro groups in that it does not seem to require the participation of a flavin co-factor.

1.2.5.2 Dioxygenase-catalyzed initial reaction

The catabolism of aromatic hydrocarbons by aerobic bacteria generally requires the activation of the molecules by the addition of two hydroxyl groups to the ring. The reactions are catalyzed by dioxygenase enzymes that introduce two atoms of molecular oxygen on adjacent carbon atoms (Gibson and Subramanian, 1984) **(Fig.1.7).** In substituted aromatic compounds, the introduction of the hydroxyl groups can lead to spontaneous elimination of the substituent and rearomatization of the ring: for example, toluene dioxygenase catalyzes the elimination of hydroxyl groups from phenol (Spain et al., 1989). Removal of aromatic nitro groups by dioxygenase enzymes was first reported as a result of studies on transformation of 2,6-dinitrophenol by *Alcaligenes eutrophus* (Ecker et al., 1992). Nitrobenzene, used extensively as the starting material for synthesis of aniline, is converted to catechol by a dioxygenase as the first step in its mineralization by a *Comomonas* sp. isolated from an aerobic waste-treatment plant (Nishino and Spain, 1995). The inducible nitrobenzene dioxygenase was found to be non specific and catalyzed the oxidation of a variety of nitrophenols, dinitrobenzene and nitrotoluenes (Spain, 1995). A *Pseudomonas* strain isolated from contaminated soil by selective enrichment grew on 2-nitrotoluene as the sole source of nitrogen and carbon (Haigler et al., 1994). The catabolic pathway involves an initial dioxygenase attack at the 2,3 position of the molecule to form 3-methyl catechol and release of nitrite. The 3-methyl catechol was degraded by a typical meta cleavage pathway. Purification of the enzymes allowed rigorous proof that the insertion of molecular oxygen and release of nitrite involves a dioxygenase mechanism, and that the rearomatization of the ring does not require a separate enzyme. Strains of *Pseudomonas* and *Comomonas* were found to convert 3-nitrobenzoate to protocatechuate by means of a dioxygenase attack at the 3,4 position with subsequent elimination of nitrite (Nadeau and Spain, 1995). Haigler and Spain (1991) investigated the ability of seven bacterial strains containing toluene degradative pathways to oxidize nitrobenzene.

Fig. 1.7 Dioxygenase catalyzed initial reaction in biodegradation of nitroaromatic coumpounds



Cells of *Pseudomonas putida* F and *Pseudomonas* sp. strain JS150 converted nitrobenzene to nitrocatechol in presence of $^{18}O_2$ suggesting a dioxygenase mechanism. *Pseudomonas mendocina* converted nitrobenzene to a mixture of MNP and PNP. *Pseudomonas picketti* PK01 converted nitrobenzene to 3-and 4-nitrocatechol via MNP and PNP which were slowly degraded to unidentified metabolites. They also observed that nitrobenzene did not serve as an inducer for the enzyme that catalyzed its oxidation, clearly indicating that nitrobenzene ring is subjected to initial attack by both mono and dioxygenase enzymes. Mineralization of a nitroaromatic compound via a dioxygenase initial attack was first reported as a result of studies with *Pseudomonas* sp. strain DNT grown on 2,4-DNT by a dioxygenase enzyme that was very similar to that of naphthalene dioxygenase (Suen et al., 1994). It adds hydroxyl groups to the 4- and 5-positions on the ring of 2, 4-DNT, and the nitro group is eliminated non-enzymatically as nitrite (Spanggord et al., 1991). 4-Methyl-5-nitrocatechol (MNC) produced by 2,4-DNT dioxygenase is the substrate for a monooxygenase that catalyses the replacement of the nitro group and elimination of nitrite. The constitutive enzyme, partially purified from cells of *Pseudomonas* sp. strain DNT, converts MNC to 2-hydroxy-5-methyl-quinone (Haigler et al., 1994), the reaction mechanism being similar to that described for other enzymes that catalyze the removal of nitro group from nitrophenols (Spain et al., 1979,

Zeyer and Kocher, 1988) and other electron-withdrawing groups from substituted phenols (Xun et al., 1992) or carboxylic acids (Hussain et al., 1980).

1.2.5.3 Reduction of the aromatic ring

The electron withdrawing properties of the nitro group cause the aromatic ring of poly nitroaromatic compounds to be highly electron deficient and resistant to microbial attack. Lenke et al. (1992) discovered an alternate mechanism of transformation involving reduction of the aromatic ring. They isolated strains of *Rhodococcus erythropolis* that use 2,4-dinitrophenol as the carbon, energy and nitrogen source. The isolates released nitrite from 2,4-dinitrophenol with transient accumulation of significant amount of 4,6-dinitrohexanoate. Presence of enzymes able to catalyse the reduction of the aromatic ring and accumulation of 4,6-dinitro-hexanoate suggested that the aliphatic compound was a dead end metabolite.

Resting cells of *Rhodococcus erythropolis* grown on 2,4-dinitrophenol released nitrite from picric acid, and spontaneous mutants could use picric acid as the nitrogen source (Lenke and Knackmuss, 1992). Initial reactions by cells and cell extracts showed the addition of a hydride ion to the aromatic ring to form a hydride-Meisenheimer complex. Addition of a second hydride ion led to the eventual formation of 2,4,6-trinitrocyclo-hexanone which decomposed to form 1,3,5-trinitropentane upon acidification and extraction. In contrast, protonation of the hydride-Meisenheimer complex led to the enzyme catalyzed rearomatization of the molecule and elimination of nitrite, which could be assimilated by bacteria along with 2,4-dinitrophenol generated during the process. Three reactions of hydride-Meisenheimer complex have been demonstrated in bacteria. The complex can (a) spontaneously decompose to the parent compound (b) be reduced to aliphatic compounds or (c) rearomatize by the addition of a proton and elimination of nitrite.

1.2.5.4 Partial reduction of the nitro group

Very early reports on the biodegradation of 2-nitrobenzoate (Cain, 1966; Ke et al., 1959) and 4-nitrobenzoate (Cartwright and Cain, 1959) provided evidence for the partial

reduction of the nitro group and the release of nitrogen and ammonia. Bacteria able to grow on MNP have been isolated (Germanier and Wuhrman, 1963) and the initial steps in the degradation pathway were found to be reductive rather than oxidative. A *Pseudomonas putida* that grew on ONP and MNP as sole sources of carbon and nitrogen was isolated from soil (Zeyer and Kearney, 1984) and was found to degrade ONP and MNP releasing nitrite and ammonium respectively but was unable to degrade PNP. Enzymes involved in metabolism were found to be inducible. *Ralstonia eutropha* strain JMP134 was shown to utilize MNP as the sole source of nitrogen, carbon and energy at a concentration of <0.5mM, above which growth was inhibited and accumulation of oxygen sensitive metabolites occurred. The conversion of 4-hydroxybenzoate to 3,4-dihydroxybenzoate has been identified as a key reaction in the degradative pathway of 4-nitrobenzoate (Groenewegen and de Bont, 1992) and 4-nitrotoluene (Haigler and Spain, 1993; Rhys-Williams et al., 1993). This novel enzymatic reaction leads to simultaneous elimination of ammonia and has also been observed in the degradation of MNP by *Pseudomonas putida* B2 (Meulenberg et al., 1996). Nishino and Spain (1993) identified an enzyme which converts hydroxylaminobenzene to 2-aminophenol in the degradative pathway of nitrobenzene by *Pseudomonas pseudoalcaligenes* JS45. This intramolecular reaction is known as Bamberger rearrangement (Bamberger, 1894; Shine, 1967; Sone et al., 1981) in which hydroxyl amino-aromatic compounds rearrange to aminophenols under mildly acidic conditions. The non-enzymatic rearrangement yields predominantly 4-aminophenol, whereas the enzyme (hydroxylaminobenzene mutase) directs the production of predominantly (>99%) 2-aminophenol. The 2-aminophenol thus produced by the initial steps in the pathway is degraded by a dioxygenase that catalyses the opening at the 1,6-position to produce 2-amino muconicsemialdehyde. The mechanism for degradation of this compound by *Pseudomonas pseudoalcaligenes* is not known. But enzymes in crude extracts from nitrobenzene grown cells catalyse the degradation of the ring-fission product and release of ammonia requiring NAD and indicating an oxidation of the aldehyde. The reductive pathway for degradation of nitrobenzene seems much more complex than the oxidative pathway, requiring one mole of oxygen and one mole of NADH to convert nitrobenzene to central metabolic intermediates and release ammonia.

In contrast, the oxidative pathway requires two moles of oxygen and one mole of NADH that can be regained if the 2-hydroxy muconicsemialdehyde, undergoes an NAD-dependent oxidation to oxalocrotonate (Nishino and Spain, 1995). If the isolate is to use the nitrite released by the oxygenolytic reaction as its nitrogen source, three additional moles of NADPH would be required for the reduction of nitrite to ammonia. Hence the more complex reductive pathway for nitrobenzene reduction seems to be well adapted to exploit the condition of an oxygen-limited ecosystem. Analogous enzyme catalyzed reactions have been reported in animals (Sternson and Gammans, 1975) and in yeast (Corbett and Corbett, 1981) but not in bacteria. The implications of the Bamberger like rearrangement in biochemistry have been discussed extensively by Corbett and Corbett (1995). In contrast to the above, *Ralstonia eutropha* JMP134 (Schenzle et al., 1997) converted hydroxyl aminobenzene to α-aminophenol and 4-aminophenol. Correspondingly 3-hydroxyl aminophenol as a metabolite of MNP underwent an enzyme catalyzed rearrangement to aminohydroquinone which was acetylated to N-acetylaminohydroquinone under anaerobic conditions. Acetylation of anilines has been demonstrated to be an important detoxification mechanism by microorganisms (Bollag and Russel, 1976, Engelhardt et al., 1977; Tweedy et al., 1970). Schackmann and Müller (1991) described a nitro reducing activity for MNP generating 3-aminophenol and 3-N-acetylamino-phenol as dead end metabolites by resting cells of *Pseudomonas* sp. strain CBS3. Zhao et al. (2000) using *Pseudomonas putida* 2NP8 proposed a pathway for MNP degradation and evidence for ammonia release postulated on hydroxyl aminobenzene transformation wherein 3-hydroxyl amino-phenol, reduction product produced by MNP nitroreductase is converted possibly to two intermediates - aminohydroquinone and 4-aminocatechol, via ortho and para-Bamberger rearrangement respectively. These are oxidized to imines which on hydrolysis form quinones and subsequently are reduced leading to the formation of 1,2,4-benzenetriol. Meulenberg et al. (1996) identified 1,2,4-benzenetriol as an intermediate and observed ammonia during nitroreductase-initiated MNP transformation by *Pseudomonas putida* B2 but under anaerobic conditions. Schenzle et al. (1997) observed a Bamberger rearrangement type of conversion of 3-hydroxyl aminophenol to amino hydroquinone during aerobic conversion of MNP but did not investigate the

release of ammonia. It was also observed that MNP grown cells of *Pseudomonas putida* produced ammonia, 2-amino-phenol, 4-aminophenol, 4-benzoquinone, N-acetyl-4-aminophenol, N-acetyl-2-aminophenol, 2-aminophenoxazine-3-one, 4-hydroquinone and catechol from hydroxyl aminobenzene. Ammonia, N-acetyl-2-aminophenol and 2-aminophenoxazine-3-one were produced by the same cells from 2-aminopehnol.

1.3 OBJECTIVE OF THE PRESENT WORK

The present study deals mainly with degradation of three mononitrophenol isomers - ortho-nitrophenol, (ONP) meta-nitrophenol (MNP) and para-nitrophenol (PNP) (Table 1.2) by bacterial cultures. Biodegradation is frequently equated with the catabolism of particular organic compounds by a single microbial strain. However it is becoming increasingly apparent that biodegradation in the natural environment is carried out by mixed bacterial communities which have been shown to be more effective even in laboratory conditions. Since soil harbours a number of microorganisms capable of degrading a variety of organic compounds, our first objective was to obtain a mixed microbial consortium from a contaminated soil sample and later enrich the consortium in the presence of mono-nitrophenol isomers. The consortium or its individual culture would be first acclimated before initiating degradation studies to enable induction of enzymes required for degradation. Different concentrations of the substrate would be employed to assess the tolerance level of the consortium and of the individual culture.

Most information about degradation pathways rests on studies made on single microbial strains, hence the microbial consortium would be constituted and each component culture isolated and identified. The potential of each individual culture would be assessed since the choice of the most competent or a combination of culture is important.

Degradation of mononitrophenol isomers using crude extract preparations of the consortium would also be studied to detect the initial enzymes involved during the degradation pathway.

Biodegradation of mononitrophenol isomers can proceed via an oxidative or reductive pathway, hence a biochemical study of the degradation pathway followed by a consortium and a single culture would be conducted by extensive NMR spectroscopy.

Table 1.2 CHEMICAL AND PHYSICAL CHARACTERISTICS

Property	**2-Nitrophenol**	**3-Nitrophenol**	**4-Nitrophenol**
Molecular weight	139.11	139.11	139.11
Color	Light yellow	Yellow crystals to tan	Colorless to light yellow
Physical point	Crystalline solid	Crystalline solid	Crystalline solid
Melting point	44 - 45 °C	96 - 98°C	113 - 114 °C
Boiling point	216 °C	194 °C	287°C
Dissociation constant (pKa)	7.21 - 7.23	8.4	7.08 - 7.18
Odour	Slightly aromatic	Slight odour	Slight odour
Solubility Distilled water Organic solvents	 1.4 g/l Soluble in benzene,CS_2, alkali hydroxides, ethyl ether and acetone	 Slight 0.1- 1% Soluble in benzene,CS_2, alkali hydroxides, ethyl ether, choloroform and acetone	 16 g/l Soluble in toluene, ethanol, choloroform. ethyl ether and alkali hydroxides

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS

The following chemicals and their sources of procurement are listed below.

Hi Media Laboratories, India

Yeast extract, Peptone, Beef extract, Agar, Brain heart infusion broth and agar, Hugh Leifson medium, Kohn tube medium, MR- VP medium- Buffered glucose broth, Nitrate broth, Sugar fermentation basal medium, Grams stain

Merck, India

Acetonitrile, Potassium hydrogenphosphate

Qualigens, India

Acetone, sodium chloride, sulphanilic acid, ammonium chloride, ammonium nitrate, succinic acid, methanol, phenol

RANBAXY LABORATORIES, INDIA

Sodium dihydrogenphosphate, disodium hydrogenphosphate, glacial acetic acid, diethyl ether, potassium ferricyanide

Sarabhai M.Chemicals, India

Manganese sulphate

S.d.Fine Chemicals, India

Sodium molybdate, magnesium sulphate, chloroform

Glaxo Laboratories, India

Potassium chloride, mercuric iodide, cupric chloride

BDH Ltd., England

o- Cresol

Naardan, Holland

m- Cresol, p- Cresol

Sisco Research Laboratories Pvt. Ltd., India

4- Aminoantipyrene, p- Nitrophenol

Loba-Chemie Indoaustranal Co., India

Calcium nitrate, o- Nitrophenol

N.R. Chemicals, India

m- Nitrophenol

2.1.1 Media

The basal mineral medium (M5 medium), with the following composition was used for enrichment as well as for growing the bacterial consortium. This medium was slightly modified for degradation and enzymatic studies.

M5 medium : g/l

NaH_2PO_4	0. 792
$Na_2HPO_4.2H_2O$	2. 563
NH_4NO_3	0. 25
$MgSO_4$. 7 H_2O	0. 2
Ca $(NO_3)_2$	0. 1
Trace elements	1 ml.

Trace elements solution contained in g/l.

Fe SO_4. $2H_2O$	1. 0
$MnSO_4$. H_2O	1. 0
Na_2MoO_4. $2H_2O$	0. 25
$CuCl_2$. H_2O	0. 25
Conc. H_2SO_4	5 ml.

pH of the M5 medium was adjusted to 7.5 and was autoclaved for 20 min at 121°C. M5 medium was supplemented with sodium succinate (1%) and yeast extract (0.5%) when increased cell yields were required.

2.1.2 Culture conditions

Growth and degradation experiments were carried out in sterile M5 medium with the required test substrate supplemented with sodium succinate and yeast extract or aromatic compounds such as nitrophenols, phenol, cresols or acetate respectively. All the experiments were conducted in 500ml Erlenmeyer flask maintained on a rotary shaker (Environ shaker 3597-1, Labline Instruments, USA) and at room temp (28 – 30°C) in dark conditions.

Nutrient agar medium containing (g/l), peptone, 5.0; beef extract, 3.0; NaCl 5.0; agar 20 (pH – 7.2) was used for plating, isolation and purification of the individual strains of the microbial consortium. The individual isolates and the consortium were routinely maintained on nutrient agar slants and plates. They were also maintained in liquid M5 medium supplemented with yeast extract and sodium succinate or mononitrophenol isomers
ONP, MNP and PNP.

2.2 ANALYTICAL PROCEDURES

2.2.1 Growth

Bacterial growth was monitored turbidometrically. A known volume of the culture broth was centrifuged at 10,000 x g, the cell pellet obtained was washed thoroughly with M5 medium, suspended in the same volume of M5 and optical density determined at 600nm using a Genesys spectrophotometer.

2.2.2 Estimations

2.2.2.1 Estimation of phenol and o- and m- cresol by colorimetry

A modified 4-aminoantipyrene colorimeteric method based on the procedure of Lacost et al. (1959) was followed.

Materials

4- Aminoantipyrene

Potassium ferricyanide

Borate buffer – 6.2g of boric acid powder and 7.0g potassium chloride were dissolved in 800ml distilled water. To this 64ml of 1N NaOH was added and solution was made upto 1L and pH adjusted to 9.2 – 9.4.

Method

To 10ml of diluted sample, 0.5ml of borate buffer, 0.1ml of 1.5% 4-amino-antipyrene and 0.1ml of 10% potassium ferricyanide solutions were added. The color developed was measured at 505nm. The results were computed from a standard calibration prepared using the respective standard compound.

Estimation of p- cresol by HPLC

Residual p-cresol in the culture supernatants was analysed by reverse phase high performance liquid chromatography (HPLC), (Shimadzu LC– 6 A, Japan) with a C–18

column (150 x 4.6mm). The mobile phase used was methanol – water – acetic acid (60 : 35 : 5 by volume). The flow rate was 1ml. min^{-1} and detection was by UV absorbance at a wave length of 278 nm.

2.2.2.2 Estimation of nitrophenols and identification of their metabolites in reaction mixtures.

Estimation of residual nitrophenol in reaction mixtures was done by spectrophotometry and chromatographic methods. The identification of metabolites during the course of nitrophenol degradation was done by NMR spectroscopy.

Estimation of nitrophenols

The nitrophenol concentration in reaction mixtures was spectrophoto-metrically measured in a Genesys spectrophotometer (Spectronic instruments, USA.) using a known volume (1ml) of the culture filtrate at the following wavelengths.

o – Nitrophenol (ONP) 412nm
m – Nitrophenol (MNP) 272nm
p – Nitrophenol (PNP) 401nm.

For a reaction mixture containing a mixture of all three isomers a wavelength of 272nm was applied or the mixture was monitored at all the three wavelengths. Nitrophenol concentration was also determined by high pressure liquid chromatography by monitoring the degradation at a wavelength of 254nm. The absorbance (spectrophotometric) and peak area values (HPLC) were plotted against concentrations respectively. From the straight lines obtained, a linear least square analysis was performed and the slope values obtained were used for measuring the concentration of compounds in the test solution (correlation coefficient = 0.99).

Materials

ONP, MNP, PNP

M5 medium

Acetonitrile

Glacial acetic acid

C18 column

Water

Spectrophotometric calibration

A stock solution of the individual isomers was prepared in M5 medium and different concentration of nitrophenols in the range of 2.5–145 μg/ml were photometrically measured at the respective wavelengths. A standard curve was obtained by plotting the optical densities against the concentration employed **(Fig. 2.1, Fig 2. 2, Fig 2.3)**.

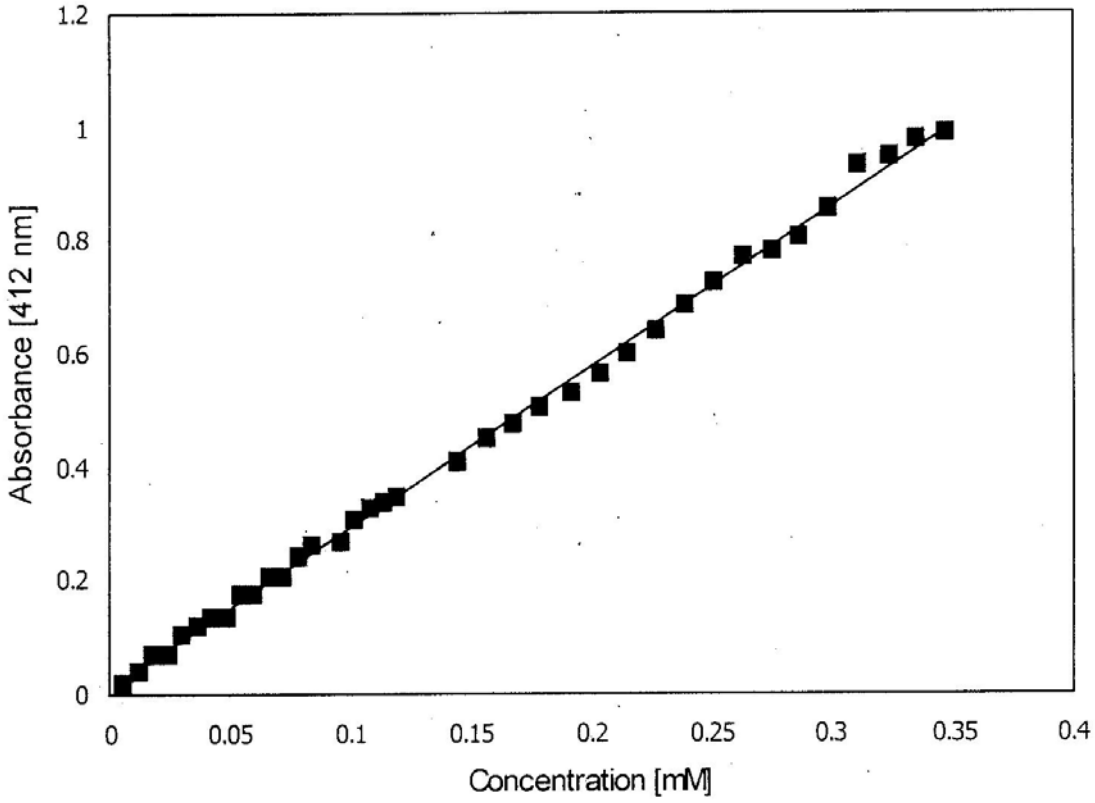


Fig. 2.1 Spectrophotometric calibration of standard ONP. Experimental details are described in Section 2.2.2.2. -■- ONP

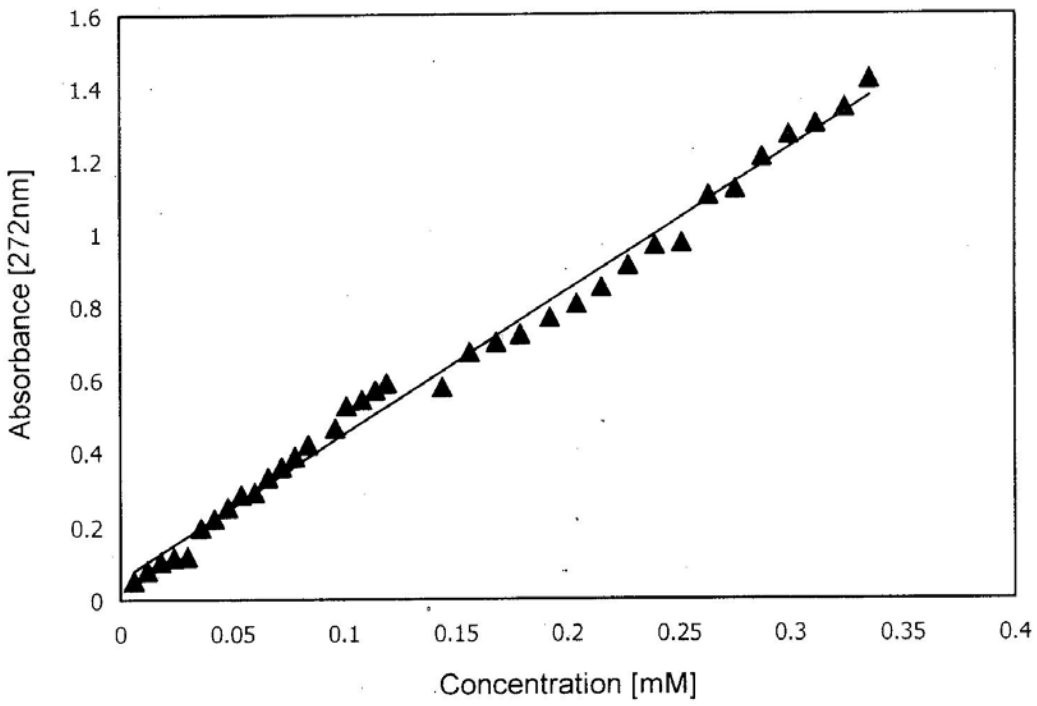


Fig.2.2 Spectrophotometric calibration of standard MNP. Experimental details are described in Section 2.2.2.2. -▲- MNP

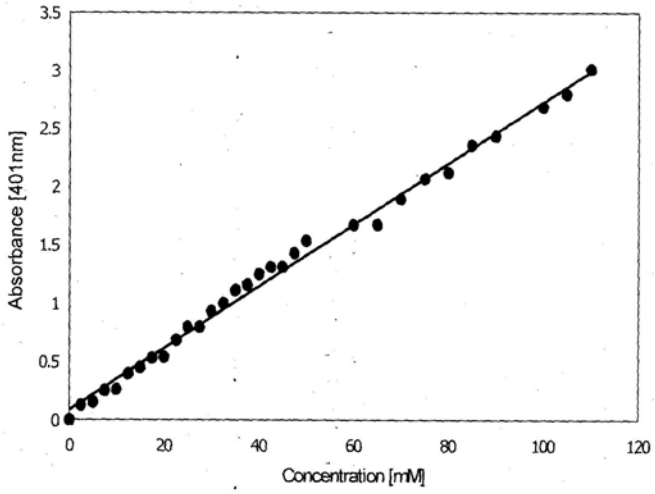


Fig.2.3 Spectrophotometric calibration of standard PNP. Experimental details are described in Section 2.2.2.2
-●- PNP

HPLC calibration

The HPLC analysis was performed on a 25cm Bondapak C18 column (Shimadzu SPD – 10A). Different concentrations of ONP, MNP and PNP separately were injected and eluted with water: acetonitrile (65:35) and 0.1% acetic acid. Solvents were eluted at the rate of 1ml/min and compounds were monitored at a wavelength of 254nm. Peak areas were plotted against concentration which gave a calibration curve enabling quantification of data from reaction mixtures

(Fig. 2.4, Fig 2.5, Fig 2.6)

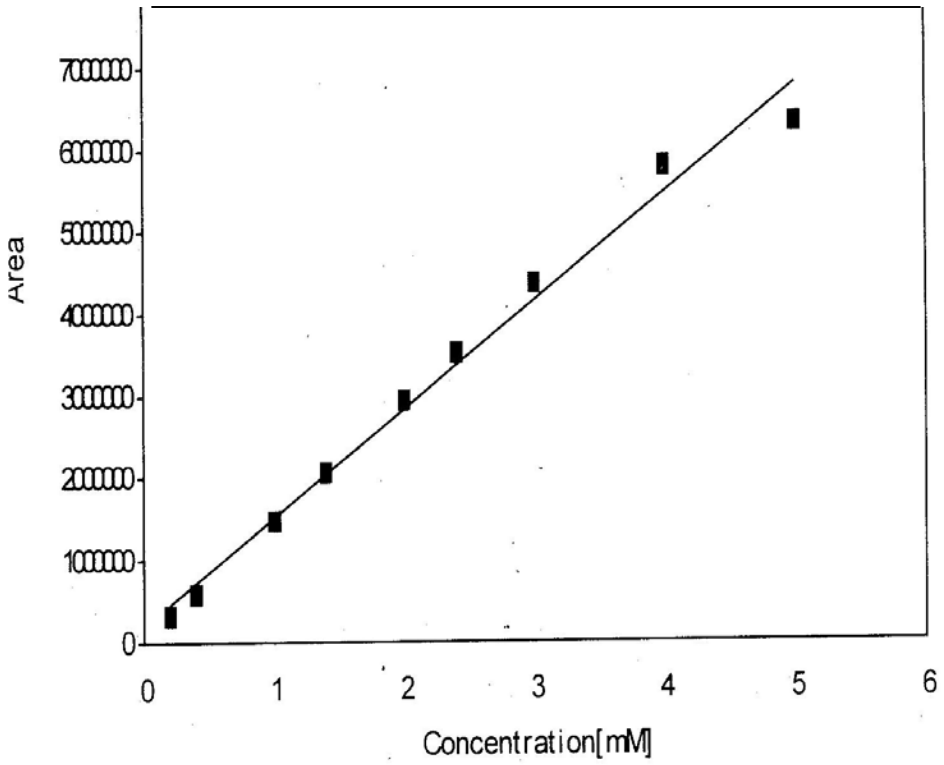


Fig. 2.4 Calibration of standard ONP by HPLC. Experimental details are described in Section 2.2.2.2. -■- ONP

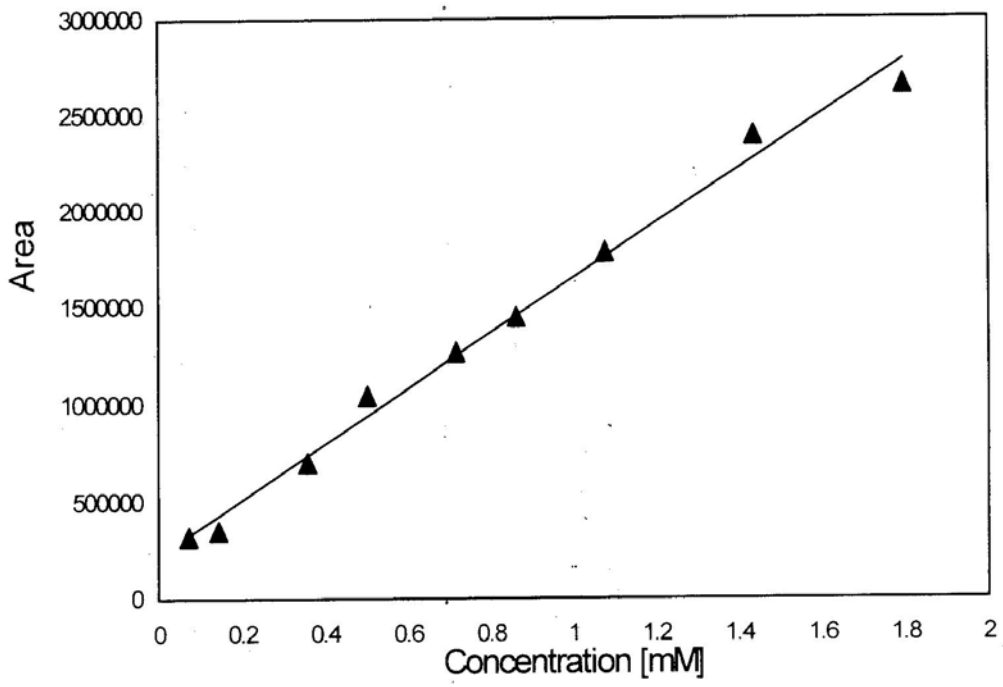


Fig.2.5 Calibration of standard MNP by HPLC. Experimental details are described in Section 2.2.2.2. -▲- MNP

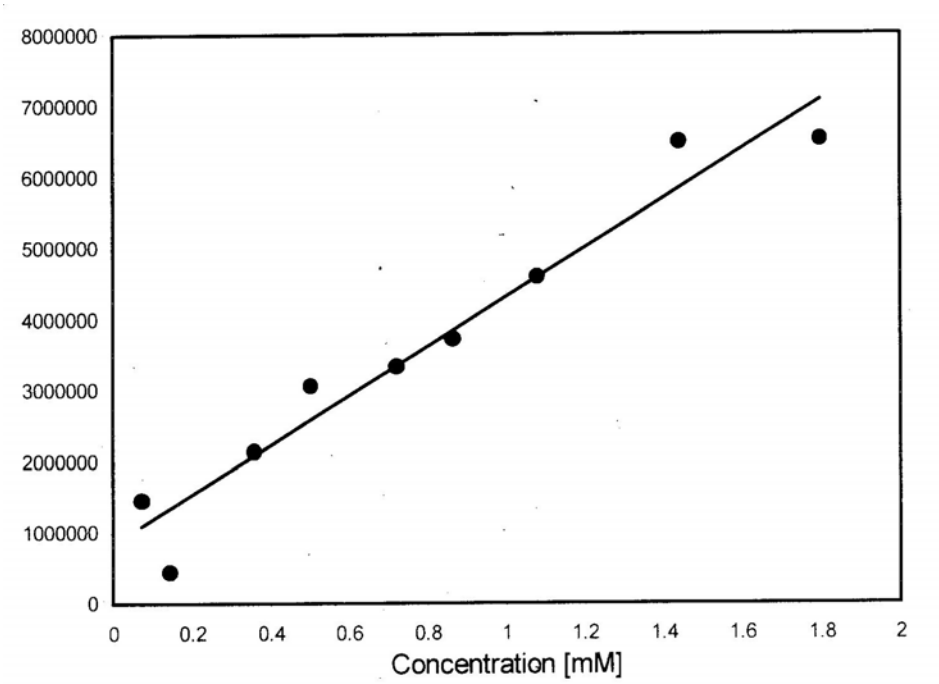


Fig.2.6 Calibration of standard PNP by HPLC.
Experimental details are described in Section 2.2.2.2
-●-PNP

2.2.2.3 NMR studies

A Brüker WH 500 operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C was used for recording the spectra. Spectra were recorded at room temperature after dissolving the reaction mixture extracts in $CDCl_3$ and expected metabolite concentration was in the range 0.5 to 20 mg. ^{1}H and ^{13}C NMR and Two-Dimensional Heteronuclear Multiple Quantum Coherence Transfer (2D HMQCT) spectra were recorded for the samples. In cases where concentrations were high (>20 mg) 2D HMQCT spectra were recorded.

^{1}H NMR

Typically a region from 0- 15ppm was scanned. About 500-1000 scans were accumulated for each spectra. The signals were referenced to TMS within ± 0.01 ppm.

2D HMQCT

The spectra contained ^{1}H data in one dimension and ^{13}C data in the other dimension. The carbon signals from carbon atom to which a proton is attached were seen clearly. A 6000 Hz spectral width, 2.7s acquisition time, pulse width 83.2μs and 6μs pulses with a delay of 1.0μs were employed. Number of scans were 16 for each trace. For ^{1}H, a 8.8 μs pulse was used. A - 1 Hz line broadening was employed.

2.2.2.4 Estimation of ammonia

Ammonia was estimated using Nesslers reagent and quantitative data was obtained by preparing a calibration curve using standard ammonium solution.

Materials

Mercuric iodide
Potassium iodide
Sodium hydroxide
Ammonium chloride

Method

Preparation of Nesslers reagent (Standard Methods, APHA, 17th ed., 1985)

100gm HgI_2 and 70 KI were dissolved in a small quantity of water and was slowly added to 160g NaOH dissolved in 500ml water. This mixture was then made up to 1l. The reagent was stored in rubber- stoppered borosilicate glassware and away from sunlight to maintain reagent stability for upto a year.

Stock ammonium solution: 3.819g anhydrous NH_4Cl at 15°C was dissolved in ammonia free water and made up to 1000ml. 1ml of this solution contained 1mg N= 1.214mg NH_3. 10ml of the stock solution was made upto 1000ml with water, 1ml – 10μgN= 12.14μg NH_3.

Standard calibration

Different aliquots of 5ml sample containing NH_3 concentration in the range of 0.24-8.25μg and 0.1ml Nesslers reagent made up by ammonia free water were measured spectrophotometrically. The optical densities observed at 400nm were plotted against concentration employed to get a standard curve

(Fig 2.7).

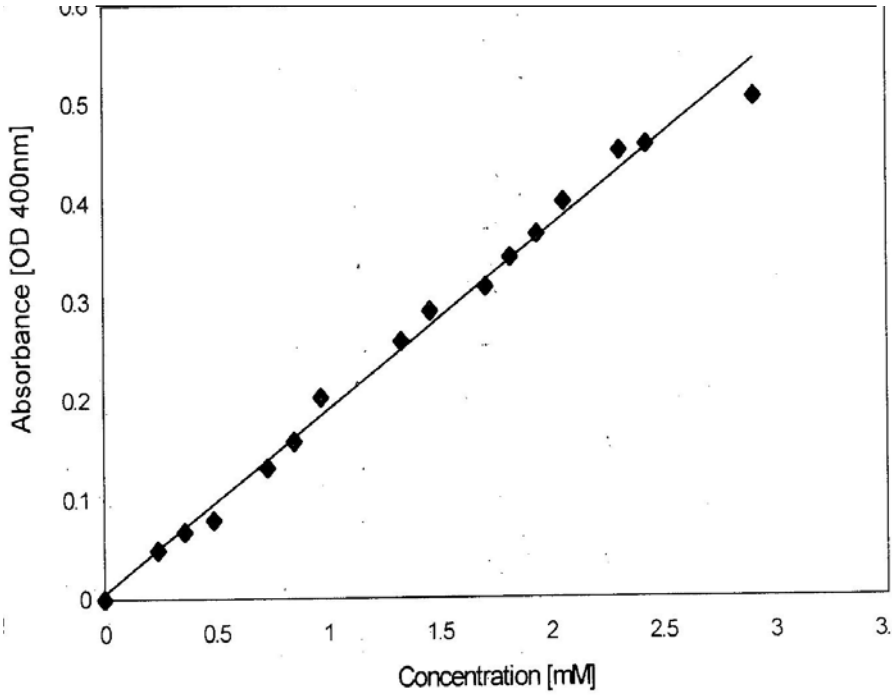


Fig. 2.7 Spectrophotometric calibration of standard Ammor
Experimental details are described in Section 2.2.2.4
-◆- Ammonia

2.2.2.5 Estimation of nitrite (Montgomery and Dymock, 1996)

Nitrite release from reaction mixtures was quantified from a standard curve prepared using a standard nitrite solution.

Materials

Sodium nitrite

Sulphanilic acid

Potassium hydrogensulphate

N- (1- Naphthyl) ethylenediamine hydrochloride.

Method

Preparation of reagents

Reagent A: Sulphanilic acid solution

Potassium hydrogensulphate	27. 2 g/l
Sulphanilic acid	3. 46 g/l.

Reagent B: 0.4% N (1-napthyl) ethylenediamine hydrochloride

Standard sodium nitrite solution

1.2325gm of sodium nitrite was dissolved in 250ml freshly distilled water. A standard solution was made using 5ml of the stock and making it to 500ml with distilled water. 1ml of this solution contained 0.0493 mg of nitrite.

Calibration

Different volumes of the standard solution containing nitrite in the range of 0.065–19.7□g were measured spectrophotometrically. The optical densities observed were plotted against concentration of NO_2 and a standard curve obtained **(Fig 2.8)**. A linear least square analysis was performed to get the regression data from which nitrite concentration in the test samples was determined (correlation coefficient = 0.99).

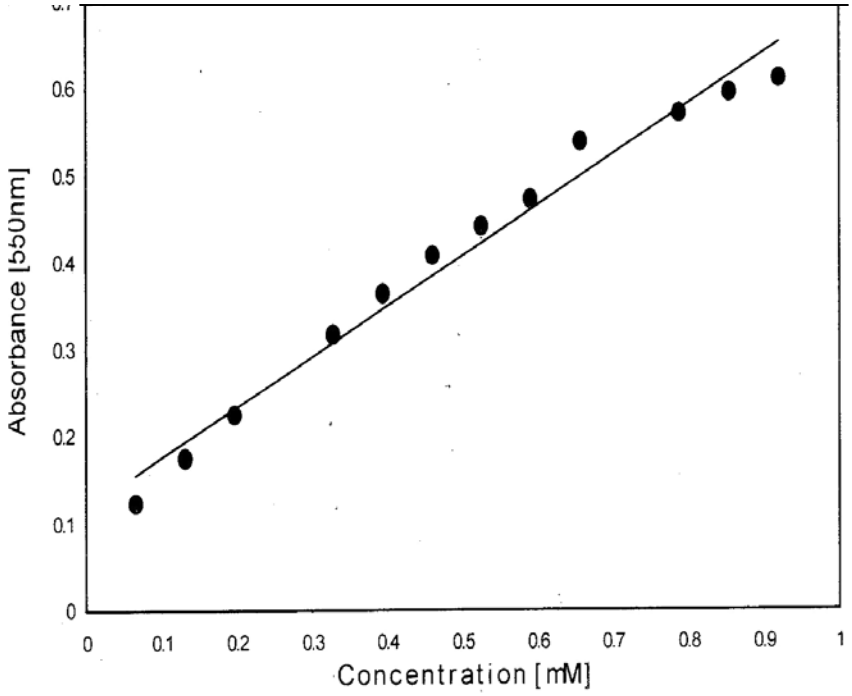


Fig.2.8 Spectrophotometric calibration of standard Nitrite. Experimental details are given in section 2.2.2.5

-●- Nitrite

2.3 BACTERIOLOGY

2.3.1 Isolation of nitrophenol degrading consortium.

Materials

Contaminated soil, M5 medium, phenol, nitrophenol isomers, nutrient agar, yeast extract, sodium succinate.

Method

On enrichment of a soil sample collected from the vicinity of a pharmaceutical industry using phenol as the feed stock and synthesizing various phenolic derivatives, a microbial community got established after four consecutive transfers in 500ml Erlenmeyer flasks which were maintained on a rotary shaker at room temp containing

1.06mM of phenol as the sole source of carbon and energy to obtain sufficient biomass. The microbial culture obtained by enrichment with phenol was thoroughly washed with M5 medium. It was then further enriched with a mixture of nitrophenol isomers (ONP, MNP and MNP) at a total concentration of 0.071mM. The nitroaromatic compounds were supplied as sole source of carbon and energy. The microbial consortium was able to degrade 0.071mM ONP completely by 72h, MNP and PNP by 48h when used separately. Acclimation of this consortium to individual nitrophenol isomers by five repeated transfers decreased the time required for degradation. The acclimated culture was able to degrade the isomers separately by 24h.

2.3.2 Resolution of the microbial consortium

The liquid enrichments were streaked on to fresh nutrient agar plates and colonies were chosen after 72h of incubation at room temp in dark conditions. Morphologically dissimilar colonies were purified by restreaking them on to fresh nutrient agar plates repeatedly. A total no of eight different colonies were observed and were maintained as a consortium as well as individually on nutrient agar plate, M5 agar and medium containing nitrophenols and also as suspensions in M5 medium supplemented with yeast extract and sodium succinate.

2.3.3 Characterization of the microbial isolates

Individual colonies bearing morphological dissimilarities were streaked on nutrient agar plates and slants and the eight colonies were designated as SNP-1, SNP-2, SNP-3, SNP-4, SNP-5, SNP-6, SNP-7 and SNP-8. Each culture was subjected to microscopic and several biochemical tests. The isolates were studied by standard taxonomical procedures and characterization was based on the following tests and identification done according to Bergey's Manual of Determinative Bacteriology (1994).

All glassware, media and other material used in bacteriological studies were either wet sterilized or dry sterilized. Wet sterilization was carried out at

121 ºC for 20 minutes in an autoclave and dry sterilization at 180 ºC for 4h in a Hot Air Oven. The media used were prepared as per manufacturer's instruction.

2.3.3.1 Materials

The diluent was 0.85% normal saline, which was dispensed in requisite quantities in suitable glass containers and autoclaved.

Grams stain

Crystal violet: 2.0g crystal violet and 0.8g ammonium oxalate were dissolved in 20ml ethyl alcohol (95%) and 80ml distilled water, respectively and the two solutions were then mixed. The prepared stain was filtered and stored in a clean and dry glass stoppered bottle.

Lugole's iodine (mordant): was prepared by mixing 1g of iodine and 2.0g potassium iodide in 300ml distilled water.

Safranin (counter stain): Safranin O (2.5g) was mixed with 100ml of 95% ethyl alcohol to get a stock solution. Whenever required 10ml of the stock solution was mixed with 90ml of distilled water for use as counter stain.

Bacteriological media

Arginine dehydrolase broth: g/l

Peptic digest of animal tissue	1.0
Sodium chloride	5.0
Dipotassium hydrogenphosphate	0.3
L-arginine	10.0
Bromocresol purple	0.016
Final pH	7.2 $\pm$ 0.2

Requisite quantity was dissolved in distilled water, pH adjusted and dispensed at the rate of 5ml into test tubes (15 x 125nm) and autoclaved.

Brain heat infusion (BHI) broth and agar: g/l

Peptic digest of animal tissue	10.08
Calf brain	12.08
Beef heart infusion	5.0
Dextrose	2.0
Sodium chloride	5.0
Disodium dihydrogenphosphate	2.5
Final pH	7.2 $\pm$ 0.2

The requisite quantity of dehydrated medium was dissolved by boiling in water, dispensed in 10ml amounts in test tubes (18 x 10mm) and autoclaved.

BH1 agar medium was prepared by using agar at a strength of 1.5% in BH1 broth medium. The agar medium was boiled to dissolve the agar dispensed in requisite quantities in Erlenmeyer conical flasks of suitable capacity as well as in 10ml amount in test tubes (18 x 150mm) plugged with cotton and autoclaved. After autoclaving, tubes containing BH1 agar were kept in a slanting position, so as to have 1" butt .

Hugh Leifson medium: g/l

Peptone	2.0
Sodium chloride	5.0
Glucose	10.0
Dipotassium hydrogenphosphate	0.3
Bromothymol blue	0.05
Agar	2.0
Final pH	7.2 $\pm$ 0.2.

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in 3ml volume in the test tubes (12 x 75mm) and autoclaved.

Kohn Two tube medium No. 1: g/l

Beef extract	2.0
Peptone	15.0
Yeast extract	2.0
Dextrose	1.0
Mannitol	10.0
Phenol red	0.05
Agar	15.0
Final pH	7.2 $\pm$ 0.2

Prior to use, to the molten and tempered sterile medium, 25ml of 40% membrane filtered urea solution was added, mixed well and 1" butt and slants were prepared using sterile glass test tubes.

Kovacs reagent: g/l

p-Dimethylaminobenzaldehyde	5.0
Amyl alcohol	75.0ml
Concentrated Hydrochloric acid	25.0ml

The reagent was prepared by first dissolving p-dimethyl amino-benzaldehyde in amyl alcohol and later adding HCl to the mixture.

MR-VP medium-Buffered glucose broth: g/l

Buffered peptone	7.0
Dextrose	5.0
Dipotassium hydrogenphosphate	5.0
Final pH	7.2 $\pm$ 0.2.

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in 10ml amounts in the test tubes (18 x 150mm) and autoclaved).

Voges proskauer reagent

VP reagent consists of two solutions

Solution A		*Solution B*	
α-napthol	5g	Potassium hydroxide	40g
Absolute alcohol	100ml	Creatine	0.5g
		Distilled water	100ml.

Nitrate broth : g/l

Beef extract	3.0
Peptone	5.0
Potassium nitrate	1.0
Final pH	7.3 $\pm$ 0.2

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in 5ml amounts in the test tubes (18 x 150mm) and autoclaved.

Reagents: Culture broth was tested for nitrate using two solutions.

Solution 1		*Solution 2*	
Sulphanilic acid	8.0g	α-naphthol	5.0 g
5N Acetic acid	1000ml	5N Acetic acid	1000ml.

Nutrient broth and agar: g/l

Peptic digest of animal tissue	5.0
Beef extract	3.0
Sodium chloride	8.0
Final pH	7.3 $\pm$ 0.2

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in 10ml amounts in the test tube (10 x 150 mm) and autoclaved. Nutrient agar medium was prepared using agar at a strength of 1.5% in nutrient broth medium. The agar medium was boiled to dissolve the agar dispensed in requisite quantities in Erlenmeyer conical flasks of suitable activity plugged with cotton.

Plate count agar : g/l

Casein enzymic hydrolysate	5.0
Yeast extract	2.5
Dextrose	1.0
Agar	15.0
Final pH	7.0 ± 0.2

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in appropriate amounts in Erlenmeyer conical flasks and autoclaved.

Simmon citrate medium: g/l

Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bromthymol blue	0.08
Agar	15.0
Final pH	6.8 ± 0.2

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in 8ml amounts in test tubes (15 x 125mm) and autoclaved. After autoclaving the medium was allowed to solidify so as to form slants with 1" butt.

Tryptone broth medium : g/l

Tryptone	10.0
Sodium chloride	5.0
Final pH	7.2 ± 0.2

The requisite quantity of dehydrated medium was dissolved by boiling in water, dispensed 5ml in test tube (18 x150mm) and autoclaved.

2.3.3.2 Methodology

Ammonia from arginine

The prepared arginine dihydrolase broth tubes were inoculated with a loopful of 20h old culture, mixed well and observed for color change in the medium due to ammonia production, as reflected by the indicator bromo thymol blue (light green to dark blue).

Catalase production

Test cultures were grown freshly on BH1 agar slants on to which were added a few drops of 3% (v/ v) hydrogen peroxide. Culture tubes were observed for the formation of nascent oxygen in the form of bubbles which was indicative of a positive reaction for catalase production.

Citrate utilisation

This test was carried out in Simmon's citrate medium. Individual test cultures were streaked on to the prepared slants. Inoculated slants were incubated for 24-48h at 37°C. Positive reaction for citrate utilization by the culture was indicated by change of colour of medium to deep blue (alkaline reaction).

Gelatin hydrolysis

Nutrient agar was prepared with 8% gelatin and plates were pre- poured. A loopful of 20h old broth culture of individual organisms was spotted into portions of the prepared plates and incubated for 24–48h at 37°C. Incubated plates were examined for gelatin hydrolysis by pouring a mixture of 1.5% mercuric chloride and 10% hydrochloric acid solution over the individual culture. The formation of a clear transparent zone around the growth area indicates positive reaction for gelatin hydrolysis.

Grams stain

The heat fixed smear of the individual presumed bacterial isolates prepared on a clean glass slide was stained with crystal violet for 1min, followed by washing off excess stain with water. Then Lugol's iodine solution was added and allowed to react for 1 minute. After washing off iodine with water, the smear was treated with 95% ethanol for 30

seconds so as to remove the excess crystal violet. Finally the smear was counter stained with safranin, allowed to react for 30 seconds, washed with water, dried and examined under oil immersion of a compound microscope. Gram positive cells appear as violet colored, while gram negative cells as pink colored. Beside, the cell morphology was also recorded.

Indole production

Individual test cultures were inoculated into prepared tryptone broth tubes and incubated for 24h at 37°C. To each of the inoculated tubes was added 0.2-0.3ml of Kovac's reagent. The formation of a dark red color in the surface layer of the culture broth indicates a positive test for indole.

Methyl red and Voges Proskauer (MR-VP) reaction

These two tests were performed using MR–VP broth. Test cultures were inoculated individually into the broth medium and incubated for 24h and 37°C. The culture broth was divided into two parts, one part was used for MR reaction and the other for VP reaction.

Test for motility and oxidation/ fermentative reaction

Stab inoculation of the test culture individually were performed in the sterile tubes of Hugh Leifson medium. Each culture was inoculated in duplicates. To one set of the inoculated tubes, a few drops of sterile liquid paraffin were added to overlay the agar medium (anaerobic condition). The other set of tubes were kept without any additions. Both the sets of tubes were inoculated at 37°C for 24–48h. Inoculated tubes were observed for the spreading of the culture growth from the line of inoculation, which indicates the motile nature of the culture. Acid production from glucose was indicated by color change of the medium from light green to yellow. Positive tubes for acid production under aerobic condition indicate the oxidative nature, while positive tubes under anaerobic condition indicate fermentative nature.

Nitrate reduction

Individual test culture was inoculated into the prepared nitrate broth and incubated for 24h at 37°C. To 5ml of the 24h old culture was added two drops each of Solution 1 and 2 of the nitrite testing reagent Development of orange/ brick red color indicated nitrate reduction to nitrite.

Oxidase production

A speck of freshly grown culture at 37°C was smeared on the edge of a piece of filter paper, onto which was added a drop of the reagent (1% aqueous solution of N,N-dimethyl-p-phenylenediamine). The edge of the culture smear was observed for color change in 30 seconds. Violet coloration indicated oxidase positive reaction.

Starch hydrolysis

A loopfull of 20h old broth culture of individual organisms was spotted onto portions of pre-poured plates of plate count agar with 1% soluble starch and incubated at 37°C for 24-48h. Incubated plates were exposed in a glass chamber saturated with iodine vapors, to read for the positive/negative action on starch.

Urease production

This test was carried out using Kohn two test tube medium No.1. Individual test cultures were inoculated into the prepared slants by making a stab in the butt and streaking on the slant. Inoculated tubes were incubated for 24–48h at 37°C. Positive urease reaction (i.e. alkaline) was indicated by a deep cerise colour of the whole medium.

The individual test cultures on primary staining showed differences in their morphological appearance. Isolate SNP-1 exhibited spore formers with the spores being sub-terminal. Isolates SNP-2–SNP-7 showed coccobacilli nature of cells and there was no poly-β-hydroxybutyrate accumulation. Cells of isolate SNP-8 exhibited cells in tetrad arrangement. The cultural and biochemical characteristics associated with these isolates are presented in individual tables, Tables 2.1 (SNP-1), Table 2.2-2.7 (SNP-2, SNP-3, SNP-4, SNP-5, SNP-6, SNP-7) and Table 2.3 (SNP-8).

Table 2.1 Characteristics of isolate SNP-1

Character	
Grams stain	+
Oxidase test	+
Catalase test	+
Oxidative test	+
Fermentative	+
Motility	+
Citrate utilization	+
MR reaction	+
VP reaction	+
Nitrate reduction	+
Arginine dihydrolase	+
Urease production	+
Indole production	–
Gelatin hydrolysis	+
Starch	–
Growth at 37°C	+
Growth in 7% NaCl	+

Identified species- ***Bacillus licheniformis***

Table 2.2 Characteristics of isolates SNP 2-7

Character	SNP-2	SNP-3	SNP-4	SNP-5	SNP-6	SNP7
Grams stain	–	–	–	–	–	–
Oxidase	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+
Oxidative	+	+	+	+	+	+
Fermentative	–	+	–	–	–	–
Motility	+	+	+	+	+	+
Citrate utilization	+	+	–	+	+	–
MR reaction	–	+	–	–	–	–
VP reaction	–	+	–	–	–	–
Nitrate reduction	+	+	–	+	+	–
Arginine dihydrolase	+	–	+	+	+	+
Urease production	+	–	+	+	+	+
Indole production	–	–	–	–	–	–
Gelatin hydrolysis	+	+	–	–	–	–
Starch	–	–	–	–	–	–
Growth at 37°C	+	+	+	+	+	+
Growth at 41°C	–	+	–	+	+	–
Growth at 4°C	–	–	–	–	–	–

Identified species

SNP- 2 ***Xanthomonas maltophila***

SNP- 3 ***Serratia liquefaciens***

SNP- 4 ***Pseudomonas putida***

SNP- 5 & 7 ***Pseudomonas* sp.**

SNP- 6 ***Psuedomonas alcaligenes***

Table 2.3 Characteristics of isolate SNP 8

Character	
Grams stain	+
Oxidase test	+
Catalase test	+
Oxidative	+
Fermentative	+
Motility	–
Citrate utilization	–
MR reaction	–
VP reaction	–
Nitrate reduction	+
Arginine dihydrolase	+
Urease production	+
Indole production	–
Gelatin hydrolysis	+
Starch	–
Growth at 37°C	+
Xylose fermentation	+
Cellulose	–
Melibiose	–

Identified species-***Sarcina maxima*** .

CHAPTER 3

MICROBIAL DEGRADATION OF MONONITROPHENOLS BY A CONSORTIUM

3.1 INTRODUCTION

Microbial communities are likely to have an extremely important role to fulfill in the degradation of simple and complex natural products and environmentally foreign compounds (xenobiotics) (Gibson, 1984). It is a commonly accepted observation that often the rate of biodegradation of a particular compound is faster in nature, host of heterogenous communities, than in pure cultures of organisms isolated from that environment. Many microbial communities clearly show that relationships between the populations confer beneficial effects which make the associations more successfull than any of the individual populations alone (Slater, 1978, 1979, 1979). Microbial degradation of organic compounds is often investigated in the laboratory by using mixed culture systems obtained from the environment (Baughman et al., 1980; Boethling and Alexander, 1979; Larson, 1979; Pritchard et al., 1979).

A microbial consortium was obtained from a contaminated soil sample for conducting degradation studies of mononitrophenol isomers. The catabolic potential of this consortium, obtained by enrichment, with nitrophenols was tested in degrading ONP, MNP and PNP separately and the simultaneous degradation of all the three isomers was also checked. Effect of pre-exposure to compounds such as phenol and cresols on the consortium's capability in degrading nitrophenols, utilization of ONP, MNP and PNP as sole sources of carbon and nitrogen and the impact of varying concentrations of these isomers on the consortium's ability were some of the experiments conducted.

Gunner and Zuckerman (1968) were among the first to describe the importance of synergistic metabolic activity between an *Arthrobacter* sp. and *Streptomyces* sp. involved in the degradation of the insecticide, diazinon. Any organic compound in

question may act as a carbon source and support the growth of a mixed culture. It is now firmly established that many microorganisms growing at the expense of one substrate, may be able to transform a different substrate in a reaction or sequence of reactions, which are not directly associated with that organism's energy production, carbon assimilation and biosynthesis or growth processes (Horvath, 1972; Alexander, 1979) leading to a mechanism of co-metabolism. Parathion, a widely used infecticide has been found to be degraded by microbial communities dependent upon a co-metabolic step (Munnecke and Hsieh, 1974, 1975, 1976). Daughton and Hsieh (1977) observed in a microbial consortium of four Pseudomonads, that, *P. stutzeri* hydrolyzed parathion to diethylthiophosphate and PNP. Neither product could separately support the growth of *P. stutzeri.* A second species, *P. aeruginosa* could not grow on parathion but grew on one of the products, PNP. Thus these two organisms acting together enabled at least part of parathion molecule to be converted to biomass, a process initially dependent on a co-metabolic step. Growth of mixed cultures and stable microbial communities establishes a situation in which organisms with different genetic background have the opportunity to exchange genetic information. The evolution of certain classes of microbial community may be an important prerequisite to the evolution of novel degradative pathways as a result of exchange and rearrangement of genetic information from different gene pools.

3.2 RESULTS

3.2.1 Degradation conditions

The conditions employed for degradation studies are given below. The basal mineral medium (M5 medium), culture medium and conditions were the same as mentioned earlier (Section 2.1.1 & 2.1.2). An increased biomass obtained by supplementing the M5 medium devoid of NH_4NO_3 (nitrogen source) with 1% succinate and 0.5% yeast extract was employed. Harvested and washed cells were induced with 0.1mM of the substrate separately for a period of 24h. Thereafter the cells were once again washed thoroughly and inoculated at OD 600nm of 0.1-0.6 into flasks containing 0.1-0.3mM mononitrophenol isomers individually in separate flasks, each in triplicates. Growth,

residual substrate, ammonia and nitrite estimation were done regularly at an interval of 6h for varying periods of incubation for the three isomers. Growth, ammonia and nitrite release were monitored spectrophoto-metrically at specified wavelengths (Sections 2.2.1; 2.2.2.4; 2.2.2.5 respectively), whereas residual substrate was analyzed by both HPLC and spectrophotometry (Section 2.2.2.2)

3.2.2 Degradation of o-Nitrophenol

At 0.1mM ONP as substrate, the biomass as measured spectrophoto-metrically showed an irregular growth curve up to 40h. Thereafter it showed a steady decrease. This indicated that in the presence of 0.1mM substrate not much growth could be registered. Although 0.1mM ONP was employed, the ONP concentration estimated spectrophotometrically at 412nm was very less. The reason for this may probably be due to the absorption of ONP on to the cell wall. An initial concentration of only 0.07mM was detected. Within a period of 12h, 67.5% decrease in the substrate concentration was observed. The control for the same period practically showed very little change in concentration **(Fig 3.1 and 3.2)**. About 90% degradation of ONP was observed by 54h. HPLC analysis also showed decrease in ONP concentration after 6h **(Fig 3.3 and Fig 3.3a)**. No ammonia could be detected in the culture filtrate during the course of degradation but a maximum of 0.009mM nitrite was observed at 6h which remained constant up to 50h and then declined coinciding with decrease in substrate concentration and subsequent disappearance **(Fig 3.4)**.

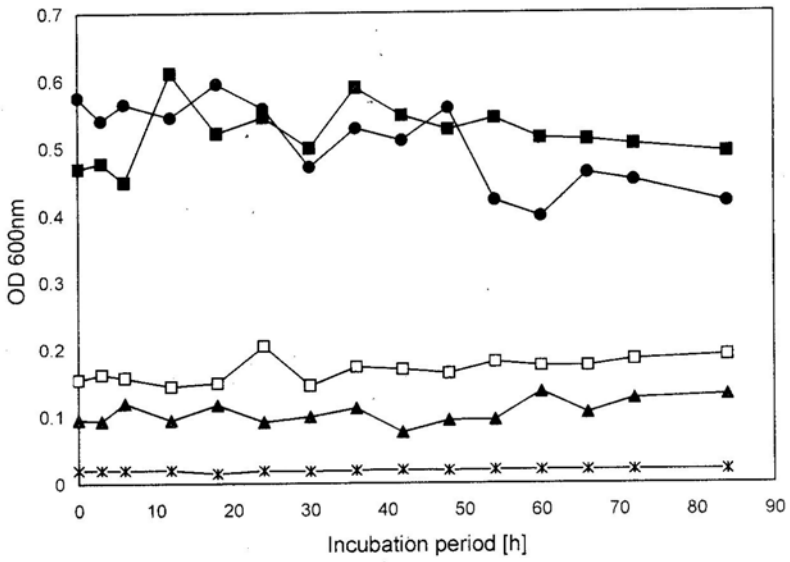


Fig. 3.1 Biomass estimation during degradation of subtoxic levels of ONP, MNP, PNP and their mixture. Experimental details are given in Section 2.2.2.1. -■-ONP, -▲- MNP, -●-PNP, -□- Mix NP's and -*- control

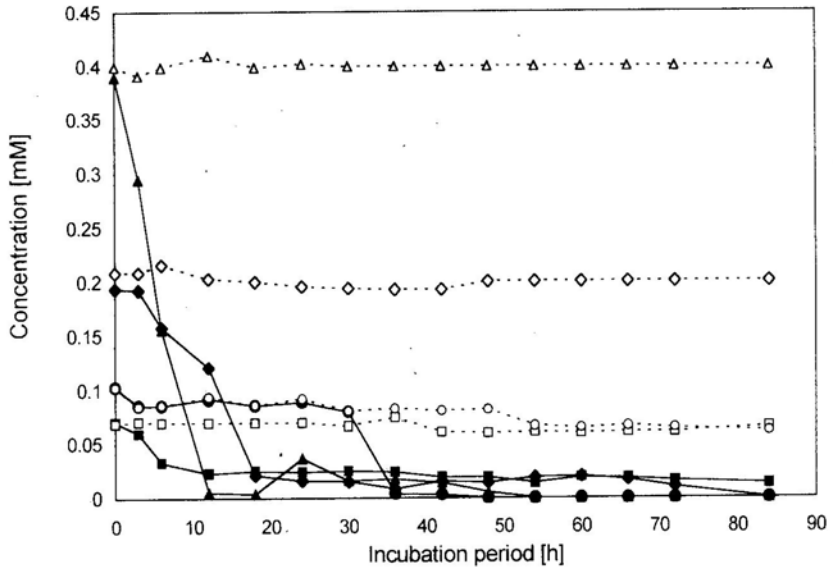


Fig. 3.2 Degradation pattern of subtoxic concentration of ONP,MNP, PNP and Mix NPs. Experimental details are described in Section 2.2.2.2.
Spectrophotometric residual analysis: c-control;
-■-ONP , -□-(c); -▲- MNP,-Δ-(c); -●- PNP, -o-(c);
-◆-Mix NPs, -◊-(c)

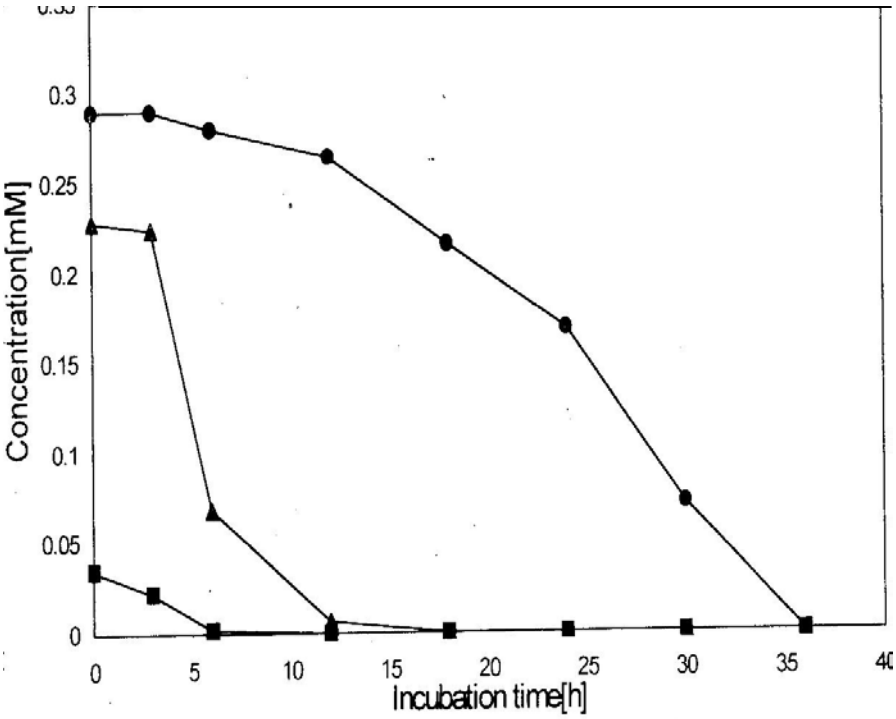


Fig. 3.3 Degradation pattern of subtoxic concentrations of ONP, MNP & PNP. Residual analysis by HPLC. Experimental details are given in Section 2.2.2.2. -■-ONP, -▲- MNP, -●-PNP

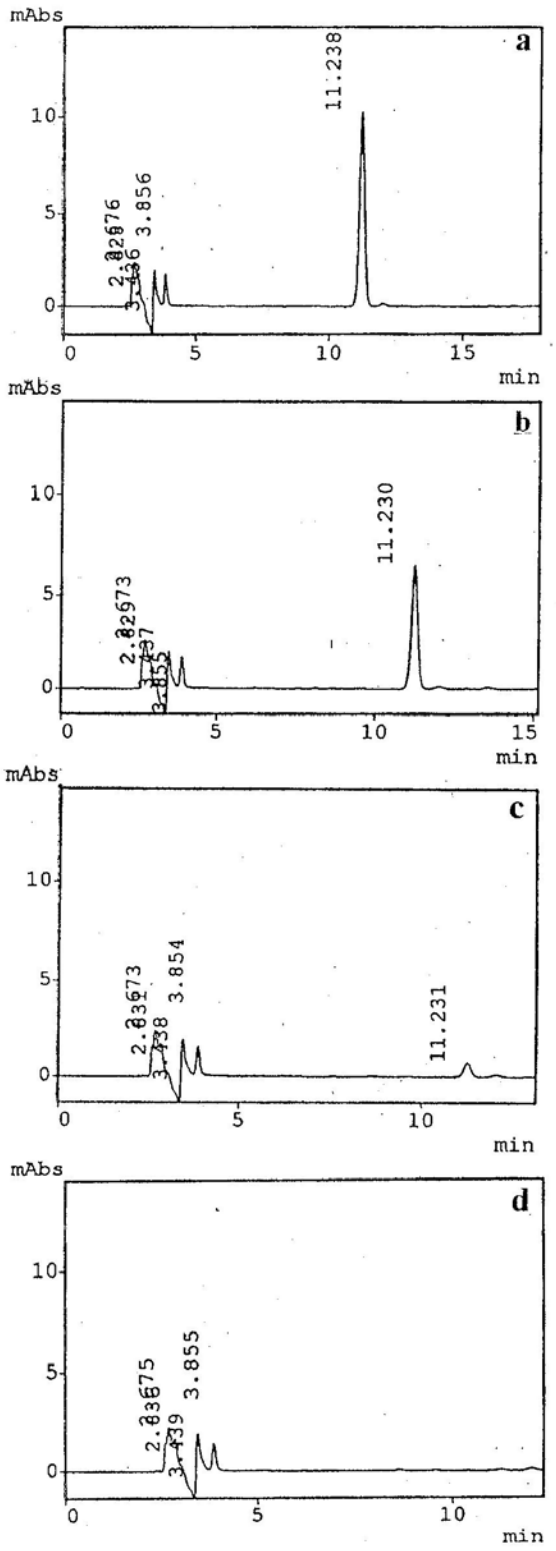


Fig. 3.3a HPLC profile during the degradation of subtoxic concentrations of ONP at (a) 0h (b) 6h (c)18h (d)24h Experimental details are given in Section 2.2.2.2

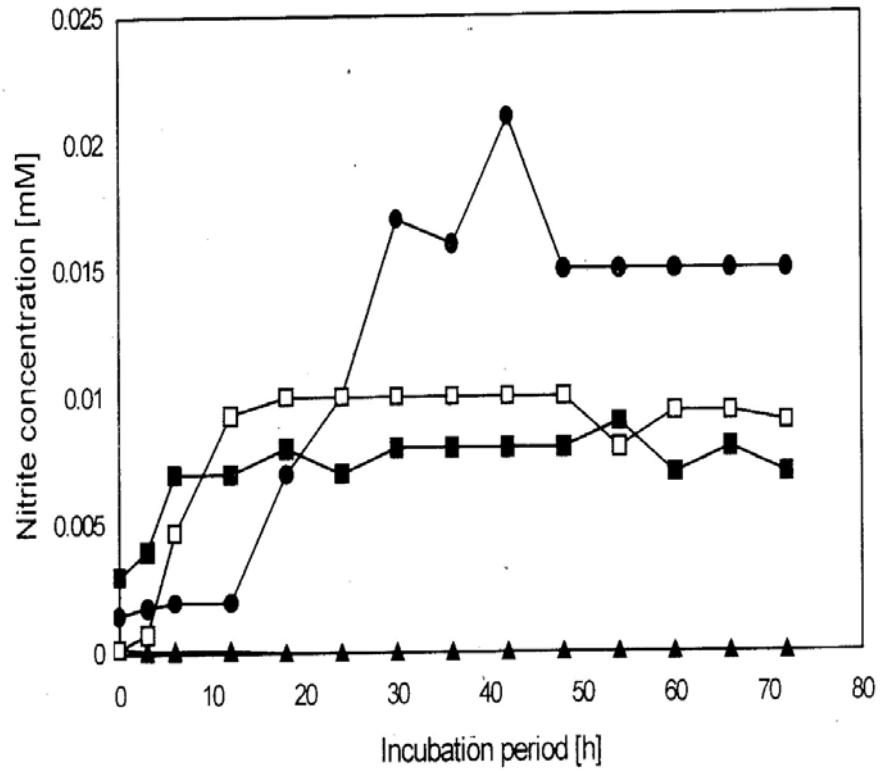


Fig. 3.4 Progress curve of nitrite release during degradation of of subtoxic concentrations of individual mononitrophenol isomers by the consortium in nitrogen free M5 medium at room temperature. Experimental details given in Section 2.2.2.5
-■- ONP grown cells, -▲- MNP grown cells, -●- PNP grown cells, -□- cells grown on Mix NPs

3.2.3 Degradation of m-Nitrophenol

To flasks containing MNP as the substrate, cells at an initial OD of 0.1 at 600nm were added. In the latter stages after 60h an increase in growth corresponding to an increase in OD 600nm of 0.14 was observed **(Fig 3.1).** Degradation monitored by UV at 272nm showed 98% reduction in substrate concentration from 0.39mM to 0.005mM which

was observed after 12h **(Fig 3.2).** This was confirmed by HPLC where no substrate could be detected beyond 12h **(Fig 3.3 & Fig 3.3b)**. Interestingly no nitrite could be detected in the culture filtrate at any stage during substrate utilization. Ammonia was detected after 6h **(Fig 3.5)**. A gradual increase in the concentration of ammonia (0.026mM) was observed from 6h which reached a maximum of 0.068mM at 55h. Presence of ammonia in the culture filtrate clearly indicated that MNP was degraded via a reductive pathway unlike ONP which showed the release of nitrite due to an oxidative pathway.

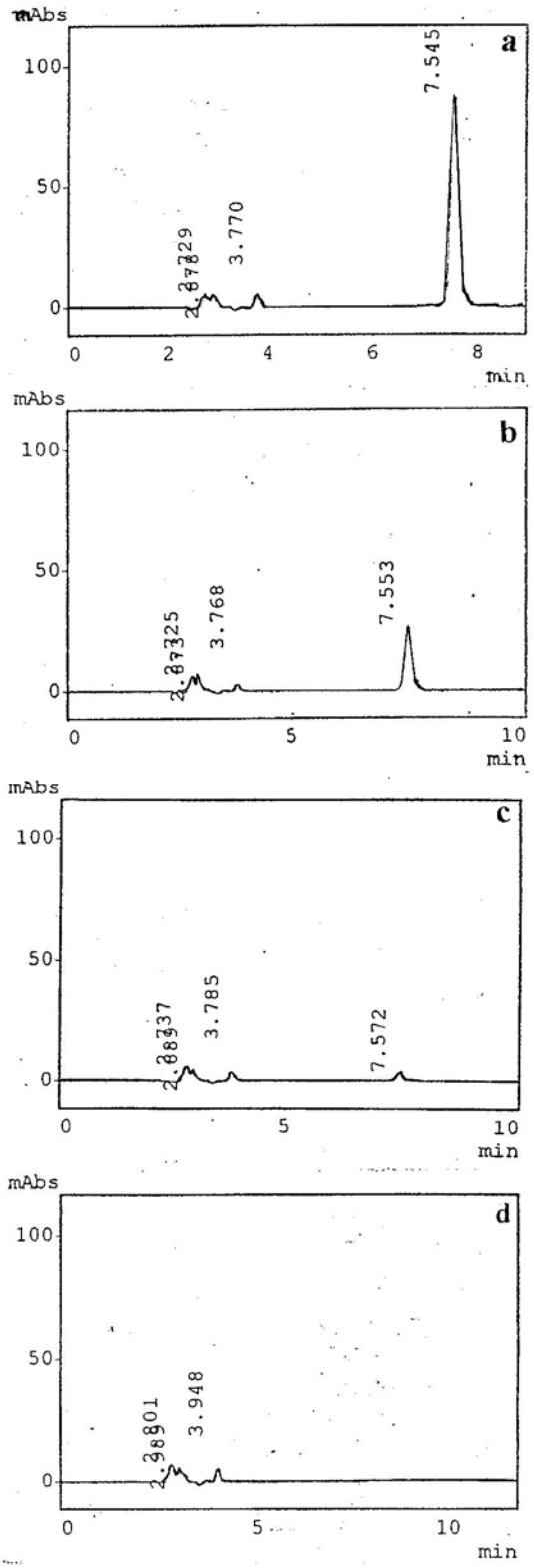


Fig. 3.3b HPLC profile during the degradation of subtoxic concentrations of MNP at (a) 0h (b) 3h (c)6h (d)12h Experimental details are given in Section 2.2.2.2

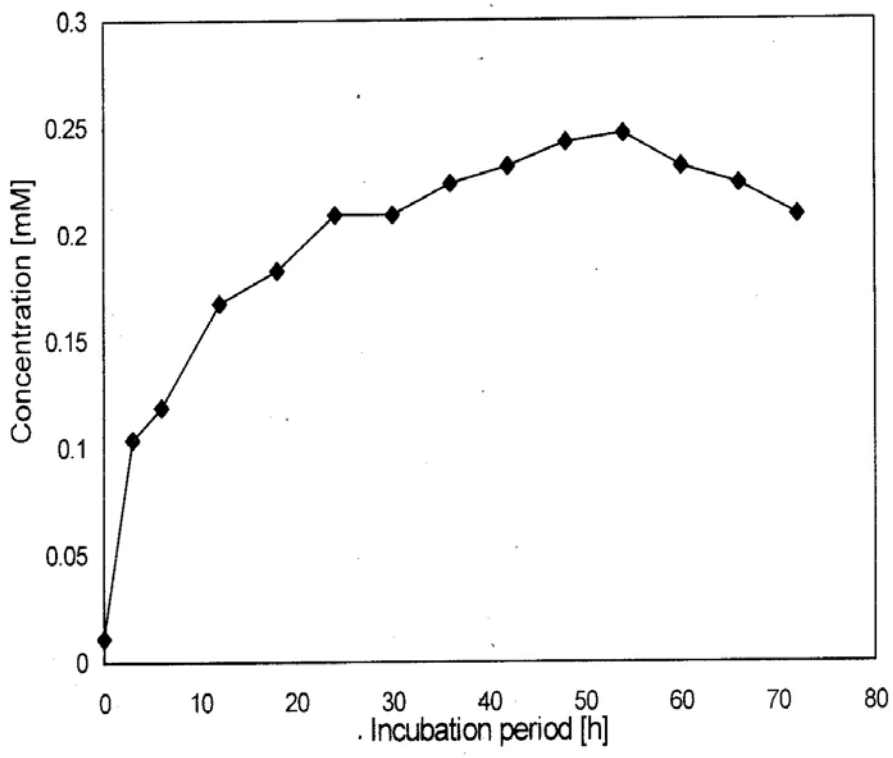


Fig. 3.5 Progress curve of Ammonia production during MNP degradation by the nitrophenol degrading consortium in nitrogen free M5 medium at room temperature. Experimental details are described in Section 2.2.2.4 -◆- Ammonia

3.2.4 Degradation of p-Nitrophenol

There was poor growth in cells with PNP as substrate. Cells were inoculated at an initial OD of 0.57 at 600nm. A gradual decrease in biomass with time was observed **(Fig.3.1)**. However, PNP degradation as monitored spectrophotometrically showed a gradual decrease up to 36h, after which it was rapid and complete by 48h **(Fig 3.2)**. HPLC data also consolidated the observation **(Fig 3.3 and Fig 3.3c**). Nitrite release was observed as

during ONP degradation. Among the three isomers, maximum concentration of nitrite release (0.02mM) was observed in the culture filtrate supplemented with PNP as shown in **(Fig.3.4).** A lag in the nitrite release was also observed up to 12h, thereafter gradually increasing and reaching a maximum at 42h. No ammonia could be detected at all.

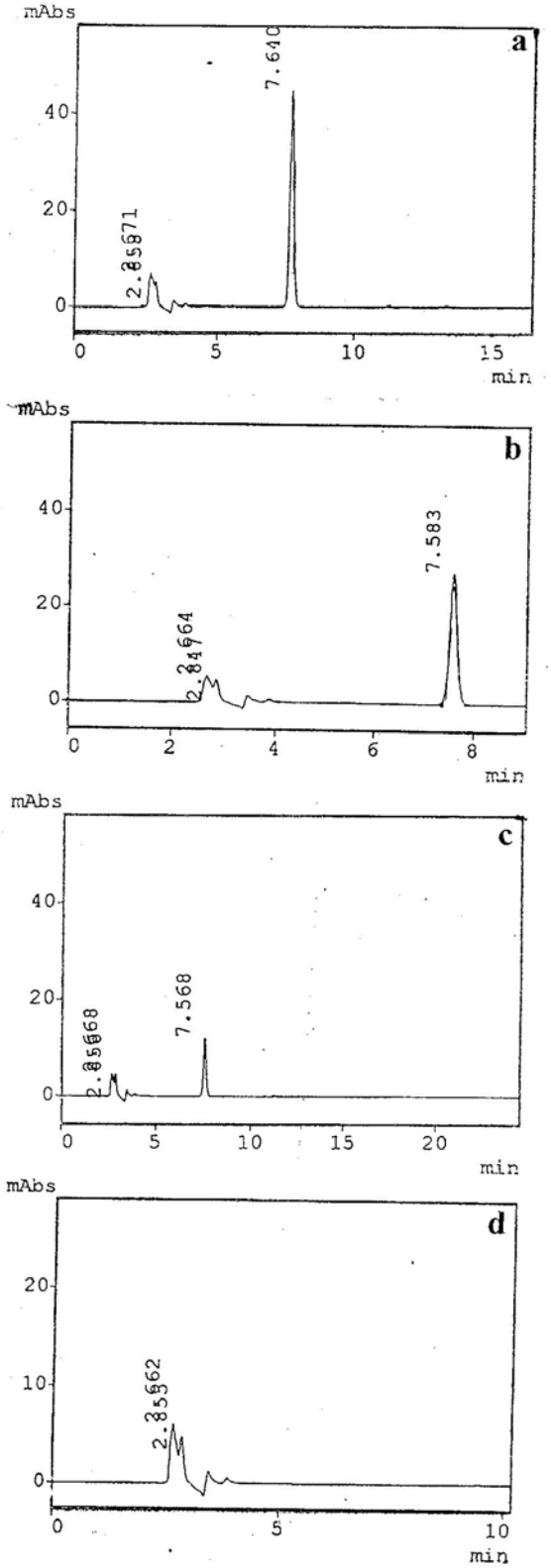


Fig.3.3c HPLC profile during the degradation of subtox concentrations of PNP at (a) 0h (b) 3h (c)6h (d)18h. Experimental details are given in Section 2.2.2.2

3.2.5 Simultaneous degradation of ONP, MNP and PNP

The concentration of each isomer was taken at 0.1mM amounting to a total of 0.3mM when a mixture of ONP, MNP and PNP was considered for degradation studies. Biomass of 0.15 at OD 600nm was considered. There was no significant cell growth even up to 80h **(Fig 3.1)**. The residual substrate analysis was carried out at 272nm spectrophotometrically which was a wavelength at which all the nitrophenol isomers showed absorbance. A 98% reduction in substrate was observed at 18h which remained constant upto 72h **(Fig 3.2)**. It was not possible to analyze the degradation of the individual isomers in a mixture by UV spectroscopy. Hence HPLC analysis was performed. **Fig 3.6**and **Fig 3.3d** shows the degradation pattern of the individual isomers. In a mixture, ONP showed complete degradation at about 24h, maximum decrease in MNP concentration was observed by 6h and around 98% of PNP was degraded by 18h Differential rates of degradation by the consortium indicates that MNP was degraded faster than ONP and PNP. The initial concentration of ONP, MNP and PNP as analyzed by HPLC were 0.04mM, 0.03mM and 0.21mM respectively. From the data obtained by HPLC, it was inferred that complete disappearance of ONP, MNP and PNP occurred at 24h, 6h and 18h respectively. About 0.01mM nitrite was found to be released from the mixture of nitrophenol isomers employed **(Fig.3.4)**. Detection of ammonia was not possible in the mixture as the concentration of MNP, the probable source of ammonia, employed was comparatively less.

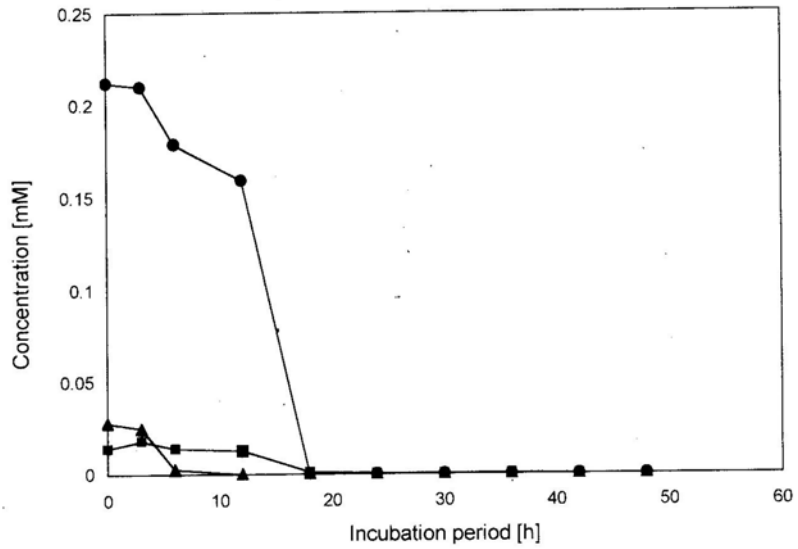


Fig. 3.6 Degradation pattern of a mixture of ONP, MNP and PNP. Experimental details are given in Section 2.2.2.2. Residual analysis by HPLC
-■-ONP, -▲- MNP, -●-PNP

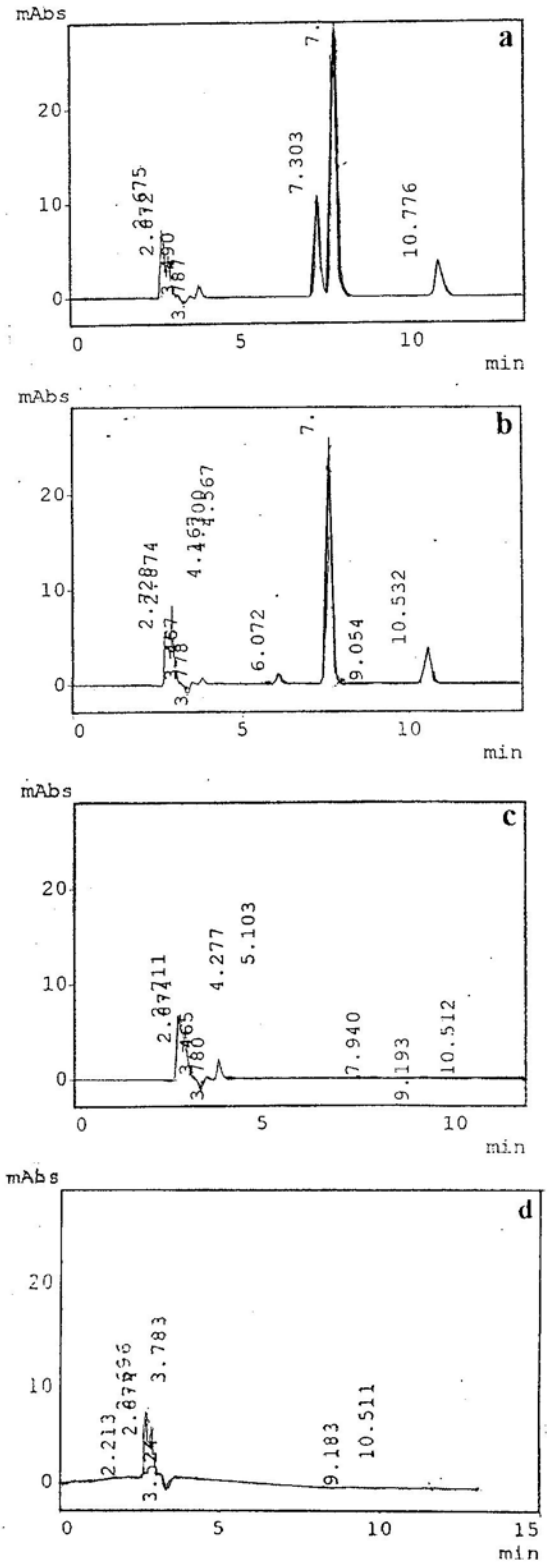


Fig. 3.3d HPLC profile during the degradation of subtoxic concentrations of Mix NPs at (a) 0h (b) 6h (c)18h (d)24h. Experimental details are given in Section 2.2.2.2

The results indicate that the available consortium was capable of degrading ONP, MNP and PNP separately using them as sole sources of carbon, energy and nitrogen sources, though remarkable growth was absent due to the early disappearance of substrate. Several mononitroaromatic compounds, including nitrophenols, are readily utilized by a variety of microorganisms as carbon, energy and nitrogen sources (Higson, 1992; Marvin-Sikkema and de Bont, 1994; Spain, 1995). The release of nitrite from the breakdown of ONP and PNP indicates that the consortium was following an oxidative mechanism to degrade these substrates and release of ammonia from MNP indicating a reductive mechanism. This clearly proves that the consortium is constituted of different cultures following different degradative mechanisms, which is an advantage especially in effluent treatment systems, which usually contain a mixture of compounds. Mixed cultures have been shown to be the most effective, than single microorganisms in degrading target molecules (Piper et al., 1996). This consortium was obtained from soil samples collected from around a phenol utilizing pharmaceutical company.

3.2.6 Effect of pre-exposure to other substrates on the degradation of nitrophenols

Majority of the phenol degraders were shown to degrade cresols, and other aromatic compounds (Babu et al., 1995, Haigler et al., 1992). The present nitrophenol degrading consortium was tested for its ability to utilize phenolic compounds such as phenol and the isomers of cresol and also sodium acetate, as carbon sources. The effect of pre-exposure of the consortium to these compounds on the degradation of the three isomers was also studied. Similar culture medium and conditions as described in Sections 2.1.1 and 2.1.2 were employed and the concentration of the phenolic compounds was maintained at 0.1mM and sodium acetate at 0.5%. The consortium was incubated for 96h. A lag was observed up to 24h thereafter which growth was observed. The cells were harvested, washed and freshly reinoculated. The OD 600nm of the cultures grown with phenol, o-, m- and p-cresol and acetate was 0.45, 0.48, 0.5, 0.6 and 0.78 respectively. Lowest growth was observed with o-cresol. Hanne et al. (1993) have observed that ONP does not induce PNP degrading pathway of

Achromobacter aurescens TW17, but gets induced well with para-substituted aromatics including 4-chlorocatechol.

The degradation of individual isomers of nitrophenol by the consortium pre-grown with phenol and cresol isomers, as well as with a combination of phenol (1mM) and all the isomers of nitrophenol (0.1mM) was studied. Pre-growth of the consortium on these substrates drastically affected the rates of degradation of all the isomers of nitrophenol, complete degradation of all the isomers (0.1mM) took not less than 96h **(Fig 3.7**, **Fig 3.8**, **Fig 3.9**, **Fig 3.10**, **Fig 3.11**, **Fig 3.12)**. It is possible that the composition of the consortium changed during the pre-growth on these substrates, due to their preferential utilization by some members, thus suppressing the growth of the active nitrophenol degrading members of the consortium. However the effect of the above substrates on the pathways of degradation of different nitrophenol isomers was varied. Pre-growth on acetate deprived the consortium of its ability to degrade ONP and affected the degradation of other isomers (Fig.3.7). There was a lag of 72h and 24h observed in culture containing PNP and MNP respectively. Very little degradation of ONP was observed even up to 96h. Although the consortium could utilize o-cresol as a substrate, it did not induce the ONP degrading pathway, as indicated by very poor degradation (Fig.3.8). Similarly m-cresol also failed to induce this pathway (Fig.3.9), whereas p-cresol seems to have induced the pathway of degradation of all these isomers, the induction of PNP being the best (Fig.3.10). Degradation of MNP was faster than that of PNP by the consortium grown on m-cresol (Fig.3.9). PNP degradation was the fastest when cells pre-exposed to induction by PNP in the case of cells pre exposed to p-cresol , which showed a lag of 48h (Fig.3.10). Pre-growth of the consortium on phenol, in presence of all three isomers of
nitrophenol affected the induction of PNP pathway (Fig.3.11), although not to the same extent as with pre-growth on phenol alone (Fig.3.12).

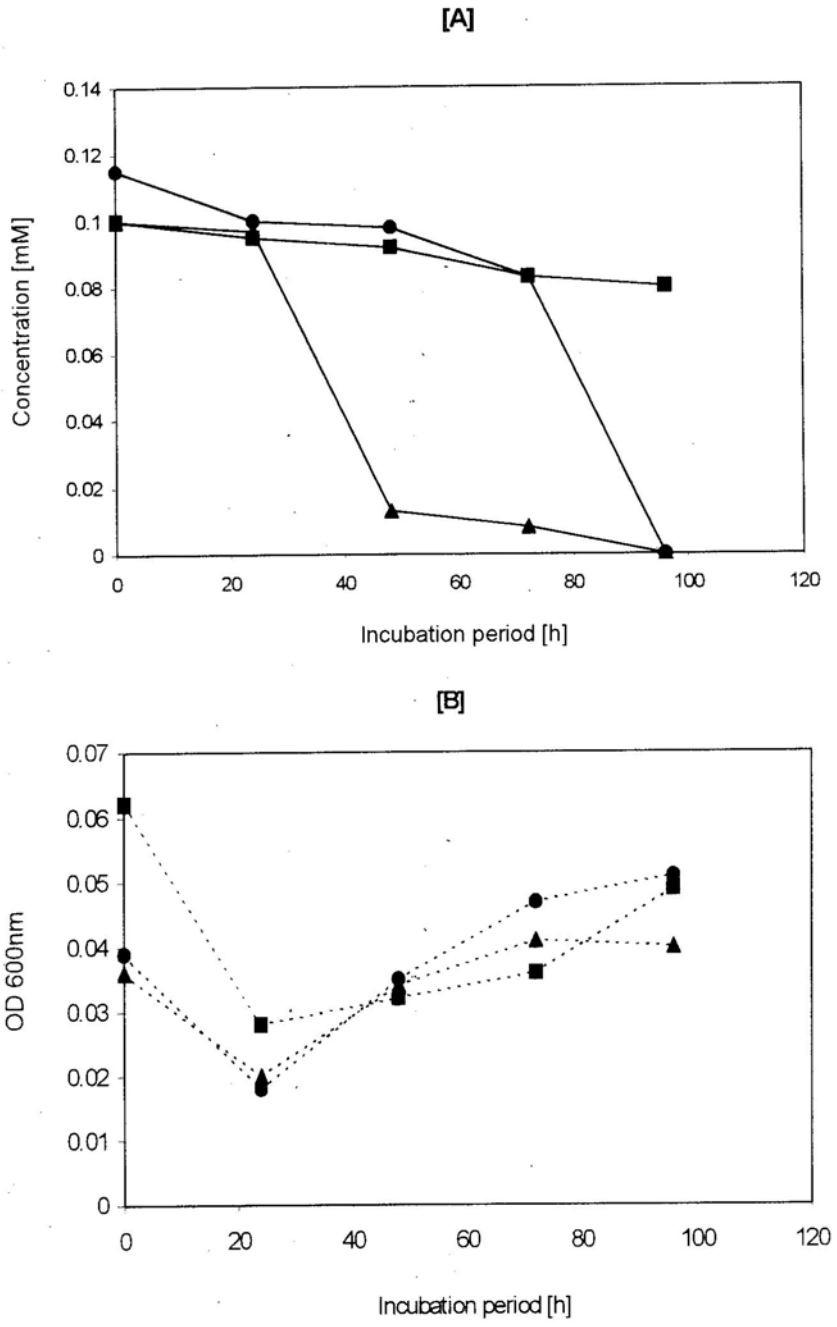


Fig. 3.7 Degradation of 0.1mM mononitrophenols by the consortium inducedwith 0.5% sodium acetate.
Spectrophotometric [A] Residual analysis by HPLC and [B]Biomass estimation.
Experimental details are described in Section 2.2.2.2.
-■-ONP, -▲- MNP, -●-PNP

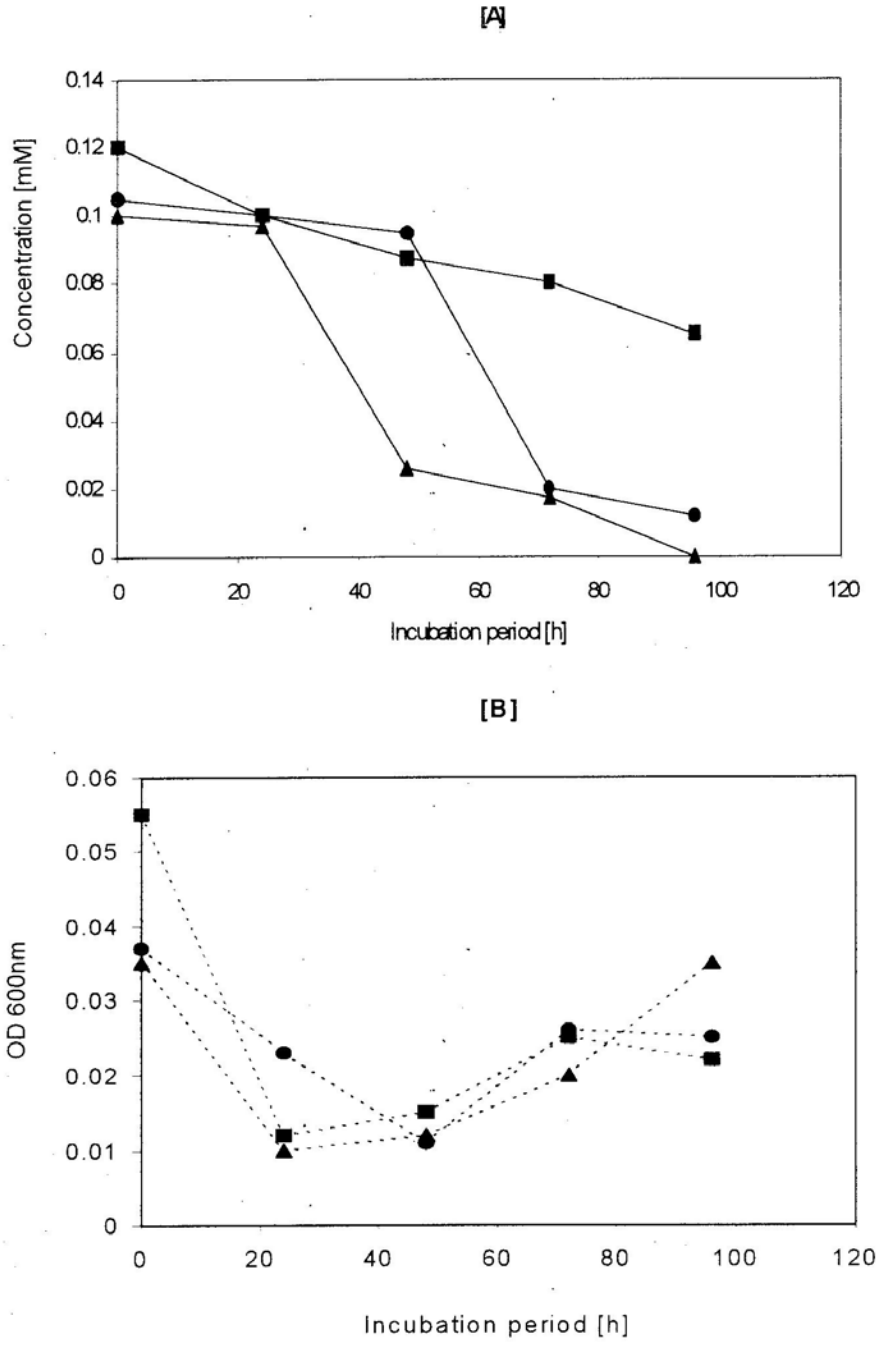


Fig. 3.8 Degradation of 0.1mM mononitrophenols by the consortium induced with o- cresol.
Spectrophotometric [A]Residual analysis by HPLC and [B]Biomass estimation.
Experimental details are described in Section 2.2.2.2
-■-ONP, -▲- MNP, -●-PNP

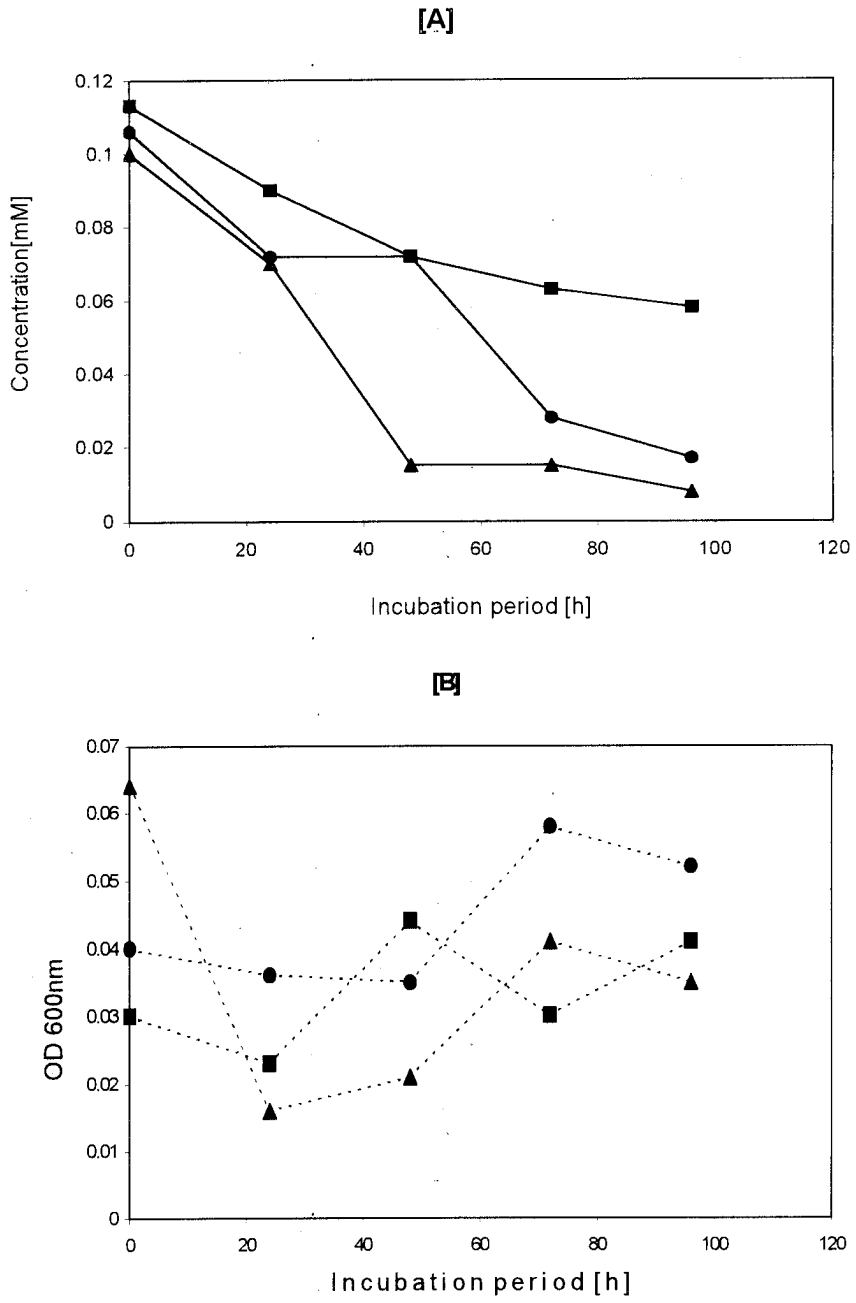


Fig. 3.9 Degradation of 0.1mM mononitrophenols by the consortium induced with m- cresol.
Spectrophotometric [A]Residual analysis and
[B] Biomass estimation
Experimental details are described in Section 2.2.2.2
-■-ONP, -▲- MNP, -●-PNP

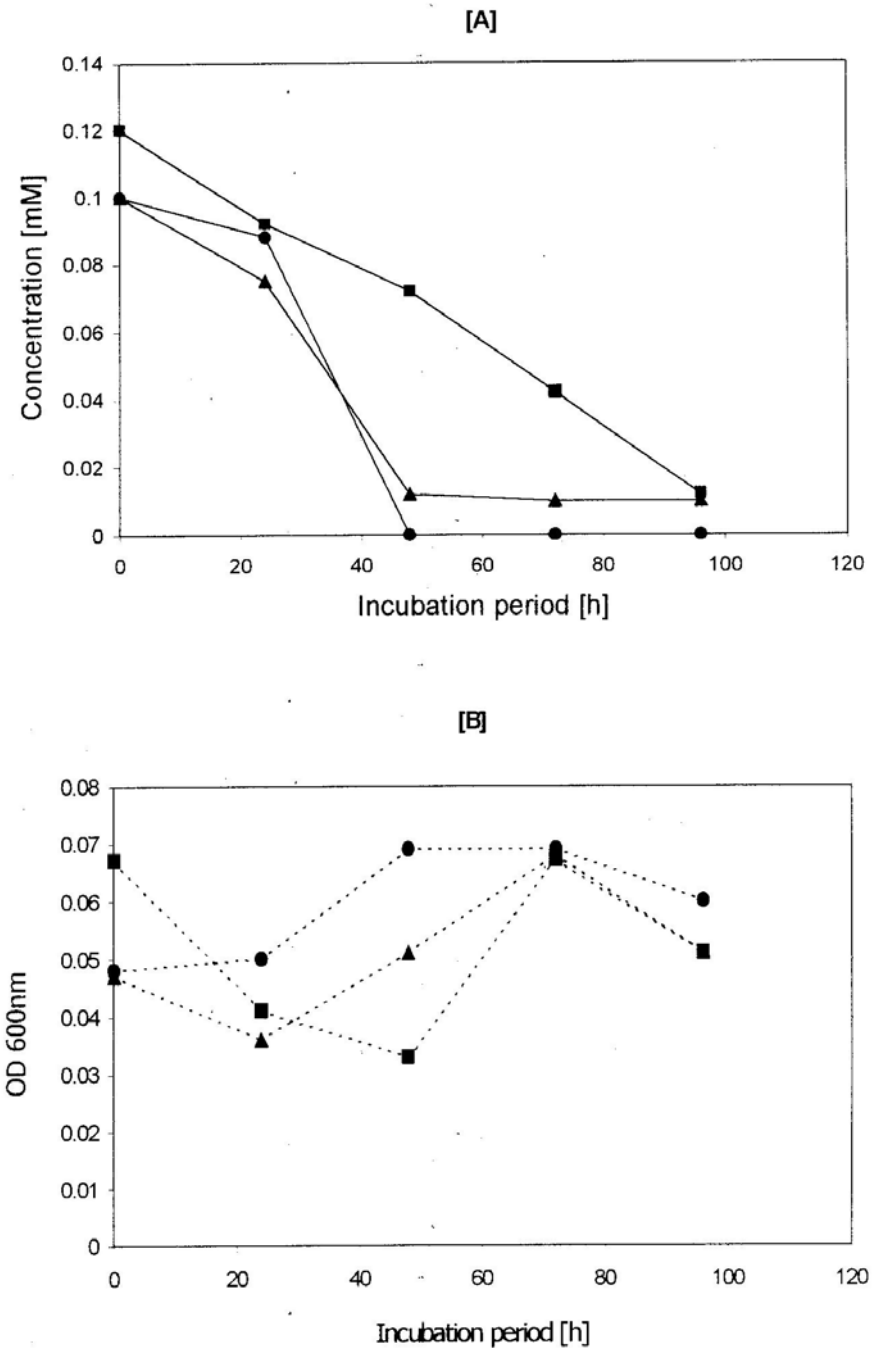


Fig. 3.10 Degradation of 0.1mM mononitrophenols by the consortium induced with p- cresol.
Spectrophotometric [A] Residual analysis by HPLC and [B] Biomass estimation.
Experimental details are described in Section 2.2.2.2
-■-ONP, -▲- MNP, -●-PNP

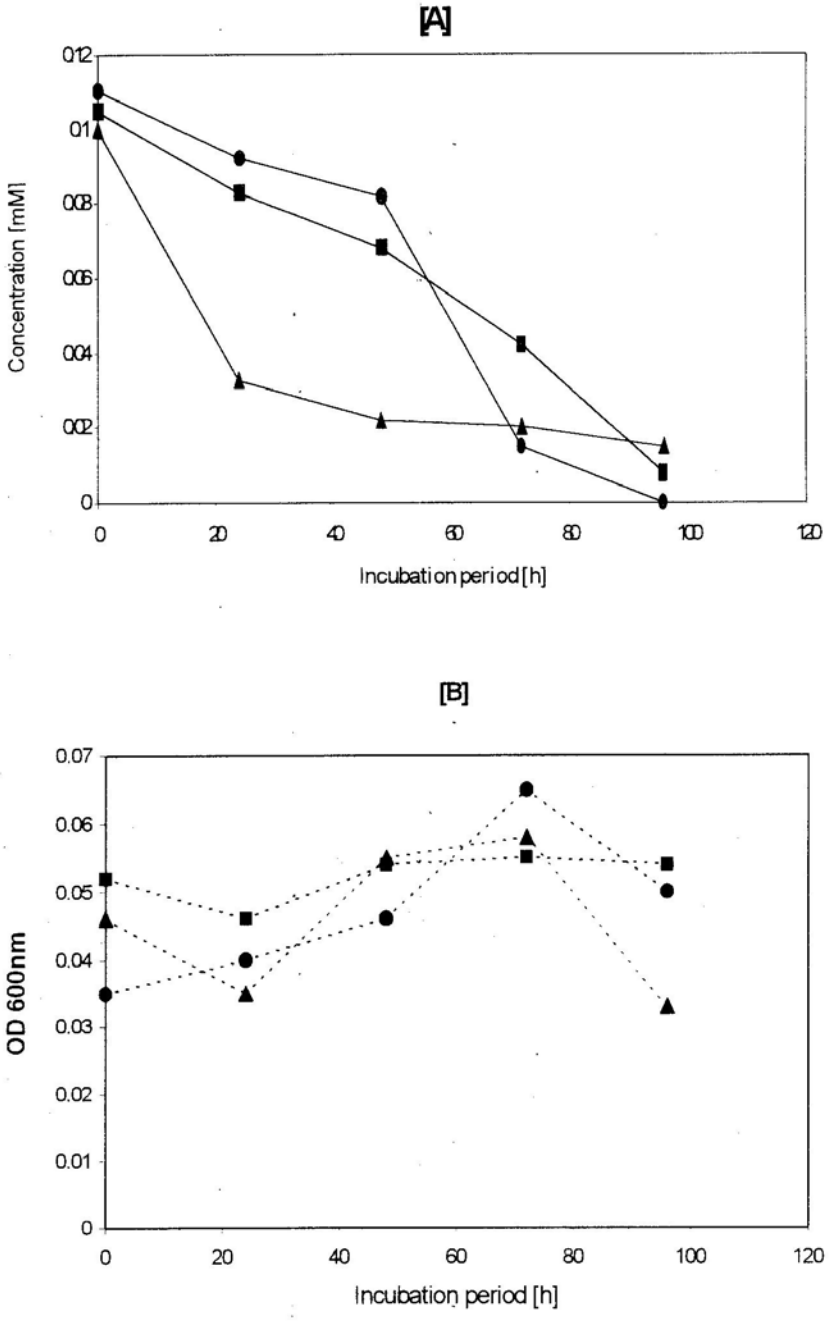


Fig. 3.11 Degradation of 0.1mM mononitrophenols by the consortium induced with1mM phenol and 0.1mM Mix NPs. Spectrophotometric [A]Residual analysis by HPLC and [B]Biomass estimation.
Experimental details are described in Section 2.2.2.2
-■-ONP, -▲- MNP, -●-PNP

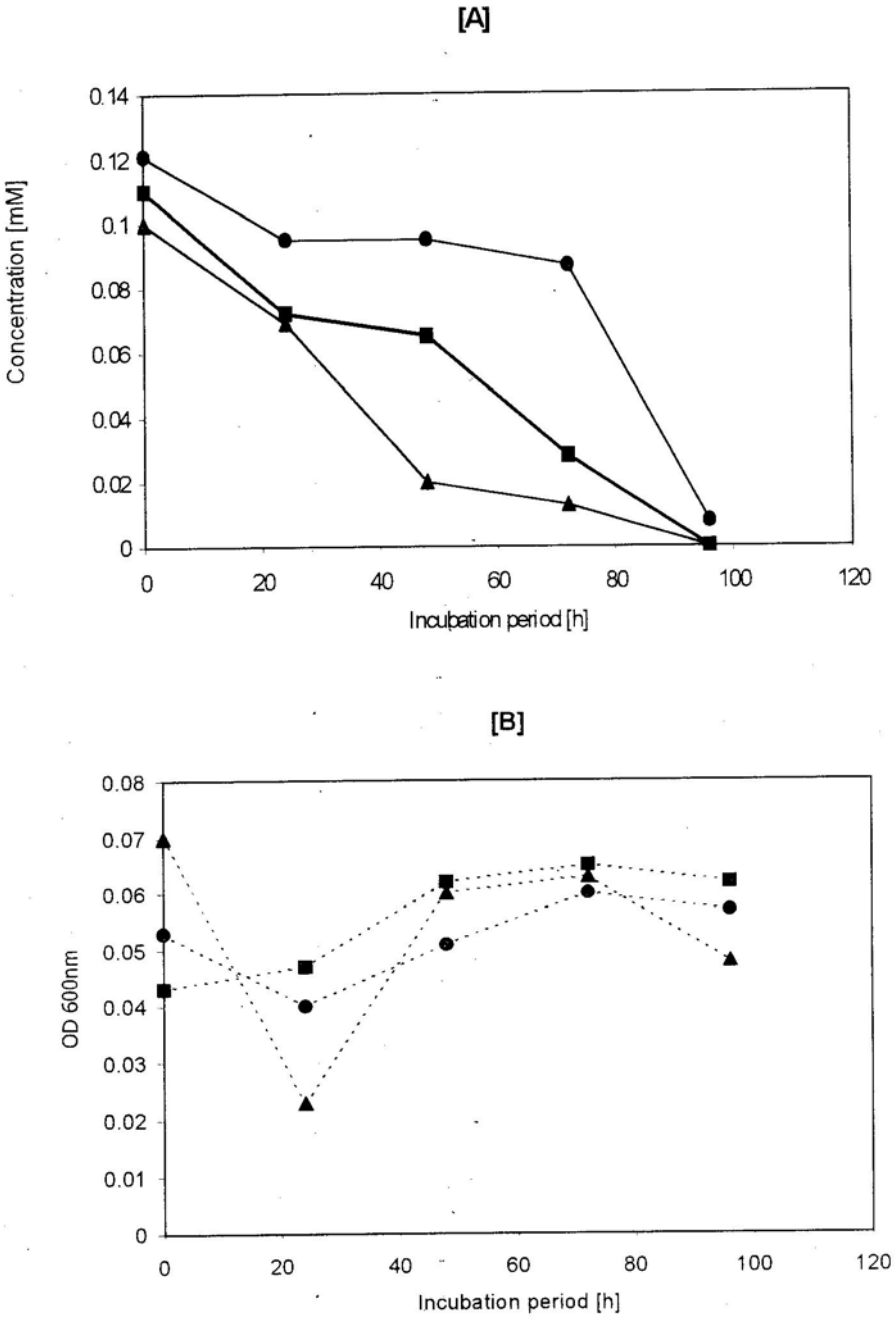


Fig. 3.12 Degradation of 0.1mM mononitrophenols by the consortium induced with phenol.
Spectrophotometric [A]Residual analysis by HPLC and [B] Biomass estimation
Experimental details are described in Section 2.2.2.2
-■-ONP, -▲- MNP, -●-PNP

Degradative enzymes can be induced by the substrate itself or by compounds similar to the enzyme substrate (Abril et al., 1989). Hanne et al. (1993) have shown that PNP degradation by *A. aurescens* TW17 was induced by PNP, 4-nitrocathechol, 3-methyl-4-nitropheonol or MNP. Also ONP and nitrobenzene did not induce the PNP pathway, indicating the strict requirement of a nitrophenol as an inducer, that too with the nitro group in para or meta position. On the other hand, PNP degradation by *Nocardia* sp. strain TW2 was induced by PNP, 4-nitrocatechol, phenol, MNP or p-cresol, but not by 3-methyl-4-nitrophenol. Pentachlorophenol degrading *Sphingomonas* sp. UG30 and *Sphingomonas chlorophenolica* strains RA2 and ATCC39723 degraded PNP (Leung et al, 1997).

In the present study, pre-exposure of the consortium to a non-aromatic substrate, sodium acetate resulted in the retardation of the degradation of nitrophenol isomers (Fig.3.7). The pattern of growth of the consortium on all the three nitrophenol isomers, after pre-growth on the above substrates, was similar. There was a fall in biomass as indicated by a drop in OD 600nm values during the first 24-48h, which later picked up (Fig.3.8-3.12). This slow increase in growth was probably due to the induction of cells by respective substrate. A similar phenomenon was also observed by Horakov and Kotouchkova (1996) with a PNP-degrading bacterium, *Corynebacterium* sp. strain 8/3. This strain required pre-incubation with 50mg/l of PNP for 72h, to achieve sufficient growth, before inoculation into higher concentration of PNP (150 mg/l) and during which a partial suppression of growth was observed.

3.2.7 Effect of induction on degradation of mono-nitrophenol isomers

Adaptation of microorganisms can play a major role in determining biodegradation rate (Felset et al., 1981; Fournier et al., 1981, Simon et al., 1979; Spain et al., 1980, Torrtensson et al., 1975). There are three ways by which adaptation can occur upon exposure of the population to a new substrate: (i) induction of specific enzymes not present (or present at low levels) in the population before (ii) selection of new metabolic capabilities produced by genetic changes and (iii) increase in the number of organisms able to catalyze a particular transformation (Spain et al., 1980). The third type of change

often follows one of the first two. Rate constants determined by using adapted population (Larson, 1979) may not be comparable with those determined by using unadapted population (Baughman et al., 1980). Bayly and Barbour (1984) reported that expression of genes which code for degradation of aromatic compounds is often inducible rather than constitutive.

In the present work the nitrophenol degrading consortium was initially grown under the same media and culture conditions (Section 2.1.1 & 2.1.2). The cells were harvested after sufficient biomass production and washed thoroughly. To test whether ONP, MNP and PNP degradation is inducible in the consortium employed in the present work, the time required to degrade the substrate by pre-exposed and non-preexposed cultures was compared. The biomass was divided into two sets for each isomer. To only one set, the isomers (separately) were added at a concentration of 0.1mM to obtain exposed cells. The reaction was stopped at 50% decrease in substrate concentration. The culture was harvested and cells washed. The non-preexposed cells and pre-exposed cells were incubated at room temperature on the shaker, and with ONP, MNP and PNP separately in both sets of cells at an initial concentration of 0.1mM. The change in substrate concentration was monitored spectrophotometrically at their respective wave lengths (Section 2.2.2.2).

The culture was incubated for 48h. Pre-exposed cells could degrade ONP within 24h **(Fig 3.13A)** whereas non-preexposed cells showed a reduction of only 51% at 48h. MNP pre-exposed cells brought about complete degradation of substrate within 24-48h with only 7.5% reduction at 48h in **(Fig 3.13B)** medium inoculated with non-preexposed cells. Interestingly non-preexposed cells were able to bring around 52% reduction in PNP concentration within 24h, though 100% degradation was observed by pre-exposed cells within the same period **(Fig.3.13C)**.

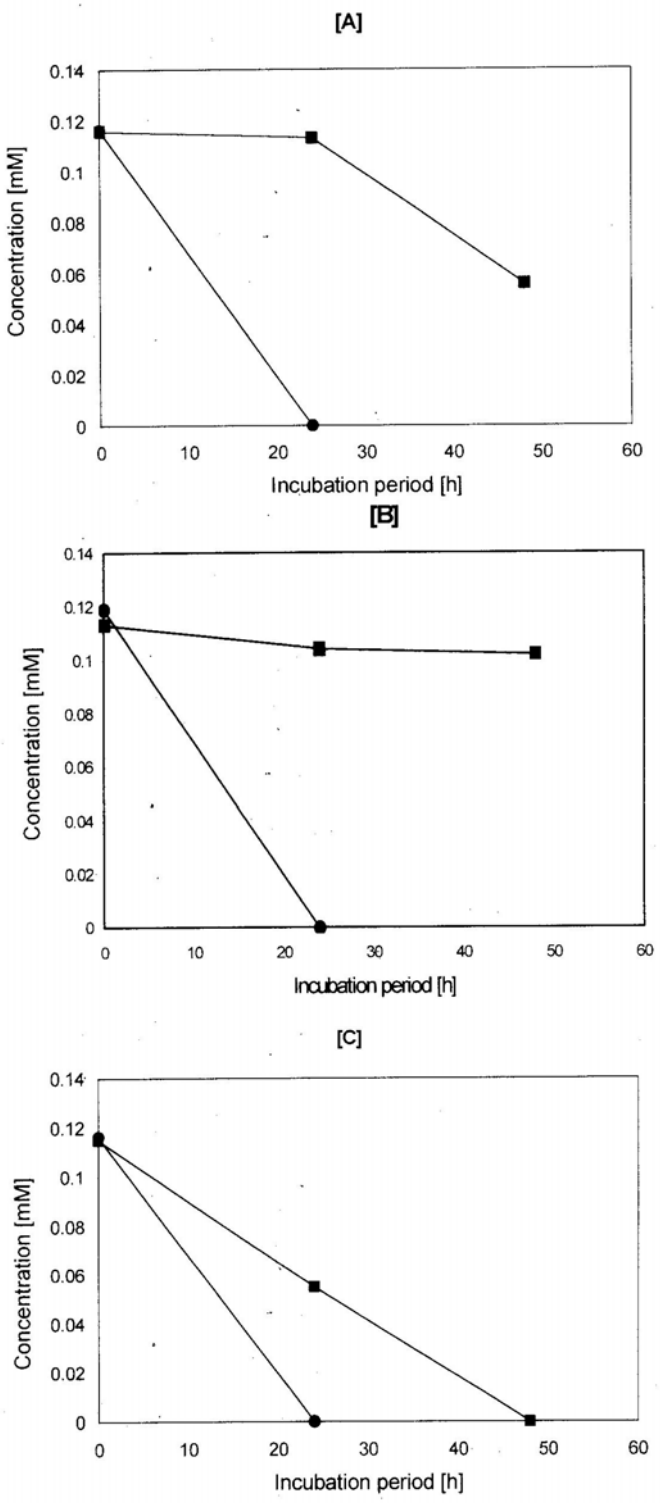


Fig. 3.13 Effect of induction and time course of degradation of 0.1 mM [A] ONP [B] MNP & [C] PNP] by pre-exposed [-● -] and non- exposed [- ■ -] cells of the nitrophenol degrading consortium. Experimental details are described in Section 2.2.2.2.

Inducible degradation of ONP and MNP was also reported by Zeyer and Kearney (1984). It proved that induction was a favourable process for rapid biodegradation of complex organic compounds though the induction periods varied for each of the isomers (MNP> ONP> PNP). The initial lag of 24h shown by non pre-exposed cells in initiating degradation was probably being used by the cells for induction. The absence of growth in both sets of flasks of all the isomers during test period may be attributed to presence of toxins or lack of an adequate supply of essential nutrients which may delay the increase in the numbers of the degrading organisms. A comparatively low concentration of 0.1mM of the substrate was chosen for induction to avoid the toxicity of the substrate at high concentration.

Grover (1967) observed that the acclimation period for degradation, in soil, of herbicide Picloram increased as its concentration increased. Similar observations were also made by Schenzle et al. (1997); Hanne et al. (1993); Wiggins and Alexander (1988). Tseng and Yang (1995) used an activated carbon biofilm acclimated with ONP, MNP and PNP for 2.5yrs in the anaerobic treatment of synthetic wastewater and found para-nitrophenol to be most toxic of all the mononitrophenols.

3.2.8 Utilization of mononitrophenol isomers as nitrogen and carbon sources

In order to confirm the utilization of ONP, MNP and PNP as the sole source of nitrogen, carbon and energy by the consortium used in the present degradation studies, flasks containing basal mineral medium with and without an additional nitrogen in the form of NH_4NO_3 along with the isomers (individually) were incubated at room temperature on a shaker. Growth was monitored spectrophotometrically by measurement of turbidity at 600nm. Because of the toxicity of the substrate, an ideal concentration of 0.3mM was chosen for the test.

As shown in **Fig 3.14B**, the utilization of ONP co-related with an increase in cell density up to 60h of incubation. Similar results were obtained in medium supplied with MNP and PNP. The cell growth observed in the above experiment carried out with nitrophenol isomers with and without NH_4NO_3 showed only a marginal difference in

biomass, the medium containing NH_4NO_3 showed slightly higher biomass than the medium without it. This showed that nitrophenol isomers could be used as C and N sources.

Degradation of individual isomers also showed the same behaviour. Medium inoculated with the consortium with or without NH_4NO_3 showed similar rates of degradation **(Fig.3.14A).** The only exception was PNP which showed gradual degradation in the absence of an external N-source in form of NH_4NO_3 whereas degradation was rapid in medium in the presence of NH_4NO_3.

In all the cases a lag period of 8-12h was observed before any apparent growth. Bruhn et al. (1987) have shown utilization of nitro-aromatic compounds as nitrogen sources by bacteria. Similar observations were made by Schenzle et al. (1997) when they used MNP as a sole source of nitrogen, carbon and energy for a bacterium *Ralstonia eutropha* JMP134.

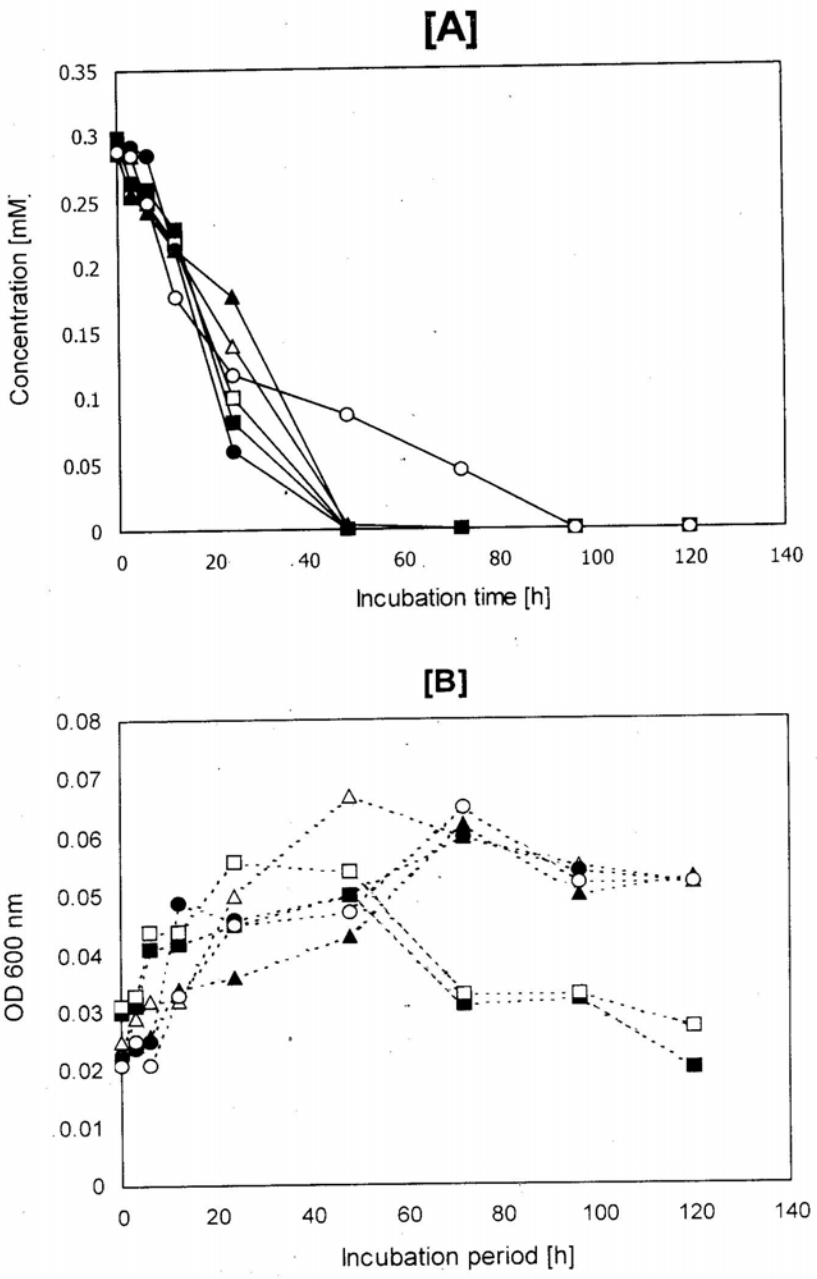


Fig.3.14 Degradation of 0.3mM mononitrophenol isomers in nitrogen [n] and nitrogen free medium.
[A] Biomass estimation [A] Residual substrate analysis. Experimental details are described in Section 2.2.1, 2.2.2.2. -■- ONP[n], -□- ONP, -▲- MNP[n], -Δ- MNP, -●- PNP[n], -o- PNP

3.2.9 Degradation of varying concentrations of the three isomers of mononitrophenol

The ability of the consortium to degrade various concentrations of the three isomers of nitrophenol (ONP, MNP and PNP) separately, was tested. The inoculum, for this purpose was prepared by incremental feeding of the respective isomer, at a rate of 0.07mM/day for several days till sufficient biomass was obtained. This method of inoculum preparation also helped the culture get acclimated to the substrate. Cells were added to each flask to obtain an initial OD of 0.1 to 0.25 at 600nm.

Among the different concentration of (0.1-2mM) ONP tested the consortium was able to degrade up to 1.5mM **(Fig.3.15A)**. A sudden fall in the concentration by 24h was observed in all cases except in medium containing 1.7mM ONP. This was followed by a lag, the duration of which depended on the concentration of the substrate. The lag period ranged from 24h (0.2mM) to 192h (1.5mM). A corresponding lag in the growth was also observed **(Fig.3.15B)**. 25% of 1mM substrate was degraded by 24h followed by no apparent change for a period of 96h. Complete disappearance was observed only after 192h. It took around 264h for the consortium to completely degrade 1.5mM of ONP. A concentration of 1.7mM of ONP proved to be toxic to the cells as indicated by the drop in optical density at 600nm.

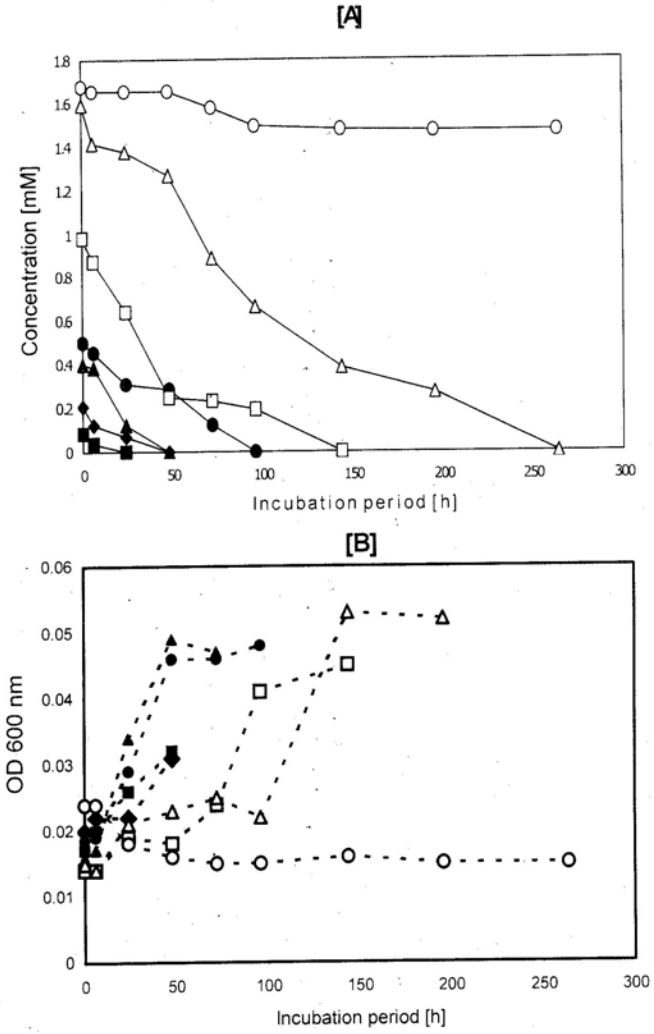


Fig. 3.15 Degradation pattern of subtoxic to toxic concentrations of ONP by the nitrophenol degrading consortium .
[A].Residual substrate analysis. [B]. Biomass estimation.
Experimental details are described in Section 2.2.1& 2.2.2.2
-■- 0.1mM, -◆- 0.2mM, -▲- 0.4 mM, -●- 0.5mM, -□-1mM, -Δ- 1.6mM, -o- 1.7mM.

Concentrations ranging from 0.25mM to 2.0mM of MNP were chosen to be degraded by the consortium already well induced by MNP. Complete degradation of the concentration (0.25mM-1.5mM) was observed by 48-160h accompanied by growth **(Fig.3.16A**and **Fig.3.16B).** The initial lag period was longer in case of medium supplied with MNP ranging from 24-96h depending upon the concentration. At 2mM, only a partial disappearance of the compound was observed as in ONP but in case of medium with MNP, the contents of the flask turned brown after 48h which deepened further on prolonged incubation. This may probably be due to the oxidation of MNP to its quinonic form.

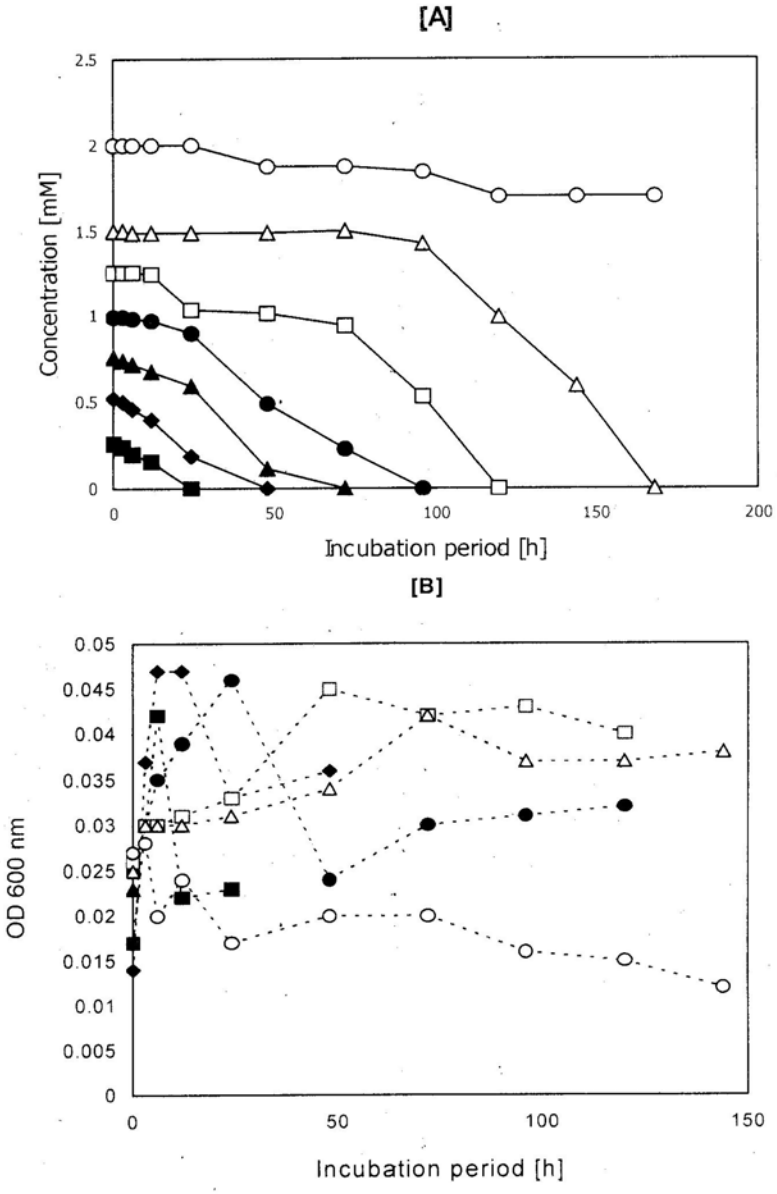


Fig. 3.16 Degradation pattern of subtoxic to toxic concentrations of MNP by the nitrophenol degrading consortium .
[A] Residual substrate analysis [B] Biomass estimation
Experimental details are described in Section 2.2.1 & 2.2.2.2
-■- 0.3mM, -◆- 0.5mM, -▲- 0.75mM, -●- 1.0mM,
-□-1.3mM, -Δ- 1.5mM, - o- 2.0mM

Figure 3.17A shows the degradation of different concentrations of PNP by the consortium (0.25-3mM). The degradation of 0.75mM was complete by 24h, 1mM by 48 hr, 1.25mM and 1.5mM by 144 hr. Corresponding growth was observed at concentrations up to 1.5mM but no growth was observed at higher concentration **(Fig.3.17B)**. Only partial degradation was observed for higher concentrations and was highest in case of MNP. Degradation of 1.5mM of MNP took 168h whereas the same concentration of PNP was degraded by 144h. Degradation of ONP at the same concentration was much slower taking 264h. In other words, the degradability of the three isomers at toxic concentrations was in the order PNP> MNP> ONP.

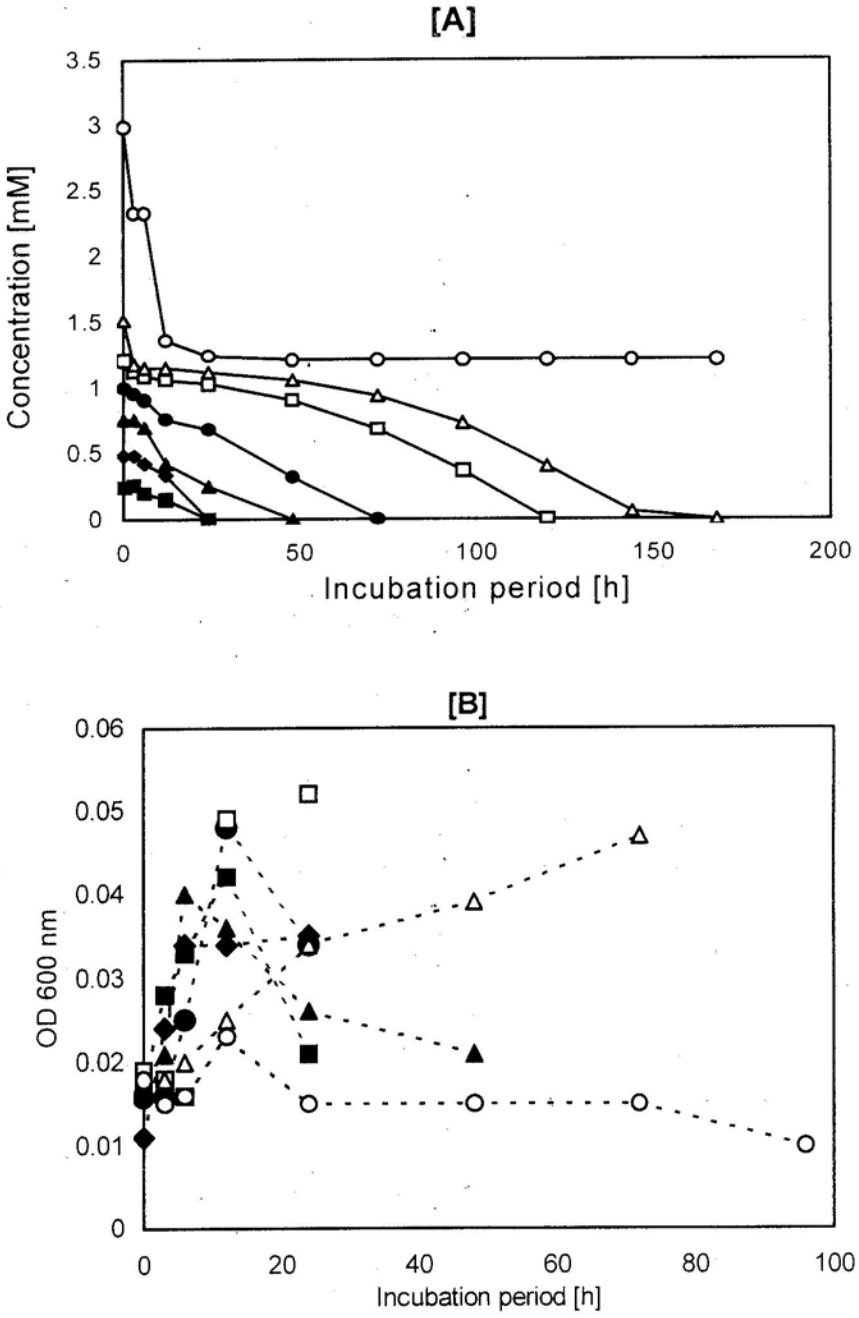


Fig. 3.17 Degradation pattern of subtoxic to toxic concentrations of PNP by the nitrophenol degrading consortium .
[A] Residual substrate analysis. [B]Biomass estimation
Experimental details are described in Section 2.2.1 & 2.2.2
-■- 0.3mM, -◆- 0.5mM, -▲- 0.75mM, -●- 1.0mM,
-□- 1.3mM, -Δ- 1.5mM, - o- 2.0mM

3.3 DISCUSSION

Mixed cultures have been to shown to be the most effective in degrading target molecules (Pieper et al., 1996). Several studies have reported that natural bacteria readily degraded mononitrophenols in soil. The mixed culture for the present study was obtained by enrichment from soil samples collected from around a phenol utilizing company. The consortium was able to degrade phenol and cresol isomers in addition to mononitrophenols. The consortium was defined and was found to be constituted of eight morphologically different bacterial cultures which were able to degrade all the mononitrophenol isomers separately as well as their mixtures.

Zaidi and Imam (1996) reported the degradation of PNP by six non-indigenous bacteria capable of degrading phenolic compounds in pure culture when inoculated into industrial wastewater. Simpson and Evans (1953) isolated strains of microbes from sewage that could use either ONP or PNP but not both, nitrite was released and the organisms were induced respectively to form catechol and hydroquinone. The consortium used in the present study could use

ONP and PNP and released nitrite in the process whereas ammonia was released during MNP degradation indicating the presence of organisms following oxidative and/ or reductive mechanisms which is beneficial especially in bioremediation of sites contaminated with heterogenous wastes. While ONP and PNP was degraded by an oxidative pathway, MNP was found to be degraded by a reductive pathway. Peres et al. (1998) reported the degradation of nitrobenzene and aniline by a mixed bacterial culture that showed two antagonistic activities - reductive and oxidative. The consortium's ability to degrade nitrophenol isomers and cresol isomers is in confirmation of the report that organisms capable of degrading one aromatic compound may degrade several other related compounds (Haigler et al.,1992; Babu et al., 1995). Zaidi and Imam (1996) reported degradation of PNP by six non-indigenous bacteria capable of degrading phenolic compounds in pure cultures when inoculated into industrial wastewater samples. The consortium used in the present study was defined and found to have eight bacterial cultures (Section 2.3.2). A mixed bacterial culture was capable of degrading methyl parathion to its final oxidation products, CO_2 and H_2O, but only in the

presence of a second carbon source such as glucose or yeast extract (Ou Li-Tse and Anil Sharma, 1989) whereas no additional sources was added to the culture medium employed in the present degradation studies.

All the degradation studies were conducted in phosphate medium as the addition of phosphorus has been shown to enhance bacterial decomposition of PNP (Jones and Alexander, 1988b; Ramadan et al., 1990). Nitrogen in the form of ammonium nitrate was used only in the initial enrichment of the consortium and degradation studies were conducted in ammonium free medium to enable the mixed cultures to use mononitrophenol isomers as the sole source of nitrogen and it was observed that the absence of an additional N-source hardly influenced the rate of degradation. Similar effect was observed by Ray et al. (1999) during the aerobic degradation of PNP.

The mineralization of many organic compounds in different environments is preceded by an acclimation period which is the length of time between the addition of a compound and the onset of its detectable mineralization (Jones and Alexander, 1988a). All the experiments during the present study were conducted after 24-48h acclimation time. Pre-exposed cells degraded all the isomers comparatively faster than non-exposed cells. A minimum lag of 6-12h for the various concentrations used was observed. The lag period observed in case of degradation of isomers by non-preexposed cells may probably be due to the time required for induction of necessary enzymes. Similar observations were done by Hanne et al. (1993) who found that non-exposed cells of *Arthrobacter aurescens* TW17 and *Nocardia* sp. TW2 took 1-2h longer to degrade PNP. Substrate concentration of 0.05-0.1mM was used for induction before initiation of mineralization studies. Higher the concentration of substrate used during degradation, longer was the induction time required. Wiggins (1988) observed a reduction in the number of active organisms and an increase in the acclimation period with increasing substrate levels. Death of cells was not observed even at high concentrations (1.0-5.0mM) indicating the tolerance of the consortium to changing concentrations or the toxicity of any intermediary metabolites. No accumulation of either

ammonia or nitrite occurred in the culture medium but a decline in their concentration with progress of time indicated their utilization by the consortium.

CHAPTER 4

DEGRADATION STUDIES BY INDIVIDUAL CULTURES OF A NITROPHENOL DEGRADING CONSORTIUM

4.1 INTRODUCTION

A consortium could exhibit important, perhaps, novel mechanisms of biodegradation. Many isolated communities clearly show that relationships between the populations confer beneficial effects, which make the association more successful than any of the individual populations alone (Slater, 1978, 1979, 1979). Not ruling out the importance of microbial consortium studies, resolution of the consortium to its constituent cultures becomes necessary. The primary aim is to obtain an easily growing microorganism in sufficient quantities to determine the biodegradation mechanisms by elucidating the catabolic sequence, purifying and identifying intermediate metabolites, assaying and characterizing the enzymes involved and determining the factors controlling the regulations of pathway expression.

Resolution of the individual cultures from the consortium was carried out as described in Section 2.3.2. About eight cultures were identified to be present in the consortium and the individual cultures were isolated and characterized (Section 2.3.3) as *Bacillus licheniformis* (SNP-1), *Xanthomonas maltophila* (SNP-2), *Serratia liquefaciens* (SNP-3), *Psuedomonas putida* (SNP-4), *Pseudomonas* sp. (SNP-5), *Pseudomonas alcaligenes* (SNP-6), *Pseudomonas* sp. (SNP-7) and *Sarcina maxima* (SNP-8). The cultures were maintained on M5 agar plates and also in suspension (M5 medium) supplied with the nitrophenol isomers. Each culture was tested for its degradation ability with a low concentration of ONP and PNP (0.1mM). All the eight cultures were individually grown under the same culture conditions as with the consortium. The cultures were incubated for a period of 72h. The extent of degradation of the mononitrophenol isomers by the individual isolates and the single culture are

shown in terms of percentage with respect to their initial absorbance values corresponding to 0.1mM concentration at 272nm.

4.2 RESULTS

4.2.1 Degradation of o-Nitrophenol and p-Nitrophenol by individual cultures

The substrate reduction was monitored spectrophotometrically. Cultures SNP-1, 2, 3, 4, 7 and 8 brought about 50% reduction in substrate concentration by 72h in case of ONP **(Figs. 4.1 and 4.2**, **Fig 4.3 and 4.4**, **Fig 4.7 and 4.8**) whereas cultures SNP-5 and SNP-6 showed only 16% and 26.1% degradation **(Figs. 4.5 and 4.6)**. Cultures when grown in presence of PNP showed varying degrees of degradation. The percentage of degradation for SNP-1, 2, 3, 4, 5, 6, 7, 9 were 9.9, 36, 30, 33, 17, 13, 15 and 30% respectively. Degradation of MNP could not be studied.

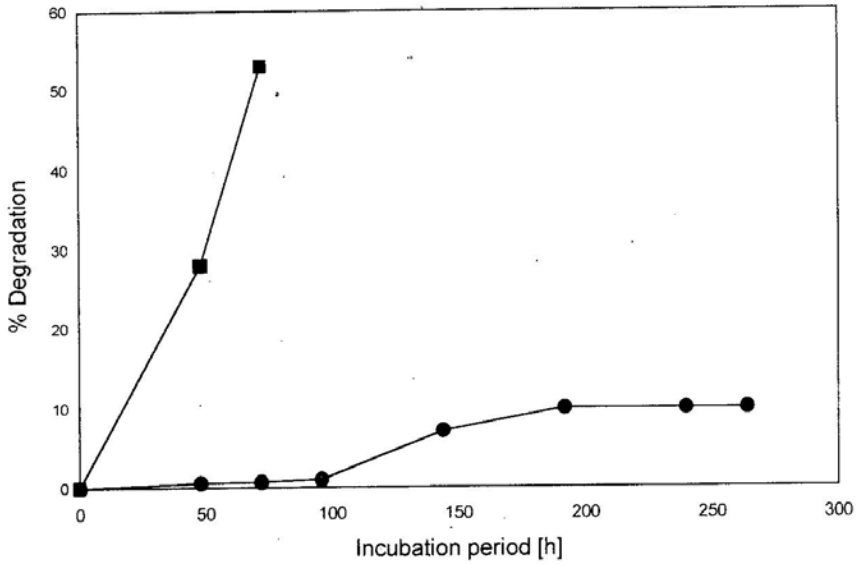


Fig. 4.1 Percent removal of substrate [0.1mM] by *Bacillus licheniformis* (SNP-1).
Experimental details are given in Section 2.2.2.2.
-■-ONP, -●- PNP

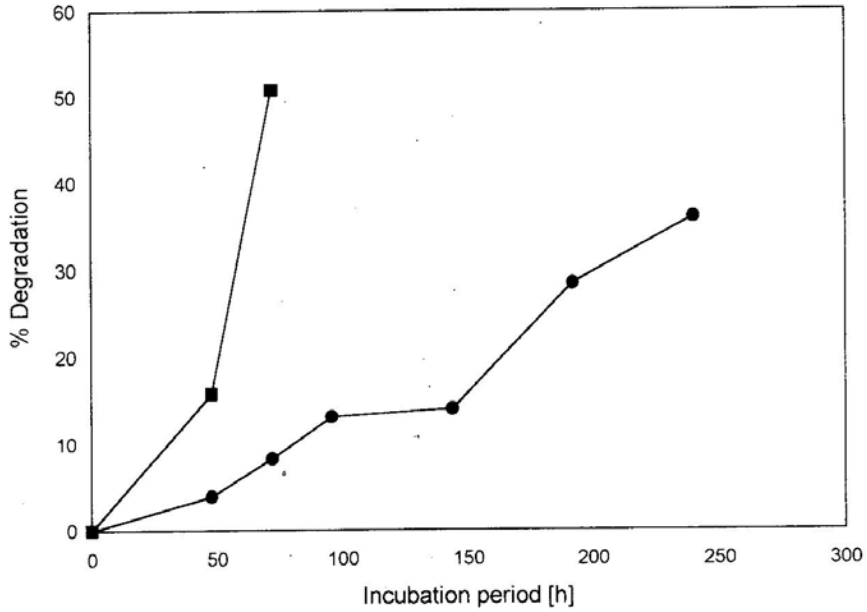


Fig. 4.2 Percent removal of substrate [0.1mM] by *Xanthomonas maltophila* (SNP-2).
Experimental details are given in Section 2.2.2.2.
-■-ONP, -●- PNP

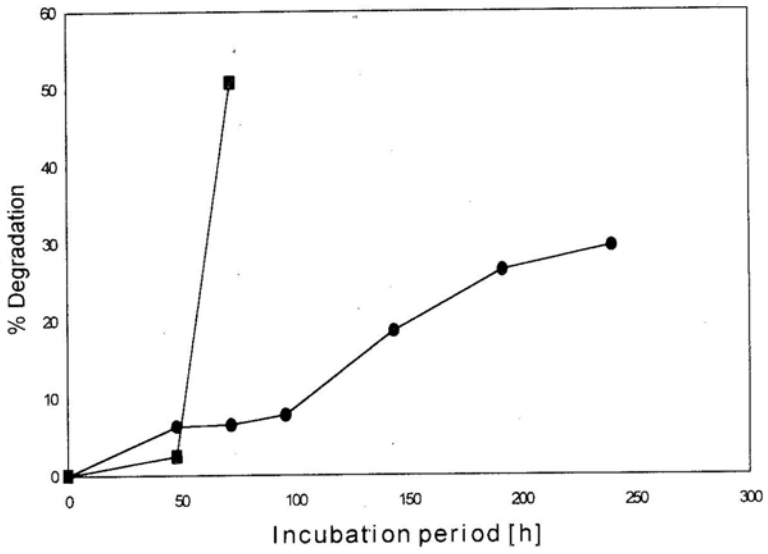


Fig. 4.3 Percent removal of substrate [0.1mM] by *Serratia liquefaciens* (SNP-3)
Experimental details are given in Section 2.2.2.2.
-■-ONP, -●- PNP

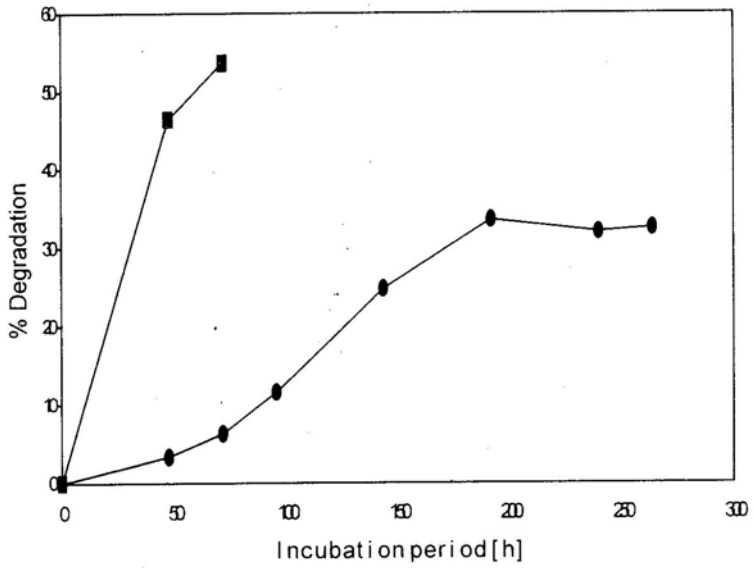


Fig. 4. 4 Percent removal of substrate [0.1mM] by *Pseudomonas putida* (SNP-4).
Experimental details are given in Section 2.2.2.2.
-■-ONP, -●- PNP

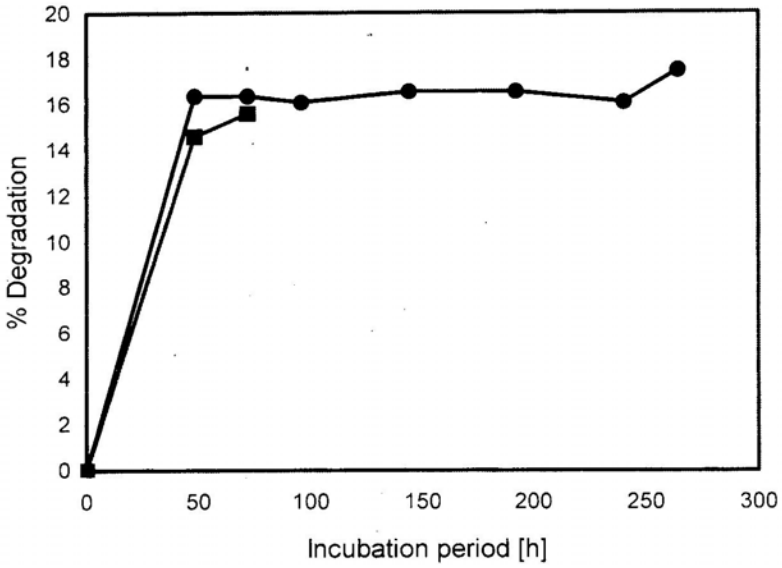


Fig. 4.5 . Percent removal of substrate [0.1mM] by *Pseudomonas* sp. (SNP-5). Experimental details are described in Section 2.2.2.2.
-■-ONP, -●- PNP

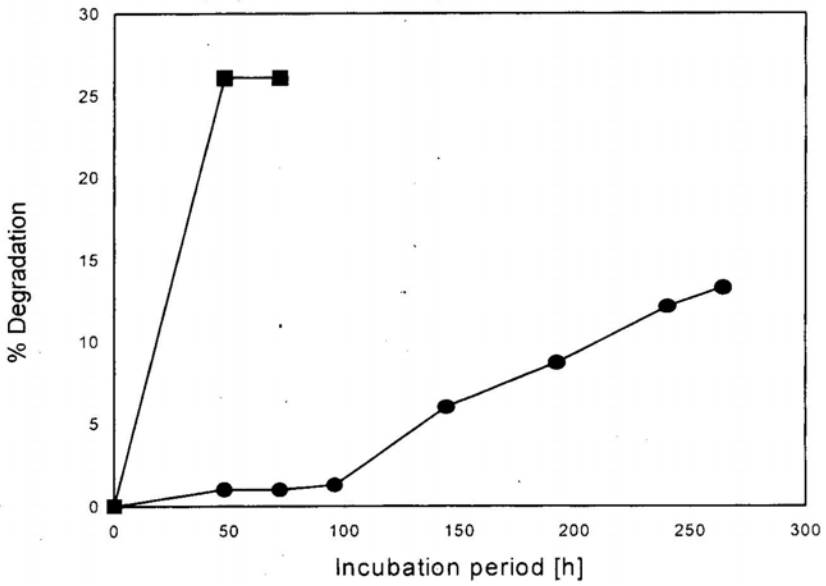


Fig. 4. 6 Percent removal of substrate[0.1mM] by *Pseudomonas alcaligenes* (SNP-6). Experimental details are given in Section 2.2.2.2. -■-ONP, -●- PNP

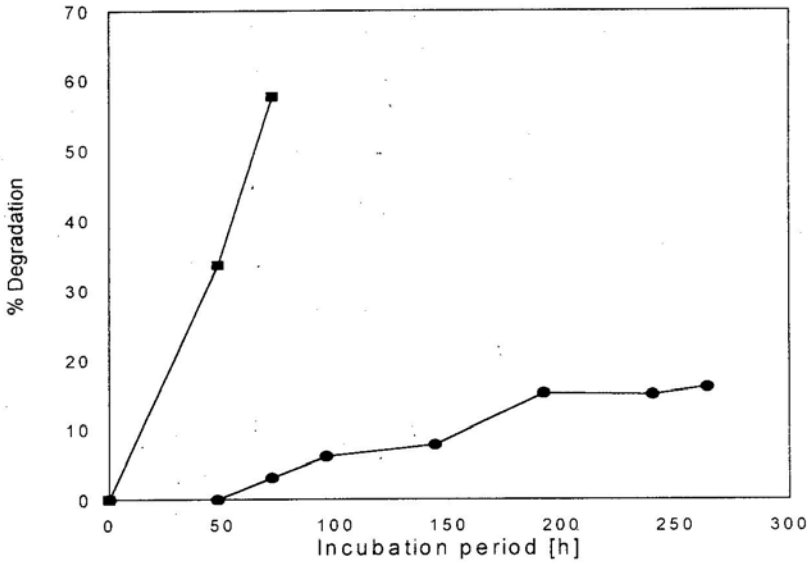


Fig. 4. 7 Percent removal of substrate [0 .1mM] by *Psuedomonas* sp.(SNP-7). Experimental details are described in Section 2.2.2.2. -■-ONP, -●- PNP

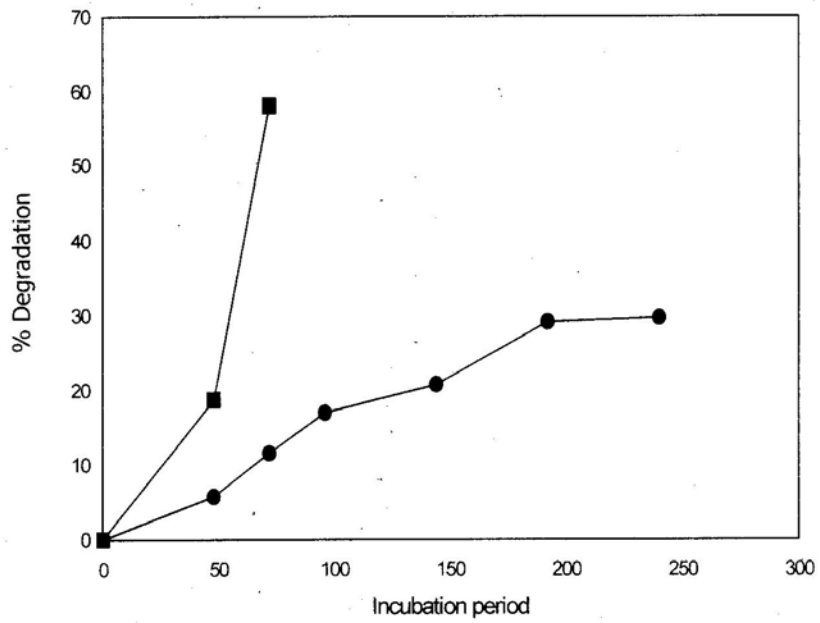


Fig. 4.8 Percent removal of substrate [0.1mM] by *Sarcina maxima* (SNP-8). Experimental details are described in Section 2.2.2.2. -■- ONP, -●- PNP

4.2.2 Simultaneous degradation of mononitrophenol isomers by individual cultures

An attempt to study the simultaneous degradation of the isomers by individual culture was carried out. The individual cultures were incubated with 0.1mM of a mixture (each isomer ~ 0.033mM) of the nitrophenol isomers, and degradation was measured based on the changes in absorbance at a wavelength of 272nm.

The percentage of degradation by following the changes in absorbance at 272nm were as follows. Cultures SNP-2, SNP-5 and SNP-6 showed 100%, 97% and 89% degradation within 48, 72 and 96 hours **(Fig.4.10**, **Fig 4.13** and **Fig 4.14**) respectively. They were followed by SNP-3, which showed 50% degradation **(Fig 4.11)**. SNP-8 and SNP-1, showed 49% and 39% **(Fig 4.16** and **Fig 4.9)** respectively while SNP-4 and SNP-7 could bring about only 21% and 19% **(Fig 4.12 and Fig 4.15)** respectively. The 100% increase in absorbancies at 272nm in medium inoculated with culture SNP-2, SNP-5 and SNP-6 could also imply that these cultures were capable of completely breaking down MNP to its metabolites. In culture medium that showed 50% increase in absorbance (SNP-3, SNP-5 and SNP-8) there was a sudden decrease within the next 24h (Fig 4.11, 4.13, 4.16). The increase in the absorbance could be due to the accumulation of a metabolite that absorbs at 272nm.The cultures when inoculated into ONP and PNP separately showed a decrease in absorbance at 412nm and an increase at 401nm respectively. But when the same cultures were inoculated into media containing a mixture of ONP, MNP and PNP, an alternate increase and decrease in absorbance values were observed.

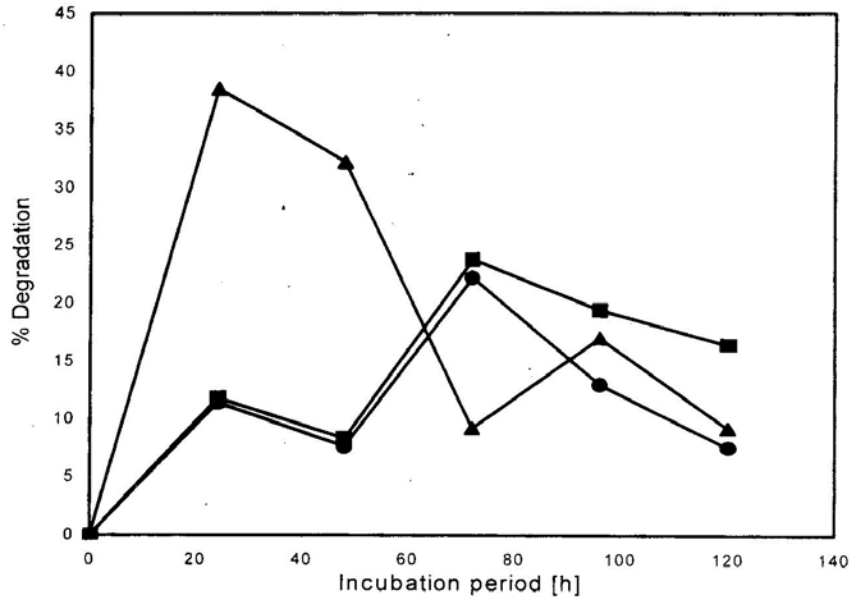


Fig. 4.9 Degradation pattern of a mixture of mononitrophenol isomers [0.1mM] by SNP-1. Experimental details are described in Section 22.2.2: -■- ONP, -▲- MNP, -●- PNP

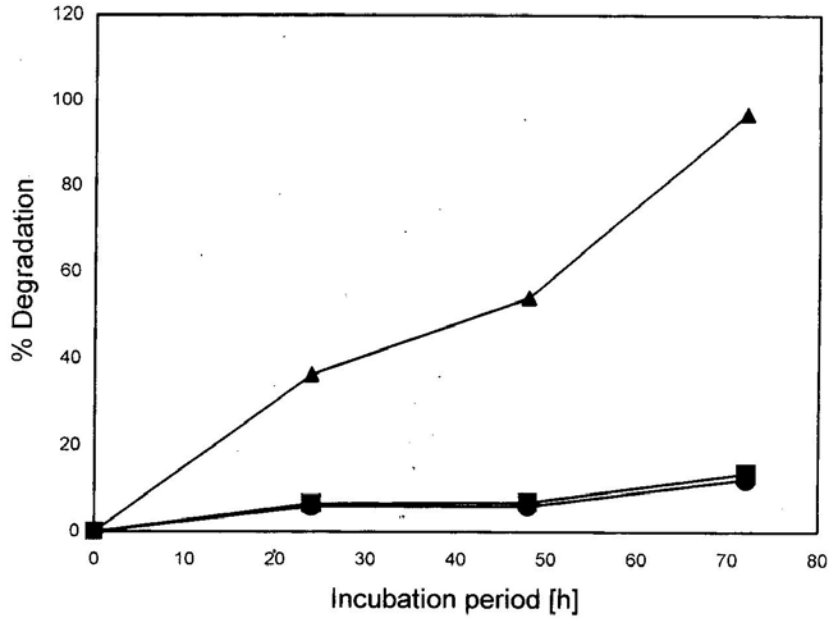


Fig.4.10 Percent removal of mononitrophenols isomers from a mixture [0.1mM] by SNP-2. Experimental details are described in Section 2.2.2.2. -■- ONP, -▲- MNP, -●- PNP

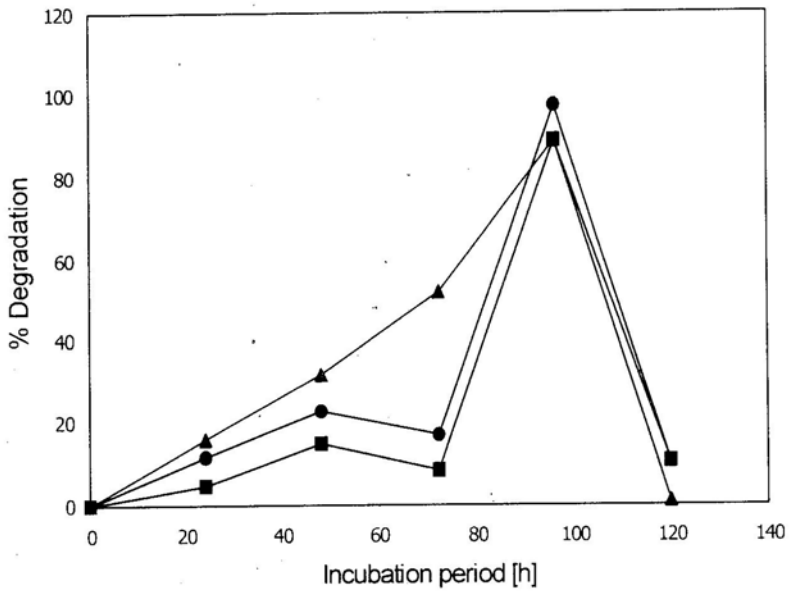


Fig.4.13 Degradation pattern of a mixture of mononitrophenol isomers [0.1mM] by SNP-5. Experimental details are described in Section 2.2.2.2. -■-ONP, -▲- MNP, -●-PNP

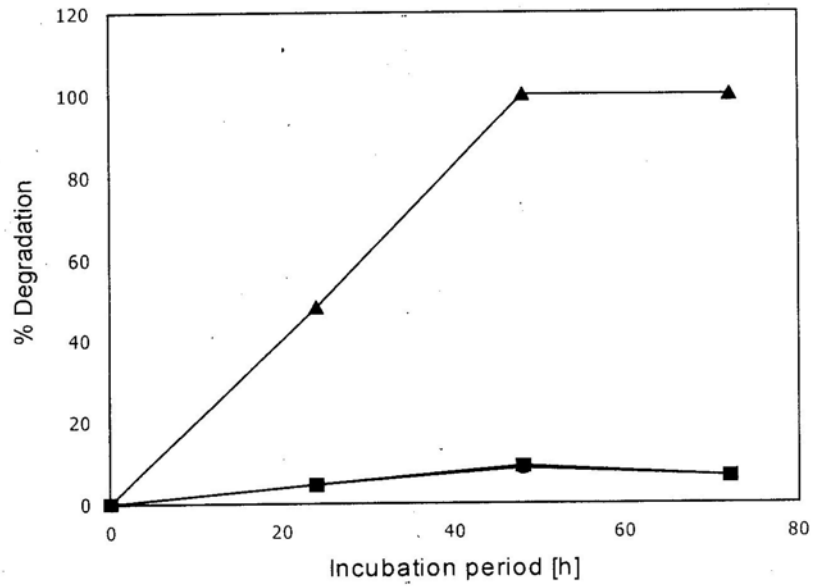


Fig.4.14 Degradation pattern of a mixture of mononitrophenol isomers [0.1mM] by SNP-6. Experimental details are desscribed in Section 2.2.2.2. -■-ONP, -▲- MNP, -●-PNP

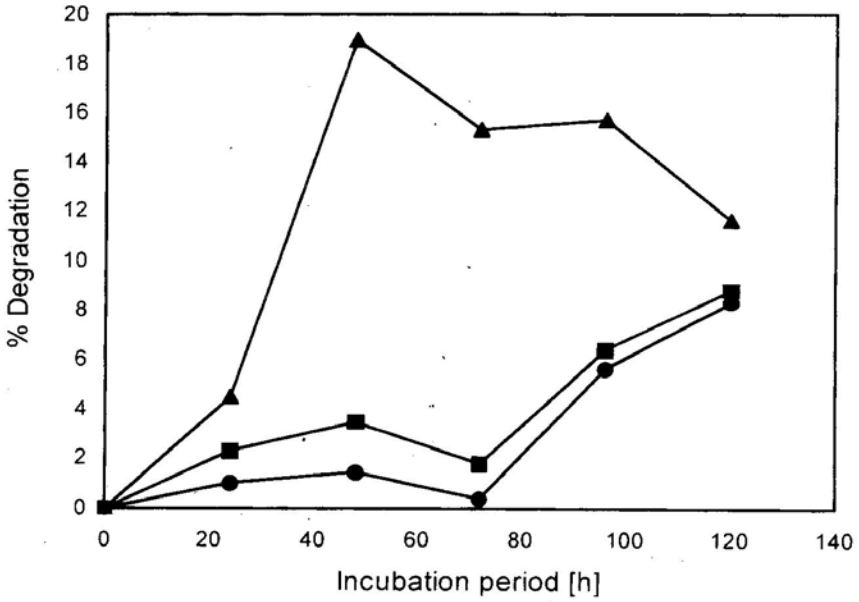


Fig.4.15 Degradation pattern of a mixture of mononitrophenol isomers [0.1mM] by SNP-7. Experimental details are desscribed in Section 2.2.2.2. -■-ONP, -▲- MNP, -●-PNP

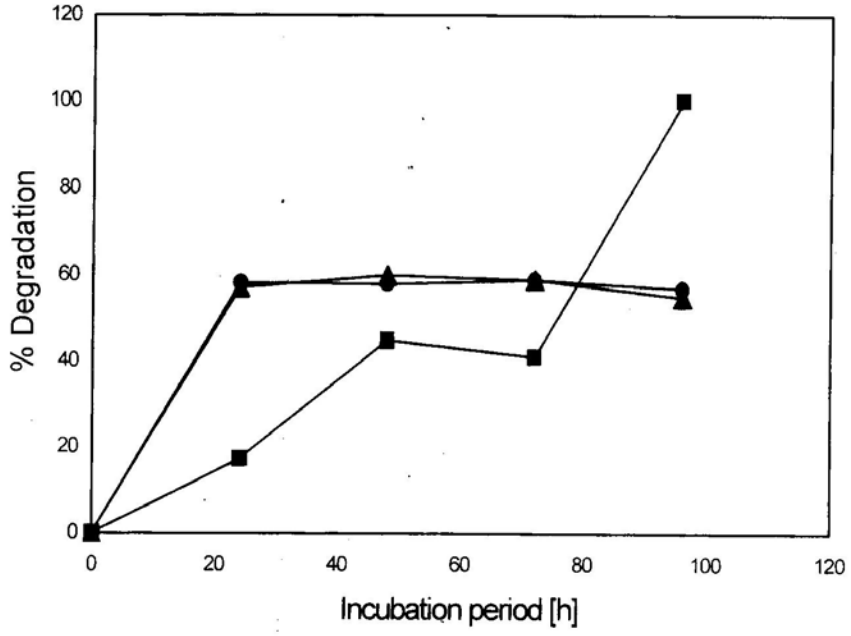


Fig.4.16 Degradation pattern of a mixture of mononitrophenol isomers [0.1mM] by SNP-8. Experimental details are desscribed in Section 2.2.2.2. -■-ONP, -▲- MNP, -●-PNP

4.2.2 Catabolic potential of a single culture, Sarcina maxima [SNP-8]

The catabolic potential of a single culture SNP-8 identified as *Sarcina maxima* was tested as no reports exist regarding mononitrophenol degradation studies using this culture.

The culture was pre -exposed to ONP, MNP and PNP separately before initiating the experiments by incremental feeding of substrate from a concentration of 0.05mM-0.1mM to facilitate induction. The culture medium was as described in Section 2.1.1. The nitrophenol isomers were supplied as a single source of nitrogen, carbon and energy at a concentration 0.1mM. All experiments were conducted separately and in triplicates. Induction process was carried out for 24-48 hrs until 50% reduction in substrate concentration was observed. For all the experiments with SNP-8, volume of cells were equated to an optical density of 0.5 at 600nm which showed CFU equivalent of $2.3x10^{13}$ /ml.

*Sarcina maxima (*SNP-8) brought a 67.6% reduction in ONP concentration by 24h that is from an initial concentration 0.071mM to 0.023 mM **(Fig.4.17)**. PNP degradation (60%) was observed between 48 to 96h bringing down the concentration from 0.191mM to 0.075mM (Fig.4.17). Degradation of MNP (50%) was observed at 60h after a long lag (Fig.4.17). As observed with the consortium studies, a stoichiometric release of nitrite was not observed in culture filtrate of ONP or PNP grown cells but extremely low nitrite was released from ONP induced medium **(Fig.4.18)**. But stoichiometric amount of ammonia was released from cells in M5 medium supplied with only MNP, as shown in **Fig 4.19**. HPLC data also showed 35.45%, 70% and 74% decrease in substrate concentration of ONP, MNP and PNP respectively **(Fig.4.20).**

Degradation of ONP and PNP accompanied by the release of nitrite and the release of ammonia from MNP proves that this organism shows both oxidative and reductive mechanisms which is advantageous especially in bioremediation of industrial effluents which usually are a heterogeneous mixture.

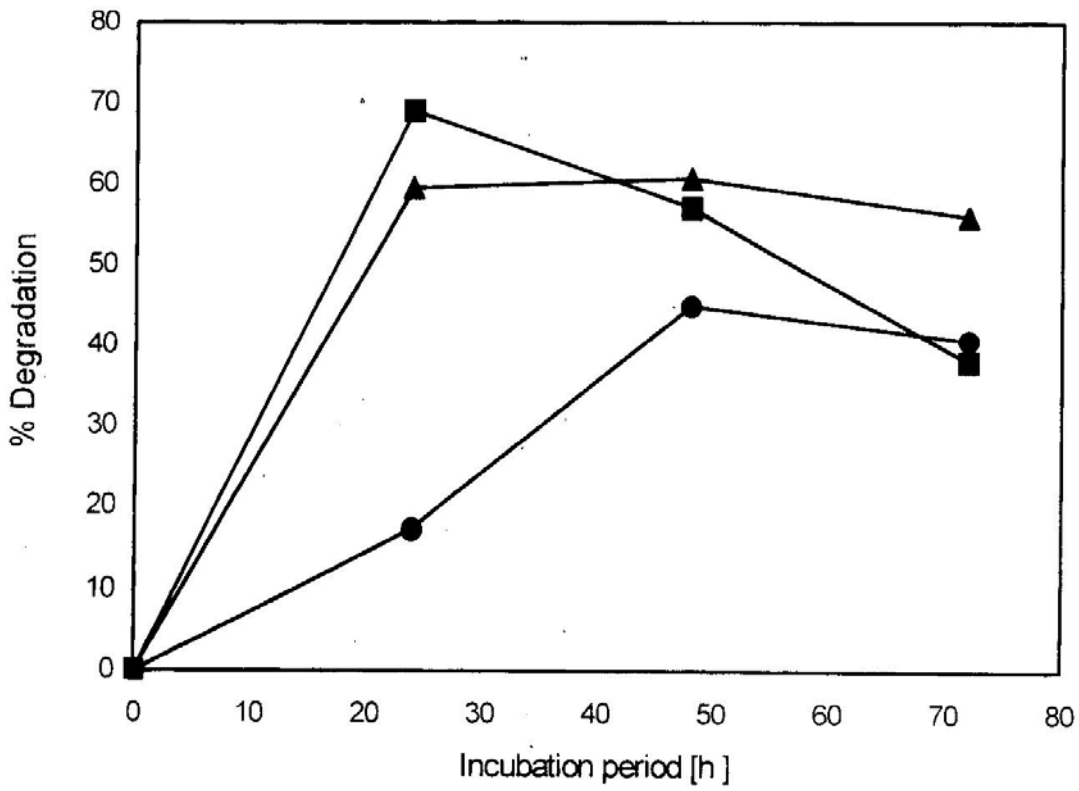


Fig. 4.17 Degradation of 0.1mM substrates by well induced cells of *Sarcina maxima* (SNP-8).
Experimental details are given in Section 2.2.2.2.
-■- ONP, -▲- MNP, -●- PNP

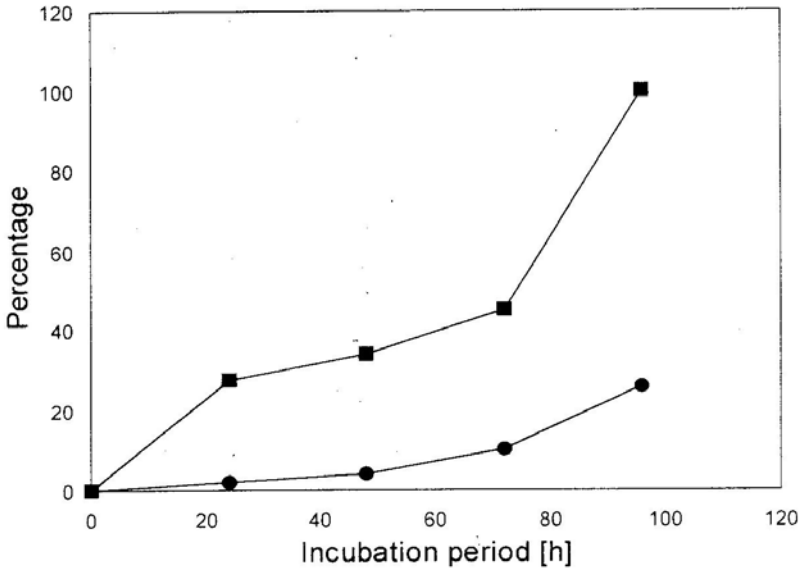


Fig. 4.18 Percentage of ONP (0.1mM) degradation and nitrite release by Sarcina maxima. Experimental details are given in Section 2.2.2.2 and 2.2.2.5
-■ - ONP, -●- nitrite

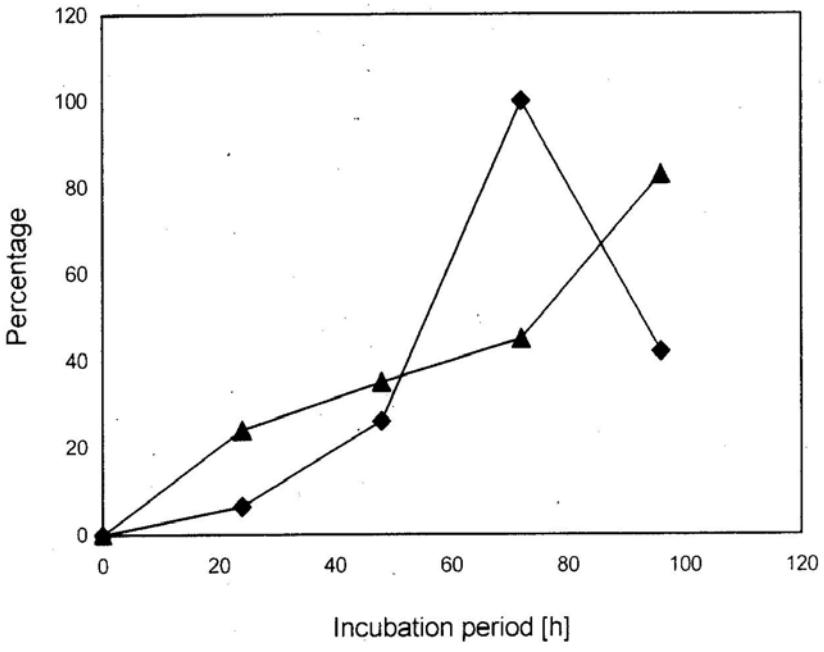


Fig. 4.19 Percentage of MNP (0.1mM) degradation and ammonia release by Sarcina maxima. Experimental details are given in Section 2.2.2.2 and 2.2.2.4.
-▲- MNP, -◆- Ammonia

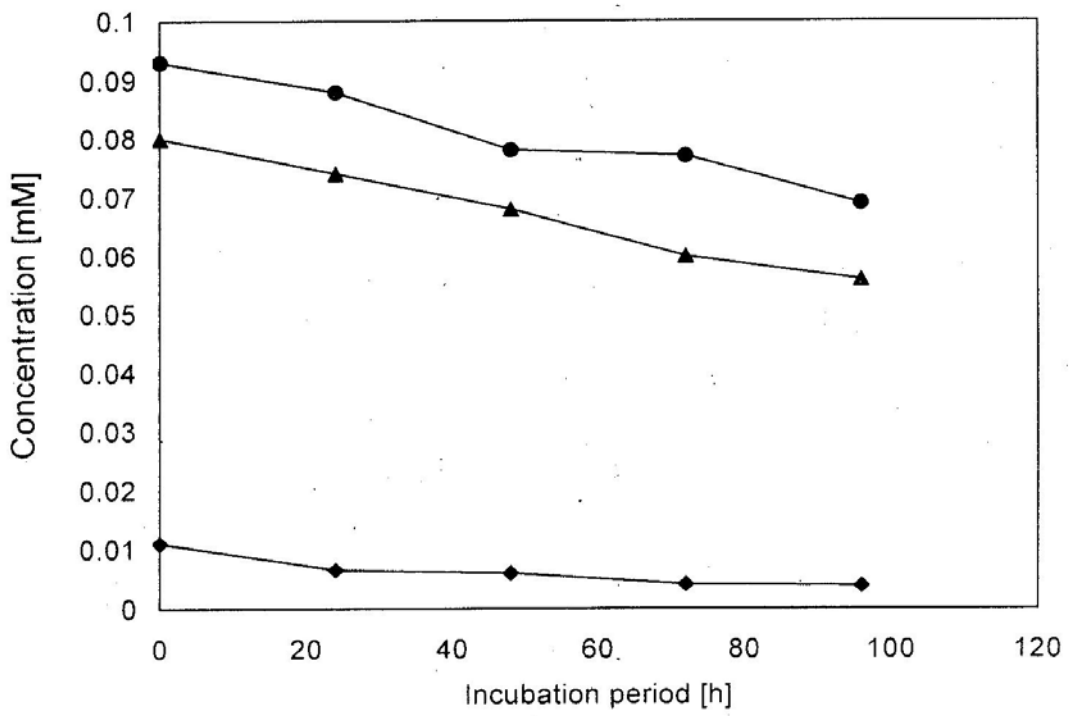


Fig. 4.20 Degradation of [0.1mM] individual mononitrophenols by SNP- 8. Residual analysis by HPLC. Experimental details are described in Section 2.2.2.2. -■- ONP, -▲- MNP, -●- PNP

4.2.3 Degradation of varying concentrations of ONP, MNP and PNP

Bacterial isolate, *Sarcina maxima* (SNP-8) was subjected to exposure of different concentrations of the substrate to check the toxicity level. The culture was increased in biomass by growing in 1% succinate and 0.5% yeast extract and later harvested by centrifugation and washed. The washed cells were induced with 0.05mM-0.1mM substrate for 48h and then washed and reinoculated into fresh M5 medium with 0.2mM and 0.5mM substrate separately as sole carbon, nitrogen and energy (CFU/ml - $2.3x10^{13}$).

As observed earlier with 0.1mM concentration of substrate, culture *Sarcina maxima* degraded ONP faster compared to other substrates. The percentage of degradation of 0.2mM substrate in ONP, MNP and PNP supplied medium were 72%, 20% and 7% respectively though **(Fig. 4.21B)** 0.22mM was not toxic as viable colonies were obtained on plating the culture upon nutrient agar. The culture was unable to use other substrates, except ONP as growth substrate. Since the induction period was longer, 50% reduction in substrate concentration (0.062mM to 0.028mM) was seen at 12h and 72% reduction by 18h, which remained constant up to 36h-42h. A 20% degradation of MNP was observed after a lag of about 6 days. PNP degradation was poorest of all, which showed only a 7% change in absorbance (Fig. 4.21B). Culture filtrate from ONP grown cells was spectrophotometrically analyzed for ammonia and nitrite. No ammonia was detected but there was a sudden surge of nitrite (though not stoichiometric) from 48h onwards that is only after 72% of substrate was degraded **(Fig. 4.21A)** proving the activity of the initial hydroxylating enzyme and the formation of probably catechol with nitrite production.

Cells induced for a comparitively shorter period when inoculated into medium containing 0.2mM of a mixture of the substrate brought about 34% degradation by 144h. But cells induced with 0.1mM of mixture of nitrophenol isomers for around 72h were capable of showing 49% degradation of 0.5mM mixture by 96h, further confirming the need for induction. **(Fig 4.21C).** There was no increase in optical density at 600nm as the concentration of 0.5mM was high.

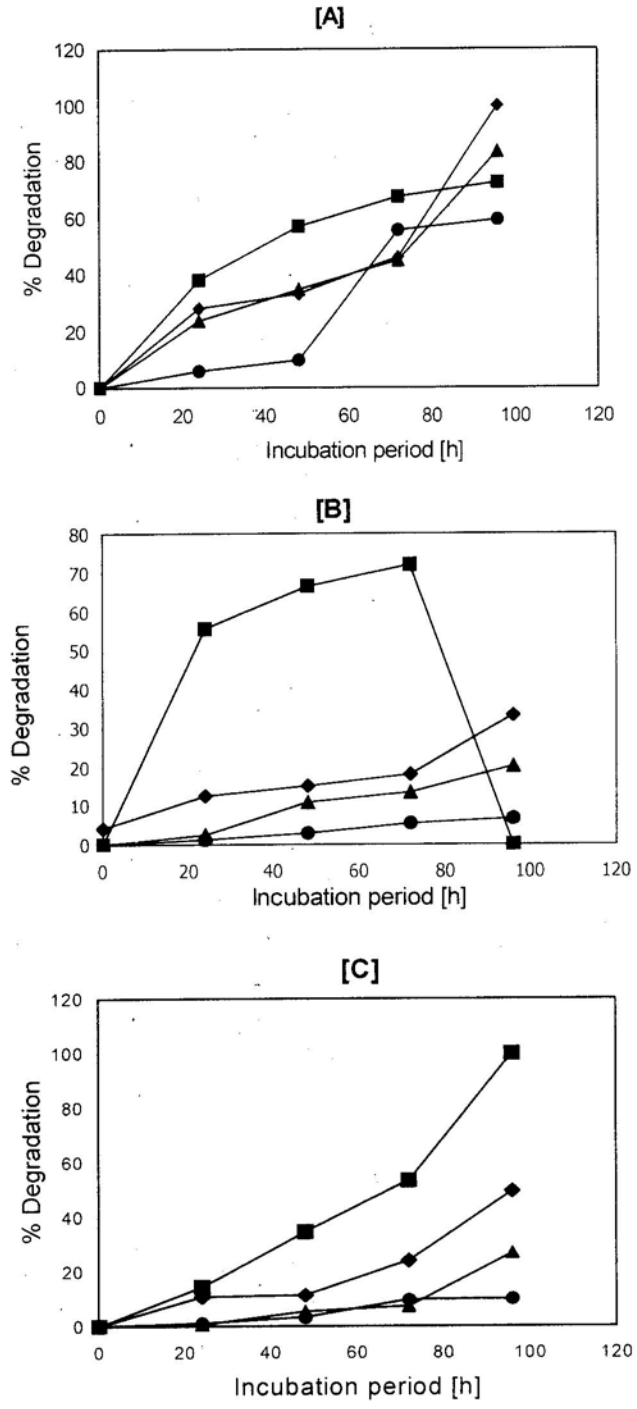


Fig. 4.21 Percent degradation of [A]0.1 mM [B] 0.2 [C] 0.5 mM individual mononitrophenols and their mixture by the SNP- 8. Experimental details are described in Section 2.2.2.2.
-■- ONP, -▲- MNP, -●- PNP, -◆- Mix NPs

4.2.4 Simultaneous degradation of a mixture of nitrophenols by bacterial isolate, SNP-8

Yeast extract and succinate grown cells of *Sarcina maxima* were inoculated into M5 medium for induction with a mixture of the nitrophenol isomers. The culture was induced from 24-72h and cells at CFU - $2.3x10^{11}$/ml were used for degradation studies. An attempt for the first time was made to study the degradative capability of the culture in M5 medium supplied with a mixture of ONP, MNP and PNP as sole sources of carbon, nitrogen and energy. The changes in the absorbancies were monitored spectrophotometrically at regular intervals of time. Three different concentrations (0.1mM, 0.3mM and 0.5mM) of the mixture of the isomers containing equal amounts of ONP, MNP and PNP were considered.

The culture filtrate obtained from M5 medium with 0.1mM of a mixture of the isomers was analyzed at three different wavelengths 272nm, 401nm and 412nm for MNP, PNP and ONP. A gradual increase in absorbance at 272nm was observed from 18h which increased to a 100% at 72h and maintained up to 120h indicating the accumulation of a metabolite which absorbed at 272nm . But at 401nm and 412nm, a 57% and 58% reduction in substrate concentration was observed respectively. Since the concentration of each substrate was low in the mixture (0.033mM), there was no detection of nitrite. No ammonia production was observed. HPLC analysis showed **(Fig. 4.22)** more than 50% degradation of all the substrates by 96h.

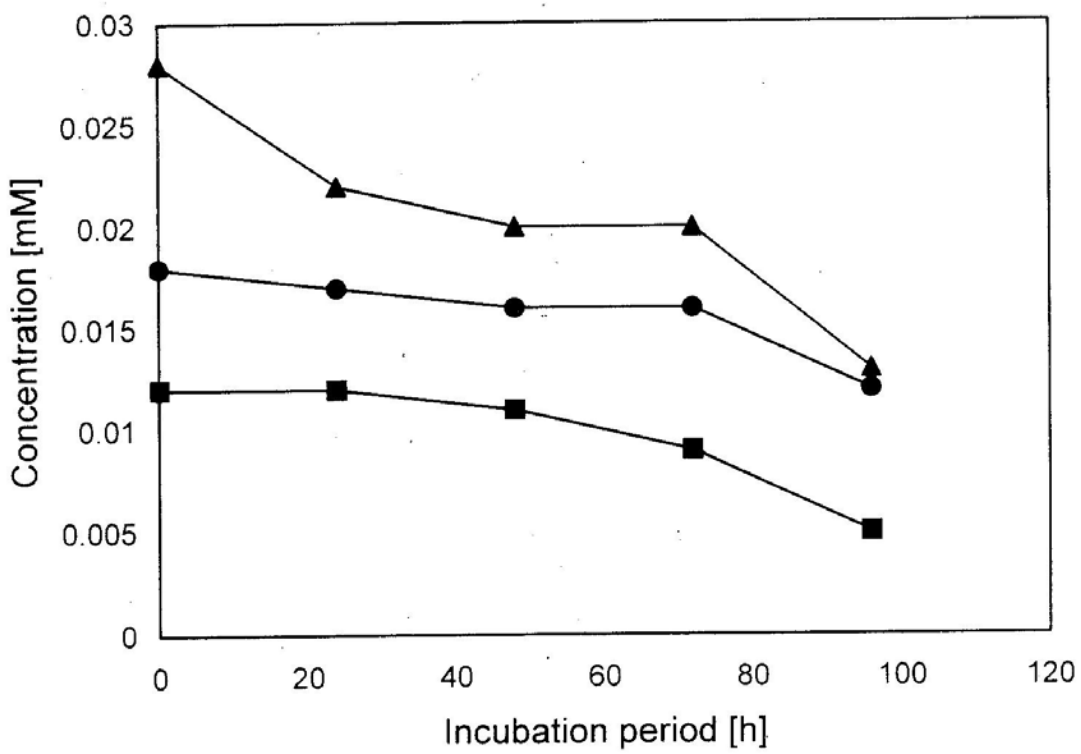


Fig. 4.22 Degradation of a mixture of mononitrophenol isomers by SNP- 8. Residual substrate analysis by HPLC . Experimental details are given in Section 2.2.2.2

-■-ONP, -▲- MNP, -●- PNP

4.3 DISCUSSION

Bacteria can oxidatively remove nitro groups from ONP (Suzuki et al., 1991, Zeyer and Kearney, 1984) MNP (Zeyer and Kearney, 1984) and PNP (Raymond and Alexander, 1971; Spain and Gibson, 1991; Spain et al., 1979). *Psuedomonads* are one of the common bacterial genus among the soil microorganisms which have been successfully employed in degradation studies. Five of the bacterial cultures which were isolated from the consortium were found to belong to the genus *Pseudomonas*. The cultures were able to degrade ONP, MNP and PNP individually though to varying degrees. It was interesting to note that though all the cultures were able to degrade ONP individually,

the duration was longer when the individual isolates were used as a consortium to degrade the same isomer. This indicated that behavioural changes play an important role in degradation by mixed culture systems. The ONP, MNP and PNP degrading bacterial culture for the present study were isolated from phenol contaminated soil and were identified as *Bacillus licheniformis* (SNP-1), *Xanthomonas maltophila* (SNP-2), *Serratia liquefaciens* (SNP-3), *P. putida* (SNP-4), *Psuedomonas* sp. (SNP-5), *P. alcaligenes* (SNP-6), *Psuedomonas* sp. (SNP-7) and *Sarcina maxima (*SNP-8). Isolate *Bacillus licheniformis* (SNP-1) was a very poor PNP degrader. The other three cultures capable of degrading 50% ONP and 30% PNP were *Xanthomonas maltophila, Serratia liquefaciens* and
P. putida,

Xanthomonas sp. and *Pseudomonas* sp. isolated from a pesticide disposal site degraded Parathion in two stages- hydrolysis of parathion to PNP and later further degradation of PNP (Tchelet et al., 1993). Zablotowicz et al. (1999) isolated a *Sphingomonas* sp. strain UG30 from a PCP contaminated soil which could mineralize both PNP and PCP. The cultures used in the present study were successful in degrading even a mixture of the mononitrophenol isomers. Few reports exist regarding the biodegradation of two isomers of nitrophenols. Of the eight cultures, SNP-2, SNP-5 and SNP- 6 (Fig. 4.2, 4.5 and 4.6) brought a 100%, 97%, and 89% degradation of all the three isomers in a mixture whereas SNP-3, SNP-8 and SNP-1 (a very poor PNP degrader) showed 50%, 49% and 39% reduction of MNP concentration respectively (Fig. 4.3, 4.8 and 4.1). Only 21% and 19% of MNP degradation was observed in case of
SNP-4 (Fig. 4.4) and SNP-7 (Fig. 4.7).

Present degradation studies either by the consortium or the individual cultures were conducted in medium wherein the mononitrophenol isomers were supplied as sole sources of nitrogen, carbon and energy. *Pseudomonas putida* B2 was able to grow on ONP as the sole source of carbon and nitrogen and converted it to nitrite and catechol which was further degraded (Zeyer et. al., 1986). A strain of *Pseudomonas cepacia*,

isolated from industrial sludge was capable of using either PNP, 2,4-dinitrophenol (DNP), 4,6 dinitrocresol or 2-nitrobenzoic acid as its sole N-source, with succinic acid as primary C-source (Mohammed et al., 1992).

Detailed degradation studies were conducted using an unreported culture *Sarcina maxima* (SNP-8) which was able to use ONP, MNP and PNP separately as well as a mixture. *Pseudomonas putida* 2NP8 (Zhao and Ward, 2000) utilized ONP with production of nitrite and MNP with formation of ammonia. In the present study, accumulation of nitrite during degradation of ONP/PNP indicated an initial monooxygenase attack and accumulation of ammonia during MNP degradation suggested an initial reductive reaction. This also suggests the presence of both oxidative and reductive mechanisms in a single organism. Though the consortium took a longer time to degrade ONP, SNP- 8 degraded ONP more rapidly than MNP and PNP. Pre-exposed cells of *Sarcina maxima* degraded 49% of 0.5mM ONP whereas non-exposed cells brought about only 34% reduction of 0.2mM substrate further proving the need for induction (Section 3.2.6). Induced and washed cells degraded more than 50% of around 0.1mM, 0.3mM and 0.5mM of ONP, MNP and PNP accompanied by varying lag periods before initiation of mineralization. The lag period increased with increasing substrate concentration. Previously Grover (1967) had observed that the acclimation period for degradation of the herbicide picloram in soil increased as its concentration increased. Lag phases ranging from 2-42 days have been reported followed by faster PNP degradation (Heitkamp et al., 1990). Though there was absence of growth at high concentration, toxicity was not observed in the present study due to the high biomass ($2.3x10^{13}$ CFU/ml) involved in degradation studies. The cells on completion of degradation of concentration as high as 0.5mM were found to be viable thus ruling out toxicity. Similar observation were made by Uberoi and Bhattacharya (1997) in methanogenic cultures when supplied with ONP and PNP.

Sarcina maxima could be subjected to longer induction periods to successfully degrade higher concentration of mononitrophenols and other related aromatic compounds as evident by their degradation by the consortium. Another possibility in

increasing the degradative ability of all the culture of the consortium is to check the efficiency in the presence of an additional, easily utilizable carbon source or any inorganic nutrients. For example, addition of phosphorus has been known to enhance the degradation rate and also minimize the acclimation period in lake water (Jones and Alexander, 1998b). However this aspect was not tested in the present work. Thus this study has clearly brought out the efficiency of individual cultures in degrading mononitrophenol isomers individually or collectively.

CHAPTER 5

MICROBIAL ENZYMES IN THE DEGRADATION OF MONONITROPHENOLS BY THE CONSORTIUM

5.1 INTRODUCTION

Aerobic microorganisms use diverse biochemical reactions to initiate the degradation of nitroaromatic compounds. Reactions that attack the nitro substituents can be grouped into two general categories: either oxidative or reductive (Reiger and Knackmuss, 1995). With mono- or di-nitro substituted aromatic compounds the preferred route for their initial degradation is hydroxylation carried out by mono- or dioxygenases, a class of enzymes which catalyze the fixing of oxygen into organic molecules. The end result of the oxygenase reactions are hydroxyl or carbonyl compounds which are normally more water soluble than the parent compound and can be broken down by a large number of microorganisms.

Oxidative reactions of nitroaromatic compounds normally result in replacement of the nitro group by an -OH group, with nitrite release. Reductive reactions, predominantly in compounds with greater than two nitro substituents on the aromatic ring involve the reduction of the nitro-substituent first to nitroso (NO), and then to hydroxylamino (NHOH) followed by an amino (NH_2) derivative prior to further processing with the release of ammonium ion. Monooxygenases incorporate one atom of the oxygen molecule into the organic substrate while the second oxygen atom goes to form water. Dioxygenases incorporate both atoms of the oxygen molecule into the substrates, except some such as naphthalene dioxygenase which carry out dioxygenation as well as monooxygenation. These enzymes participate in the oxidative metabolism of a wide variety of chemicals of pharmaceutical, agricultural and environmental significance.

5.1.1 Dioxygenases

Two types of dioxygenases have been reported -

1. Ring - hydroxylating dioxygenases
2. Ring - cleaving dioxygenases

The present study centered mostly around ring cleaving dioxygenases. These enzymes have iron as the prosthetic group (in the active site) which participate in catalysis, hence also referred to as pyrocatechase. These enzymes do not have a co-factor requirement in contrast to ring-hydroxylating dioxygenases. Dihydroxyl substituted aromatic compounds (e.g. catechol) or hydroxyl group in para position relative to the other hydroxyl group (gentisate) can be cleaved by ring cleaving dioxygenases. Cleavage can occur in the bond joining carbons containing two hydroxyl groups (ortho or intradiol) or in the bond proximal to the carbon containing one of the two hydroxyl groups (meta or extradiol).

Ortho cleavage

This type of intradiol cleavage occurs between two adjacent hydroxyl groups, for example, catechol 1,2-dioxygenase also named as Pyrocatechase I. This enzyme contains a ferric ion (Fe^{3+}) as the prosthetic group. It oxidizes catechol to cis, cis-muconic acid (Eltis et al., 1993; Gibson, 1993) which is further metabolized to form a major intermediate, β-ketoadipate, which is fed into the TCA cycle for further metabolism. Ortho cleaving enzymes have narrow substrate specificity.

Meta cleavage

This type of extradiol cleavage occurs proximal to one of the two-carbon containing hydroxyl groups, example, catechol 2,3-dioxygenase. These enzymes contain a ferrous ion (Fe^{2+}) as the prosthetic group. Extradiol cleavage of catechol produces 2-hydroxy muconicsemialdehyde. This product undergoes further metabolism to form the key intermediate 2-keto-4-hydroxyvalerate, which is eventually metabolized to form acetoacetate, pyruvate and acetaldehyde.

5.1.2 Monooxygenases

Monooxygenases are more abundant than dioxygenases, more complex in action and can catalyse several different types of oxygen insertion reactions. This class of enzymes are also called mixed function oxidases since one atom of oxygen becomes reduced to water i.e. two reductants (substrate) are needed. Since one of the main substrate becomes hydroxylated, they are also called hydroxylases. Bacterial monooxygenases can also hydroxylate aromatic compounds. Monohydroxylation of the aromatic ring is usually carried out by monooxygenases, for example, in case of phenol, these enzymes incorporate an additional hydroxyl group to the existing hydroxyl to form catechol. Some of the monooxygenases are phenol hydroxylase, nitrophenol oxygenase etc.

5.2 RESULTS

5.2.1 Culture conditions

Initially the biomass of the nitrophenol degrading consortium was increased by growing it in M5 medium supplemented with 1% sodium succinate and 0.5% yeast extract. The culture was washed thoroughly with M5 medium and the cells were induced with the three mononitrophenol isomers (o-, m- and p-Nitrophenol) separately at a concentration of 0.8mM (Fig.5.1). Degradation of the substrate was spectrophotometrically monitored regularly at wavelengths- 412nm, 272nm and 401nm for ONP, MNP and PNP respectively (Fig. 5.2). Later the cells were harvested, suspended in a small volume of the culture filtrate. The cells were broken by sonication (7X15s). The resultant mixture was centrifuged (12000 rpm) for 30 minutes at 4^{o}C. The supernatant thus obtained was used as a crude extract to assay for enzyme activity.

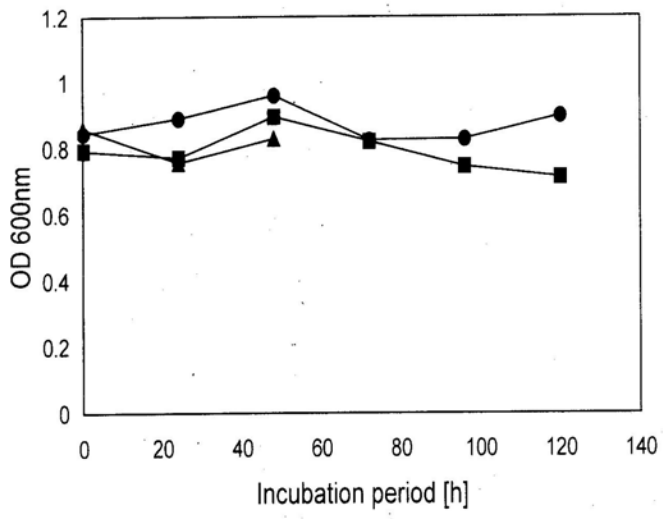


Fig. 5.1 Biomass estimation during degradation of 0.8mM individual mononitrophenol isomers Experimental details are given in Section 2.2.1.

-■- ONP, -▲- MNP, -●- PNP

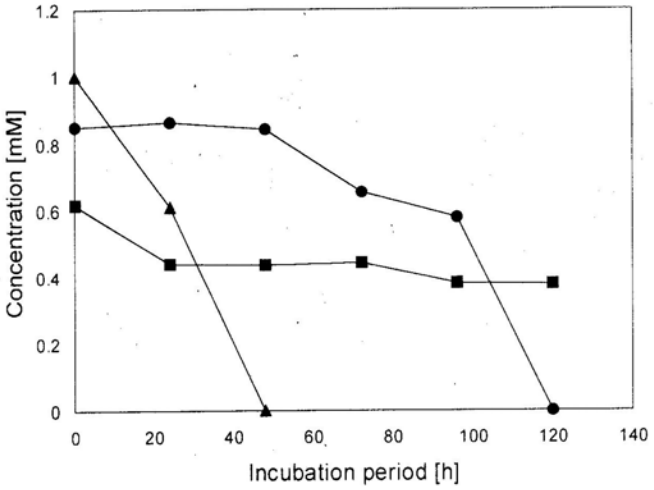


Fig. 5.2. Degradation pattern of 0.8mM of individual mononitrophenol isomers.Residual analysis by HPLC. Experimental details are given in Section 2.2.2.2.

-■-ONP, -▲- MNP, -●- PNP

5.2.1.1 Catechol 1,2-dioxygenase (Pyrocatechase I)

Pyrocatechase I was assayed according to the method of Nakazawa and Nakazawa (1970) spectrophotometrically, based on the rate of formation of cis, cis-muconic acid.

The reaction mixture in a final volume of 3 ml contained 2.89 ml of 0.1mM phosphate buffer at pH 7.5, 0.06 ml of 0.01M catechol and 0.05 ml of the enzyme (crude extract).

The enzyme kinetics was monitored at an optical density of 260nm which registers the formation of cis, cis-muconic acid (ccMuA). The optical density was recorded at 30 seconds interval for 3 minutes at 24°C (Genesys spectrophotometer). The amount of cis, cis-muconic acid was calculated from the difference in extinction co-efficients of the reaction mixture. The difference in the molar extinction co-efficients of catechol and cis, cis-muconic acid equals 16000 at 260nm under the conditions described. One unit of enzyme activity is defined as the amount which catalyzes the formation of 1μmol of cis, cis-muconic acid per minute at 24°C. Specific activity of the enzyme is defined in units as 1μmol of cis, cis-muconic acid formed per minute per mg. protein.

5.2.1.2 Catechol 2-3 -dioxygenase [metapyrocatechase]

Metapyrocatechase was assayed by the method of Nozaki (1970) by measuring the increase in optical density at 375nm caused by the formation of 2-hydroxy muconicsemialdehyde.

The reaction mixture in a final volume of 3ml contained 2.8 ml of 0.5mM phosphate buffer at pH 7.5, 0.1 ml of 0.01M catechol and 0.1 ml crude extract as enzyme.

The assay was carried out in a UV quartz cell. The reaction was initiated by the addition of the enzyme at 24°C. The rate of increase in absorbance at 375nm was followed in a spectrophotometer for 3 minutes. One unit of enzyme is defined as the

amount which oxidizes 1μmol of catechol per minute at 24°C. The molar extinction co-efficient of the product is approximately 4.4×10^4 under the assay conditions.

Crude extracts obtained from ONP induced cells showed pronounced catechol 1,2-dioxgenase activity followed by PNP and MNP induced cells and the activity units were 0.81, 0.25 and 0.15/ml of culture filtrate respectively (Fig. 5.3). Catechol 2,3-dioxygenase activity was observed though minimal (0.0198 AU/ml) in MNP induced crude extract (Fig. 5.4) only.

It was not possible to assay other enzymes involved in the degradation pathway still further. The results indicate that catechol 1,2-diooxygenase and catechol 2,3-dioxygenase were produced by the constituent cultures of the consortium on induction with ONP, MNP and PNP.

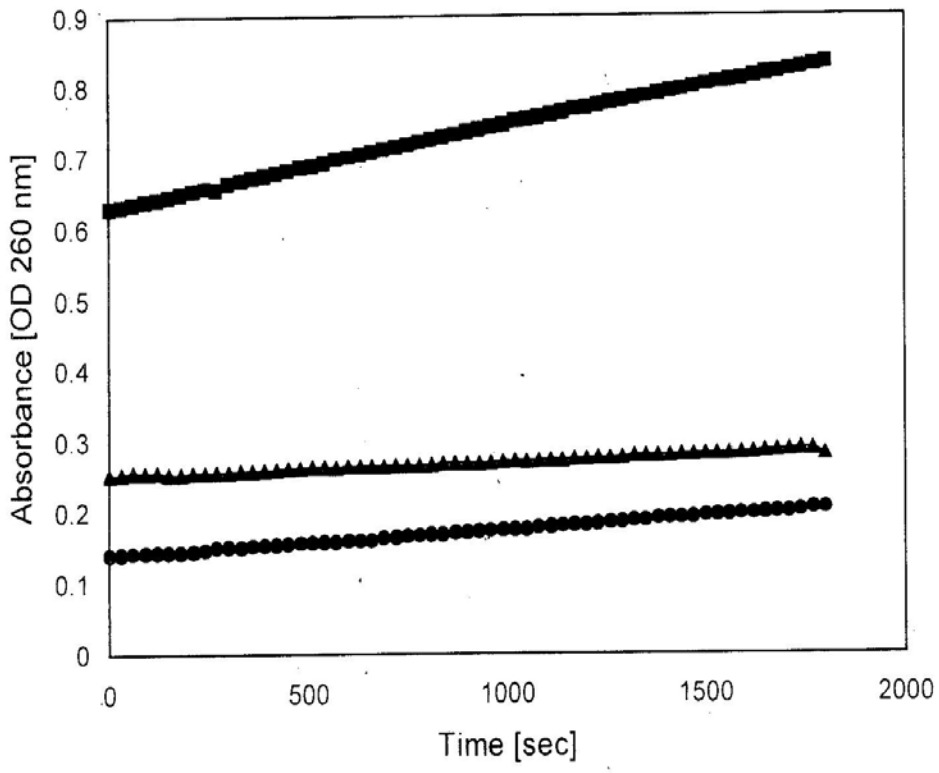


Fig. 5. 3 Catechol-1,2- dioxygenase activity as observed in cell free extracts of the nitrophenol degrading consortium when induced with ONP/ MNP or PNP . Experimental details are described in Section 5.2.1.1
-■- ONP, -▲- MNP, -●- PNP

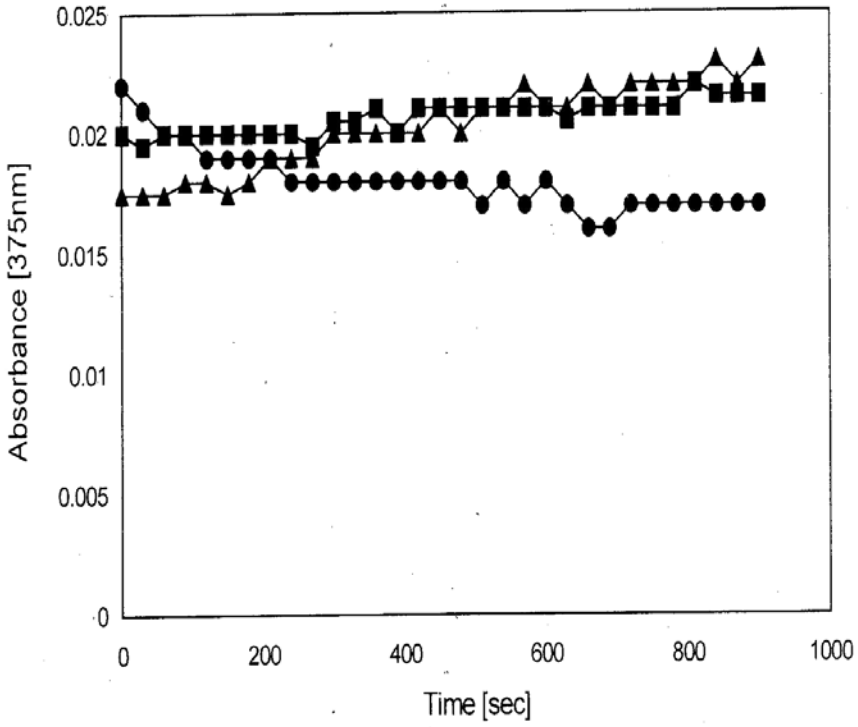


Fig. 5.4. Catechol -2.3 - dioxygenase activity as observed in cell free extracts of the nitrophenol degrading consortium when induced with ONP/ MNP or PNP Experimental details are described in Section 5.2.1.2

-■-ONP, -▲- MNP, -●- PNP

5.3 DISCUSSION

In the present study catechol dioxygenase activity was observed in crude extracts of cells induced with ONP, MNP and PNP. Catechol 1,2-dioxygenase activity was pronounced in cell extracts induced with ONP as the reaction mixture still contained some residual ONP due to its incomplete degradation even up to 120h at which the reaction was stopped for ONP MNP and PNP. Residual substrate analysis of MNP and PNP showed their disappearance by 50h and 96h respectively implying the non-availability of enough catechol for enzyme activity which may have undergone further metabolism. Maximum catechol dioxygenase activity therefore could have been achieved in crude extracts of cells induced with all isomers in the initial hours of degradation. Catechol 1,2-dioxygenase activity was found to be high towards catechol and methylated catechols (Zeyer et al., 1986). *Psuedomonas putida* B2 grown on ONP catabolized ONP to catechol by nitrophenol oxygenase which was subsequently degraded via ortho cleavage (Folsom et al., 1993). A similar nitrophenol activity was observed in the crude extracts used in the present study. An ortho cleavage pathway indicates the involvement of catechol 1,2-dioxygenase which is an ortho cleaving enzyme. Crude extracts of *Rhodobacter capsulatus* B10 and E1F1 converted PNP to 4-nitrocatechol upon the addition of NADPH and showed catechol 1,2-dioxygenase activity similar to the activity observed during the present study but without the requirement of NADPH (Roldon et al., 1997). It is interesting to note that *R. capsulatus* did not metabolize PNP in dark and could tolerate concentrations up to 0.5mM which is in contrast to the behaviour of the mixed culture system used in the present study wherein all the experiments by the consortium as well as the single isolate *Sarcina maxima* were conducted in dark and the consortium showed a concentration tolerance $>$0.5mM.

Catechol 2,3-dioxygenase activity could be observed in crude extracts of MNP induced cells only. Extracts of nitrobenzene (an analogous compound) grown culture JS765 showed high catechol 2,3-dioxygenase activity that was not abolished by heating to 60^{o}C for 10 minutes. The ring cleavage product had an absorbance maximum at

375nm consistent with findings in the present study, which was for γ-hydroxy muconicsemialdehyde.

CHAPTER 6

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPIC STUDIES OF THE MICROBIAL DEGRADATION OF MONONITROPHENOL ISOMERS

6.1 INTRODUCTION

Nuclear magnetic resonance (NMR) is a branch of spectroscopy based on the fact that atomic nuclei oriented by a strong magnetic field absorb electromagnetic radiation at characteristic frequencies. The parameters that can be measured on the resulting spectral lines can be interpreted in terms of molecular structure, conformation, molecular motion and other rate parameters. The usefulness of NMR to the chemist and biologist stems in large measure from the finding that nuclei of the same element in different chemical environments give rise to distinct chemically shifted signals. The fact that proton appears in most organic compounds and in many inorganic ones as well has resulted in a concentration of effort upon this particular nucleus, termed as ^{1}H NMR spectroscopy. Another isotope used in NMR studies is carbon-13 which has an advantage over ^{1}H NMR in that it provides direct observation of molecular backbone of carbon containing functional groups with no attached protons and of carbon reaction sites of interest (Farrar & Becker, 1971). The importance of NMR rests on the fact that it provides a much greater wealth of different clues on question of structure, dynamics and function than other methods especially in solution.

Both proton and carbon-13 nuclear magnetic resonance spectroscopy was employed in the present degradation studies using reaction mixtures of the consortium as well as of a single bacterial culture, Sarcina maxima (SNP-8).

Most reports regarding ONP degradation give proof of an initial ortho cleavage forming catechol, which is converted to cis, cis- muconic acid through a series of steps

following a β-ketoadipate pathway. PNP degradation via production of hydroquinone and p-benzoquinone with further degradation via

β-ketoadipate pathway has been proposed by Chauhan et al. (2000) using *Arthrobacter protophormiae* RKJ100. Kadiyala and Spain (1999) reported the conversion of PNP to 4-nitrocatechol subsequently getting oxidized to 1,2,4-trihydroxybenzene by *Bacillus sphaericus* JS905. Initial studies on MNP degradation metabolites were based on the degradation of a closely related nitroaromatic compound, nitrobenzene. Comparatively, few reports exist regarding MNP degradation pathway and the identification of intermediate pathways by NMR studies (Schenzle et al., 1997). Intensive NMR studies were undertaken as described in this work to decipher the degradation pathways of ONP, MNP and PNP using a consortium, which was a daunting challenge, and an unreported culture, *Sarcina maxima.*

6.2 RESULTS

The samples of nitrophenol isomers after degradation by the microbial consortium and *Sarcina maxima* were subjected to intensive ^{1}H NMR and 2D HMQCT studies. Compounds identified by ^{1}H NMR and 2D HMQCT along with the NMR data for all the nitrophenol isomers are shown in Table 6.1-6.6. Since sufficient concentration of nitrophenol isomers were used, isolated residue after evaporation of the extracted solvent, contained detectable amounts of metabolites to give NMR spectra with a very good signal-to-noise ratio. Further the coupling pattern of spins were clearly identifiable, enabling unequivocal assignments of signals in most of the cases. This was also aided by areas of signals and chemical shift values of the non- exchangeable protons.

6.2.1 Degradation by microbial consortium

6.2.1.1 o-*Nitrophenol*

Unlike MNP and PNP, a large number of metabolites were detected in the culture media of ONP degraded by the consortium **(Table 6.1 and Fig. 6.1a & Fig 6.1b)**. Residual

undegraded ONP was also detected. The metabolites detected were - catechol (6.58ppm, 7.22ppm and OH- 7.82ppm), cis, cis-muconic acid (7.30ppm, 6.9ppm and COOH- 4.3ppm), γ-hydroxy muconic-semialdehyde (7.92ppm, 7.06ppm and 7.41ppm), maleylacetate (7.69ppm, 7.52ppm, and CH_2- 4.13ppm) and β-ketoadipate (CH_2 at 4.18 ppm and 4.4ppm). The detection of the above mentioned metabolites indicated that the consortium exhibited the degradation pathway proposed by Zeyer and Kearney (1984) and Zeyer and Kocher (1988).

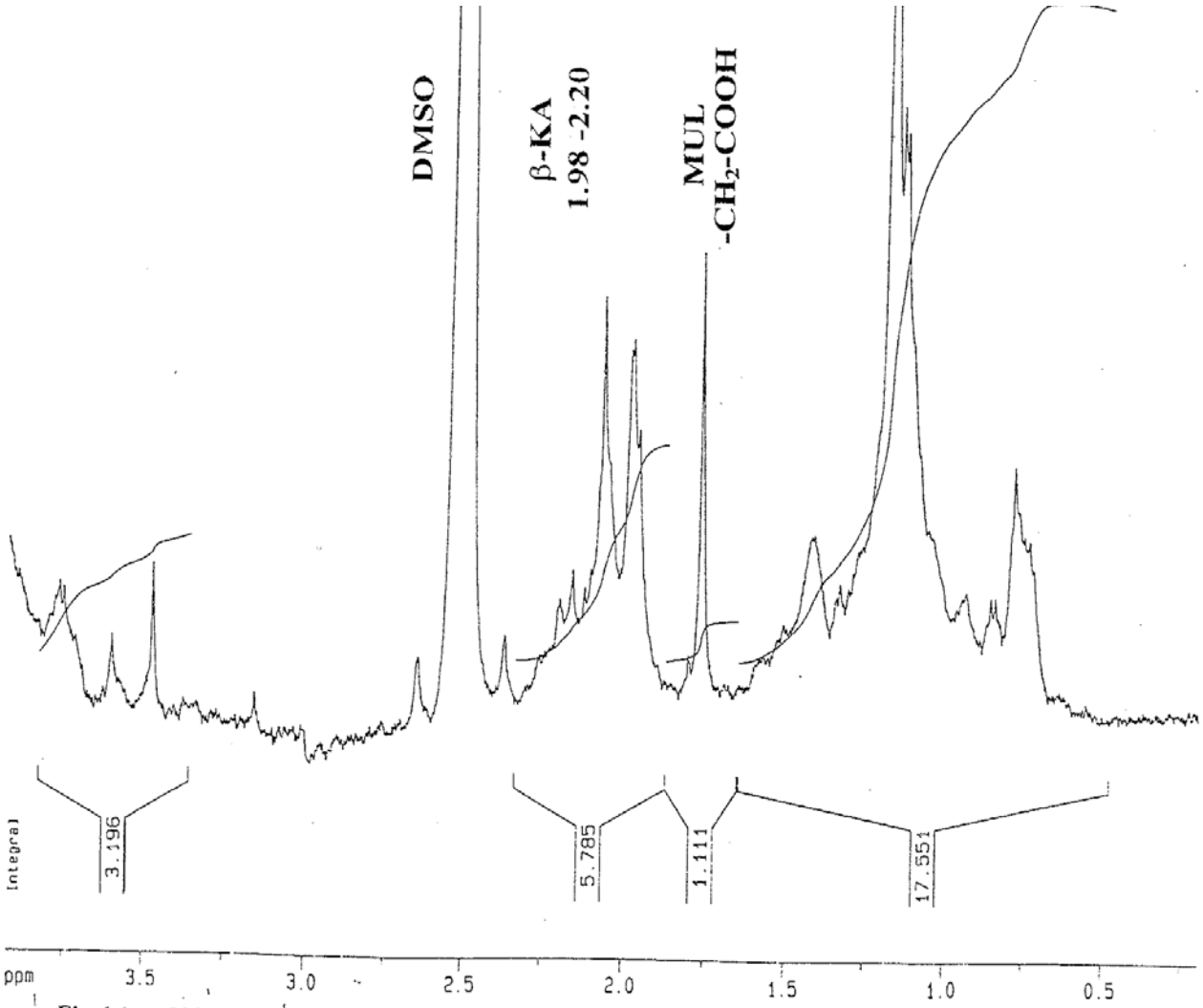


Fig 6.1a 500 MHz ^{1}H NMR spectrum showing the region for 0-4.0ppm of the reaction mixture obtained by the degradation of o-Nitrophenol by the microbial consortium. Experimental details are described in Section 2.2.2.3. β-KA→ β-ketoadipate, MUL-muconolactone, DMSO-dimethyl sulphoxide

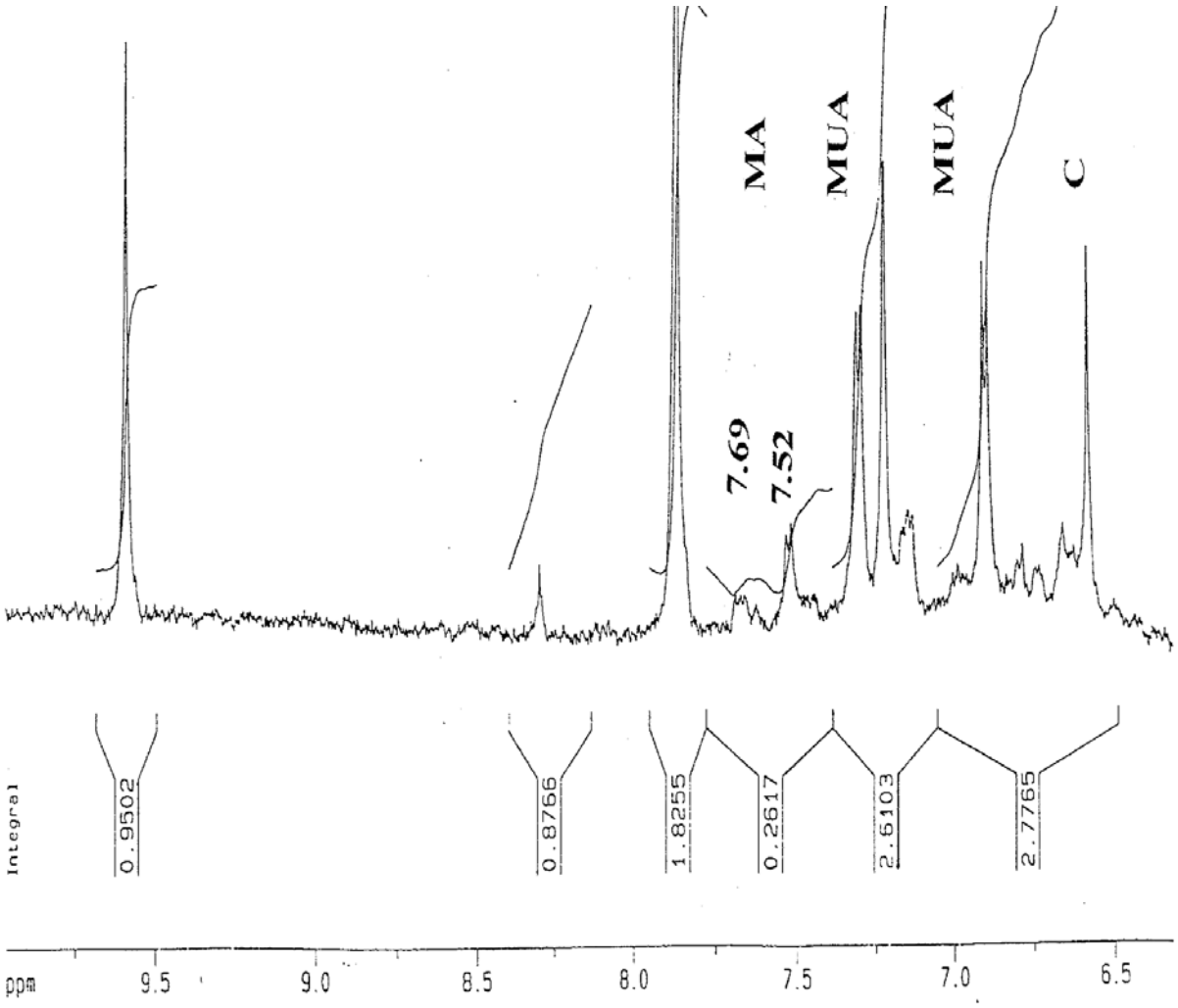
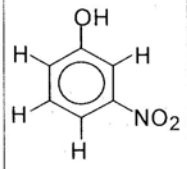
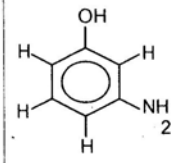
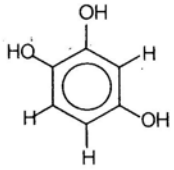
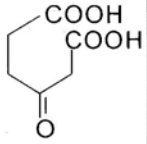


Fig 6.1b 500 MHz ^{1}H NMR spectrum showing the region for 6.5- 9.5ppm of the reaction mixture obtained by the degradation of o-Nitrophenol by the microbial consortium. Experimental details are described in Section 2.2.2.3. MUA-cis, cis-muconic acid, MA-maleyl acetate, C-catechol

6.2.1.2 m-*Nitrophenol*

The detection of 4-aminocatechol in the NMR spectra of MNP degraded sample clearly indicates that it came from 3-hydroxyl aminophenol (Table 6.2 and Fig 6.2a & Fig 6.2b). Aromatic protons at 6.89, 6.92 and 7.05ppm with a broad signal at 8.5ppm for the NH_2 group confirm the formation of 4-aminocatechol. The formation of 1,2,4-benzentriol was inferred from the observation of aromatic proton signals at 6.66ppm, 6.62ppm (d, 8.3 Hz) and 7.0 ppm (d, 8.3 Hz) and OH signals at 8.26ppm. β-ketoadipate detected from its characteristic CH_2 signals was the other metabolite. The detection of above mentioned metabolites indicates that the degradation of MNP is probably by the pathway proposed by Zhao et al., (2000).

Table 6.2 ^{1}H NMR data of the degradation of MNP by the consortium

Compound	Structure	H- 2	H- 3	H- 4	H- 5	H- 6	Others
m-Nitrophenol		7.72 (s)		7.82 (d, 8.1, 1.8)	7.42 (d, 8.1)	7.2 (8.1, 1.8)	OH 6.3 (Br)
4-aminocatechol			6.89		6.92	7.05	8.5 (Br NH_2)
1,2,4 - bezenetriol			6.66 (s)		6.62 (d, 8.3)	7.00 (d, 8.3)	OH (8.26)
β- ketoadipate							$-CH_2-$ (2) 3.95 -CH2- (4, 5) 1.95- 2.25

s- singlet, **d**- doublet, **Br**- broad

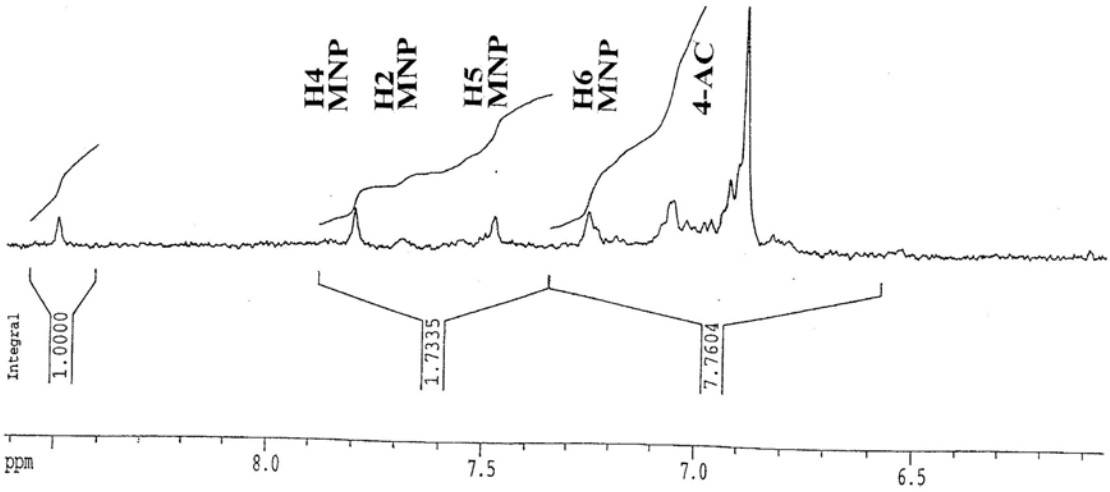


Fig 6.2a 500 MHz ^{1}H NMR spectrum showing the region for 6.0- 8.5ppm of the reaction mixture obtained by the degradation of m-Nitrophenol by the microbial consortium. Experimental details are described in Section 2.2.2.3. MNP→ m-Nitrophenol, AC- aminocatechol.

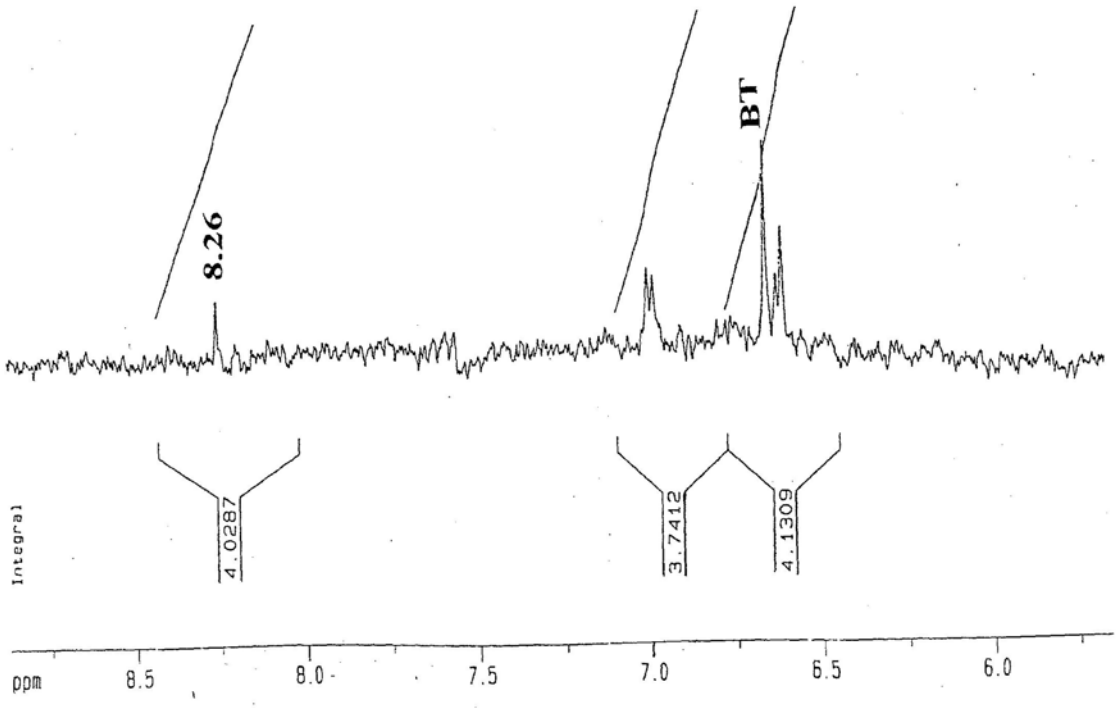
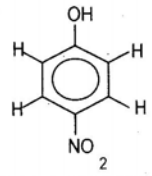
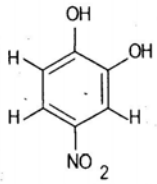
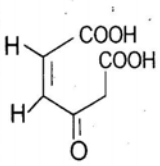
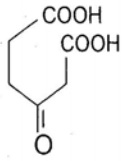


Fig 6.2b 500 MHz ^{1}H NMR spectrum showing the region for 5.5- 9.0ppm of the reaction mixture obtained by the degradation of m-Nitrophenol by the microbial consortium. Experimental details are described in Section 2.2.2.3 BT- 1,2,4-benzenetriol

6.2.1.3 p-*Nitrophenol*

Degradation of PNP by the consortium appears to proceed through the degradative pathway reported in case of *Moraxella* sp. (Spain and Gibson, 1991; Spain and Gibson, 1979) and *Arthrobacter* sp. (Hanne et al., 1993). The metabolites detected in ^{1}H NMR spectra are 4-nitrocatechol, maleyl acetate and β-ketoadipate **(Table 6.3 and Fig. 6.3a & Fig 6.3b).** While maleyl acetate is characterized by the aromatic protons at 7.72ppm and 7.58ppm, β-ketoadipate is characterized by the chemical shift values of 4 - CH_2 groups at 2.1ppm -2.35ppm and 4.1ppm and their characteristic coupling pattern. Identification of 4-nitrocatechol indicated that the pathway does not proceed through the conversion of p-benzoquinone to hydroquinone from PNP. Detection of 4-nitrocatechol also indicated the other metabolites to probably be 1,2,4-benzenetriol, $\tilde{\gamma}$-hydroxy muconicsemialdehyde, maleyl acetate and β-ketoadipate which was confirmed by their detection in the NMR spectra. The other metabolites could not be identified convincingly.

Table 6.3 ^{1}**H NMR data of the degradation of PNP by the consortium**

Compound	Structure	H- 2	H- 3	H- 4	H- 5	H- 6	Others
p-Nitrophenol		H- 2, H- 6 (6.82)			H- 3, H- 5 (8.9) 7.99		
4-nitrocatechol			8.15 (s)		8.3 (d, 10.1)	7.3 (d, 10.1)	
Maleylacetate				7.72	7.58		$-CH_2-$ 4.35 (m)
β- ketoadipate							$-CH_2-$ (2) 4.1 (m) -CH2- (4, 5) 2.1 - 2.35 (m)

s- singlet, **d**- doublet, **m** -multiplet, **Br**- broad

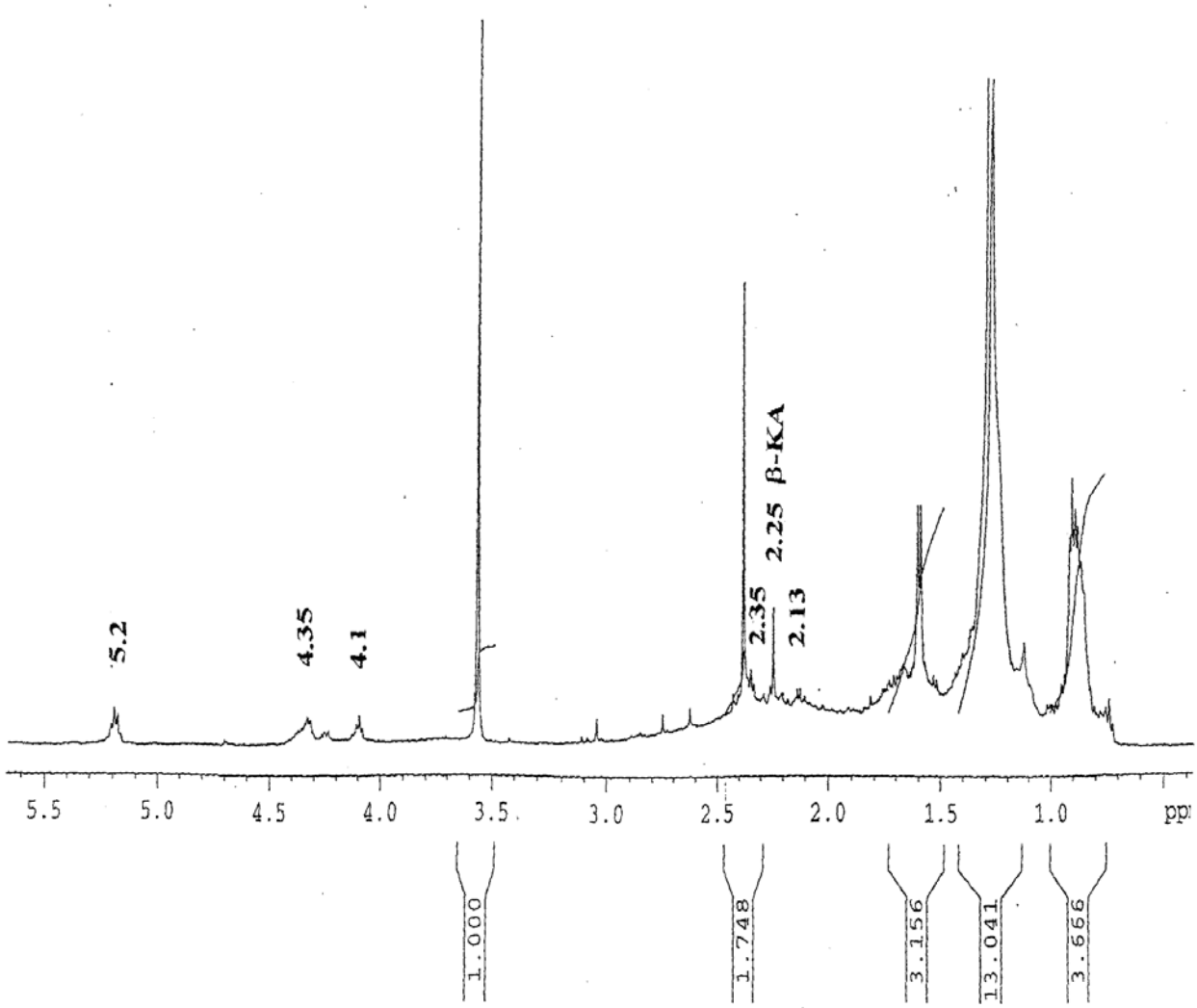


Fig 6.3a 500 MHz ^{1}H NMR spectrum showing the region for 0-5.5ppm of the reaction mixture obtained by the degradation of p-Nitrophenol by the microbial consortium. Experimental details are described in Section 2.2.2.3. β-KA→ β-ketoadipate

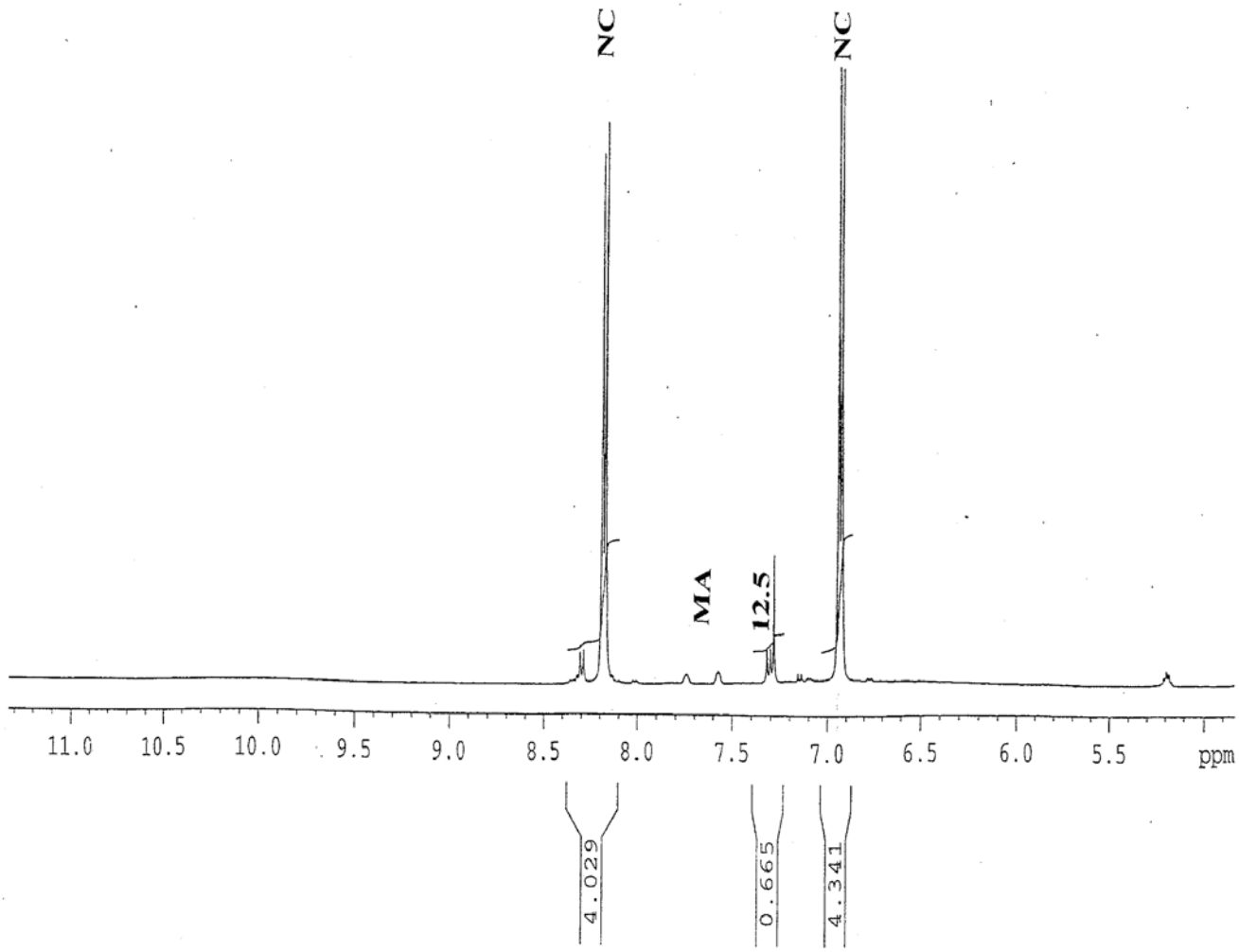


Fig 6.3b 500 MHz ^{1}H NMR spectrum showing the region for 5.5-11.0ppm of the reaction mixture obtained by the degradation of p-Nitrophenol by the microbial consortium. Experimental details are described in Section 2.2.2.3. NC-nitrocatechol, MA-maleylacetate

6.2.2 Degradation by the bacterial isolate (SNP-8)

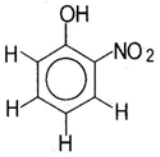
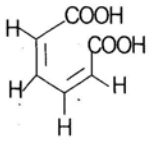
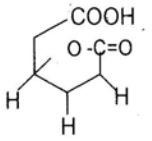
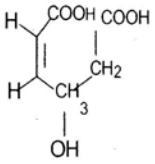
Since *Sarcina maxima* was found to degrade all the nitrophenol isomers (Chapter-4), an elaborate degradative study by this organism was carried out with all the three nitrophenol isomers as substrates. Generally the degradative products by SNP-8 contained a large amount of undegraded nitrophenols in all the three cases. However, the metabolites detected were in sufficient amounts and a careful examination of NMR signals indicated that they were from initial metabolites of the degradative pathway followed in case of all the three nitrophenol isomers. In these cases, two-dimensional HMQCT spectral data gave unequivocal assignments of several metabolites.

6.2.2.1 o-*Nitrophenol*

^{1}H NMR spectra of ONP degraded sample **(Table 6.4 and Fig. 6.4a, Fig 6.4b & Fig 6.4c)** indicated the presence of a new metabolite, β-hydroxy maleylacetate (7.16ppm, 7.56ppm and -CH- 5.85ppm and CH_2-2.25ppm). The coupling pattern indicated the presence of highly coupled -CH group at 5.85ppm. The other metabolites detected were cis, cis-muconic acid (7.38 and 7.29ppm) and muconolactone (7.74ppm, 5.05ppm,

-CH-O-4.25ppm and -CH_2-2.05-2.25ppm). Observation of β-hydroxy maleylacetate showed that there is no deviation to the general degradative pathway followed for ONP degradation proposed by Zeyer and Kearney (1984), Zeyer et al., (1986) and Zeyer and Kocher (1988). Also it confirms that formation of maleylacetate from γ-hydroxy muconicsemialdehyde should go through the formation of β-hydroxy maleylacetate.

Table 6.4 NMR data on the degradation of o-Nitropenol by *Sarcina maxima*

Compound	Structure	H- 2	H- 3	H- 4	H- 5	H- 6	Others
o-Nitrophenol			8.13 (d, 7.5)	7.02 (t, 7.5)	7.61 (t, 8.2, 1.3)	7.18 (d, 8.2)	OH 10.6
Cis, cis-muconic acid		H-2, H-6 7.38	H-3, H-4 7.29				
Muconolactone		7.74 (d, 9.2, 5.0)	5.05 (dd, 17.2, 9.0)				-CH-O- 4.25 (m 10.1, 6.1) -CH_2- 2.05- 2.25 (m)
β- hydroxy maleylacetate				7.61 (d, 7.3)	7.56 (d, 7.3)		-CH- 5.85 (dt, 9.2) -CH2- 2.25 (m)

d- doublet, **dd**- doublet of a doublet, **t**- triplet, **dt**- doublet of a triplet, **m**- multiplet

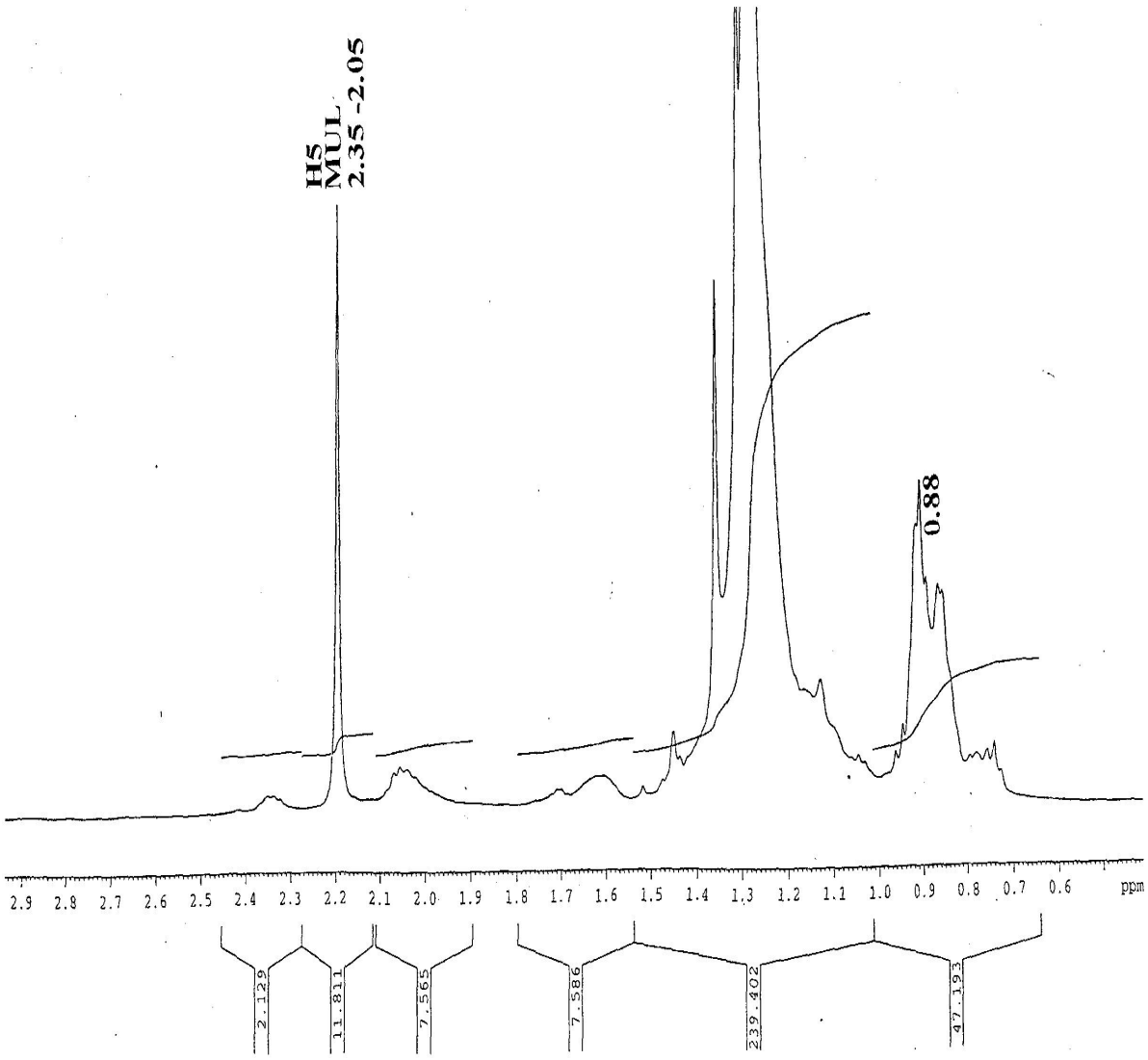


Fig 6.4a 500 MHz ^{1}H NMR spectrum showing the region for 0-2.9ppm of the reaction mixture obtained by the degradation of o-Nitrophenol by *Sarcina maxima*. .Experimental details are described in Section 2.2.2.3. MUL-muconolactone

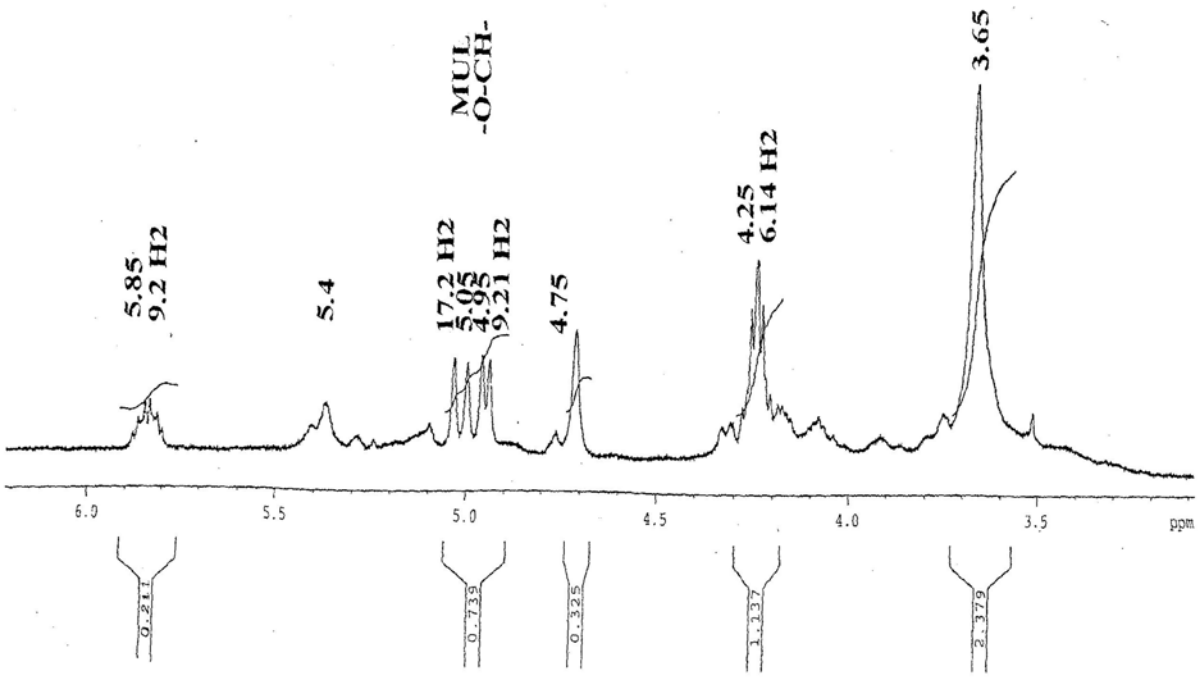


Fig 6.4b 500 MHz ^{1}H NMR spectrum showing the region for 3.0-6.0ppm of the reaction mixture obtained by the degradation of o-Nitrophenol by *Sarcina maxima*. Experimental details are described in Section 2.2.2.3. MUL- muconolactone

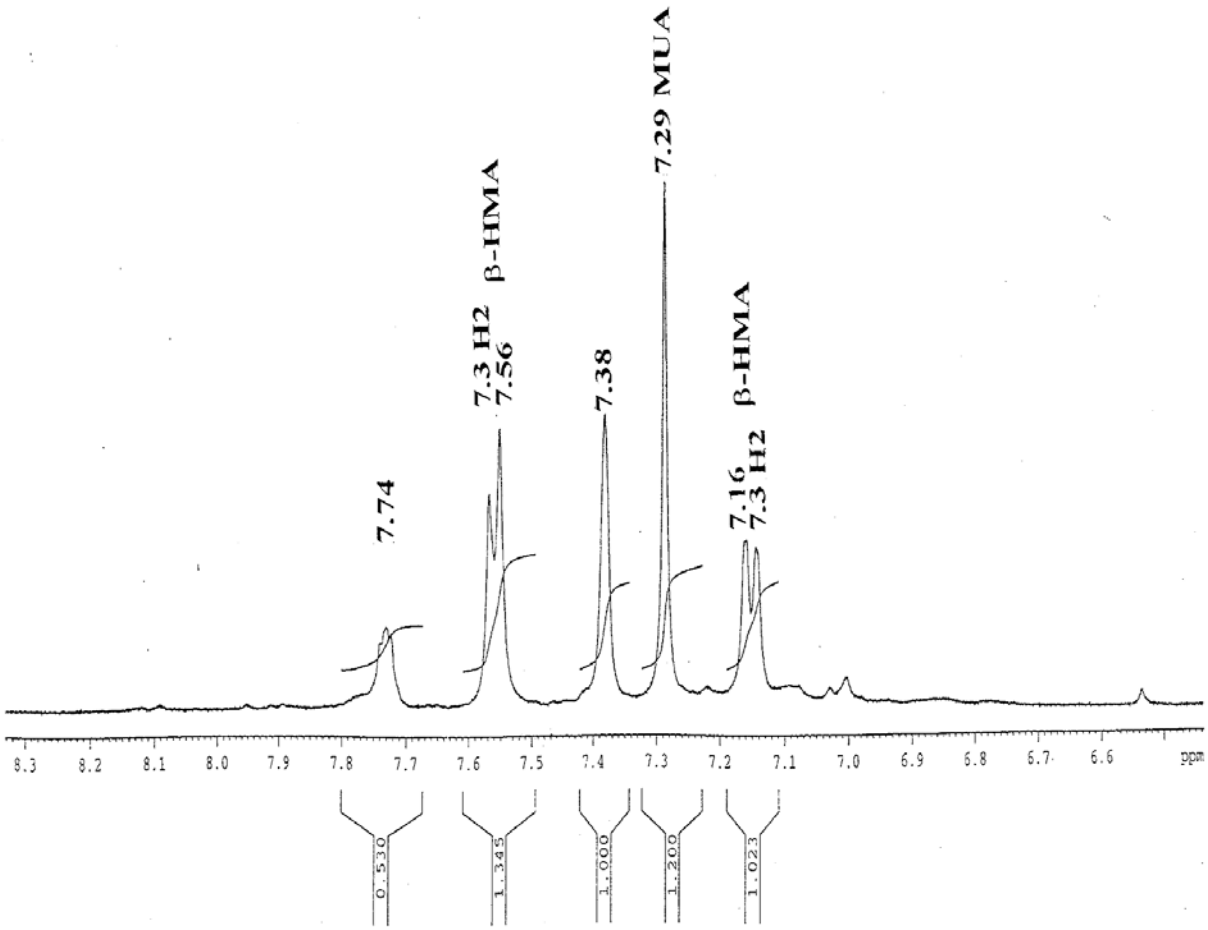


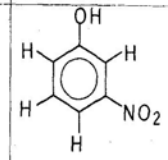
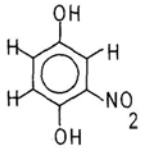
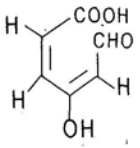
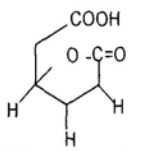
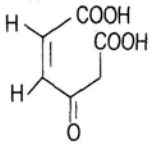
Fig. 6.4c 500 MHz ^{1}H NMR spectrum showing the region for 6.0- 8.0ppm of the reaction mixture obtained by the degradation of o-Nitrophenol by *Sarcina maxima*. Experimental details are described in Section 2.2.2.3. MUA- cis, cis-muconic acid , βHMA→ β-hydroxy maleylacetate

6.2.2.2 m-*Nitrophenol*

In case of MNP, a new metabolite 2-nitrohydroquinone (7.52ppm, 7.58ppm, 7.11ppm and OH- 7.59ppm) was detected **(Table 6.5 and Fig 6.5a & Fig 6.5b)**. The signals did not correspond to any metabolite reported so far. The other metabolites detected were γ-hydroxy muconicsemialdehyde (7.29, 7.06 and 7.42ppm), muconolactone (7.58 and 4.89ppm) and maleylacetate (7.63, 7.51 and -CH_2- 3.98ppm). Observation of 2- nitrohydroquonine indicated that the degradative pathway may be slightly different than that proposed by Zhao and Ward (2000) and Zhao et al. (2000).

2-nitrohydroquinone can undergo one-electron reduction to give 2-hydroxyl aminohydroquinone, which can form 1,2,4- benzenetriol to follow the regular degradative pathway thereafter.

Table 6.5 NMR data on the degradation of m-Nitropenol by *Sarcina maxima*

Compound	Structure	H- 2	H- 3	H- 4	H- 5	H- 6	Others
m-Nitrophenol		7.72 (s)		7.82 (d, 8.1, 1.8)	7.42 (d, 8.1)	7.2 (8.1, 1.8)	OH 6.3 (Br)
2- nitrohydroquinone			7.52 (s)		7.58 (d)	7.11 (d, 8.9)	OH 7.59
γ -hydroxy muconic semialdehyde		7.29 (d, 3.0)		7.06 (dd, 8.9, 3.0)	7.42 (d, 8.9)		
Muconolactone		7.58 (d)	4.89 (dd, 4.3, 10.7)				- CH- O 4.13 (m) 7.1 Hz $-CH_2-$ 2.25 (m)
Maleyl acetate				7.63 (dd, 10.0, 5.0)	7.51 (dd, 10.0, 5.0)		

s- singlet, **d**- doublet, **dd**- doublet of a doublet, **Br**- broad, **m**- multiplet

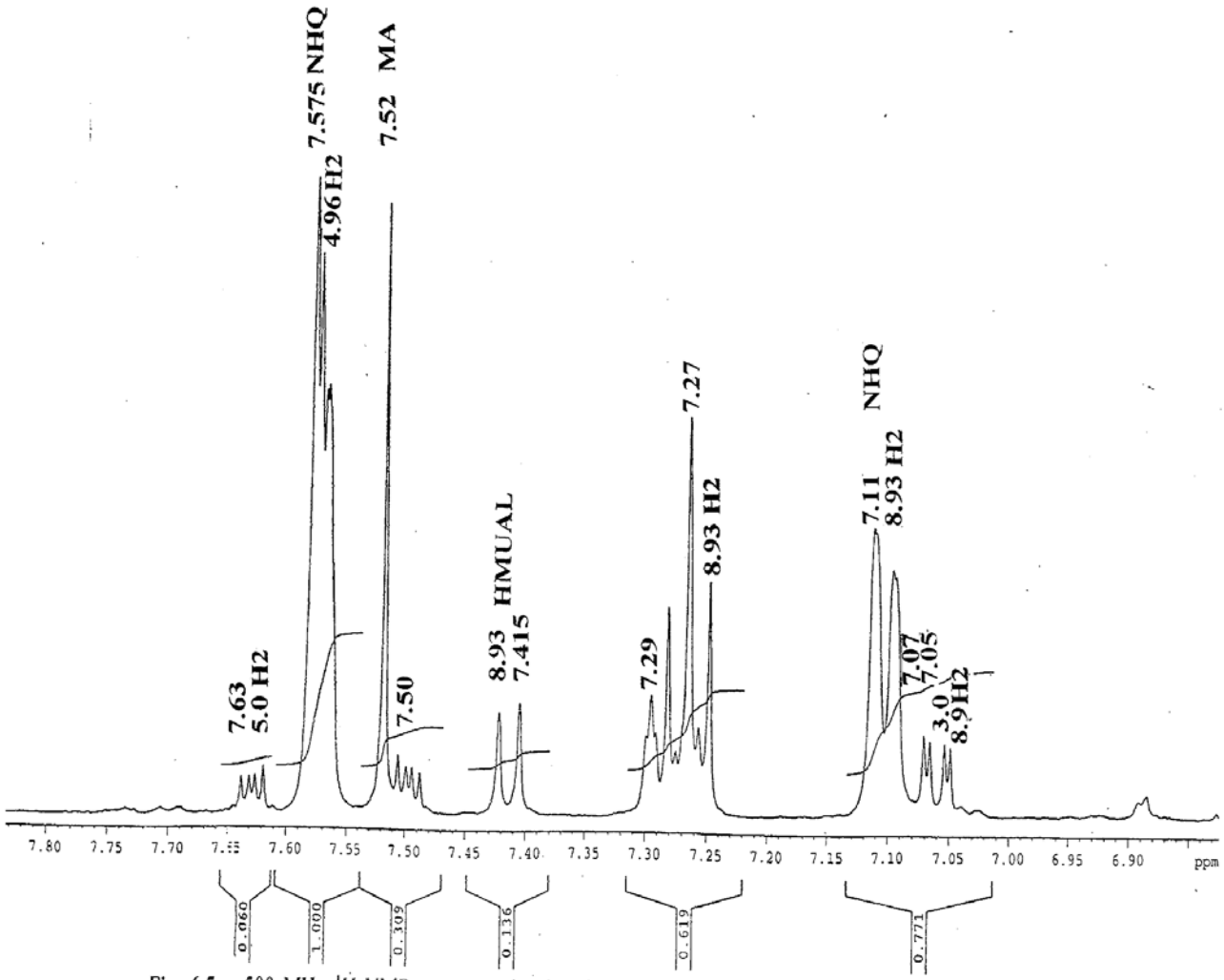


Fig. 6.5a 500 MHz ^{1}H NMR spectrum showing the region for 6.85-7.80ppm of the reaction mixture obtained by the degradation of m-Nitrophenol by *Sarcina maxima*. Experimental details are described in Section 2.2.2.3. NHQ-nitrohydroquinone, MA-maleyl acetate, HMUAL→γ-hydroxy muconicsemialdehyde.

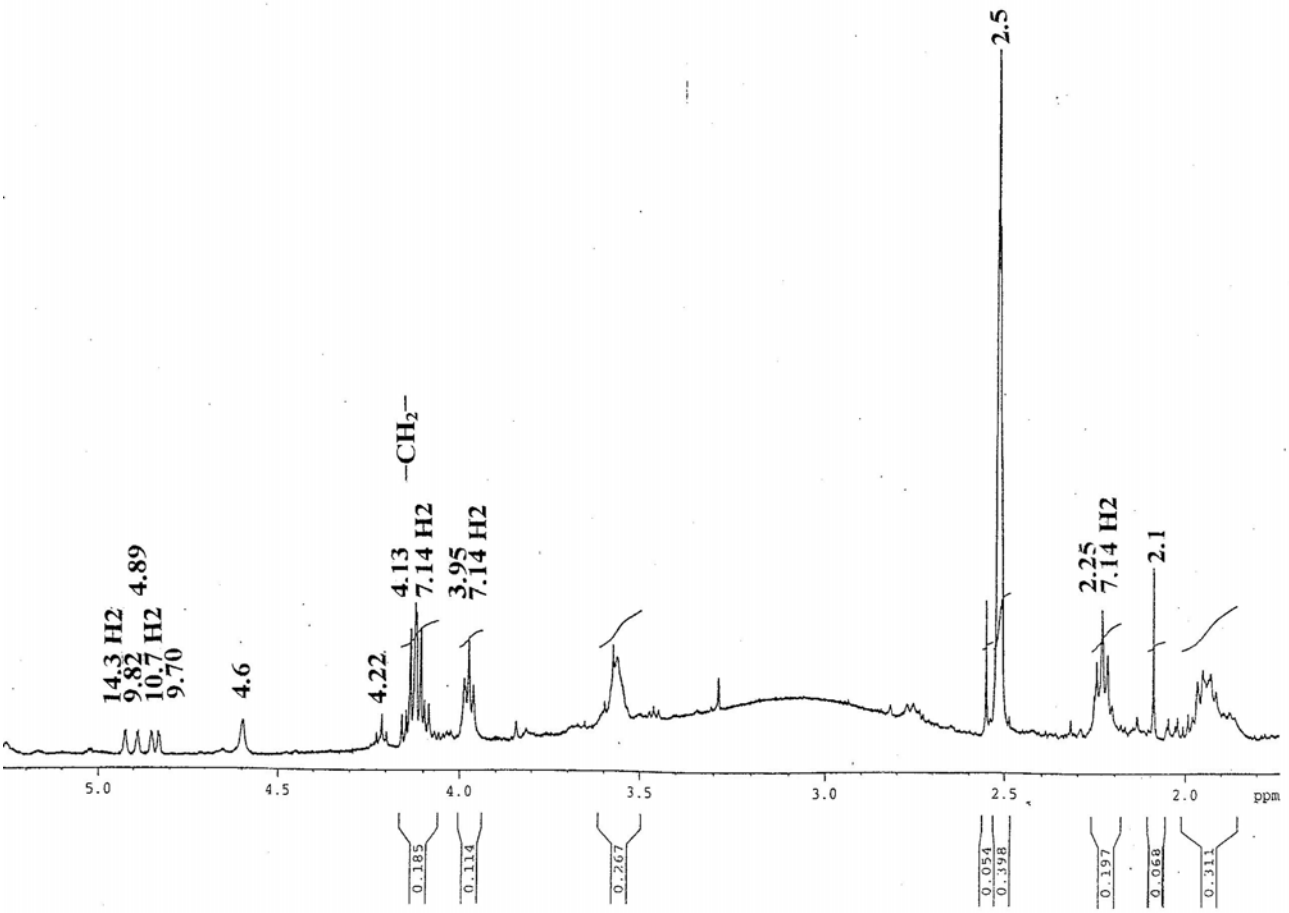
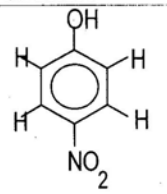
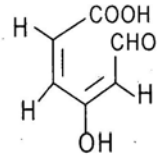
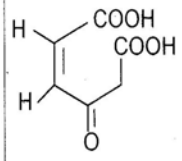
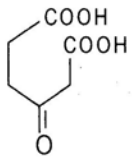


Fig. 6.5b 500 MHz ^{1}H NMR spectrum showing the region for 2.0-5.0ppm of the reaction mixture obtained by the degradation of m-Nitrophenol by *Sarcina maxima*. Experimental details are described in Section 2.2.2.3

6.2.2.3 p-*Nitrophenol*

Besides the signals from undegraded PNP (6.82 and 7.99ppm for aromatic protons and 115ppm and 124ppm for aromatic carbons), the other metabolites detected were maleylacetate (7.63ppm, 7.51ppm, and CH_2 -4.13ppm, carbon 130 and 127ppm), γ-hydroxy muconicsemialdehyde (7.29ppm, 7.07ppm and 7.41ppm) and β-ketoadipate (-CH_2 at 3.95ppm, 1.95ppm and carbon- 39.0ppm; **Table 6.6 and Fig.6.6a, Fig 6.6b, Fig 6.6c, Fig 6.6d).** Carbon-13 signals from 2D HMQCT were quite confirmatory in deducing the aromatic and aliphatic nature of the metabolites clearly. Unlike in the case of the consortium, 4-nitrocatechol was not detected. The degradation may be expected to go through p- hydroquinone formation as proposed by Spain and Gibson (1979) and Spain and Gibson (1991).

Table 6. 6 NMR data on the degradation of p-Nitropenol by *Sarcina maxima*

Compound	Structure	H- 2	H- 3	H- 4	H- 5	H- 6	Others
p-Nitrophenol		H-2, H-6 6.82 (d, 10.1 Hz) ^{13}C= 115.0			H- 3, H- 5 7.99 (d, 10.1 Hz) ^{13}C= 115.0		
γ-hydroxy muconic semialdehyde		7.29 (s)		7.07 (d, 9.0)	7.41 (d, 9.0)		
Maleylacetate				7.63 (dd, 10.0, 5.0) ^{13}C= 130	7.51 (dd, 10.0, 5.0) ^{13}C= 127		-CH2- 4.13 (m) ^{13}C= 66
β - ketoadipate							-CH_2- (4, 50 1.95- 2.25) -CH_2- (2) 3.95 (m) ^{13}C= 39.0ppm

s- singlet, **dd**- doublet of a doublet, **d**- doublet, **Hz**- hertz, **m**- multiplet

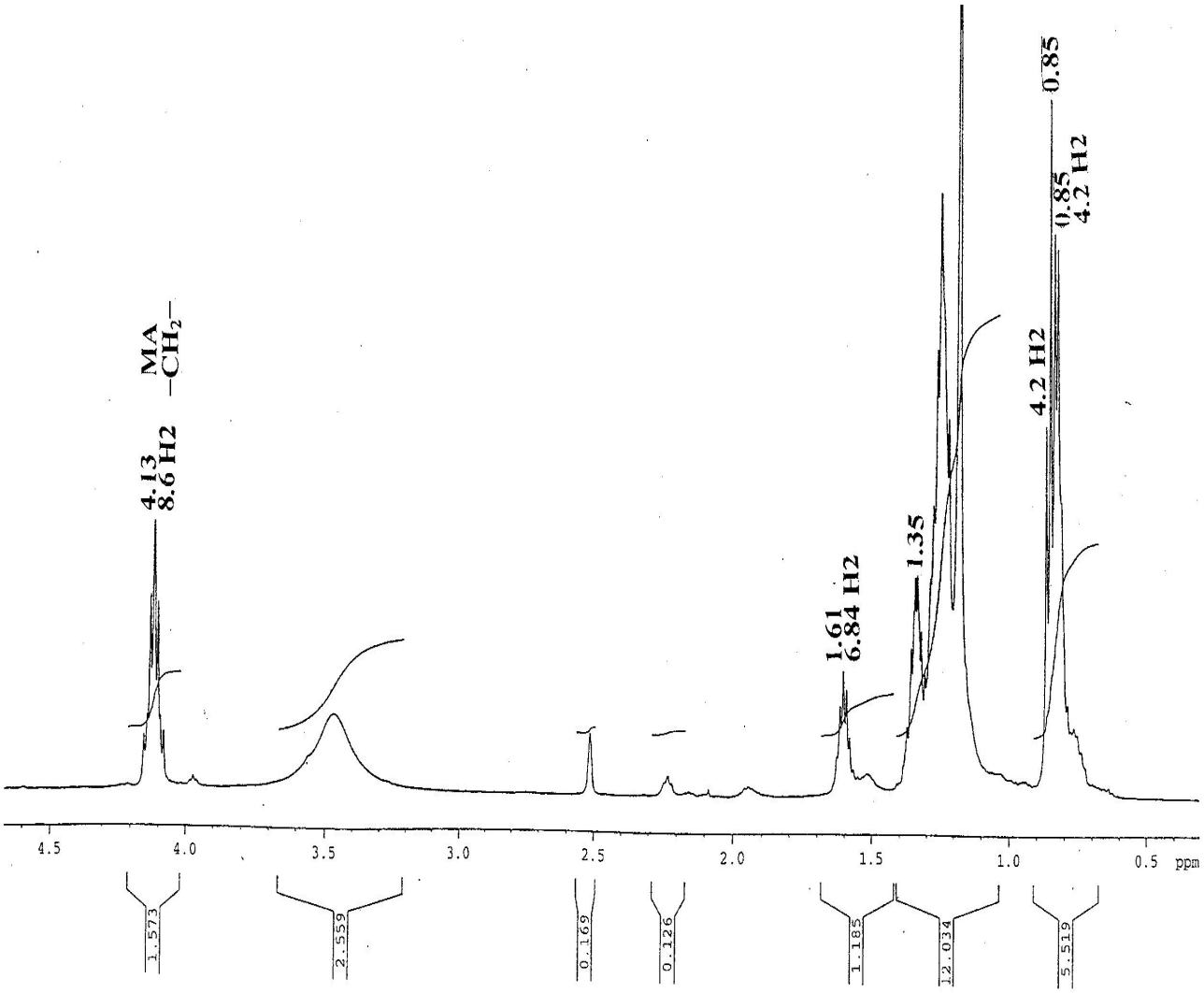


Fig. 6.6a 500 MHz ^{1}H NMR spectrum showing the region for 0.5- 4.5ppm of the reaction mixture obtained by the degradation of p-Nitrophenol by *Sarcina maxima*. Experimental details are described in Section 2.2.2.3. MA- maleyl acetate

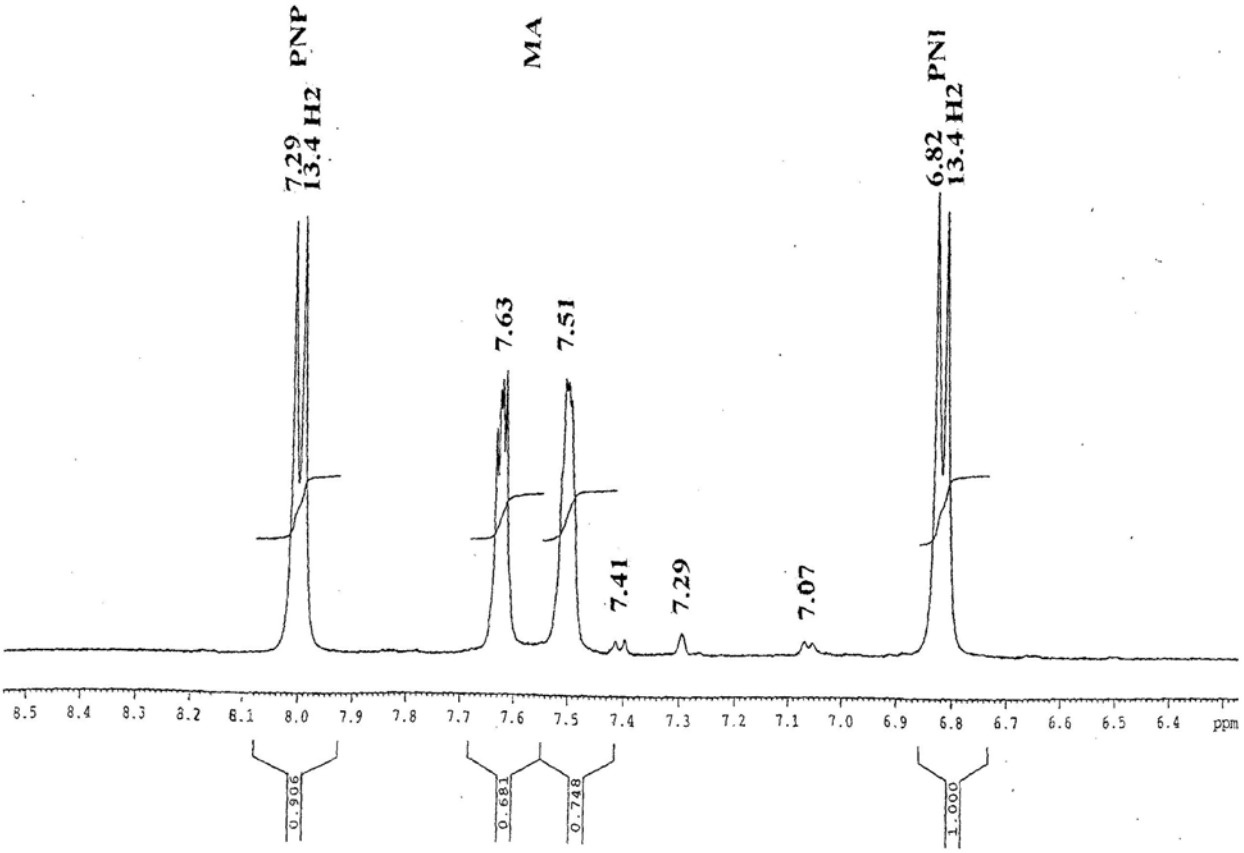


Fig. 6.6b 500 MHz ^{1}H NMR spectrum showing the region for 6.4- 8.5ppm of the reaction mixture obtained by the degradation of p-Nitrophenol by *Sarcina maxima*. Experimental details are described in Section [illegible] PNP - p-nitrophenol, MA- maleyl acetate

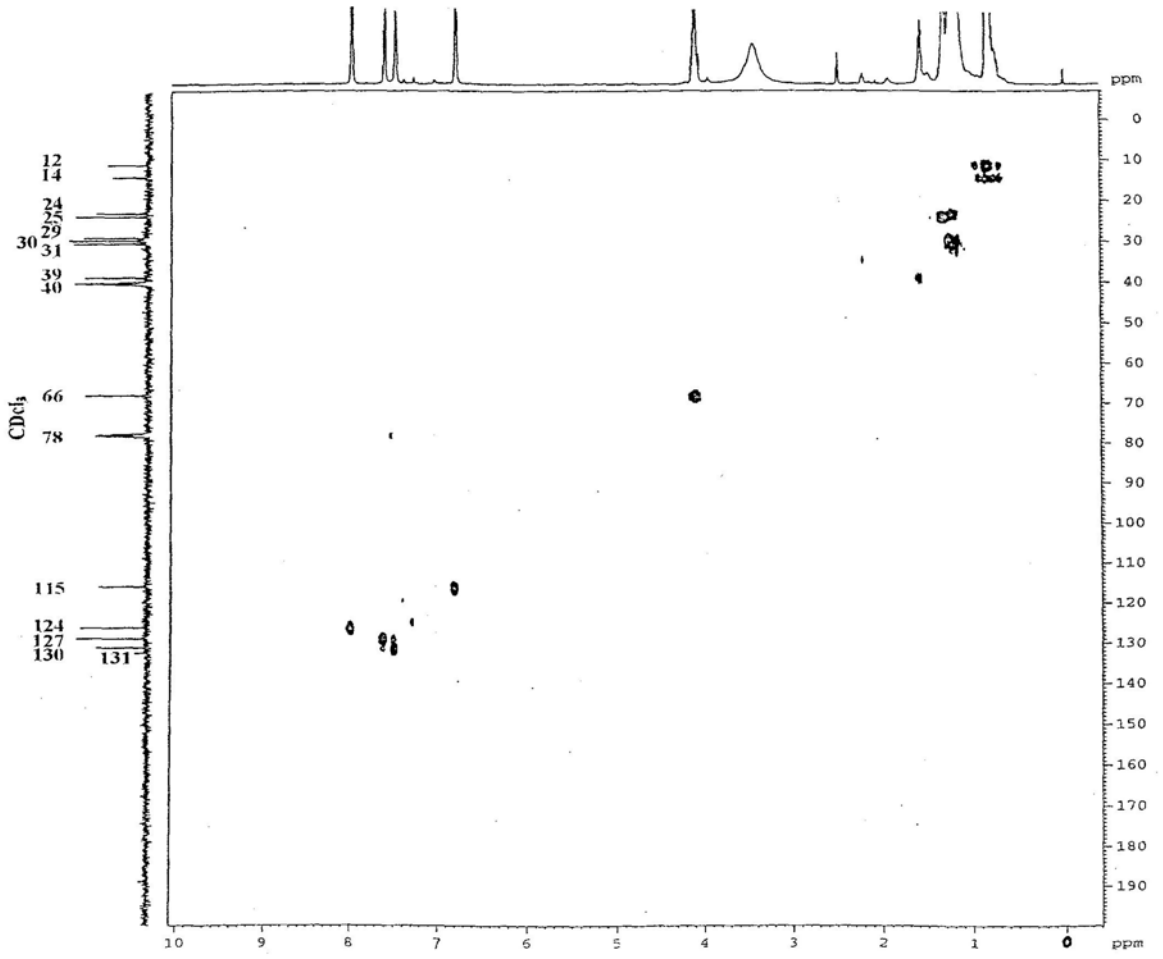


Fig 6.6c 2D HMQCT spectrum showing the region for 0-10ppm of the reaction mixture obtained by the degradation of p-Nitrophenol by *Sarcina maxima*. Experimental details are described in Section 2.2.2.3

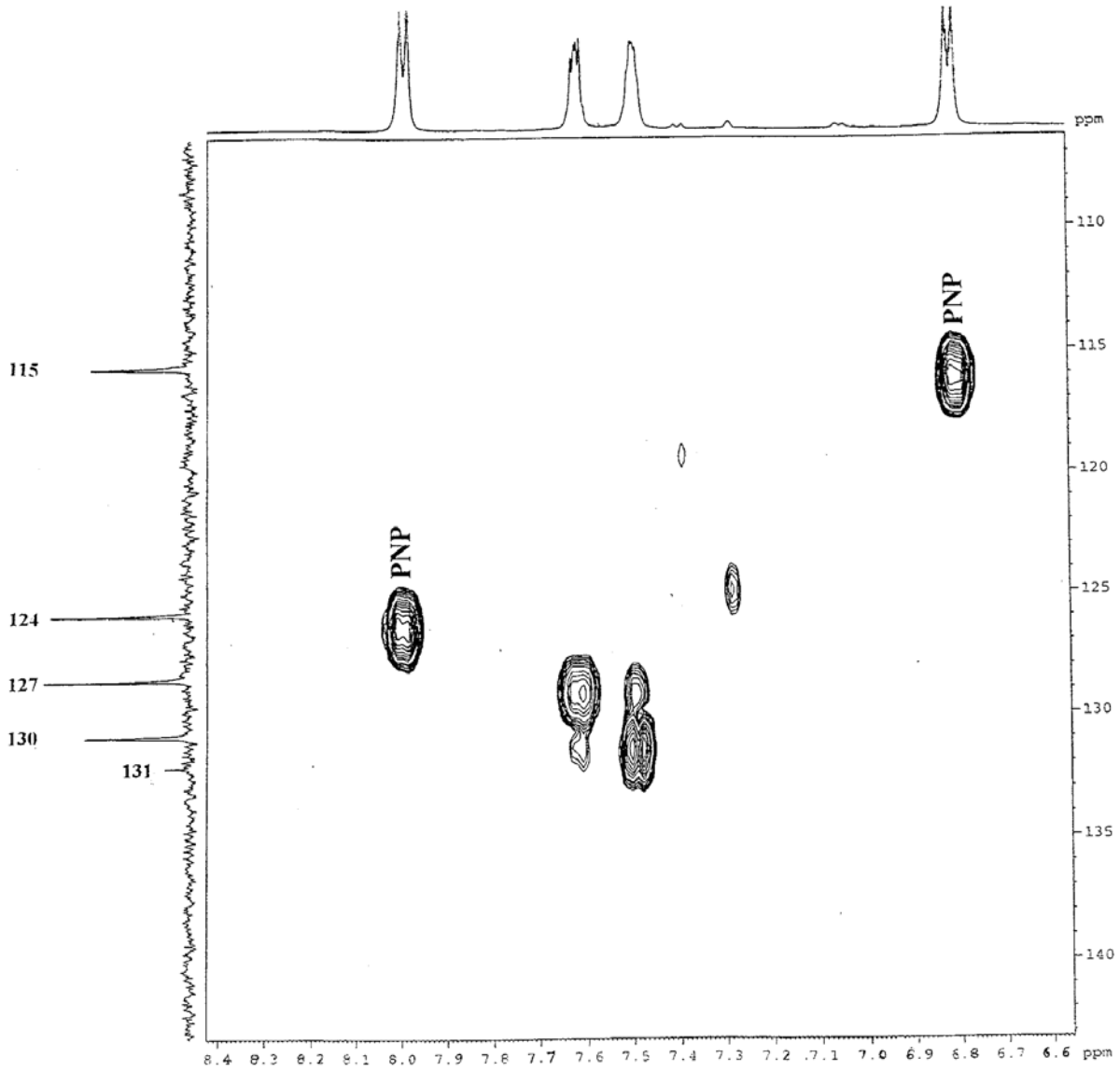


Fig. 6.6d 2D HMQCT spectrum showing the region for 0-5.5ppm of the reaction mixture obtained by the degradation of p-Nitrophenol by *Sarcina maxima*. Experimental details are described in Section 2.2.2.3. PNP→ p-Nitrophenol

6.3 DISCUSSION

Due to lack of sufficient concentration of metabolites in the sample, especially in case of degradation by the consortium, satisfactory 2D HMQCT spectra could not be detected. In such case only normal ^{1}H NMR spectra were recorded. Identification of metabolites both in the case of degradation by consortium and *Sarcina maxima* were made from the data obtained by one and two dimensional (2D HMQCT) spectra. The splitting patterns were helpful in identifying some of the metabolites like 2-nitrohydroquinone, β-hydroxy maleylacetate, cis, cis-muconic acid, muconolactone, 4-nitrocatechol, 1,2,4-benzenetriol, γ-hydroxy muconic-semialdehyde, β-ketoadipate, 4-aminocatechol, 3-hydroxyl aminophenol, 2-hydroxyl aminophenol, catechol, 4-aminocatechol and maleylacetate. The chemical shift values of these signals were reflective of their micro-environments like -O-CH- or -CH=CH- groups. The coupling pattern like doublet of a triplet, multiplet and few coupling constants measured also helped in clearly identifying the signals with respect to their neighbouring proton nuclei.

As mentioned earlier the intermediates in the pathway of ONP degradation have been well identified and enough evidence exists regarding an initial monooxygenase catalyzed reaction leading to catechol which is further metabolized to cis,cis-muconic acid and β-ketoadipate consistent with the NMR proof from the present study. In reaction mixtures of the bacterial culture *Sarcina maxima*, a new metabolite β-hydroxy maleylacetate was identified and it is proposed that γ-hydroxy muconicsemialdehyde is converted to maleylacetate via the new metabolite identified.

Interesting observations were made in the reaction mixtures of the consortium and *Sarcina maxima* induced with MNP. Metabolites identified suggested two different pathways during the degradation of MNP. The mixed culture system seemed to degrade MNP to β-ketoadipate via β-hydroxyl aminophenol whereas the single culture degraded MNP via 2-nitrohydroquinone. Schenzle et al., (1997) found that cell extracts of MNP grown cells of *Ralstonia eutropha* JMP134 converted MNP to trace amounts of 3-hydroxyl aminophenol. Only one oxygen sensitive metabolite was formed whose ^{1}H NMR signals were δ 6.15ppm (d, 8.1Hz, 4H), δ 6.25ppm (d, 8.1Hz, 6H), δ6.32ppm

(broad singlet 2H), δ 6.90ppm and a low field proton (5H) was split into a triplet by two ortho couplings. Under anaerobic conditions resting cells of *R.eutropha* gave a new metabolite which did not give prominent signals like for 3-hydroxyl aminophenol. The metabolite displayed a singlet at 2.13ppm typical for an acetyl group in the ^{1}H -NMR spectrum. Further, three multiplets in the aryl proton region integrating each for one H appearing at δ <7.0ppm led to the identification of N-acetylaminohydroquinone. Also hydroxyl aminobenzene, a structural analog of hydroxyl aminophenol was converted to 2-aminophenol and 4- aminophenol but under anaerobic conditions. However in the present study with the consortium, 4- aminocatechol was identified as the metabolite of 3- hydroxyl aminophenol under aerobic conditions which was further converted to β-ketoadipate via 1,2,4-benzenetriol. Meulenberg et al., (1996) identified 1,2,4-benzenetriol as an intermediate of nitro reductase initiated MNP transformation by *P. putida* B2 under anaerobic conditions.

A parallel pathway during MNP metabolism by bacterial culture *Sarcina maxima* was observed wherein 2-nitrohydroquinone was detected as the initial metabolite followed by others such as γ-hydroxy muconicsemialdehyde, maleylacetate etc. A similar pathway was proposed by Zhao et al. (2000) for MNP metabolism based on studies with analogous hydroxyl aminobenzene which was converted to hydroxyl aminophenol which was later reduced to aminohydroquinone and 4-aminocatechol. These products were oxidized to imines and later to 1,2,4- benzenetriol. No imines could be detected in the present study.

The consortium and the single culture followed two different pathways in the degradation of PNP also. The consortium degraded PNP to β-ketoadipate via the formation of 4- nitrocatechol, 1,2,4-benzenetriol and maleylacetate. Earlier, Raymond and Alexander (1971) had suggested the conversion of PNP to 4-nitrocatechol by a *Flavobacterium* species. Jain et al. (1994) suggested 4- nitrocatechol and 1,2,4-benzenetriol to be involved in the degradative pathway of PNP metabolism. However in the reaction mixture of *Sarcina maxima,* no nitrocatechol was detected but the NMR signals showed the presence of

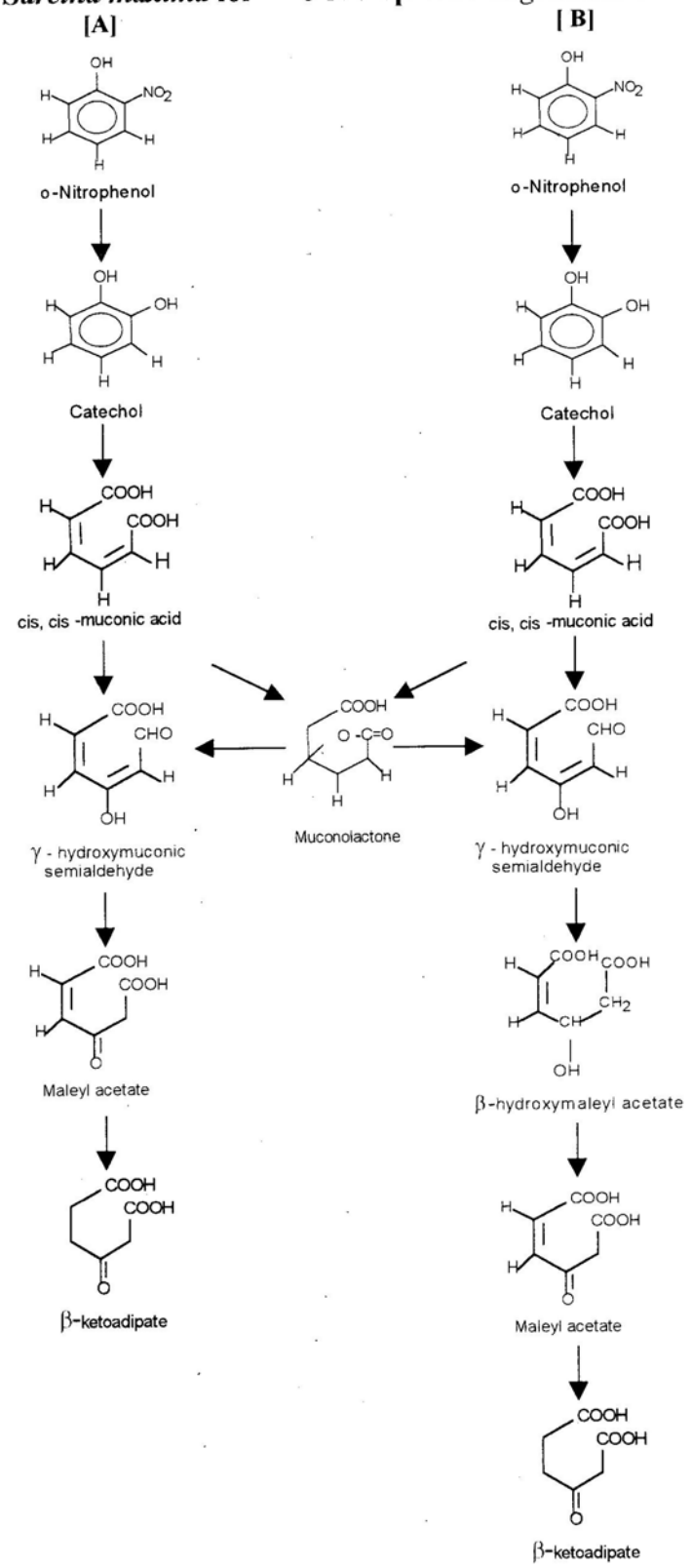
γ-hydroxy muconicsemialdehyde, maleylacetate and β-ketoadipate. Therefore in *Sarcina maxima,* PNP degradation is proposed to go through hydroquinone formation as proposed by Spain and Gibson (1991) during their studies on PNP degradation by a *Moraxella* sp. where they reported the conversion of hydroquinone to 1,2,4-benzenetriol and maleylacetate in the absence of NADPH. All the degradation studies during the present work were carried out in the absence of any co-factors.

Based on intensive NMR studies conducted during the present work, **Scheme 6.1, Scheme 6.2** and **Scheme 6.3** show the proposed pathways followed by ONP, MNP and PNP during their degradation by the consortium and the bacterial culture *Sarcina maxima*.

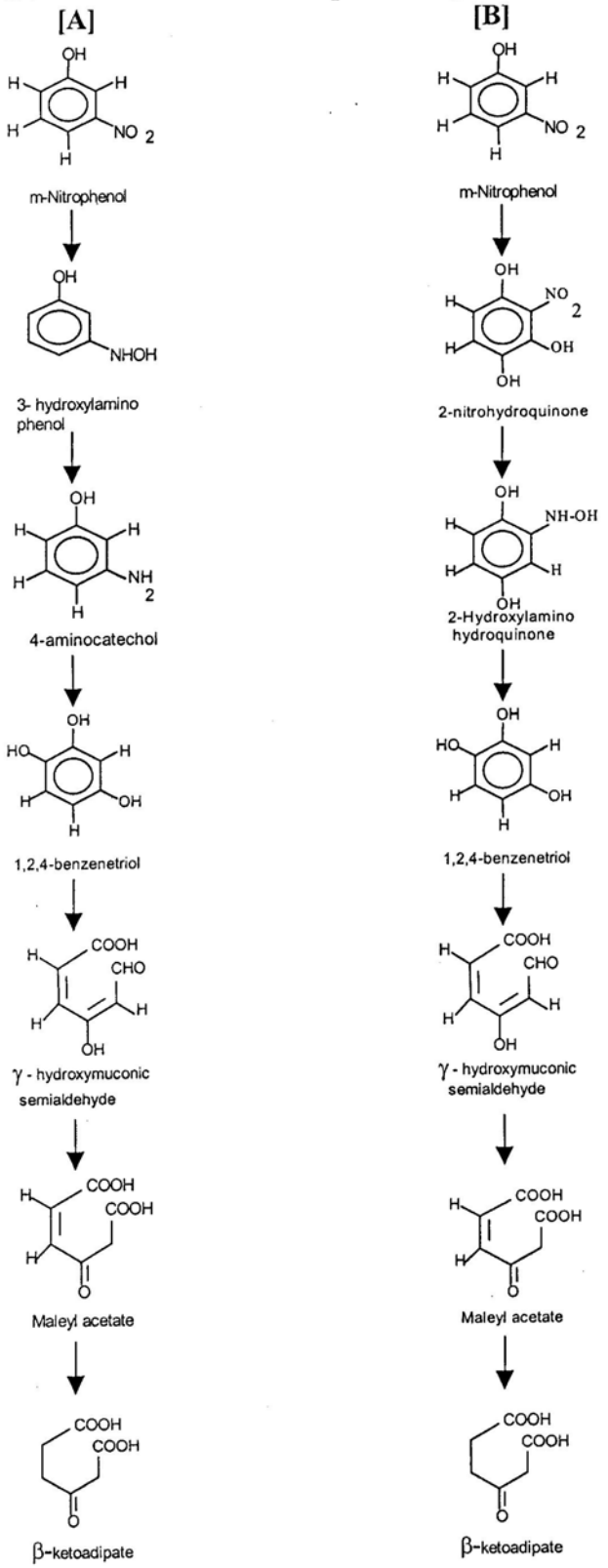
The microbial consortium employed is a group eight microorganisms probably present in different proportions. Also each potential organism may degrade the three nitrophenol isomers to different extents. The degradative action may also be synergetic aided by different microorganisms at various stages.

Identification of new metabolites indicating new pathways followed by the constituent organisms of a mixed bacterial culture system explains the effectiveness of NMR spectroscopy in biodegradation studies.

Scheme 6.1 Pathway followed by the [A] Consortium [B] *Sarcina maxima* for o-Nitrophenol degradation

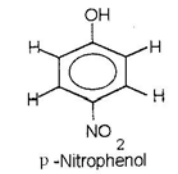


Scheme 6.2 Pathway followed by the [A] Consortium [B] *Sarcina maxima* for m-Nitrophenol degradation

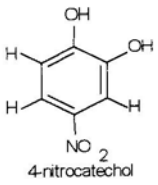


Scheme 6.3 Pathway followed by the [A] Consortium [B] *Sarcina maxima* for p-Nitrophenol degradation

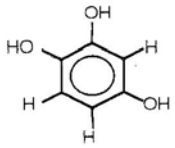
[A]



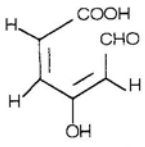
p -Nitrophenol



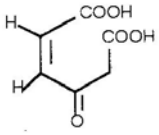
4-nitrocatechol



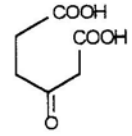
1,2,4-benzenetriol



γ- hydroxymuconic semialdehyde



Maleyl acetate

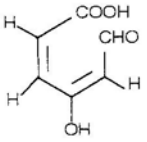


β-ketoadipate

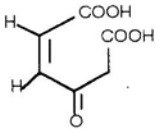
[B]

p -Nitrophenol

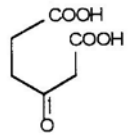
p-hydroquinone



γ- hydroxymuconic semialdehyde



Maleyl acetate



β-ketoadipate

CHAPTER 7

CONCLUSIONS

The mixed bacterial culture used in the present study was obtained from a soil sample polluted with phenolic compounds and was found to consist of eight different bacterial cultures. The consortium on enrichment and long periods of acclimation was found to degrade all the mononitrophenol isomers (ONP, MNP and PNP) individually as well their mixture, though, to varying degrees after a minimum lag of 6-12h. The rate of MNP degradation being generally the fastest followed by PNP and ONP. Simultaneous degradation also showed rapid disappearance of MNP. Nitrite was released during the degradation of ONP and PNP indicating an oxidative mechanism whereas production of ammonia during MNP breakdown indicated a reductive pathway. This type of activity was observed during degradation of mononitrophenol isomers by the consortium and a single bacterial isolate. Release of nitrite is probably a defense mechanism against the toxicity of the compound. Production of ammonia from nitroaromatic compounds avoids the production of potentially toxic amines in the environment. Toxic concentrations (> 0.5mM) were tolerated by the consortium and successful degradation ranging between 0.5mM-5mM were recorded using the consortium as evident by the viability of the cells and the identification of metabolites. The lag period increased with increasing substrate concentration. At very low concentrations, the long acclimation periods may be the result of slow growth of the mineralizing organisms. However at high concentrations, the toxicity of the compound may reduce the number of active organisms and increase the acclimation period and this may also be accounted for the apparent low growth observed throughout the experimental study. The presence of toxic intermediates, their accumulation or even an inadequate supply of essential nutrients may account for the delay in growth. Induction experiments revealed that pre-exposed cells brought about early substrate disappearance which was a favourable process for rapid biodegradation.

Strong dioxygenase activity was observed in cell extracts of the consortium induced with mononitrophenol isomers. Monooxygenase activity was also observed in partially degraded crude cell extracts of ONP and PNP induced cells. Pronounced catechol 1,2-dioxygenase activity in ONP and PNP induced cell extracts clearly indicated an ortho cleavage of the substrates. In contrast MNP induced cell extracts showed only catechol 2,3-dioxygenase activity which is an enzyme involved in meta cleavage. The ring cleaving enzyme had an absorbance maxima at 375nm consistent with hydroxy muconicsemialdehyde. None of the enzymes involved seemed to require any additional co-enzymes or manganese ions as no such compounds were added during the course of study.

Resolution of the consortium revealed the presence of eight bacterial culture which were characterized and identified as-*Bacillus licheniformis* (SNP-1), *Xanthomonas maltophila* (SNP-2), *Serratia liquefaciens* (SNP-3), *Psuedomonas putida* (SNP-4), *Pseudomonas* sp. (SNP-5), Pseudomonas *alcaligenes* (SNP-6), *Psuedomonas* sp. (SNP-7) and *Sarcina maxima* (SNP-8). All the cultures were capable of degrading ONP and PNP and also brought about simultaneous degradation of a mixture of the mononitrophenol isomers to varying degrees indicating a concerted effort towards breakdown of the substrate. Among them an unreported culture *Sarcina maxima* (SNP-8) was studied in detail for its ability in transforming the substrates. The culture followed both oxidative and reductive mechanisms in degrading the isomers.

Intensive NMR studies revealed the pathways followed by the consortium and the bacterial isolate *Sarcina maxima* based on the metabolites identified. ONP was degraded by the consortium and *Sarcina maxima* with catechol as the initial metabolite asserting the involvement of monooxygenase activity. Muconolactone was a common intermediate though not a dead end metabolite. γ-hydroxy muconicsemialdehyde and β-hydroxy maleylacetae, a new metabolite hitherto unreported was identified in the reaction mixture of the consortium and the individual culture respectively.

A variation between the consortium and *Sarcina maxima* could be observed in the initial catabolism of MNP. The consortium seemed to breakdown MNP to 4-aminocatechol which undergoes enzyme catalyzed removal of nitro group to give β-ketoadipate via 1,2,4-benzenetriol. Alternatively *Sarcina maxima* converted MNP to 2-nitrohydroquinone, not substantially proved in previous reports. The 2-nitrohydroquinone can undergo one-electron reduction to give 2-hydroxyl aminohydroquinone which is further degraded to ammonium and later mineralized by ring cleaving enzymes.

The initial reaction in the degradation of PNP by the consortium was clearly a monooxygenase catalyzed hydroxylation of the ring forming 4-nitrocatechol which undergoes an oxygenase catalyzed removal of the nitro group with the formation of 1,2,4-benzenetriol as evident by the enzyme activity and presence in the reaction mixture. In contrast 4-nitrocatechol was not detected in the reaction mixture of *Sarcina maxima*, instead γ-hydroxy muconicsemialdehyde, maleylacetate and β-ketoadipate were observed. Therefore PNP breakdown by this culture was expected to go through the formation of p-hydroquinone.

The investigation aided substantially in understanding the degradative ability and pathway followed by the consortium and the bacterial culture *Sarcina maxima.*

SCOPE

Because of the environment problems caused by nitroaromatic compounds, a potent microbial consortium or a combination of competent constituent cultures of a consortium could be employed in bioremediation technologies of natural water, soil and in treatment of industrial and sewage waste water.

- Nitroaromatic degrading microorganism may also be applied in the biocatalytic production of industrially valuable compounds from relatively cheap substrates which may be difficult to synthesis chemically.

- Enzymes in the degradative pathway could be employed in degrading related nitroaromatic compounds as some of them possess broad substrate specificity and such reactions may also lead to the formation of selected products

- Whole cells or cell preparations could be used in the production of biosensors employed in the study of pesticide residue analysis.

- The substrate range of bacteria may be broadened by genetic manipulation of the degradative pathway in order to treat closely related compounds

- Adaptation and tolerance to several related compounds could be used as an advantage in the treatment of a particular mixture of pollutants.

- Cultures able to tolerate varying concentrations and culture conditions could be employed in fermentors with IBT (immobilized bacteria technology) to withstand high chemical loading.

- Employment of a consortium with two antagonistic activities-reductive and oxidative, is an advantage and can especially be used in treatment of heterogeneous wastes.

SUMMARY

Nitroaromatic compounds are pollutants resulting from numerous industrial and agricultural activities. Some compounds are produced by incomplete combustion of fossil fuels, others are used as synthetic intermediates in the manufacture of dyes, plasticizers, pesticides, explosives and solvents As a consequence, nitroaromatic compounds have become pollutants in rivers, wastewaters, groundwaters, soils and the urban atmosphere.

The presence of the nitro group causes such compounds to be more resistant to biodegradation than the unsubstituted analogs and they tend to accumulate in the environment and cause deleterious effects to the biological systems due to their toxicity. Fresh and marine waters, sewage and soils possess highly diverse microbial communities that exhibit degradative capacities and species within these communities transform many organic compounds aerobically or anaerobically. A mixed bacterial system isolated from a contaminated soil sample was employed during the present study on individual and simultaneous degradation of mononitrophenol isomers ONP, MNP and PNP. The study is recorded in the following seven chapters as follows-

Chapter 1 consists of a brief introduction to nitroaromatic compounds and detail review of literature regarding the biodegradation of these compounds both aerobically and anaerobically by various microorganisms and specially bacteria. Emphasis is also laid on the different mechanisms involved in degradation. Factors that affect biodegradation such as carbon and nitrogen sources, inorganic nutrients, acclimation period, inoculum size, substrate concentration, adaptation, varying pH and temperature conditions have been dealt with. It includes a brief introduction the characteristics of the mononitrophenol isomers and the objective of studying their degradation.

Chapter 2 encompasses all the materials and methods employed during the degradative studies of mononitrophenol isomers. Analytical procedures like calibrations and estimation of growth of cultures, estimation of phenol, cresols, nitrophenol isomers, ammonia and nitrite have been dealt with. A separate section , Bacteriology, deals with the enrichment, isolation of mixed bacterial system and its constituent cultures, their characterization and identification.

Chapter 3 deals mainly with degradation using a consortium. The mixed bacterial culture successfully degraded individual isomers of mononitrophenol and their mixture. The consortium degraded subtoxic concentrations ($<$ 0.5mM) of ONP, MNP and PNP by 54h, 12h and 36h on short periods of induction. Nitrite was released during ONP and PNP degradation indicating an oxidative mechanism whereas production of ammonia during MNP breakdown suggested a reductive mechanism. Simultaneous degradation of a mixture of the isomers occurred by 18h during which more than 90% substrate disappearance was observed. Differential rates of degradation of the three isomers from a mixture indicated that MNP was catabolized faster than PNP and ONP as evident by HPLC. Only nitrite release could be recorded during simultaneous degradation of subtoxic concentrations probably due to the presence of two sources of nitrite production in the form of ONP and PNP in the mixture. Pre-growth of the consortium on related aromatic compounds such as isomers of cresol and phenol drastically affected the rate of degradation of all the mononitrophenol isomers. Pre-growth on non-aromatic compound such as sodium acetate resulted in retardation of degradation of ONP, MNP and PNP. Pre-exposed cells of the consortium degraded ONP, MNP and PNP by 24h whereas non-exposed cells could catabolize only 50% in the same period. Presence of an additional nitrogen source (NH_4NO_3) did not affect the degradation rates. The consortium could tolerate toxic concentrations (0.5mM-3mM) and bring about complete degradation of 1.5mM concentrations.

Chapter 4 highlights the degradative abilities of the individual bacterial cultures of the consortium. Eight bacterial cultures were isolated from the consortium and they were identified as *Bacillus licheniformis* (SNP-1)*, Xanthomonas maltophila* (SNP-2)*, Serratia*

liquefaciens (SNP-3), *Pseudomonas putida* (SNP-4), *Pseudomonas* sp. (SNP-5), *Psuedomonas alcaligenes* (SNP-6), *Psuedomonas* sp. (SNP-7) and *Sarcina maxima* (SNP-8) of which *Sarcina maxima*, an unreported culture was employed for degradation studies.

All the cultures except SNP-4 and SNP-7 showed around 50% reduction in substrate concentration by 96h. Cultures SNP-2, SNP-5 and SNP-6 brought around 90% reduction in substrate concentration within 96h followed by SNP-3, SNP-8 and SNP-1 which degraded 50% of the mixture. SNP-4 and SNP-7 could degrade only about 20% of the isomers. Well induced cells of *Sarcina maxima* exhibited both oxidative and reductive mechanisms in degrading mononitrophenol isomers as evident by the release of nitrite and ammonia respectively. Interestingly cells induced for a long periods could degrade around 50% of 0.5mM of a mixture of the isomers while cells induced for comparitively short periods showed only 34% reduction in substrate concentration.

Chapter 5 includes the assays adopted to study the enzymatic activities of cell extracts of the mixed bacterial culture. Dioxygenase activities were observed in cell extracts of the consortium induced with ONP, MNP and PNP separately. Pronounced catechol 1,2-dioxygenase activity was observed in ONP and PNP induced cell extracts indicating an ortho cleavage pathway in their degradation during which catechol was oxidized to cis, cis-muconic acid. Catechol 2,3-dioxygenase activity could be recorded only in MNP induced cell extracts and an absorbance maxima at 375nm consistent with the formation of γ -hydroxy muconicsemialdehyde was observed

Chapter 6 is an extensive study on the biochemistry of the pathways employed by a mixed bacterial culture and one of its constituent culture *Sarcina maxima*, hitherto unreported, using NMR spectroscopy. Intensive ^{1}H NMR and 2D HMQCT studies revealed the pathways followed by the degrading consortium and the single culture *Sarcina maxima*. ^{1}HNMR spectra of the ONP sample degraded by the consortium revealed the presence of metabolites such as catechol, cis, cis-muconic acid, γ -hydroxy

muconicsemialdehyde, maleylacetate and β-ketoadipate. The spectra of ONP reaction mixture degraded by *Sarcina maxima* showed that formation of maleylacetate from γ-hydroxy muconicsemialdehyde should go through a new metabolite β-Hydroxy maleylacetate, hitherto unreported. A deviation in MNP degradation of the consortium and the single culture was observed. The consortium seemed to breakdown MNP to 4-aminocatechol indicating it came from 3-hydroxyl aminophenol, 1,2,4-benzenetriol and β-ketoadipate were the other metabolites. *Sarcina maxima* seemed to convert to 2-nitrohydroquinone as indicated by its presence along with γ-hydroxy muconicsemialdehyde, muconolactone and maleyl acetate. The pathway followed by the consortium during PNP degradation was by the formation of 4-nitrocatechol, maleylacetate and β-ketoadipate which was confirmed by the ^{1}H NMR spectra. Both ^{1}H NMR and Carbon-13 signals from 2D HMQCT confirmed the presence of maleylacetate, γ-hydroxy muconicsemialdehyde and β-ketoadipate in the PNP reaction mixture of S*arcina maxima*. The pathway is expected to go through the formation of p-hydroquinone as the initial metabolite as no 4-nitrocatechol was detected.

Chapter 7 draws conclusions from the degradation studies of mononitrophenol isomers carried out by the mixed bacterial culture and the individual culture *Sarcina maxima* (SNP-8) and lists the scope for future research in the field of biodegradation studies.

REFERENCES

Abril MA, Michan C, Timmis KN and Ramos JL (1989). Regulator and enzyme specificities of the TOL plasmid encoded upper pathway for degradation of aromatic hydrocarbons and expansion of the substrate range of the pathway.
J. Bacteriol. 171: 6782-90

Alexander M (1967). The breakdown of pesticides in soils in "Agriculture and the Quality of our Environment" (Brady NC, ed.), *Amer. Assoc. Advan. Sci.,* Washington D.C. 331

Alexander M (1979). In "Microbial degradation of Pollutants in Marine environments" (Bourquin AW and Pritchard PH, eds.), *United States* E*nvironment Protection Agency,* Gulf Breeze, Flor. p. 67

Ali Sadat S and Walia SK (1996). Biodegradation of nitrobiphenyl and nitrotoluene by *Pseudomonas putida*. *Abstr. Gen. Meet. Am. Soc. Microbiol.*
(Vol 15, No. 11); (1995) 95, Meet, 437

Angermaier L and Simon H (1983). On nitroaryl reduactase activities in several C*lostridia*. *Hoppe Seylers Z. Physiol. Chem*. 364: 1653-64

APHA (1989). Standard methods for the examination of water and wastewater. 17th ed. *American Public Health Association.* Washington D.C.

Babu KS, Ajith-Kumar PV and Kunhi AAM (1995). Mineralization of phenol and its derivatives by *Pseudomonas* sp. strain CP4. *W. J. Microbiol Biotechnol*.
11: 661-64

Bamberger E (1894). Über das Phenylhydroxy amine. *Chem. Ber*. 27: 1548-57

Baughmann GL, Paris DF and Stun WC (1980). Quantitative expression of biotransformation rate. pp. 105-111. In Maki AW, Dickinson KL and Cairns J. Jr (ed).

Biotransformation and fate of chemicals in the aquatic environment. *American Society for Microbiology*. Washington, D.C.

Baumann E and Herter E (1877-78). *Hoppe-Seyl. Z*. 1: 244. In Robinson et al., (1951) Studies in Detoxication.39. Nitro Compounds. *Biochem. J.* 50: 221

Bayly RC and Barbour MG (1984). The degradation of aromatic compounds by the meta and gentisate pathways. pp. 253-294. In Gibson DT (ed). Microbial degradation of organic compounds *Marcel Dekker*, Inc., New York

Beland FA, Heflich RH, Howard PC and Fu PP (1985). The in vitro metabolic activation of nitropolycylic aromatic hydrocarbons. In *Polycyclic hydrocarbons and Carcinogenesis, ACS Symposium Series*, ed. Harvey RG, pp. 371-96. Washington, DC: *Am. Chem. Soc*.

Bhushan B, Chauhan A, Samanta SK and Jain RK (2000). Kinetics of biodegradation of p-Nitrophenol by different bacteria. *Biochem. Biophys. Res. Commun*. 274: 626-30

Blasco R and Castillo F (1992). Light dependent degradation of nitrophenols by the phototrophic bacterium Rhodobacter capsulatus E1F1. *Appl. Environ. Microbiol*. 58: 690-95

Blasco R and Castillo F. (1993). Characterization of a nitrophenol reducatse from the phototrophic bacterium *Rhodobacter capsulatus* EIFI. *Appl. Environ. Microbiol.* 59: 1774-78

Boethling RS and Alexander M (1979). Effect of concentration of organic chemicals on their biodegradation by natural microbial communities.
Appl. Environ. Microbiol. 37: 411-18

Bollag JM and Russel S (1976). Aerobic versus anaerobic metabolism of halogenated anilines by a *Paracoccus* sp. *Microbiol. Ecol*. 3: 65-73

Boopathy R and Kulpa CF (1992). Trinitrotoluene (TNT) as a sole nitrogen source for a sulphate-reducing bacterium *Desulfovibrio* sp. (B. Strain) isolated from an anaerobic digestor. *Curr. Microbiol*. 25: 235-41

Boopathy R and Kulpa CF (1993). Nitroaromatic compounds serve as nitrogen for *Desulfovibrio* sp. (B strain). *Can. J. Microbiol*. 39: 430-33

Boopathy R, Kulpa CF and Wilson M (1993). Metabolism of 2,4,6-trinitrotoluene (TNT) by *Desulfovibrio* sp. (B strain). *Appl. Microbiol. Biotechnol*. 39: 270-75

Bruhn C, Lenke H and Knackmuss H.-J. (1987). Nitrosubstituted aromatic compounds as nitrogen source for bacteria. *Appl. Environ. Microbiol*. 63: 208-10

Bryant C and De Luca M (1991). Purification and characterization of an oxygen-insensitive NAD(P)H nitro reductase from *Enterobacter cloacae*. *J. Biol. Chem*. 266: 4119-25

Bumpus JA, Tatarko M (1994). Biodegradation of 2,4,6-trinitrotoluene by *Phanerochaete chrysosporium*, identification of initial degradation products and the discovery of a TNT metabolite that inhibits lignin peroxidases.
Curr. Microbiol. 28: 185-90

Cain RB (1966). Induction of anthranitite oxidation during the metabolism of ortho-nitrobenzoate by certain bacteria. *J. Gen*. *Microbiol*. 42:197-217

Cain RB (1966). Utilization of anthranilic and nitrobenzoic acids by *Nocardia opaca* and a *Flavobacterium. J. Gen. Microbiol*. 42: 219-35

Callahan MA, Slimak MW, Gabel NW, May JP, Fowler CF, Freed JR, Jennings P, Durfee RL, Whitmore FC, Maestri B, Mabey WR, Holt BR and Gould C (1979). Water related environmental fate of 129 priority pollutants. EPA report- 440/4-79-029. *U.S. Environmental Protection Agency*, Washington, D.C.

Cartwright NJ and Cain RB (1959). Bacterial degradation of nitrobenzoic acids. *Biochem. J*. 71: 248-61

Chauhan A, Chakraborti AK and Jain RK (2000). Plasmid-encoded degradation of p-nitrophenol and 4-nitrocatechol by *Arthrobacter protophormiae*.
Biochem. Biophy. Res. 270: 733-740

Chauhan A, Samanta SK and Jain RK. (2000). Degradation of 4-nitrocatechol by *Burkholderia cepacia*: a plasmid-encoded novel pathway.
J. Appl. Microbiol. 88: 764-72

Corbett MD and Corbett BR (1981). Metabolism of 4-chlorinitrobenzene by the yeast *Rhodosporidium* sp. *Appl. Environ. Microbiol*. 41: 942-49

Corbett MD and Corbett BR (1995). Bioorganic chemistry of the arylhydroxyxylamine and nitrosoarene functional groups. In Spain JC (ed.) 1995. Biodegradation of nitroaromatic compounds. *Plenum Press*. New York, N.Y.

Cortez L, Chapalamadugu SN and Chaudhry GR (1989). The biodegradation of methylparathion and p-nitrophenol by a *Pseudomonas. Abstr. Annu. Meet. Am. Soc. Microbiol*. 89 Meet. 274

Crawford RL. (1995). Biodegradation of nitrated munition compounds and herbicides by obligately anaerobic bacteria. In Spain JC (ed.). Biodegradation of nitroaromatic compounds. pp. 87-98. *Plenum Press*, New York, N.Y.

Daughton CG and Hsieh D (1977). Parathion utilization by bacterial symbionts in a chemostat. *Environ*. *Microbiol.* 34: 175-84

Dickel O and Knackmuss H.-J (1991). Catabolism of 1,3-dinitrobenzene by *Rhodococcus* sp. QT-1. *Arch. Microbiol*. 157: 76-79

Dimkov R and Topalova Y (1994). Dependence of the microbiological degradation of aryl-containing xenobiotics on their concentration.
Acta-Microbiol. *Bulg.* 29: 9-16

Donlon BA, Razo-Flores E, Lettinga G. and Field JA (1996). Continuous detoxification, transformation and degradation of nitrophenols in upflow anaerobic sludge blanket (UASB) reactors. *Biotechnol. Bioeng*. 5: 439- 49

Dunnivant FM, Schwarzenbach RP and Macalady DL (1992). Reduction of substituted nitrobenzenes in aqueous solutions containing natural organic matter. *Environ Sci. Technol*. 26: 2133-41

Ecker S, Widmann T, Lenke H, Dickel O, Fischer P, Bruhn C and Knackmuss H-J. (1992). Catabolism of 2,6-dinitrophenol by *Alcaligenes eutrophus* JMP 134 and JMP 222. *Arch. Microbiol*. 158: 149-54

Eltis LD, Hofman B, Hecht H-J, Lunsdorf H, and Timmis KN (1993).
Purification and crystallization of 2,3-dihydrobiphenyl 1,2-diooxygenase.
J. Biol. Chem. 268: 2727-32

Engelhardt G, Wallnöfer P, Fuchsbichler G and Baumeister W (1977). Bacterial transformations of 4-chloroaniline. *Chemosphere*. 6: 85-92

Farrar CT and Becker ED (1971). Pulse and Fourier Transform NMR. Introduction to theory and methods. *Academic Press*, Inc., New York

Felset A, Maddox JV and Bruce W (1981). Enhanced microbial degradation of carbofuran in soils with histories of Furadan use. *Bull. Environ. Contam. Toxicol.* 26: 781-88

Fernando T, Bumpus JA and Aust SD (1990). Biodegradation of TNT (2,4,6-trinitrotoluene) by *Phanerochaete chrysosporium. Appl. Environ Microbiol*.
56: 1666-71

Fewson CA (1981). Biodegradation of aromatics with industrial relevance, pp. 141-79. In Leisinger T and Look AM (ed.), Microbial degradation of xenobiotics and recalcitrant compounds. *Academic Press*, London, United Kingdom

Folsom BR, Stierli R, Schwarzenbach RP and Zeyer J (1993). Comparison of substituted 2-nitrophenol degradation by enzyme extracts and intact cells. *Environ. Sci. Technol*. 28: 306-11

Fournier JC, Cadaccioni P and Soulas G (1981). Soil adaptation to 2,4-D degradation in relation to the application rates and the metabolic behaviour of the degradaing microflora. *Chemosphere*. 10: 977-84

Funk SB, Roberts DT, Crawford DL and Crawford RL (1993). Degradation of trinitrotoluene (TNT) and sequential accumulation of metabolic intermediates by an anaerobic bioreactor during its adaptation to a TNT feed. Abstr 93rd Annu. Meet, *Am. Soc. Microbiol*. p. 421

Funk SB, Roberts DJ, Crawford DL and Crawford RL (1993). Initial phase optimization for bioremediation of munition-contaminated soils.
Appl. Environ. Microbiol. 59: 2171-77

Germanier R and Wuhrman K (1963). Über den aeroben mikrobielen Abbau aromatischer Nitroverbindugen. *Pathol. Microbiol*. 26: 569-78

Gibson DT (1984). Microbiol degradation of organic compounds. ed. pp. 181-252. *Marcel Dekker*, New York

Gibson DT and Subramanian V (1984). Microbial degradation of organic compounds. ed. DT Gibson, pp.181-252. *Marcel Dekker*, New York

Gibson DT (1993). Biodegradation, biotransformation and the Belmont. *Journal of Industrial Microbiol.* 12: 1-12

Golab T, Bishop CE, Donoho AL, Manthey JA and Zornes LL (1975). Behaviour of ^{14}C oryzalin in soil and plants. *Pestic. Biochem. Physiol.* 5: 196-204

Golab T, Allthaus WA and Wooten HL (1979). Fate of [^{14}C] trifluralin in soil. *J. Agric. Food. Chem*. 27: 163-79

Gorontzy T, Küver J and Blotevogel KH (1993). Microbial transformation of nitroaromatic compounds under anaerobic conditions. *J. Gen. Microbiol*.
139: 1331-36

Groenewegen PEJ and de Bont JAM (1992). Degradation of 4-nitrobenzoate via 4-hydroxyl aminobenzoate and 3,4-dihydroxybenzoate in *Comamonas acidovorans* NBA-10. *Arch. Microbiol*. 158: 381-86

Grosjean D (1985). Reactions of o-cresol and nitrocresol with NO_2 in sunlight and with ozone-nitrogen dioxide mixtures in the dark. *Environ. Sci. Technol*.
19: 968-74

Grover R (1967). Studies on the degradation of 4-amino-3,5,6-trichloropicolinic acid in soil. *Weed Res*. 7: 61-67

Gunner HB and Zuckermann BM (1968). *Nature.* 217: 1183. . In Gibson DT (ed.) 1984, Biodegradation and the significance of microbial communities. Microbilogy series. Vol. 13, *Marcel Dekker Inc.* New York and Basel.

Haigler BE and Spain JC (1991). Biotransformation of nitrobenzene by bacteria containing Toluene degradative pathways. *Appl. Environ. Microbiol.* 57: 3156- 62

Haigler BE, Pettigrew CA and Spain JC (1992). Biodegradation of mixtures of substituted benzenes by *Pseudomonas* sp. strain JS150. *Appl. Environ. Microbiol.* 58: 2237-44

Haigler BE and Spain JC (1993). Biodegradation of 4-nitrotoluene by *Pseudomonas* sp. strain 4NT. *Appl. Environ. Microbiol.* 59: 2239-43

Haigler BE, Nishino SF and Spain JC (1994). Biodegradation of 4-methyl-5-nitrocatechol by *Pseudomonas* sp. strain DNT. *J. Bacteriol.* 176: 3433-37

Haigler BE, Wallace WH and Spain JC (1994). Biodegradation of 2-nitrotoluene by *Pseudomonas* sp. strain JS42. *Appl. Environ. Microbiol.* 60: 3466-69

Hanne LF, Kirk LL, Alger D, Woodruff T and Casadevall M (1991). Characterization of soil actinomycetes capable of degrading para-nitrophenol. *Abstr. Gen. Meet. Am. Soc. Microbiol*. 91 Meet, 303

Hanne LF, Kirk LL, Appel SM and Narayan AD (1992). Induction of p-nitrophenol (PNP) degradation in actinomycetes by structural analogs of PNP.
Abstr. Gen. Meet. Am. Soc. Microbiol. 92. Meet. 360

Hanne LF, Kirk LL, Appel SM, Narayan AD and Bains (1993). Degradation and induction specificity in Actinomycetes that degrade p-nitrophenol. *Appl. Environ. Microbiol*. 59: 3505-08

Hartter DR (1985). The use and importance of nitroaromatic chemicals in the chemical industry in "Toxicity of Nitroaromatic compounds" (Rickert DE. ed.) Chemical Industry Institute of Toxicology Series. *Hemisphere*. Washington. DC. pp. 1-14

Heijmann CG, Holliger C, Glauss MA, Schwarzenbach RP and Zeyer J (1993). Abiotic reduction of 4-chloronitrobenzene to 4-chloroaniline in a dissmilatory iron-reducing enrichment culture. *Appl. Environ. Microbiol*. 59: 4350-53

Heitkamp MA, Camel V, Reuter TJ and Adams WT (1990). Biodegradation of p-nitrophenol in an aqueous waste stream by immobilized bacteria. *Appl. Environ. Microbiol*. 56: 2967-73

Higson FK (1992). Microbial degradation of nitroaromatic compounds. *Adv. Appl. Microbiol.* 37: 1-19

Hlavica P (1982). Biological oxidation of nitrogen in organic compounds and deposition of N-oxidised products. CRS. *Crit. Rev. Biochem.* 12: 39-101

Hoffrichter M, Günther T and Fritsche W (1993). Metabolism of phenol, chloro and nitrophenol by the *Pencillium* strain Bi. 7/2 isolated from a contaminated soil. *Biodegradation.* 3: 415-21

Horakova D and Kotouchkova L (1996) Biotransformation of 4-nitrophenol by *Corynebacterium* sp. strain 8/3. *Int. Biodeterior. Biodeg.* 37: 249

Holt JG, Kreigh NR, Sneath PHA, Staley JT and Williams ST (eds.) (1994). Bergey's Manual of Determinative Bacteriology. 9th ed. *Williams and Wilkins*, Baltimore. pp. 554-51

Horvath RS (1972). *Bacteriol Rev*. 26: 146. In Gibson DT (ed.) 1984, Biodegradation and the significance of microbial communities. Microbiology series. Vol. 13, *Marcel Dekker* Inc. New York and Basel.

Hussain M, Enstch B, Ballou DP, Massey V and Chapman PJ (1980). Flouride elimination from substrates in hydroxylation reactions catalyzed by p-hydroxybenzoate hydroxylase. *J. Biol. Chem*. 255: 4189- 97

Jain RK, Dreisbach JH and Spain JC (1994). Biodegradation of p-nitrophenol via 1,2,4-benzenetriol by an *Arthrobacter. Appl. Environ. Microbiol*. 60: 3030-32

Javanjal SS and Deopurkar RL (1994). Biodegradation of p-nitrophenol by indigenously isolated bacteria from pesticide. *Indian J. Microbiol*. 13: 125-29

Jones SH and Alexander M (1988a). Effect of inorganic nutrients on the acclimation period preceding mineralization of organic chemicals in lake water. *Appl. Environ. Microbiol.* 54: 3177-79

Jones SH and Alexander M (1988b). Phosphorus enhancement of mineralization of low concentrations of p-nitrophenol by *Flavobacterium* sp. in lake water. *FEMS. Microbiol. Lett.* 52: 121-126

Kaake RH, Roberts DJ, Stevens TO, Crawford RL and Crawford DL (1992). Bioremediation of soils contaminated with the herbicide 2-sec-butyl-4,6-dinitrophenol (Dinoseb). *Appl. Environ. Microbiol*. 58: 1683-89

Kaake RH, Crawford DL and Crawford RL (1994). Optimization of an aerobic bioremediation process for soil contaminated with the nitroaromatic herbicide: Dinoseb (2-sec-butyl-4,6-dinitrophenol). In "Applied Biotechnology for Site Remediation", ed. Henchee RE, Anderson DB, Metting Jr. FB and Sayles GD. 337-41. Boca Raton: Lewis

Kadiyala V and Spain JC (1999). A two component monoxygenase catalyses both the hydroxylation of p-nitrophenol and the oxidative release of nitrite from nitro catechol in *Bacillus sphaericus* JS 905. *Appl. Environ. Microbiol.* 24: 79 - 84

Ke YH, Gee LL and Durham NN (1959). Mechanism involved in the metabolism of nitrophenylcarboxylic acids by microorganisms. *J. Bacteriol.* 77: 593-98

Kearney PC and Kaufman DD (1976). Herbicides: chemistry, degradation and mode of action. *Marcel Dekker.* Inc., New York

Keith LH and Telliard WA (1979). Priority pollutants.1. A perspective view. *Environ. Sci. Technol*. 13: 416-23

Kinouchi T and Ohnishi Y (1983). Purification and characterization of 1-nitropyrene nitroreductases from *Bacteroides fragilis. Appl*. *Environ*. *Microbiol.* 45: 1234-41

Kotouchkova L, Vavrik J, Nemec M, Plocek J and Zdrahal Z (1997). Use of immobilised cells of the strain *Corynebacterium* sp. for 4-nitrophenol degradtion. *Folia. Microbiol*. 42: 509-12

Kumar S, Mukerji KG and Lai R (1996). Molecular aspects of pesticide degradation by microorganisms. *Crit. Rev. Microbiol*. 22: 1-26

Larson JT (1979). Estimation of biodegradation potential of xenobiotic organic chemicals. *Appl*. *Environ*. *Microbiol*. 38: 1153-61.

Lenke H and Knackmuss H-J (1992). Initial hydrogenation during catabolism of picric acid by *Rhodococcus erythropolis* HL 24-2. *Appl*. *Environ*. *Microbiol*.
58: 2933-37

Lenke H, Pieper DR, Bruhn C and Knackmuss H-J (1992). Degradation of 2-4-nitrophenol by two *Rhodococcus erythropolis* strains HL 24-1 and HL 24-2. *Appl*. *Environ*. *Microbiol*. 58: 2928-32

Leung KT, Tresse O, Errampalli D, Lee H and Trevors JT (1997). Mineralization of p-nitrophenol by pentachlorophenol degrading *Sphingomonas* sp. *FEMS- Microbiol. Lett*.16: 4107-14

Loeser C, Ait-Oubelli M and Hertel T (1998). Growth kinectics of the 4-nitrophenol degrading strain *Pseudomonas putida* PNP1. *Acta*. *Biotechnol*.
1: 29-41

Marvin-Sikkema FD and de Bont JAM (1994). Degradation of nitroaromatic compounds by microorganisms. *Appl*. *Microbiol*. *Biotechnol.* 42: 499-507

Masse R, Badr M, Ayotte C and Sylvestre M (1985). Gas chromatographic-mass spectrometric characterization of bacterial metabolites of 4-nitrobiphenyl formed in gram negative strain B206. *Toxicol*. *Environ*. *Chem*. 10: 225-46

McCormick NG, Feeherry FF and Levinson HS (1976). Microbiol transformation of □, 4,6-trinitrotoluene and other nitroaromatic compounds. *Appl*. *Environ*. *Microbiol*. 31: 949-58

McCormick NG, Cornell JH and Kaplan AM (1978). Identification of biotransformation products from □,4-dinitrotoluene. *Appl*. *Environ Microbiol*.
35: 945-48

Meijers AP and vander Lur RC (1976). The occurance of organic micropollutants in the river Rhine and the river Mass in 1974. *Water Res*. 10: 597-604

Meulenberg RM, Pepi M and de Bont JAM. (1996). Degradation of 3-nitrophenol by *Pseudomonas putida* B2 occurs via 1,□ 4-benzenetriol. *Biodegradation*. 7: 303-11

Meyer E (1905). *Hoppe Seyl. Z*. 46: 497. In Robinson et al., (1951) Studies in Detoxication.39. Nitro Compounds. *Biochem. J*. 50: 221

Michels J and Gottschalk G (1994). Inhibition of the lignin peroxidase of *Phanerochaete chrysosporium* by hydroxyl amino-dinitrotoluene, an early intermediate in the degradation of 2,4,6-dinitrotoluene. *Appl*. *Environ Microbiol*. 60: 187-194

Mitra D and Vaidyanathan CS (1984). A new 4-nitrophenol from a *Nocardia* sp. *Biochem*. *Int*. 8: 609-15

Mohammed T, Bartha R and Antonie A (1994). Biodegradation of three nitrophenols and of 2-nitrobenzoate by *Pseudomonas cepacia. Gen*. *Meet*. *Am*. *Soc*. *Microbiol;* (1994) Meet. Vol. 13, Nov 19

Montgomery HAC and Dymock JF (1961). The determination of nitrite in water. *Analyst.* 86: 414

Munnecke DM and Hsieh DPH (1974). Microbiol decontamination of parathion and p-nitrophenol in aqueous media. *Appl*. *Microbiol*. 28: 212-17

Munnecke DM and Hsieh DPH (1976). Pathway of microbial metabolism of parathion. *Appl. Environ. Microbiol*. 31: 63-69

Nadeau LS and Spain JC (1995). The bacterial degradation of m-nitrobenzoic acid. *Appl*. *Environ*. *Microbiol*. 61: 840-43

Nakazawa T and Nakazawa A (1970). Pyrocatechase (*Pseudomonas) Methods*. *Enzymol*.17A: 518-522

Nelson LM (1982). Biologically induced hydrolysis of parathion in soil isolation of hydrolyzing bacteria. *Soil Biol. Biochem.* 14: 219-22

Nishino SF and Spain JC (1990). Adaptation to biodegradation of p-nitrophenol depends on accumulation of a metabolite. *Abstr*. *Annu*. *Meet*. *Am*. *Soc*. *Microbiol*. 90 Meet, 306

Nishino SF and Spain JC (1993). Cell density dependent adaptation of *Pseudomonas putida* to biodegradation of p-nitrophenol. *Environ*. *Sci*. *Technol*. 27: 489-94

Nishino SF and Spain JC (1993). Degradation of nitrobenzene by a *Pseudomonas pseudoalcaligenes. Appl*. *Environ. Microbiol*. 59: 2520-25

Nishino SF and Spain JC (1995). Oxidative pathway for the degradation of nitrobenzene by *Comamonas* sp. strain JS 765. *Appl*. *Environ. Microbiol.* 61:2308-13

Nozaki M (1970). Metapyrocatechase (*Pseudomonas). Methods*. *Enzymol.* 17A: 522-25

O'Brien RW and Morris JG (1971). The ferrodoxin-dependent reduction of chloramphenicol by *Clostridium acetobutylicum. J*. *Gen. Microbiol.* 67: 256-71

O'Connor OA and Young LY (1989). [Medcent, NewYork Univ., New York, NY 10016, USA]. *Environ. Toxicol. Chem*. 8 (10): 853-62

Oettingen and Von WF (1949). *Nat*. *Inst*. *Hlth. Bull.* No. 190: 232. In Robinson et al., (1951) Studies in Detoxication.39. Nitro Compounds. *Biochem. J*. 50: 221

Oren A, Gurevich P and Hems Y (1991). Reduction of nitrosubstituted aromatic compounds by the halophilic anaerobic *Haloanaerobacterium praevalens* and *Sporohalobacter marismortuii*. *Appl*. *Environ. Microbiol*. 57: 3367-70

Ou LT and Sharma A (1989). Degradation of methyl parathion by a mixed bacterial culture and a *Bacilllus* sp. isolated from different soils. *J*. *Agric. Food Chem*. 37, 6: 1514-18

Parrish FW (1977). Fungal transformation of 2,□-nitrotoluene and □.4,6-trinitrotoluene. *Appl*. *Environ. Microbiol*. 34: 232-33

Pemberton JM, Corney B and Don RH (1979). Evolution and spread of pesticide degrading ability among soil microorganisms pp. 287-99. In Timmis KN and Pühler A (ed.), Plasmids of medical, environmental and commercial importance. *Elsevier/North Holland Biomedical Press*, Amsterdam, The Netherlands

Peres CM, Naveau H and Agathos SN (1998). Biodegradation of nitrobenzene by its simultaneous reduction into aniline and mineralization of the aniline formed. *Appl. Microbiol. Biotechnol*. 49: 343-49

Peterson FJ, Mason RA, Hovespian J and Holtzman JL (1979). Oxygen sensitive and insensitive nitroreduction by *Eschericia coli* and rat hepatic microsomes.
J. *Biol. Chem*. 254: 4009-12

Petrova KP and Laha S (1995). Characteristics of nitrophenols degrading bacteria, isolated from various contaminated soils. *Abstr. Gen. Meet. Am. Soc. Microbiol.* 95, Meet, 437. (Vol. 15, No. 11)

Pieper DH, Timmis KN and Ramos JL (1996). Designing bacteria for the degradation of nitro and chloroaromatic pollutants. *Natur Wisenchaften Aufsatze*. 83: 201-203

Piet GJ and Smeenk JGMM (1985). Behaviour of organic pollutants in pretreated Rhine water during dune infiltration. p.144. In Ward CH, Giger HL and McCarty PL (ed.). Ground water quality. *John Wiley and Sons. Inc*., New York

Pitts JN, Lokensgard DM, Harger WP, Fischer TS, Meija V, Schuler JJ, Scorziell GM and Katzenstein YA (1982). Identification and direct activities of 6-nitrobenza (a) pyrene, 9-nitroanthracene, 1-nitropyerene and 5H-phenanthro [4,5-bed]-pyran-5-one. *Muta*. *Res*. 103: 241-49

Prakash D, Chauhan A and Jain RK (1996). Plasmid encoded degradation of p-nitrophenol by *Pseudomonas cepacia. Biochem*. *Biophys*. *Res*. *Commun*.
224: 75-81

Preuss A, Fimpel J and Dickert G (1993). Anaerobic transformation of 2,4,6-trinitrotoluene (TNT). *Arch*. *Microbiol*. 159: 345-53

Pritchard PH, Bourquin AW, Fredrickson HL and Maziarz T (1979). System design factors affecting environmental fate studies in microcosms. pp -251-72. In Bourquin AW and Pritchard PH (ed.), Microbial degradation of pollutants in marine environments: proceedings of the workshop. *U.S. Environmental Protection Agency*, EPA-600/9-79-012. Washington, D.C.

Rafii F, Franklin W, Heflich RH and Cerniglia CE (1991). Reduction of nitroaromatic compounds by anaerobic bacteria isolated from the human gastrointestinal tract. *Appl*. *Environ*. *Microbiol*. 57: 962-68

Ramadan MA, El Tayeb OM and Alexander M. (1990). Inoculum size as a factor limiting success of inoculation for degradation. *Appl*. *Environ*. *Microbiol*. 56: 1392-96

Ramanathan MP and LalithaKumari D (1999). Complete mineralization of methylparathion by *Pseudomonas* sp. A3. *Appl*. *Environ. Biochem*. *Biotechnol*. 80:1-12

Ray P, Oubelli MA and Loser C (1999). Aerobic 4-nitrophenol degradation by microorganisms fixed in a continuously working aerated solid-bed reactor. *Appl. Microbiol. Biotechnol*. 51: 284-90

Raymond DGM and Alexander M (1971). Microbial metabolism and co-metabolism of nitrophenols. *Pest*. *Biochem*. *Physiol.* 1: 123-30

Reiger PG and Knackmuss JH (1995). Basic knowledge and perspective on biodegradation of □,4,6-trinitrotoluene and related nitroaromatic compounds in contaminated soil. In Spain JC (ed.) 1995. Biodegradation of nitroaromatic compounds. *Plenum Press*, New York: pp.1-18

Rhys-Willlaims W, Taylor SC and Williams PA (1993). A novel pathway for the catabolism of 4-nitrotoluene by *Pseudomonas. J. Gen. Microbiol.* 139: 1967-72

Robertson BK and Alexander M (1991). Influence of calcium, iron and pH on phosphate availability for microbial mineralization of organic chemicals. *Appl. Environ. Microbiol*. 58: 38-41

Robinson D, Smith JN and Williams RT (1951). Studies in detoxification of 39 nitro compounds. *Biochem. J*. 50: 221-227

Roldon MD, Blasco R, Cabellero FJ and Castillo F (1997). Degradation of p-nitrophenol by the phototrophic bacterium *Rhodobacter capsulatus*.
Arch. Microbiol. 169: 36-42

Schackmann A and Müller R (1991). Reduction of nitroaromatic compounds by different *Pseudomonas* species under aerobic conditions. *Appl*. *Microbiol*. *Biotechnol*. 34: 809-13

Schenzle A, Lenke H, Fischer P, Williams RA and Knackmuss H-J (1997). Catabolism of 3-nitrophenol by *Ralstonia eutropha* JMP 134.
Appl. *Environ*. *Microbiol*. 63: 1421-27

Schnell S and Schink B (1991). Anaerobic aniline degradation via reductive deamination of 4-amino benzoyl-Co.A in *Desulphobacterium anilini*. *Arch*. *Microbiol*.155: 183-90

Schuetzle D (1983). Sampling of vehicle emissions for chemical analysis and biological testing. *Environ*. *Health Pesrpect*. 47: 65-80

Schwarzenbach RP, Stierli R, Folsom BR and Zeyer J. (1988). Compound properties relevant for assessing the environmental partitioning of nitrophenols. *Environ*. *Sci*. *Technol*. 22: 83-92

Scow KM, Schmidt SR and Alexander M (1989). Kinetics of biodegradation of mixtures of substrates in soil. *Soil Biol*. *Biochem*. 21: 703-08

Shine HJ (1967). The rearrangement of phenylhydroxylamines. pp.182-120. In Eaborn C and Chapman NB. (ed.), Reactions mechanisms in organic chemistry. *Elsevier Publishing Co*., Amsterdam, the Netherlands

Simon-Sylvestre G and Fournier JC (1979). Effects of pesticides on the soil microflora. *Adv. Agron.* 31: 1-81

Simpson JR and Evans WC (1953). The metabolism of nitrophenols by certain bacteria. *Biochem*. *J*. 55: XXIV

Slater JH (1978). In "The Oil industry and Microbial ecosystems". (Chapter KWA and Somerville HJ, eds.), *Heyden and Son, Ltd*. London. p. 137

Slater JH (1979) in "Microbial Degradation of pollutants in Marine Environments" (Bourquin AW and Pritchard PH, eds.), *United States Environmental Protection Agency,* Gulf Breeze, Fla., p. 283

Slater JH and Somerville HJ. (1979) in *Symp. Gen. Microbiol*. 29: 221. In Gibson DT (ed.) 1984, Biodegradation and the significance of microbial communities. Microbilogy series. Vol. 13, *Marcel Dekker Inc.* New York and Basel

Sone T, Tokuda Y, Sakai T, Shinkai S and Manabe (1981). Kinetics and mechanisms of the Bamberberger rearrangement. 3. Rearrangement of phenylhydroxylamines. *J. Chem. Soc. Perkin. Trans*: 1981: 298-302

Spain JC, Wyss O and Gibson DT (1979). Enzymatic oxidation of p-nitrophenol. *Biochem.. Biophys.. Res .Commun.* 88: 634-41

Spain JC, Pritchard PH and Bourquin AW (1980). Effects of adaptation on biodegradation rates in sediment/water cores from estaurine and freshwater environments. *Appl*. *Environ*. *Microbiol*. 40: 726-34

Spain JC, Zylstra GJ, Blake CK and Gibson DT (1989). Mono-hydroxylation of phenol and 2,5-dichlorophenol by toluene dioxygenase in *Pseudomonas putida* F1. *Appl. Environ. Microbiol*. 55: 2648-52

Spain JC and Gibson DT (1991). Pathways for biodegradation of p-nitrophenol in a *Moraxella* sp. *Appl. Environ. Microbiol*. 57: 812-19

Spain JC (1995). Biodegradation of nitroaromatic compounds. *Annu. Rev. Microbiol*. 49: 523-55

Spanggord RJ, Spain JC, Nishino SF and Mortelmane KE (1991). Biodegradation of 2,4-dinitrotolune by a *Pseudomonas* sp*. Appl. Environ. Microbiol.* 57: 3200-05

Spiker JK, Crawford DL and Crawford RL (1992). Influence of 2,4,6-trinitrotoluene (TNT) concentration on the degradation of TNT in explosive contaminated soils by the white rot fungus *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* 58: 3199-3202.

Stahl JD and Aust SD (1993). Plasma membrane dependent reduction of TNT by *Phanerochaete chrysporium. Biochem. Biophys. Res. Commu.*192: 471-76

Sternson LA and Gammans RE (1975). A mechanistic study of aromatic hydroxylamine rearrangement in the rat. *Bioorg. Chem*. 4: 58-63

Suen WC, Haigler BE and Spain JC (1994). 2,4-Dinitrotoluene dioxygenase gene from *Pseudomonas* sp. strain DNT: homology to naphthalene dioxygenase. *Abstr. 94th Annu. Meet. Am. Soc. Microbiol*. p. 458

Suzuki KP, Giomi T, Kaidoh T and Itagaki E (1991). Hydroxylation of o-halogenophenol and o-nitrophenol. *J. Biochem*. 109: 348-53

Tchelet R, Levanon D, Mingelgrin U and Hennis Y (1993). Parathion degradation by a *Pseudomonas* sp. and a *Xanthomonas* sp. and by their crude enxyme extracts as affected by some cations. *Soil Biol. Biochem.* 25: 1665-71

Tokiwa H and Ohnishi Y (1986). Mutagenecity and carcinogenecity of nitroarenes and their sources in the environment. *CRC. Crit. Rev. Toxicol.* 17: 23-60

Tortensson NTL, Stark J and Goransson B (1975). The effect of repeated applications of 2, 4-D and MCPA on their breakdown in soil. *Weed Res*.
15: 159-64

Traxler RW, Wood E and Delancy JM (1974). Bacterial degradation of alpha -TNT. *Dev. Ind. Microbiol*. 16: 71-76

Tseng SK and Yang CJ (1995). The reaction characteristics of wastewater containing nitrophenol, treated using an anaerobic biological fludized bed *Water-Sci. Technol*. 30: 233-40

Tseng SK, Haung SH and Huang SK. (1996). Anaerobic digestion of nitrophenol in the presence of glucose. *Meded. Fac. Landbouwwet. Rijksuniv. Gent*. 61: 2085-92

Tuovinen K, Kalislekorhonen E, Raushel FM and Hanninen O (1994). Phosphotriesterase- A promising candidate for use in detoxification of organophosphates. *Fundam Appl. toxicol.* 23: 578-84

Tweedy BG, Loeppky C and Ross JA (1970). Metabromuron: acetylation of the aniline moiety as a detoxification mechanism. *Science*. 168: 482-83

Uberoi V and Bhattacharya SK (1997). Toxicity and degradation of nitrophenols in anaerobic systems. *Water Environ. Res*. 69: 146-56

Villanaueva JR (1964). The purification of a nitroreductase from *Nocardia V*. *J. Biol. Chem*. 239: 773-76

Valli K, Brock BJ, Joshi DK and Gold ME (1992). Degradation of 2,4-dinitrotoluene by the liginin-degrading fungus *Phanerochaete chrysosporium. Appl. Environ. Microbiol*. 58: 221-28

Weisberger EK (1978). Mechanism of chemical carcinogenesis. *Annu. Rev. Pharamcol. Toxicol.* 18: 395-415

Wiggins BA and Alexander M. (1988). Role of chemical concentration and second carbon sources in acclimation of microbial communities for biodegradation. *Appl*. *Environ*. *Microbiol*. 54: 2803-07

Wiggins BA, Jones SH and Alexander M (1987). Explanations for the acclimation period preceeding the mineralization of organic chemicals in aquatic environment. *Appl*. *Environ*. *Microbiol*. 53: 791-96

Xing XH, Inoue T, Tanji Y and Unno H (1999). Enhanced microbial adaptation to p-nitrophenol using activated sludge retained in porus carrier particles and simultaneous removal of nitrite released from degradation of p-nitrophenol. *J. Ferment. Bioeng.* 87: 372-77

Xun L, Toppe E and Orser S (1992). Diverse substrate range of a *Flavobacterium* pentachlorophenol hydroxylase and reaction stoichemetrics. *J. Bacteriol*. 174: 2898-902

Zablotowicz RM, Lung KT, Alber T, Cassidy MB, Trevors JT, Lee H, Veldhuis L and Hall JC(1999). Degradation of 2,4-dinitrophenol and selected aromatic compounds by *Sphingomonas* sp. UG 30. *Can. J. Microbiol*. 45: 840-48

Zaidi BR, Murakami Y and Alexander M (1988). Factors limiting success of inoculum to enhance biodegradation of low concentrations of organic chemicals. *Environ. Sci. Technol.* 22: 1419-25

Zaidi BR, Murakami Y and Alexander M (1989). Predation and inhibitors in lake water affect the success of inoculation to enhance biodegradtion of organic chemicals. *Environ Sci. Technol*. 23: 859-63

Zaidi BR and Mehta N (1992). Effects of alternate substrates on the degradation of toxic chemicals in industrial waste water by the inoculated bacteria. *Appl. Gen. Meet. Am. Soc. Microbiol.* 92. Meet, 359

Zaidi BR and Mehta N (1994). The role of inoculum size in the success of inoculation to enhance biodegradation of toxic chemicals at high and low concentrations. *Abstr. Gen. Meet. Am. Soc. Microbiol*. 94 Meet, 407 (Vol. 134, No. 19)

Zaidi BR and Imam SH (1995). Isolation, purification and characterization of bacterial strains capable of enhancing degradation of p-nitrophenol (PNP) when inoculated into Barcelona industrial waste water. *Abstr. Gen. Meet. Am. Soc. Microbiol*. 95, Meet, 430

Zaidi BR, and Imam SH (1996). Inoculation of microorganisms to enhance biodegradation of phenolic compounds in industrial water. Isolation and identification of three indigenous bacterial strains. *J. Gen. Appl. Microbiol.*
42: 249-56

Zaidi BR, Imam SH and Greene RV (1996). Accelerated biodegradation of high and low concentrations of p-nitrophenol (PNP) by bacterial inoculation in wastewater: the role of inoculum size on acclimation period. *Curr. Microbiol.* 33: 292-96

Zeyer J and Kearney PC (1984). Degradation of o-nitrophenol and m-nitrophenol by a *Pseudomonas putida. J. Agric. Food. Chem*. 32: 238-42

Zeyer J and Kocher HP (1988). Purification and characterization of a bacterial nitrophenol oxygenase which converts ortho-nitrophenol to catechol and nitrite. *J. Bacteriol*. 170: 1789-94

Zeyer J, Kocher HP and Timmis N (1986). Influence of para-substituents on the oxidative metabolism of ortho-nitrophenol by *Pseudomonas putida* B2. *Appl. Environ. Microbiol*. 52: 334-39

Zhao JS and Ward OP. (1999). Microbial degradation of nitrobenzene and mono-nitrophenol by bacteria enriched from municipal activated sludge. *Can. J. Microbiol.* 45: 427-32

Zhao JS and Ward OP (2000). Cometabolic transformation of nitrobenzene by 3-nitrophenol degrading *Pseudomonas putida* 2NP8. *Can.. J. Microbiol*.
46: 643-52

Zhao JS, Singh A, Huang XD and Ward OP (2000). Biotransformation of hydroxyl aminobenzene and aminophenol by *Pseudomonas putida* 2NP8 cells grown in the presence of 3-nitrophenol. *Appl. Environ. Microbiol*. 66: 2336-42

Zoetmann BCJ, Harmsen K, Linders JBHJ, Morra CFH and Slooft W (1980). Persistent organic pollutants in river water and ground water of the Netherlands. *Chemosphere*. 9: 231-49