CHARACTERIZATION OF ACTIVATED SLUDGE BACTERIA CAPABLE OF GROWTH ON TOLUENE AND OTHER AROMATIC COMPOUNDS

A thesis submitted to
Dublin City University
in fulfilment of the requirements
for the award of the degree of
Doctor of Philosophy

by

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November, 1994.

Dedicated To My Family

DECLARATION

I hereby declare that all the work reported in this thesis was carried out by me at Dublin City University during the period of February, 1991 to October 1994.

To the best of my knowledge, the results presented in this thesis originated from the present study, except where references have been made. No part of this thesis has been submitted for a degree at any other institution.

Signature of Candidate : Nazmun Nahar Date : 16/12/94

ACKNOWLEDGEMENTS

I am greatly indebted to my supervisor, Dr.Brid Quilty for her valuable advice, wholehearted aid and encouragement at all stages of the work.

To Dr. Richard O'Kennedy, Head of School of Biological Sciences, gratitude for permitting this work to be carried out in the School of Biological Sciences.

I would like to acknowledge Bord na Mona, for providing funding in support of this research and to Bristol Myers Squibb (Ireland) Ltd. for the activated sludge sample.

I would like to thank Dr. Thecla Ryan, Dr. Michael O'Connell and their postgraduate students for their help and advice with the genetic work in this project. A special word of thanks to Brain Corcocran.

I must acknowledge the help of my fellow friend, Dr. Geraldine Mulcahy for her continuous cooperation during the course of this research.

Thanks are due to the staff, technicians and all postgrads of the Biology Department, DCU, for their help and advice.

To Professor M. S. J. Hashmi and his wife, Mrs. Kashmiri Hashmi, a special word of appreciation and gratitude for their help during my stay in Ireland.

I am grateful to Professor Naiyyum Choudhury, Head of the Department of Microbiology, University of Dhaka, Bangladesh for his advice and encouragement during my stay in Ireland.

A very special thanks to my parents, brothers and sister for their continuous support and encouragement throughout my education.

Finally, I would like to express my special thanks to my husband, Dr. Mohammed Alauddin who helped greatly in the preparation of this thesis and to my daughter, Sumaiya Sabah for her patient contribution.

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ABSTRACT

Characterization of activated sludge bacteria capable of growth on toluene and other aromatic compounds.

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A total of fifteen bacteria from activated sludge were isolated for their ability to grow on aromatic substrates as the sole source of carbon and energy. They were identified as members of the genera *Pseudomonas* and *Aeromonas*. Four *P. putida* spp. To-1, To-3, To-5, Na-13 and one *A. caviae* sp.To-4 capable of good growth on toluene were selected for further study.

All five bacteria were capable of growth on toluene, benzoic acid, *m*-toluic acid, ethylbenzene, pseudocumene, *m*- and *p*-xylene and *m*- and *p*-chlorophenol. *A. caviae* To-4 alone was capable of growth on phenol and naphthalene. The growth of these bacteria was investigated under different cultural conditions - solid culture, liquid culture, directly added toluene and in the presence of other carbon sources. Growth was better when toluene was supplied in the vapour phase. The toxic effect of directly added toluene was alleviated by the addition of a readily metabolizable carbon source and by the acclimation of the cells to toluene. Strains To-4 and To-5 were selected as representative strains of the genera to investigate the removal of toluene. Both of the strains behaved similarly in respect to the utilization of toluene and the utilization of toluene was dependent on the availability of toluene and oxygen. The optimum pH for growth of all the isolates in the presence of toluene was between pH 5.8 to pH 7.4.

All five isolates showed greater *meta* activity than *ortho* activity in degrading toluene. However following growth on non-aromatic substrates the *Pseudomonas* spp. showed *meta* activity and the *Aeromonas* sp. showed *ortho* activity. A large plasmid band (85 - 120 kb) was detected in all the *Pseudomonas* spp. following growth on aromatic and non-aromatic substrates. The strain *A. caviae* To-4 did not carry any plasmid following growth on non-aromatic substrates. This strain carried a large plasmid (85 - 120 kb) only when grown in the presence of toluene. The identification of the plasmid was confirmed by dot blot hybridization using a TOL probe. Restriction analyses of the plasmid DNA of *A. caviae* To-4 and *P. putida* To-5 were compared to that of the TOL plasmid of *P. putida* NCIB 10432 and the presence of specific sequences of the TOL plasmid were confirmed by Southern hybridization using both a digoxigenin-labelled and a radioactively labelled probe. The strains To-4 and To-5 carried an identical TOL plasmid which contained some of the sequences of the TOL plasmid of *P. putida* NCIB 10432.

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1. INTRODUCTION

1.1 Hydrocarbons - a source of pollution

We live in a time where chemicals play a vital part in our everyday life. The greatest contribution of scientific knowledge to human health and comfort has come from the use of chemicals. The production and uses of synthetic organic chemicals has increased enormously during the last thirty years. The use of synthetic organic chemicals in agriculture, medicine, nutrition and hygiene has resulted in an increased mean human longevity. This has put demands on increased food and fibre production and has made man more dependent on the use of synthetic chemicals (Chakrabarty, 1982)

The input of natural and man-made compounds to our ecosystem has increased markedly in the last two decades. It has been estimated that world-wide over 63,000 chemicals are in common use and between 200 and 1000 new synthetic chemicals are marketed each year (Moriarty, 1990). Aromatic compounds abound in nature, and are a vast potential source of industrial chemicals. The majority of these compounds are formed through pyrolysis of organic matter. These include lignin and its breakdown products, cell walls from algae and algal spores. Lignin, with its complex aromatic structure, is a potential source of aromatic chemicals. In contrast aromatic hydrocarbons formed during petrogenesis ($\approx$ 150°C) usually contain alkyl substituents. These naturally occurring aromatic compounds have been supplemented in recent years by a large number of synthetic chemicals used for a wide variety of purposes in the chemical industry. When however the activities of man raises their concentration above those found naturally in the environment they are classified as environmental pollutants (xenobiotic compounds). A list of different sources of aromatic hydrocarbons are presented in Table 1 (Gibson, 1989).

Table 1: Natural and xenobiotic sources of aromatic hydrocarbons in the environment

Natural
Coal
Petroleum
Forest and prairie fires
Xenobiotic
Combustion of coal and oil
Motor vehicle emissions
Coal liquifications and gasification procedures
Oil spills
Industrial contamination

Aromatic compounds of most concern are those which are industrially produced. The potential and actual impact of these xenobiotic compounds is difficult to predict or assess. Relatively little is known about their environmental chemistry or the ability of the biota to metabolize or degrade them. A recent European Economic Community inventory listed more than 100,000 different substances, 30,000 of which were considered to be a threat to the environment because they were bioaccumulative, recalcitrant and/or toxic (McEldowney *et al.*, 1993).

The challenge we face comes primarily, not solely, from synthetic chemicals. This challenge has been defined in terms of specific priority pollutants that are of special concern because of feared carcinogenic, mutagenic or teratagenic effects. Many aromatic derivatives can pose a health hazard. Most are toxic at relatively high concentrations and some are carcinogenic at relatively low concentrations. They are biochemically active and more irritating to mucous membranes than aliphatic and alicyclic hydrocarbons in equivalent concentrations.

Aromatic hydrocarbons and their derivatives are used in increasing amounts in a number of industrial operations such as the manufacture of chemical solvents, plastics production, and other petroleum based industries. Coal and petroleum are the starting materials for the synthesis of most organic chemicals, from the mid-1880s they have been the basis for the far-reaching petrochemical industry. Most of the aromatic hydrocarbon supply (> 70%) comes from petroleum with the remaider being derived from coal and imports (Young, 1984). Toluene is one of the major by-products of these aromatic compounds. It is present in gasoline at 5 - 7% (w/w) and has a solubility of 515 mg/l in water at 20°C (U.S Public Health Service, 1989). Increased use of petrochemicals by modern society has increased the amount of aromatic hydrocarbons found in air and soil samples. In 1988 the American Environmental Protection Agency found toluene in 54% of ground water samples and 28% of surface water samples near chemical waste sites (U.S Public Health Service, 1989). Significant contamination of ground water also arises from spillage of gasoline or other petroleum-based fuels and from leakage of gasoline from underground tanks. It is also used in plastics manufacture (Caglioti, 1983) and in the chemical industry as a solvent. Therefore, it is not surprising that toluene is a common hydrocarbon pollutant found in wastewater, polluted surface water, soil etc (Arcangeli and Arvin, 1993).

Little data are available on the quantities of toxic waste produced in Ireland and its disposal. Figures for the waste arising from the production of some hazardous organic compounds in Ireland were collated by An Foras Forbatha in 1986. The data are summarised in Table 2. This table highlights the quantities of organic solvents that constitute toxic waste. As the chemical and pharmaceutical industry has grown considerably in the last decade these figures must be considered an underestimate of the situation today and indeed this is reflected by Boyle in 1986 and more recently by the Department of the Environment (1992) who showed an increase from 52,500 tonnes of toxic waste produced in 1986 to 99,400 tonnes in 1992.

Table 2: Toxic and dangerous waste arising in Ireland (1984/85)

Waste category	Waste quantities (tonnes/annum)
Organic solvents	26,940
Biocides and phyto-pharmaceutical substances	7,680
Chlorinated solvents	6,368
Phenols	600
Organic-halogen compounds	17.4
(excluding other substances referred to this listing)	

Depending on the activity responsible for the release, pollutants may be released from isolated points at high concentrations e.g. metal smelters, or at low concentrations from many diffused sources e.g. leachate from domestic disposal sites. In the case of point sources the impact of the pollutants may initially be restricted to the immediate locality of the source, the concentration of the pollutant decreasing rapidly away from the source as it is dissipated and diluted in the environment. However, the dilution and dissipation of a pollutant from a locality rarely, if ever, completely eliminates or protects the environment from possible adverse effects. The entry of organic chemicals into the environment can be described as outlined in Figure 1 (Leisinger, 1983)

In this figure four streams of organic chemicals leading to pollution are identified :

(i) Chemicals whose use lead to their entry into the environment, e.g., aerosols, propellants, pesticides, fertilizers.

(ii) Chemicals entering the environment in the effluents of municipal sewage treatment systems, e.g., hard detergents, solvents.

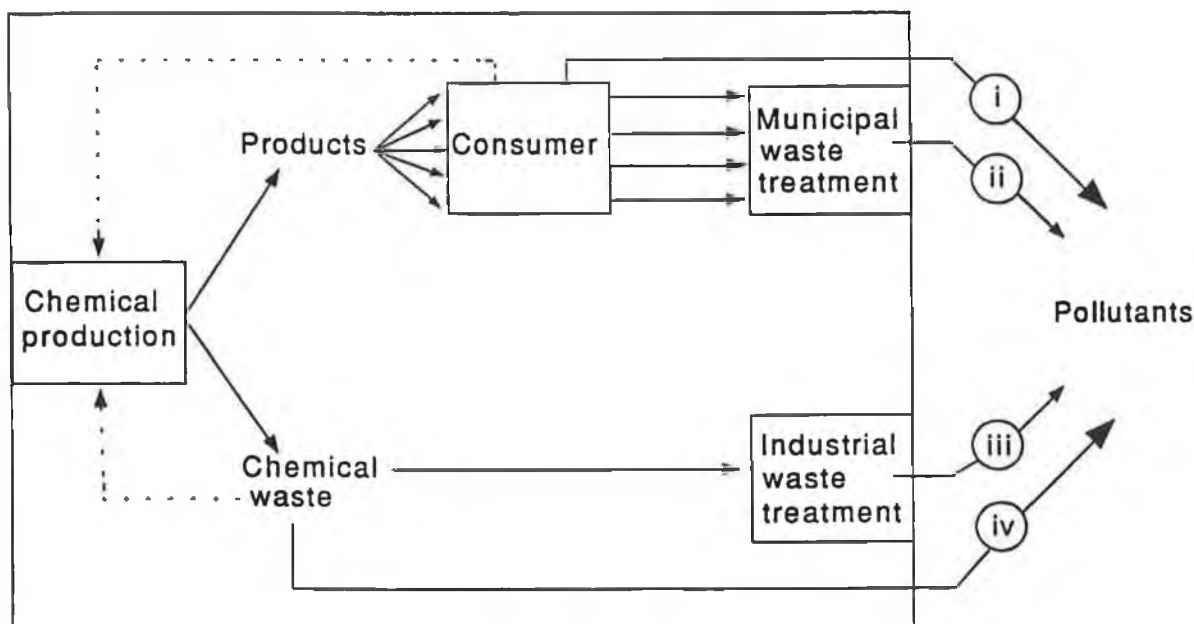


Figure 1: Release of organic chemicals into the environment

(iii) Chemicals resistant to biological degradation in industrial waste treatment systems, e.g., chlorobenzenes, aminonapthal sulfonic acids, and aromatic hydrocarbons.

(iv) Direct discharges, losses, spills and accidents leading to the entry of chemicals from production sites into the environment.

Increasing stringency of statutory regulations concerning public health and a growing involvement of mankind in environmental problems puts pressure on industry to pay more attention to the environmental aspects of industrial processes.

In recent years there has been considerable interest in the removal of these hazardous wastes which enter into the environment as a result of man's industrial and agricultural activities. There is concern among the general

public and environmentalists about traditional methods used to dispose of toxic waste such as incineration and landfill. Organic compounds can be removed from the environment by a variety of mechanisms including biodegradation, autooxidation, adsorption, sedimentation, hydrolysis and photodegradation. A number of physical, chemical and biological processes have been developed to achieve a highly efficient removal of hazardous compounds from the environment. Among these, biological processes are generally accepted to play the major role (Painter and King, 1985). The main advantages of this method are, many chemically different organic compounds can be oxidized to carbon dioxide and water; the method is cheap, reliable and kind to the environment. Among the treatment technologies biological degradation ranks the most effective. In Ireland most chemical plants treat their effluents in biological treatment systems (O'Flaherty, 1989).

Biological wastewater treatment provides a classic example of the development of successful, large-scale processes in a vital area of biotechnology resulting from the co-ordinate application of engineering and microbiology. Application of microbial degradation and removal of undesirable constituents in industrial and municipal wastewaters is not a new concept. There is a wide range of biological treatment systems in current use for the purification of domestic and industrial wastewater. As the awareness of the chemical contamination of the environment has greatly increased, much research on biological degradation of toxic chemicals has occurred. Of the many agents involved, bacteria are thought to be the most important in biodegradation processes. Bacteria are ubiquitous and their size, specific growth rates, metabolic versatility and mode of life make them eminently suitable for this function.

1.2 Aerobic biological processes involved in the treatment of industrial wastewater

Biological treatment processes for wastewater can be either aerobic or anaerobic in nature. However, the aerobic processes are most commonly used.

Waste chemicals may be volatile and consequently should be considered for treatment both in the gaseous and liquid phases. The most widely used biological waste treatment process for the liquid phase is the activated sludge process. More recently biofilters have been used for the biological treatment of the gaseous phase.

1.2.1 Activated sludge

The activated sludge process was originally developed by Ardern and Lockett in 1914 and since then has been improved through a number of engineering and operation developments. Activated sludge processes are continuous flow, dispersed, flocculated growth processes in which the bacterial flocs, described as activated sludge, are separated from the treated effluent by sedimentation in a clarifier and recycled to the aeration tank where they are recontacted with the wastewater undergoing treatment (Hamer, 1985). Successful operation is dependent on the capability of the biomass (sludge) to assimilate the waste and convert it into cells and suspended solids, and to form a floc particle that will settle (Chambers, 1982).

Ng *et al.*, (1989) pointed out that the activated sludge process is widely used for the purification of pharmaceutical wastewater. The composition of wastewater varies according to the pharmaceutical product being manufactured. They described the synthetic wastewater of a pharmaceutical company which contained a mixture of organic compounds among which were 2-ethylhexanoic acid, methylene chloride, triethylamine, methylisobutylketone, acetone and propanic acid and their biological treatment was investigated. Kilroy and Gray (1992) described four commonly used toxic solvents ethylene, glycol, methanol and isopropanol in pharmaceutical wastewater being treated by the activated sludge process.

The principal genera of bacteria found in activated sludge along with their postulated role are outlined in Table 3 (Horan, 1990). In the activated sludge process, the most widely occurring and abundant group of microorganisms are

Table 3: Reactions carried out by the principal genera of bacteria found in activated sludge

Genus	Function
Pseudomonas	Removal of carbohydrate, slime production, denitrification
Zoogloea	Slime production, floc formation
Bacillus	Protein degradation
Arthrobacter	Carbohydrate degradation
Microthrix	Fat degradation, filamentous growth
Nocardia	Filamentous growth, foaming and scum formation
Acinetobacter	Phosphorus removal
Nitrosomonas	Nitrification
Nitrobacter	Nitrification
Achromobacter	Denitrification

the bacteria, and it is this group which are most important in terms of utilizing the organic matter present in wastewater, although fungi, protozoans, and a range of other invertebrate organisms also play important but comparatively minor roles. Bacteria are the most versatile of all the organisms associated with wastewater treatment in terms of conditions under which they can thrive and the substrates they can metabolize. It is the small size of bacteria and their resultant large surface area to volume ratio which makes them so efficient, in terms of nutrient and catabolic exchange, with the liquid medium in which they are either suspended or are in contact. Their short doubling times, which can be as little as 20 minutes in pure culture, enable bacteria to rapidly take advantage of increased substrate availability compared with other organisms (Gray, 1989).

Biomass composition is determined by both the waste characteristics and

process operation parameters. The biological treatment of most waste streams depends on the 'ubiquity principle' which states that bacteria are ubiquitous and the most effective species of microorganisms for a given environment will eventually establish themselves in that environment (Zachopoulos and Hung, 1990). The bacterial population which arises in a bioreactor as a result of physiological and genetic adaptation is unique from the point of species diversity (Lange *et al.*, 1987). Large number of organisms from different species and genera may be present displaying a wide range of degradative abilities. The predominant microbes in the activated sludge process are chemoheterotrophic bacteria. The microbes best studied are those with the ability to grow as either microbial aggregates or those able to become directly associated with such aggregates so as to be recycled.

The most important hydrocarbon degrading bacteria in waste treatment systems are basically - *Pseudomonas, Aerobacter, Alcaligens, Bacillus, Corynebacterium, Comamonas, Mycobacterium* as well as others (Hamer, 1985). Among these, the genus *Pseudomonas* is dominant in aerobic sewage and wastewater treatment processes. Much of their degradative ability is due to the possession of various types of plasmids (Chakrabarty, 1976). Much research work has been carried out on the genus *Pseudomonas* and in particular the strain *Pseudomonas putida,* which carries genes for the degradation of toluene on the TOL plasmid. A number of bacteria have been reported to posses the ability to degrade toluene such as *Pseudomonas putida* (Worsey and Williams, 1975), *Pseudomonas aeruginosa* (Kitagowa, 1956), a *Pseudomonas* sp. (Claus and Walker, 1964), *Achromobacter* sp, (Worsey and Williams, 1975) and a *Nocardia* sp. (Raymond *et al.*, 1967).

A number of environmental factors affect the activity of the wastewater microbial populations and the rate of biochemical reactions generally. Of particular importance are temperature, pH, dissolved oxygen, nutrient concentration and inhibition by toxic compounds (McClure *et al.*, 1991). It is possible to control all these factors within a biological treatment system in order to ensure that microbial growth continues under optimum conditions.

Temperature has a profound effect on wastewater microorganisms, not only in governing the rate of reaction, but changes in temperature also give rise to significant alterations in the community structure. The majority of biological treatment systems operate in the mesophyllic temperature range, growing best in the temperature range 20°C - 40°C. The pH and dissolved oxygen concentration in a biological reactor can be controlled to any level by the operator. The optimum pH range for carbonaceous oxidation lies between 6.5 - 8.5 pH. A dissolved oxygen concentrations between 1 - 2 mg/l is significant for active aerobic heterotrophic microbial activity, although optimum growth is dependent on sufficient essential nutrients and trace elements being present. The optimum feed composition for effective biotreatment in industrial wastewater is a BOD_5 : N:P ratio of 100:5:1, a pH between 6.0 and 8.5 and temperature within the mesophyllic range for microbial growth (Clark and Clough, 1971).

In a system treating toxic or inhibitory waste, bacterial diversity is reduced by the increased selective pressure on the population due to the presence of inhibitory compounds. Reducing the types of bacteria present can diminish the genetic ability to adapt to changes in the environment or waste composition. As a result hazardous waste treatment processes are often plagued by upsets and are unable to degrade new compounds entering the waste stream (Lange *et al.*, 1987).

1.2.2 Biofiltration

Many of the organic chemicals present in industrial effluent are volatile and may escape biological treatment in the activated sludge process due to loss in the gaseous phase. Recently, interest has been focused on a relatively new technology for the treatment of volatile substances; the technique is known as biofiltration. Biofiltration was first developed in Germany in the late 1970's and early 1980's and has been used to deodorize gaseous effluents, and to remove toxic pollutants, including volatile organic carbons (VOC's) from industrial effluents. Biofiltration has proved to be a technique for air

pollution control for many different industrial gases. Several authors described biofilters to treat waste gas streams containing a cocktail of pollutants (Ottengraf *et al.*, 1986; Ottengraf, 1987; Joe and Dragt, 1988; Bohn, 1992).

The technique is based on the ability of microorganisms (generally bacteria, and to a small extent moulds and yeasts) to degrade organic as well as inorganic compounds to mineral end-products, like water and carbon dioxide under aerobic conditions. In the case of biofiltration, microorganisms are attached to a suitable packing material.

The microbial composition of a biofilter will vary depending on the type of pollutant being treated, the condition under which the filter is operated and its situation, and the packing materials used. The packing materials of biofilters are usually of an organic nature (peat, compost, soil etc.).

According to Joe and Dragt (1988), and Ottengraf *et al.* (1986), biofiltration compares very favourably to other methods of waste treatment of gases.

1.2.3 Bioaugmentation of waste treatment processes

Bioaugmentation or biomass enhancement is the addition of selected organisms to a treatment system in order to improve its efficiency. The abilities of the selected organisms can be improved by mutation or genetic engineering. The addition of genetically engineered microorganisms to a treatment plant constitutes a release of such organisms to the environment as most plants are open systems with final effluents being discharged into local waterways and bioreactors that are open to the atmosphere. There is considerable public and legislative opposition to the release of engineered organisms, therefore most companies producing bacterial suppliments prefer to use more traditional techniques for improving their strains (Zachopoulos and Hung, 1990).

Bioaugmentation has two main purposes : to minimize and if possible eliminate the lag period (acclimation period) and to improve decomposition of the waste (Golueke and Diaz, 1989). Bioaugmentation can increase the biological diversity and activity of a population by adding bacteria with enzymatic systems which allow degradation of previously non-biodegradable organics or by adding bacteria which have higher metabolic rates (Lange *et al.*, 1987). An increase in bacterial diversity increases the gene pool available to the population in times of stress. The ability of the organisms to transfer DNA to the indigenous population may be of equal or greater importance than the survival of the organisms itself.

The benefits obtained by the use of bioaugmentation can be summarized as follows :

- improved floc formation and settling
- increased rates of waste assimilation and versatility in substrate uptake
- decreased sludge solid yields due to a more efficient breakdown of the colloidal material
- rapid establishment or restoration of the biological activity in the waste treatment process that has experienced a start-up, an upset or chlorination of the return sludge
- increased stability and tolerance to facillating growth conditions
- odour control
- lower operating and energy costs (Chambers, 1982)

Both activated sludge and biofilters may augmented with suitable microorganisms.

1.3 Identification of *Pseudomonas* and *Aeromonas* species

1.3.1 Pseudomonads

The ubiquity of pseudomonads in natural habitats and their remarkable nutritional versatility has led to their being the subject of extensive research.

The biodegradation of various organic compounds including aromatics has been studied at the biochemical and genetic level with a view to understanding and improving the degradation of these compounds in the environment. The application of selected and adapted *Pseudomonas* species to polluted soils or water may improve the removal of pollutants from the environment and their addition to waste treatment systems may improve the efficiency to treatment before effluents are discharged to the environment.

The term pseudomonad is commonly used to described rod-shaped, Gram-negative, non-sporulated, polarly flagellated bacteria. Pseudomonads form a vast and heterogenous group of organisms. The genus *Pseudomonas*, which was created by Migula in 1894, is the most important of the many genera of bacteria possessing the above characteristics (Palleroni, 1986). Members of the genus *Pseudomonas* occupy a dominant position in the biosphere in terms of variety of habitat and the number of species in a given habitat. *Pseudomonas* species are found in large numbers in all major natural environments, terrestrial, freshwater and marine, and in many different associations with plants and animals (Clark and Slater, 1986).

Members of this genus are described in Bergey's Manual of Systematic Bacteriology (Palleroni, 1984) as being straight or slightly curved rods, 0.5 - 1 µm in the diameter and 1.5 - 5 µm in length. Species are motile by polar flagella, rarely non-motile although non-motile strains of various species are occasionally isolated from nature. *Pseudomonas* species are strictly aerobic and chemeoorganotrophic. The optimum growth temperature for most strains is 28°C but many are capable of growth in the range of 4 - 43°C.

Pigments of various chemical types are produced by many *Pseudomonas* species. They can be soluble in water and freely diffusible into culture media or they can remain associated with the cells. The best known of the soluble pigments are the fluorescent pigments of some members of the RNA group 1. The fluorescent pseudomonads include *P. aeruginosa, P. fluorescence* and *P. putida*. These pigments act as siderophores, strong iron chelators, and allow

growth in media having a low iron content. Another important soluble pigment is pyocyanin, a phenazine derivative characteristic of *P. aeruginosa.* Among the water soluble pigments that remain closely associated with cellular structures or diffuse only to a limited extent are several carotenoids and phenazine compounds.

Pseudomonas species are subdivided on the basis of RNA homology. There are five RNA groups. The best known species of the genus are included in group I. *P. aeruginosa* is by far the best known especially in relation to medical microbiology. *P. fluorescence* and *P. putida* are other well known members of RNA group 1. Both are complex species and can be subdivided into a number of biovars or biotypes. *P. pseudomellei* and *P. cepacia* belong to the RNA group II and are isolated from soil and water (Palleroni, 1986). *P. luteola* belongs to the RNA group V (Costas *et al.*, 1992).

One of the most striking properties of members of this genus is their remarkable nutritional versatility. Organic compounds readily used by *Pseudomonas* species include alcohols, aliphatic acids, amides, amines, amino acids, aromatic compounds, carbohydrates and hydrocarbons. This ability to utilize a wide range of compounds makes *Pseudomonas* species an important component of activated sludge particularly that treating wastewater derived from the chemical industry. Of the various substrates used as the sole carbon and energy source aromatic compounds are particularly interesting due to the biochemical intricacies of the various pathways by which they are metabolized (Palleroni. 1986). Pseudomonads are readily isolated that can use aromatic compounds as their major source of energy for growth since these compounds are widely distributed in natural environments.

1.3.2 Aeromonads

Aeromonas species are commonly found in a wide range of aquatic systems and foods and have been isolated from coastal waters and lakes, (Dumontet, 1990) rivers, (Blaise and Armstrong, 1973) drinking water, (Krovecek *et al.*,

1992) and a variety of foods (Buchanan and Palumbo, 1985). Motile *Aeromonas* species occur widely in water, sludge and sewage. These organisms have been isolated occasionally from apparently healthy people. However, a fecal origin can not explain the presence of these bacteria in surface water or sewage. Motile *Aeromonas* spp. have long been recognised as the causal agent of "red-leg" in amphibians and motile *Aeromonas* species were reported to be pathogenic for humans when wounds were exposed to polluted water (Davis *et al.*, 1978).

Members of this genus are described in Bergey's Manual of Systematic Bacteriology as fermentative, Gram-negative, oxidase-positive, catalase-positive, non-sporulated rods and motile by a single polarly located flagellum. They are straight rod-shaped, 0.3 - 1.0 μm in diameter and 1.0 - 3.5 μm in length. They are resistant to the vibriostatic agent 2,4-diamino-6,7-diisopropyl petridine (0/129). *Aeromonas* species are facultatively anaerobic and chemoorganotrophic, using a variety of sugars and organic acids as carbon sources. The optimum growth temperature of motile *Aeromonas* species is 28°C. Some strains can grow at 5°C. The maximum temperature at which growth occurs is usually 38 - 41°C.

Members of the genus *Aeromonas* are now clearly differentiated from members of the *Enterobacteriaceae* and from members of the genera *Pseudomonas* and *Vibrio* (Popoff, 1984)). Traditionally, the family *Vibrionaceae* comprised fermentative, catalase - negative and oxidase positive, aquatic, Gram - negative bacteria which, if motile, possessed polar flagella. Such organisms were assigned to the four genera, *Aeromonas, Plasiomonas, Photobacterium* and *Vibrio* (Baumann and Schubert, 1984). Profound taxonomic changes ensued with the advent of nucleic acid homology/sequence studies. Thus it has been proposed to transfer *Aeromonas* to the newly established family *Aeromonadaceae* (Colwell *et al.*, 1986).

Aeromonas is the type genus of the family *Aeromonadaceae* (Colwell *et al.*, 1986). The genus has hitherto included the non-motile species *A. media* (Allen

et al., 1983) and *A. salmonicida* (Popoff 1984). Motile species, which have undergone many revisions in their classifications, include *A. caviae, A. hydrophila* and *A. sobria* (Popoff, 1984).

1.4 Cultivation of bacteria on volatile aromatic compounds

It has been known for many years that several aromatic hydrocarbons can be utilized by bacteria as sole carbon and energy sources for growth. The ability of bacteria to utilize aromatic hydrocarbons for growth was first demonstrated in 1908 by Stormer, who isolated *Bacillus hexacarbivoraun* by virtue of its ability to grow with toluene and xylene. In 1913, Sohangen reported the utilization of benzene by microorganisms and one year later Wagner isolated two organisms capable of growth with toluene, benzene and xylene. Gray and Thornton (1928) isolated from soil two strains of *Mycobacterium agreste* that could use toluene, and Tausson (1929) described four species, *Bacterium toluolicum* a, b, c and d, which grew with toluene and some other benzenoid hydrocarbons as the sole carbon source. Tausson also established the conditions for the laboratory cultivation of bacteria which degraded toluene and other hydrocarbons

Kitagawa (1956) studied the oxidation of toluene and related compounds by a strain of *Pseudomonas aeruginosa* which had been grown in nutrient broth and afterwards exposed for some hours to small concentrations of toluene, benzyl alcohol, benzaldehyde or benzoic acid in phosphate buffer solutions. He concluded that toluene was possibly oxidized through this series of intermediates. Claus and Walker (1964) reported two unidentified species of *Pseudomonas* and *Achromobacter,* which grew with toluene, benzene or certain other related compounds as sole carbon source when isolated from soil. They oxidized toluene through 3-methyl catechol. The cultural conditions for bacteria growing on toluene or other liquid hydrocarbons were also described. Toluene was supplied in the vapour phase to the bacteria both for agar plates (solid culture) and shaken flasks (liquid culture).

The medium used for growth of *Pseudomonas* species is usually a minimal salts medium with the addition of an appropriate organic compounds. Slight alterations may be made depending on the experimental reason for growth but in general the composition of the minimal salts medium is as described by Stanier *et al.*, (1966). Basal inorganic media are used and when required, of agar (approx. 2%).

There are a variety of different cultural methods which can be used to grow bacteria in the presence of volatile organic compounds. The methods of Tausson which he described in "Method de Diffusioszufusses" (1929) were later described by Claus and Walker (1964) to grow bacteria on toluene.

Agar plate cultures (solid culture) were incubated in a closed desiccator, over a saturated aqueous solution of toluene with water. Thus toluene was supplied as a vapour phase saturated atmosphere in the desiccator.

For shaken-flasks cultures, a small test-tube containing liquid toluene was suspended inside the flask. Alternatively an open glass tube (5 - 6 mm) was inserted through the cotton wool plug, dipped into the medium in the flasks and a few drops of toluene introduced into this tube. Toluene thus diffused as a vapour or by solution into the medium without liquid toluene itself coming in direct contact with the bacteria.

Toluene may be supplied to larger culture (5 - 10 litres) or continuous culture apparatus as described by Skinner and Walker (1961) by aerating them with air previously saturated with water and toluene vapours. Alternatively the vapour of the hydrocarbon can be introduced by passing air from a compressor containing the hydrocarbon into the culture vessel.

Gibson (1976) initially tried to isolate bacteria by enrichment culture where the aromatic hydrocarbon was added directly to the minimal salts medium. This procedure was unsuccessful and subsequently a strain of *Pseudomonas putida* was isolated by introducing ethylbenzene vapour in the shaken-flasks

culture as the sole source of carbon. This strain could utilize benzene, toluene and ethylbenzene as sole carbon and energy source.

Toluene like many other organic solvents, is highly biotoxic when added directly to the growth medium and kills most organisms at low concentrations (0.1%) (Inoue *et al.*, 1991). It is often used therefore to sterilize microbial cultures. The physiological basis of such solvent toxicity, however, remains poorly characterised. Toxicity is apparently due to the interaction of toluene with the cytoplasmic membrane, leading to the loss of the cations Mg^{2+} and Ca^{2+} , as well as other small molecules (de Smet *et al.*, 1983).

1.5 Biodegradation

Biodegradation can be defined as the breakdown of a compound by a living organism. Ultimate biodegradation is the complete breakdown of a compound to fully oxidized simple molecules eg. CO_2, H_2O, NO_3^-, NH_4^+ with the formation of new cells (Painter and King, 1985). The extent of biodegradation of a compound in the environment can vary. The susceptibility of a compound to biodegradation is a function of a number of parameters. These include the chemical structure, the potential of the compound to associate with other organic and inorganic material in the environment and many physio-chemical factors such as pH , temperature, oxygen tension and salinity (Neilson *et al.*, 1985). As well as these many factors acclimation is an important step when considering the biodegradation of a recalcitrant compound.

Acclimation is a period of physiological, morphological and genetic adaptation of the biomass to a new environment. Adaptation can be either phenotypic or genotypic. In phenotypic adaptation the genetic information of the cell is unchanged but the degree of expression of certain genes is altered. This may be common during short term fluctuations in the environment. Genotypic adaptation involves changes in the genetic information of the cell and these changes are transmitted to daughter cells. There are two basic methods of genetic change : mutation and recombination. Mutation is a change in the

nucleotide sequence of the DNA occurring within an individual cell. Recombination is any process whereby genes from two separate cells are brought together into the one cell, for example, transformation, conjugation, transduction and genetic engineering. Genotypic adaptation may be common during chronic changes in the environment (Senthilnathan and Ganczarzyk, 1989)

The length of time required for acclimation to various pollutants can range from a few days to several weeks. As there is little or no detectable mineralization during acclimation it is important to minimize the time required. The length of the acclimation period can be affected by the presence of a secondary carbon source or the rarity of the microbes that can degrade certain chemicals. The presence of inhibitory compounds or a high enough concentration of a chemical to inhibit those microbes capable of metabolizing it will also affect the time needed for acclimation (Wiggins and Alexander, 1988). When acclimated organisms are subjected to a different environment they tend to adapt to the new environment and as a result may lose the ability to grow at the expense of the original substrate. Phenotypic adaptations are temporary and therefore more rapidly lost than genotypic adaptations. This process of adaptation of acclimated biomass may influence the biodegradability of organic pollutants which are discharged intermittently (Senthilnathan and Ganzarczyk, 1989). For such industries which produces a variety of products on staggered schedules it has been suggested that a background level of the pollutant in the influent would help to maintain the bacterial population responsible for its mineralization (Eckenfelder, 1989).

A wide range of microorganisms have been found to possess biochemical pathways for the partial or complete degradation of a number of synthetic and naturally-occurring aromatic hydrocarbons. Among the most widely suited catabolic pathways are those found in the genus *Pseudomonas*. *Pseudomonas* appeared to have a remarkable potential to evolve entire catabolic sequences for xenobiotic compounds. This evolutionary flexibility may reflect the diversity of enzymes and pathways that have evolved to

degrade naturally occurring organic compounds and may also involve genetic rearrangements within and between strains (Clarke and Slater, 1986).

1.5.1 Biodegradative pathways of aromatic compounds

Many different biodegradative pathways converge on a common intermediate - catechol. The central role of catechol in the oxidation of aromatic compounds is shown in Figure 2. Catechol undergoes subsequent degradation by one of the two pathways : the *ortho*-cleavage pathway or the *meta*-cleavage pathway. Cleavage of the bonds between adjacent carbon atoms that carry hydroxyl groups is known as "*ortho*" or "intradiol" cleavage and the pathway by which the product of such cleavage is metabolised is known as *ortho* or β-ketoadipate pathway. In the second mode of cleavage of the benzene nucleus, attack occurs between two carbon atoms, only one of which carries a hydroxyl group, the other carbon atom being either unsubstituted or substituted with other than a hydroxyl group. In this case the hydroxyl group may be either *ortho* or *para* to one another, and the enzymes catalyzing such cleavage again usually designated by the position of the carbon bond attacked. This type of cleavage is known as *meta* cleavage and the pathway by which products of such cleavage are metabolised is called the *meta* pathway. In some instances catechol is metabolized by the *ortho* pathway, in others the *meta* pathway, while some bacteria have the genetic capability to degrade it by both pathways. In the later situation, the precursor of catechol, on which the microorganisms are grown apparently determines which pathway is used or predominates (Gibson, 1984).

The *ortho* pathway dioxygenases for catechol are red and contain tightly bound ferric iron; the *meta* pathway enzymes are colourless and require ferrous iron, which is usually more easily removed (Hayaishi, 1966). The *meta* pathway predominates in nature as the majority of aromatic compounds, especially methyl-substituted aromatics are degraded via the *meta* pathway while benzoate and chlorinated aromatics are metabolised via the *ortho* pathway (Williams and Murray, 1974). The *ortho* cleavage is not normally

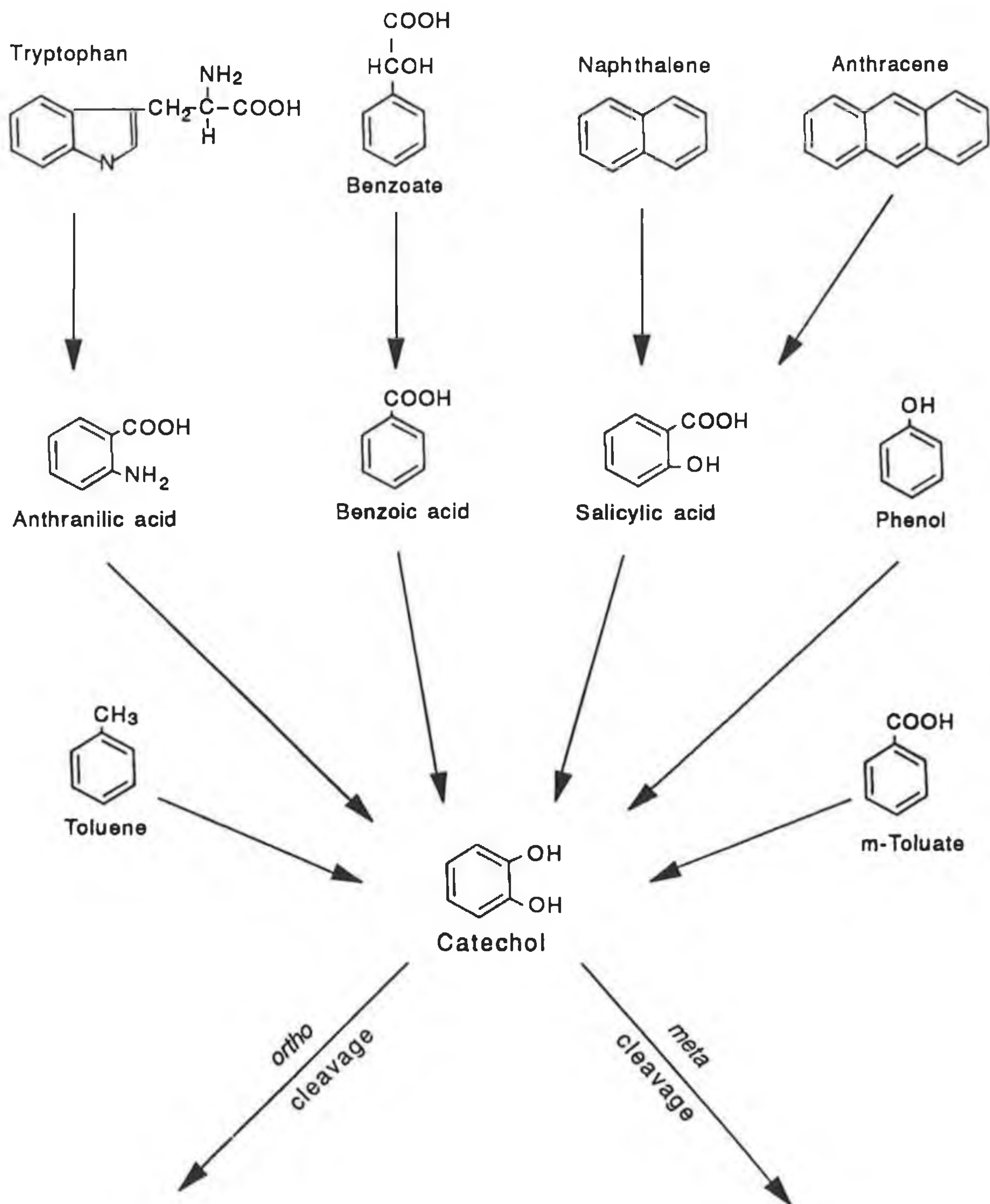


Figure 2: The cntral role of catechol in the oxidation of aromatic compounds by *Pseudomonas* spp. (Feist and Hegeman, 1969).

expressed in cells containing the plasmid encoded *meta* pathway due to the differences in the regulation of the two pathways (William and Worsey, 1976).

The induction of the *meta* pathway is substrate specific while the *ortho* pathway is product specific (Ornston and Stanier, 1966). *Cis, cis*-muconate, the initial product of the *ortho*-fission of the aromatic ring induces the enzymes of the *ortho* pathway. The enzymes of the *meta* pathway are induced by the primary substrate such as toluene, phenol etc. The activity of the *meta* enzymes prevents the accumulation of catechol and therefore the formation of *cis, cis*-muconate (Feist and Hageman, 1969).

The *Ortho*-cleavage pathway

The *ortho*-cleavage or β-ketoadipate pathway degrades catechol via β-ketoadipate to succinate and acetyl Co A (Figure 3). The structural genes for the enzymes involved are designated *cat* ABC and *pca* D and are shown in Figure 3. Initial cleavage of the aromatic ring of catechol is catalyzed by catechol 1,2-dioxygenase. This reaction is induced by the product, *cis, cis*-muconate and is also quite sensitive to catabolite repression (Wu *et al.*, 1972). Catechol 1,2-dioxygenase consists of two non-identical subunits with a single active site. The ferric form of iron is the sole co-factor (Nozaki, 1979). All three enzymes of the catechol branch share a common inducer, *cis, cis*-muconate. *Cis, cis*-Muconate lactonizing enzyme (4-carboxy methyl-4-hydroxyisocrotonolactone lyase) is a decyclizing enzyme catalyzing the conversion of *cis, cis*-muconate to muconolactone and is induced by *cis, cis*-muconate (Hegeman, 1966). Muconolactone isomerase, also induced by *cis, cis*-muconate, converts muconolactone to enol-lactone which is further metabolised to β-ketoadipate by enol-lactone hydrolase. The synthesis of muconolactone isomerase is subject to coordinate control (Ornston and Stanier, 1966). A transferase enzyme catalyzes the conversion of β-ketoadipate to β-ketoadipyl Co A which is then converted to succinate and acetyl Co A which feed directly into the TCA cycle. Enol-lactone hydrolase and transferase are induced by β-ketoadipyl Co A (Wheelis and Ornston, 1972).

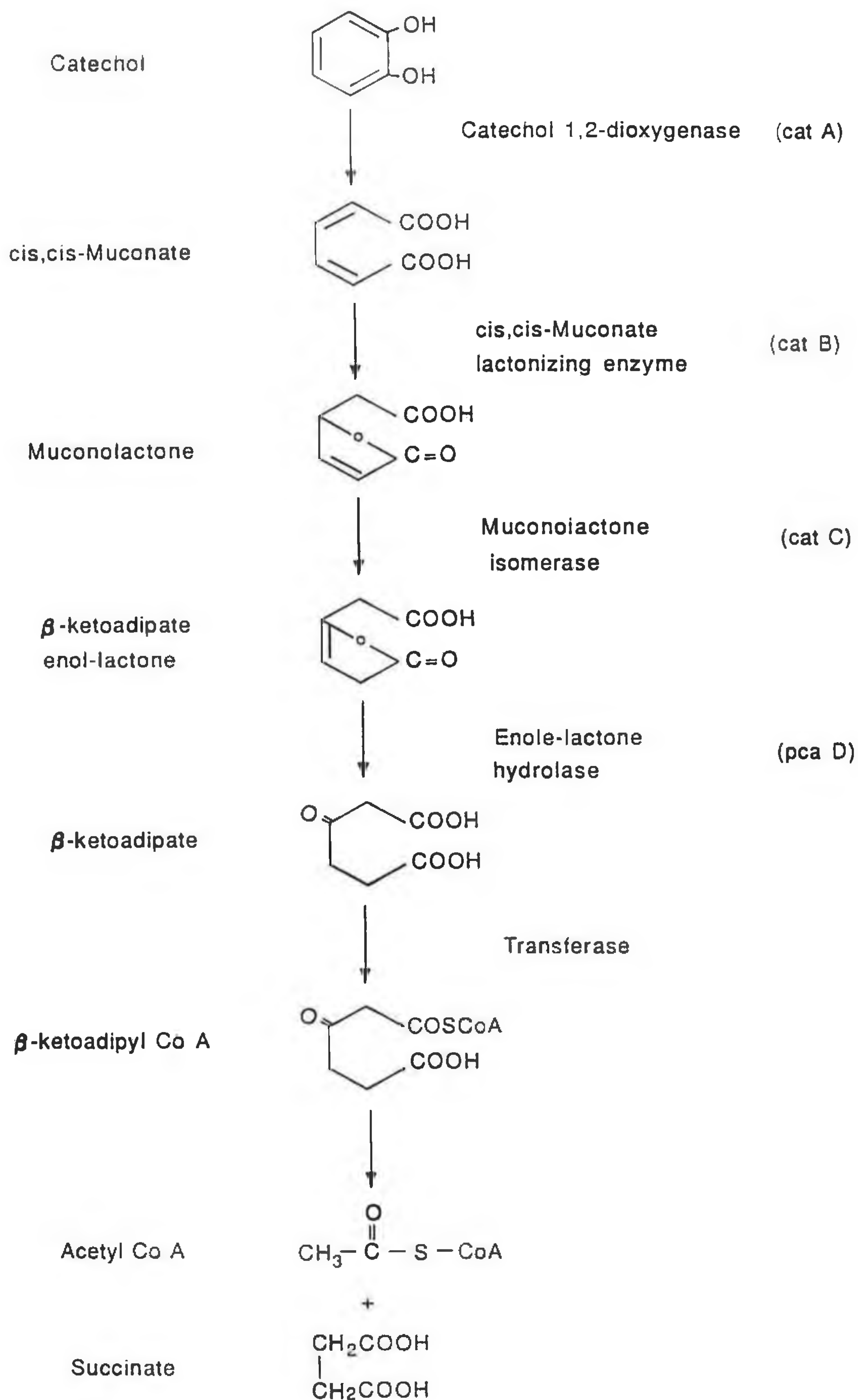


Figure 3: The *ortho* cleavage pathway in Pseudomonads (Wu *et al.*, 1972; Wheelis and Ornston, 1972)

The *ortho*-cleavage pathway is chromosomally encoded. The structural genes for muconate-lactonizing enzyme (*cat* B) and muconolactone isomerase (*cat* C) have been shown to lie in close proximity on the chromosome. These enzymes share a common inducer (*cis, cis*-muconate)and their synthesis is co-ordinately controlled. Catechol 1,2-dioxygenase, though also induced by *cis, cis*-muconate, and its structural gene (*cat* A) are subject to independent control (Wu *et al.*, 1972).

A regulatory gene, *cat* R has been located to the left of *cat* B on the *Pseudomonas putida* chromosome. Mutation studies of *cat* R suggest that it exerts positive control on the expression of *cat* B and C. Mutations in *cat* R do not affect the induction of catechol 1,2-dioxygenase. As well as being induced in the presence of *cis* , *cis*-muconate, *cat* A is also subject to independent regulation by catabolic repression. This may be due to the role catechol 1,2-dioxygenase plays in the formation of *cis, cis*-muconate which acts as inducer for the subsequent enzyme reactions (Wu *et al.*, 1972).

The *Meta*-cleavage pathway

Catechol is metabolised via the *meta*-cleavage pathway to pyruvate and acetaldehyde (Figure 4). The genes encoding the enzymes of the *meta* pathway are designated *xyl* E-K and are shown in Figure 4. As with the *ortho* pathway the initial reaction involves fission of the aromatic nucleus of catechol. This is achieved by the action of catechol 2,3-dioxygenase to produce 2-hydroxy-muconic semialdehyde. The active form of catechol 2,3-dioxygenase contains ferrous iron as a prosthetic group (Harayama and Rekik, 1990). 2-Hydroxymuconic semialdehyde can be metabolised via either the hydrolytic or dehydrogenative pathway, depending on the substrates.

2-Hydroxymuconic semialdehyde is degraded by the NAD^+-dependent dehydrogenase to a 4-oxalocrotonate. The ring fission product of 4-methylcatechol, 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate (Figure 5) is also metabolised via the dehydrogenative pathway.

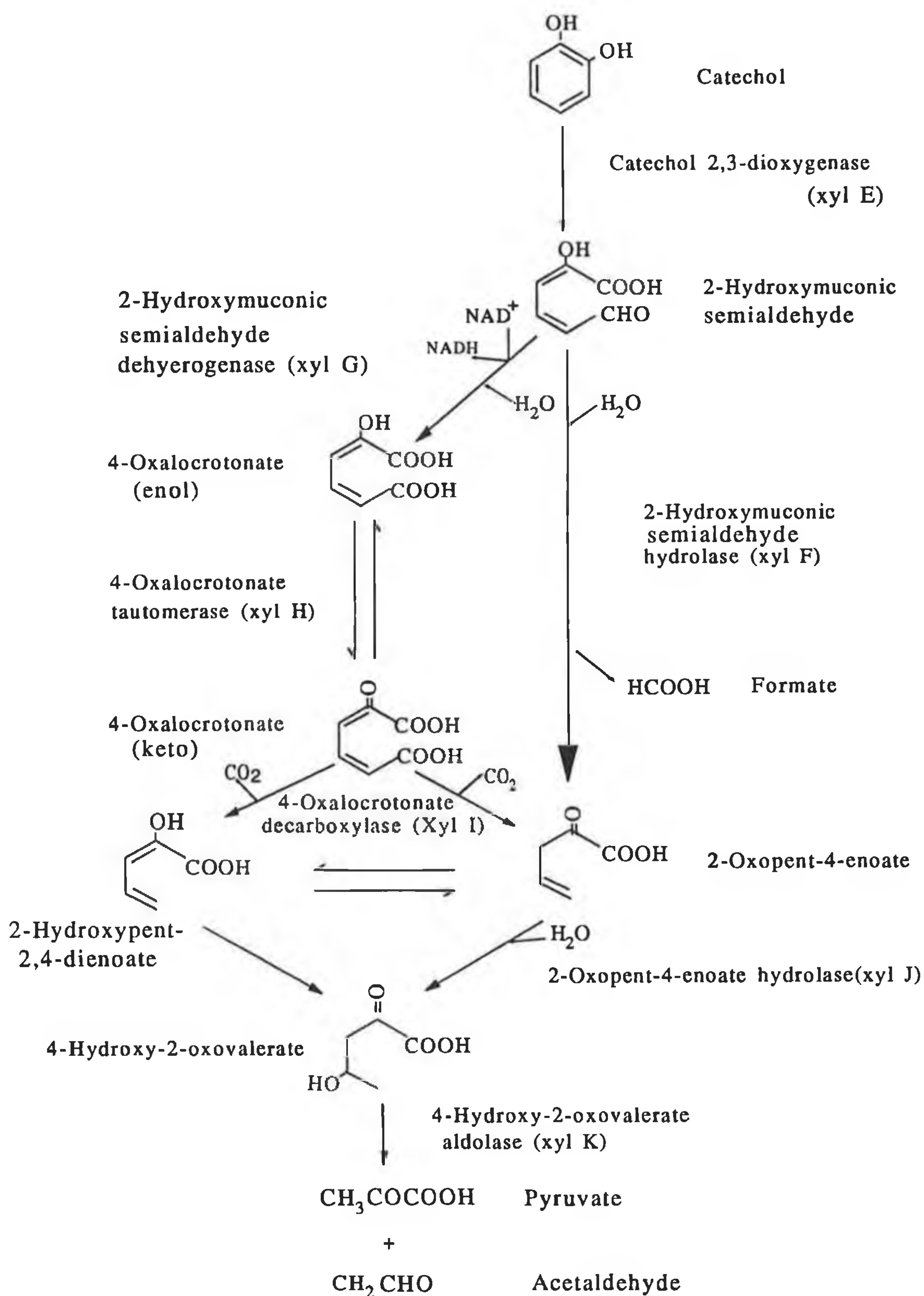


Figure 4: The *meta* cleavage pathway in Pseudomonads (Bayly and Barbour, 1984; Wigmore *et al.*, 1974; Harayama *et al.*, 1989)

The product of 3-methylcatechol cleavage, 2-hydroxy-6-oxohepta-2,4-dienoate (Figure 6), is metabolised by the hydrolase to 2-oxopent-4-enoate and formate.

The dehydrogenase is inactive against 2-hydroxy-6-oxohepta-2,4-dienoate as it lacks an oxidizable aldehyde group (Harayama *et al.*, 1989).

The product of the 2-hydroxymuconic semialdehyde dehydrogenase reaction is the enol for of 4-oxalocrotonate (2-hydroxyhexa-2,4-diene-1,6-dienoate). This is converted to the keto form (2-oxohexa-4-ene-1,6-dienoate) by 4-oxalocrotonate tautomerase. The keto form is the substrate for 4-oxalocrotonate decarboxylase resulting in the formation of 2-oxopent-4-enoate with the release of CO_2. Mg^{2+} is required for decarboxylase activity (Harayama and Rekik, 1990; Bayly and Barbour, 1984).

At this point the hydrolytic and dehydrogenative pathways converge. 2-Oxopent-4-enoate hydratase acts on 2-oxopent-4-enoate to form 4-hydroxy-2-oxovalerate. Harayama *et al.* (1989) found that the product of the decarboxylase was the enol compound 2-hydroxypent-2,4-dienoate and that this was spontaneously and reversibly transformed into 2-oxopent-4-enoate, the keto form. They found the enol form to be the substrate for the hydratase.

Their work also suggested that the hydratase and decarboxylase form a physical complex in vivo which may ensure the efficient transformation of the unstable intermediate 2-hydroxypent-2,4-dienoate. Finally, 4-hydroxy-2-oxovalerate aldolase catalyzes the conversion of 4-hydroxy-2-oxovalerate to the TCA intermediates, pyruvate and acetaldehyde.

The genes for the *meta*-cleavage pathway are organised into an operon, carried on a number of catabolic plasmids. There is a high degree of homology between the *meta*-cleavage operons of different plasmids. One of the best studied is that carried on the TOL plasmid, pWWO. The *meta* operon of pWWO comprises thirteen genes : *xyl* E, F, G, H, I, J, K which code for the structural genes of the enzymes responsible for the degradation of catechol

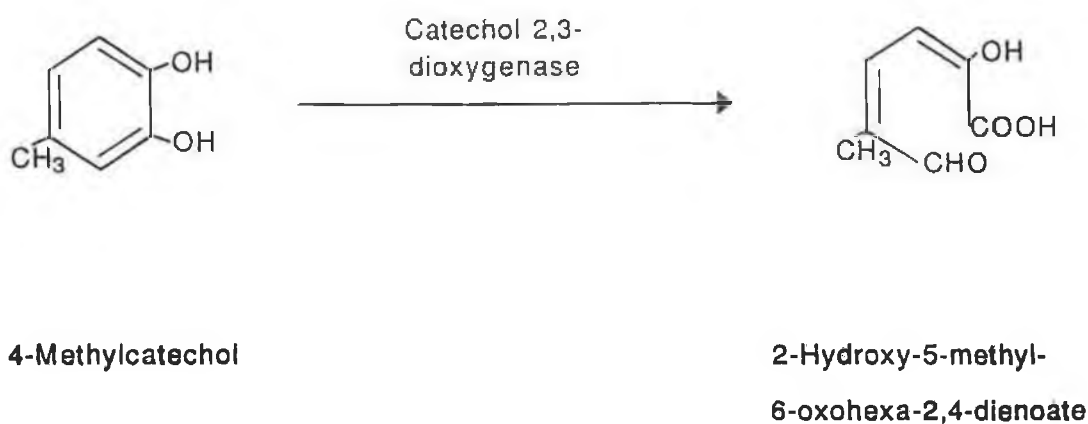


Figure 5: The cleavage of 4-methylcatechol by catechol 2,3-dioxygenase

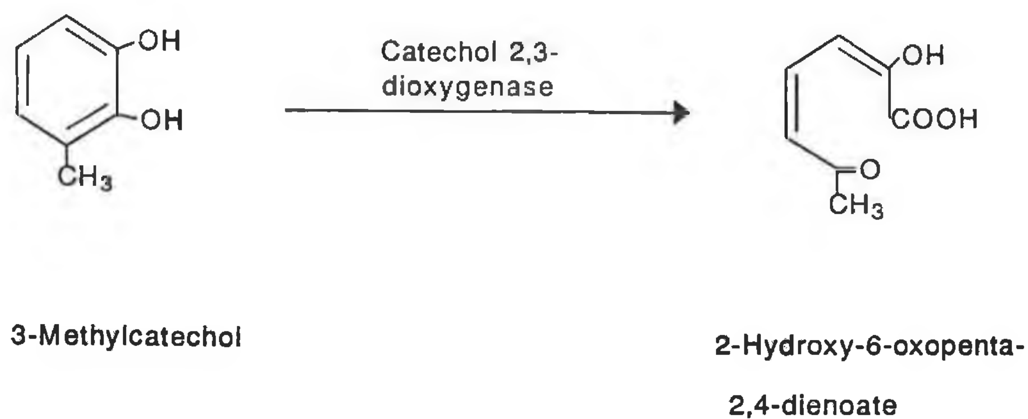


Figure 6: The cleavage of 3-methylcatechol by catechol 2,3-dioxygenase

to pyruvate and acetaldehyde as detailed in Figure 4; *xyl* X, Y, Z which encodes the 3 subunits of toluene 1,2-dioxygenase and *xyl* L which encodes 1,2-dihydroxy-3,5-cyclohexadiene-1-carboxylate dehydrogenase (these two enzymes catalyze the conversion of toluate or benzoate to catechol) ; also contained on the pWWO *meta* operon are two genes, *xyl* T and *xyl* Q, the function of which are as yet unknown. The order of these genes within the operon is as follows : *xyl* X, Y, Z, *xyl* L, *xyl* T, *xyl* E, *xyl* G, *xyl* F, *xyl* J, *xyl* Q, *xyl* K, *xyl* I, *xyl* M (Harayama and Rekik, 1990).

A promoter is located upstream of *xyl* X and the regulatory gene *xyl* S is located downstream of *xyl* H. As with many degradative pathways studied , the *meta* cleavage pathway is under positive regulation. Toluene and benzoate act as inducers of the pathway in the presence of the *xyl* S gene product. The *meta* pathway can also be induced by the substrates for the TOL upper pathway eg. toluene, benzyl alcohol, when the products of both *xyl* S and *xyl* R (regulatory gene for upper pathway) are present (Worsey *et al.*, 1978).

Over production of the *xyl* S gene product leads to constitutive expression of the *meta* pathway in the absence of inducers, indicating the existence of both an active and inactive form of the *xyl* S protein, with activation occurring in the presence of the inducing compounds. Mutation studies involving *xyl* S have revealed the possible existence of a third regulatory gene. A mutation in *xyl* S prevents induction of the *meta* pathway by m-toluate but not by unsubstituted compounds such as benzoate. Benzoate interacts with the OP2, operator-promoter region of pWWO to induce the *meta* pathway in the absence of known plasmid regulatory genes. Benzoate induction of OP2 has only been demonstrated in *Pseudomonas* spp. suggesting the involvement of a chromosomal gene, possibly a regulatory gene for the chromosomally-encoded benzoate dioxygenase (Cuskey and Sprenkle, 1988).

The entire *meta* cleavage operon of pWWO is 10 kb in size, making it one of the largest operons known in bacteria. Its size could pose a problem with regard to both transcription and stability of the mRNA for the operon. It has

been suggested that the *meta* operon was formed by the fusion of two DNA modules each possessing its own promoter. The first comprising the genes for the conversion of toluate/benzoate to catechol, *xyl* X, Y, Z, L, (T) and the second encoding the degradation of catechol, *xyl* (T), E, G, F, J, Q, K, I, H (Harayama and Rekik, 1990).

1.5.2 Catabolic Plasmids

Plasmids are genetic elements found outside the chromosome within the cell. They can replicate autonomously independent of the chromosome, and although considered non-essential for the cell, they often perform secondary functions that are vital to the cell under certain conditions. Many of the degradative pathways studied have been located on plasmids. Catabolic plasmid have been found in pseudomonads and a range of other bacteria. Since the degradative plasmids, so called because they specify enzymes responsible for the biodegradation of a variety of complex organic compounds, are rather unique to *Pseudomonas* (Gunsalus *et al.*, 1975). These plasmids occur naturally and can be either transmissible or non-transmissible.The transmissible plasmids, although transmissible among most *Pseudomonas* species, have not been shown to be transferred to members of other genera. Another interesting aspect of these plasmids is their compatibility. Excepting CAM and OCT (n-alkane degradation) and NAH/SAL (naphthalene/salicylate degradation) and TOL (toluene degradation), most degradative plasmids appear to be compatible with each other (Chakrabarty, 1976). This compatibility means that a single bacterial strain can carry a number of catabolic plasmids thus giving it a broader substrate range (Chakrabarty, 1976). Thus, they must belong to different incompatibility groups. The only degradative plasmid that has been tested in relation of compatibility characteristics with R factors is CAM, which specifies the camphor degradative pathway has been reported to be incompatible with a number of P2-group R factors and has, therefore, been assigned to group P2. Because of different compatibility characteristics, several hydrocarbon degradative plasmids can be maintained in a single bacterial strain to form a

multiplasmid strain which has an enhanced capability to utilize crude oil (Friello *et al.*, 1976).

Degradative plasmids tend to be large, up to approximately 200 kb (Kilobase pairs) for the CAM plasmid (encoding a camphor degradative pathway). The degradative genes constitute only a small portion of the plasmid, for example a 40 kb region of the TOL plasmid encodes the entire degradative pathway. The genes controlling transfer have also been located on some of these large plasmids but little is known about the function of the rest of the plasmid.

Many catabolic plasmids have been shown to share homologous regions. Most of these regions encode the degradation of catechol via the *meta*-cleavage pathway (section 1.5.1). In a number of studies, a high degree of homology has been demonstrated between the *meta*-cleavage operons of the TOL, NAH and SAL plasmids (Lehrbach *et al.*, 1983; Cane and Williams, 1986; Bayley *et al.*, 1979). Further studies strongly indicated the same gene order on the *meta* operons of the TOL and NAH plasmids (Assinder and Williams, 1988).

A large body of evidence supports the theory that many different catabolic plasmids share a common ancestry. The organization of catabolic genes in distinct units (operons) may facilitate the assembly of new degradative plasmids and pathways. Genetic instability may promote rearrangement of DNA to form new pathways which are preserved by selective pressures (Yen and Serdar, 1988). Many pathways have been described in *Pseudomonas* species for the degradation of a variety of aromatic compounds. The critical step in these pathways is the cleavage of the aromatic ring. The initial catabolic steps involve the insertion of two hydroxyl groups on the aromatic nucleus. Subsequent ring fission proceeds via *ortho* or *meta* cleavage of the hydroxylated aromatics.

An interesting example of two separate plasmids specifying the same degradative function is XYL and TOL. The plasmid is a transmissible plasmid characterized in *P.putida* (arvilla) mt-2 strain (Wong and Dunn, 1974;

Williams and Murray, 1974). The presence of the TOL plasmid allows the host cells to degrade not only *p*- or *m*-toluates, but also the corresponding xylenes (Worsey and Williams, 1975). Another plasmid , termed XYL in order to distinguish it from TOL, has been characterised in a strain of *Pseudomonas* Pxy where the xylene pathway is specified by a non-transmissible plasmid. Since *p*- or *m*-toluate is an intermediate of xylene degradation and since the degradative pathways are designated based on the nature of the primary substrate and subsequently referred to the TOL plasmid as XYL (Friello *et al.*, 1976). Excepting the difference in transmissibility and molecular sizes (Friello and Chakrabarty, 1976), XYL and TOL appears to have identical xylene degradative genes, and the regulation of the two pathways appears to be the same.

In order to gain further insight as to the extent of occurrence of such plasmids specifying a single degradative pathway, Williams and Worsey (1976) analyzed the ability of 13 bacterial cultures isolated from 9 different soil samples to metabolize *m*-toluate. All 13 strains appeared to carry the TOL plasmid similar to the one characterised in *P.putida (arvilla)* mt-2. Eight of the isolates could transfer their TOL plasmids into their own cured strains and several strains appeared to harbor nontransmissible TOL plasmids. Many of the strains also are non-fluorescent and some may not be pseudomonads.

It is thus interesting that the toluate and xylene degradative pathways appear to be specified by genes borne on plasmids in so many different types of soil bacteria. Since the XYL and TOL plasmids have now been characterised by electron microscopy, it would be interesting to isolate the plasmid DNA from all the soil bacteria harbouring the TOL plasmids and determine their genetic homology by DNA-DNA hybridization as well as electron microscope heteroduplex analysis to gain an insight into the evolution of such plasmids (Chakrabarty, 1976).

1.6 Biochemical pathways involved in the degradation of specific compounds

1.6.1 Phenol

The plasmid -encoded, multicomponent enzyme, phenol hydroxylase catalyzes the formation catechol by the hydroxylation of the aromatic nucleus of phenol as shown in Figure 7 (Shingler *et al.*, 1989). Catechol is further metabolized via the *meta*-cleavage pathway as described previously. The synthesis of phenol hydroxylase and the enzymes of the meta pathway are induced in the presence of phenol (Feist and Hegeman, 1969).

Meta-cleavage of catechol results in the formation of 2-hydroxymuconic semialdehyde which can be metabolized by either the NAD^{+} - dependent dehydrogenase or an hydrolase. In the case of phenol degradation the preferred rout seems to be via the dehydrogenase activity. However, when 2-hydroxymuconic semialdehyde accumulates in the cells this seems to "drive" it down the hydrolytic pathway (Wigmore *et al.*, 1974)

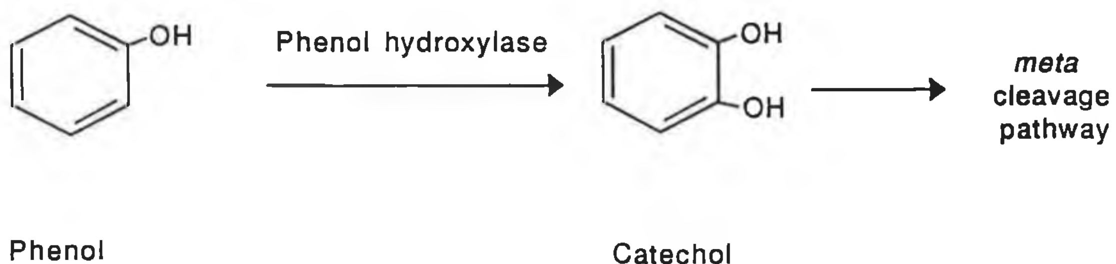


Figure 7: Hydroxylation of phenol by Pseudomonads

Some pseudomonads with the ability to metabolize phenol carry TOL, NAH, SAL or related plasmids (Wong *et al.*, 1978); Cane and Williams, 1982). A natural multiplasmid system encoding phenol, m-toluate and salicylate metabolism was isolated from *P. putida* EST1001 (Kivisaar *et al.*, 1989).

1.6.2 Naphthalene

The biochemistry of naphthalene degradation by bacteria was first studied in 1943 (Davies and Evans, 1964). The following pathway for naphthalene degradation has been demonstrated in pseudomonads (Figure 8) (Yen and Serdar, 1988). The capital letters prefixed by nah represent the genes encoding the corresponding enzymes.

The initial reaction of the pathway is catalyzed by naphthalene dioxygenase, a multi-component membrane-associated enzyme system of similar composition to toluene dioxygenase, described previously. It consists of a terminal iron-sulphur containing oxygenase (ISP_{NAP}); $reductase_{NAP}$, an iron-sulphur flavoprotein which is the initial electron acceptor, shuttling electrons from NADH to the terminal oxygenase and $ferredoxin_{NAP}$ also an iron-sulphur protein, which acts as an intermediate electron carrier. The action of these components of naphthalene dioxygenase is shown in Figure 9 (Haigler and Gibson, 1990).

The second step in the metabolism of naphthalene is the oxidation of *cis*-naphthalene dihydrodiol (*cis*-1,2-dihydroxy-1,2-dihydronaphthalene) to 1,2-dihydroxynaphthalene by *cis*-naphthalene dihydrodiol dehydrogenase. This enzyme requires NAD as an electron acceptor (Yen and Serdar, 1988). 1,2-dihydroxynaphthalene is cleaved by a dioxygenase to yield 2-hydroxychromene-2-carboxylic acid, which is subsequently converted to *cis*-2-hydroxybenzaldehyde and pyruvate in a reaction catalyzed by an aldolase. Salicylaldehyde is further oxidized to salicylate by an NAD^+-dependent dehydrogenase.

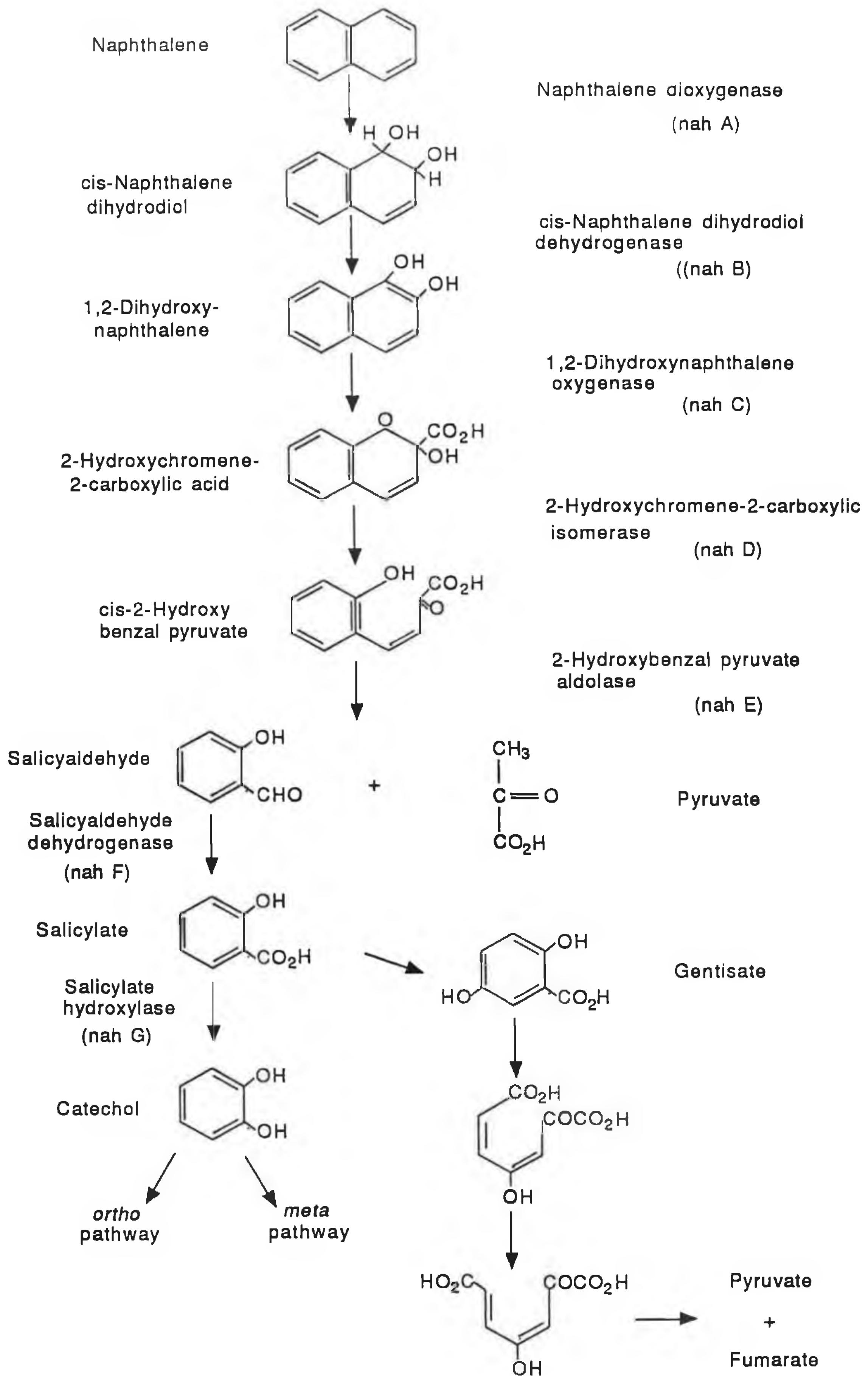
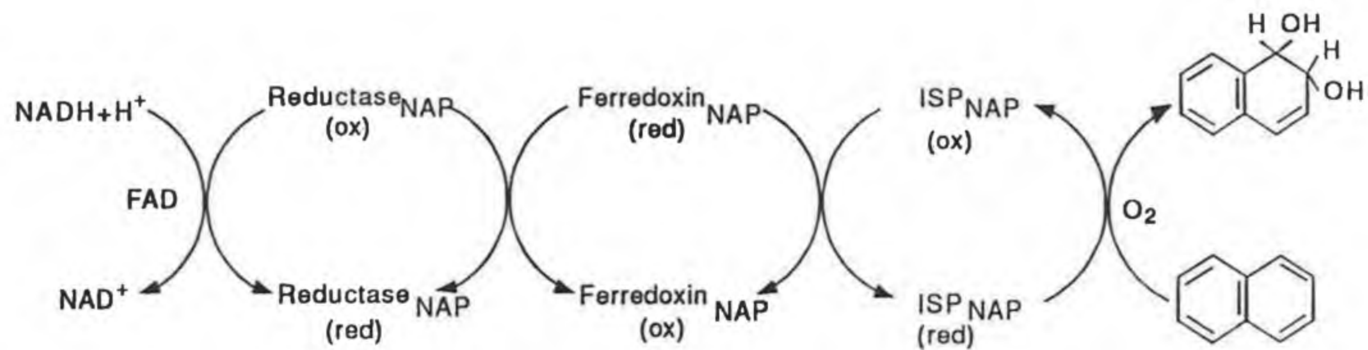


Figure 8: **Naphthalene and salicylate catabolic pathways in Pseudomonads**



NAD - Nicotinamide adenine dinucleotide
FAD - Flavin adenine dinucleotide
red - reduced
ox - oxidized

Figure 9: Proposed electron transport scheme for naphthalene dioxygenase

In most cases salicylate is converted to catechol by salicylate hydroxylase which is further metabolized via the *ortho* or *meta* cleavage pathways. Salicylate hydroxylase (monooxygenase) requires NADH or NADPH as electron donors and FAD as a cofactor (Nozaki, 1979). The formation of genetisate from salicylate has been proposed during naphthalene metabolism by *Pseudomonas fluorescens* and *Pseudomonas alcaligense* (Yen and Serdar, 1988).

A number of plasmids carrying naphthalene catabolic genes have been demonstrated in *Pseudomonas* spp. All naphthalene catabolic plasmids belong to the incompatibility groups P7 or P9. All are self-transmissible. To date all naphthalene plasmids studied encode the same upper pathway (naphthalene through salicylate) and most carry the same lower pathway for salicylate degradation including a close relationship between all naphthalene degradative plasmids (Yen and Serdar, 1988).

As indicated in Figure 8 catechol can also be metabolized via the chromosomally-encoded pathway. This can occur when mutation in the *meta* cleavage pathway allow catechol to accumulate thus inducing the *ortho* pathway. In some strains all or portions of the naphthalene catabolic pathway are encoded on the chromosome and therefore catechol is metabolized via the *ortho* pathway (Frantz and Chakrabarty, 1986). In *P. putida* NP the upper pathway enzymes are specified by chromosomal genes that can integrate into large plasmid, pDTG11 found in this strain (Yen and Serdar, 1988).

1.6.3 Toluene

A number of pseudomonads posses the ability to utilize toluene and related compounds as their sole source of carbon and energy. However, the metabolic pathways for toluene degradation is not the same in all species that have been examined. At the present time three pathways for the degradation of toluene have been reported and they are shown in Figure 10. Pathway A which involves the initial monooxygenation of a methyl group is utilized by

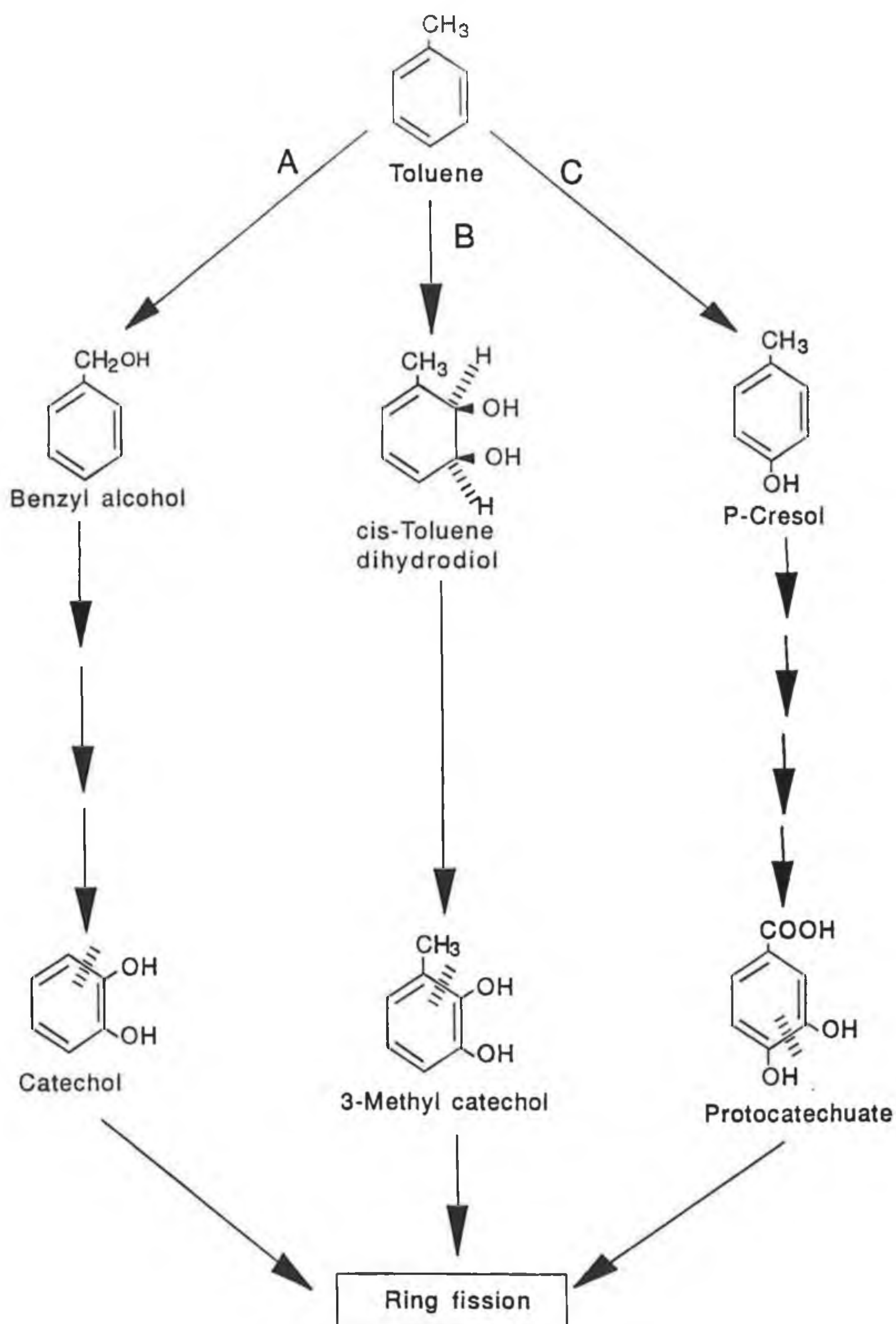


Figure 10: Pathways utilized by different strains of *Pseudomonas* for the degradation of toluene (Gibson, 1989)

Pseudomonas species that contain TOL plasmids. Pathway B is chromosomally encoded in some *Pseudomonas* responsible for toluene degradation. Pathway C is utilized by *Pseudomonas mendocina* which is unique insofar as it is the only organism reported to date that initiates the degradation of toluene by monohydroxylation at the *para*-position.

Degradative pathway encoded by the TOL plasmid

The toluene degradative pathway encoded by the TOL plasmid pWWO is presented in Figure 11.

The structural genes for the enzymes involved are designated *xyl* A, B, C, D, L. Toluene undergoes initial oxidative attack at the methyl substituent to form benzyl alcohol. The toluene mono-oxygenase systems requires NADH and FAD for this reaction (Gibson and Subramanian, 1984). Benzyl alcohol is further oxidized to yield benzaldehyde, benzoate and finally catechol, which then undergoes *meta* fission.

Benzyl alcohol dehydrogenase catalyzes the reversible oxidation of benzyl alcohol to benzaldehyde with the concomitant reduction of NAD^+. Benzaldehyde dehydrogenase then catalyzes the irreversible oxidation of benzaldehyde to benzoate with a corresponding NAD^+ reduction. Both these enzymes are dimers of identical subunits and neither contain a prosthetic group (Shaw and Harayama, 1990).

Toluene oxygenase has quite a broad substrate specificity, oxidizing toluene, *m*- and *p*-xylene, *m*-ethyltoluene and 1,3,4-trimethylbenzene. It can also oxidize *m*- and *p*-chlorotoluene at a low rate. Benzyl alcohol dehydrogenase and benzaldehyde have a broader specificity, oxidizing *m*- and *p*-methyl-, ethyl- and chloro-substituted benzyl alcohols and benzaldehydes, as well as the unsubstituted compounds.

Toluene oxygenase, therefore is the limiting step in the oxidation of chloro-

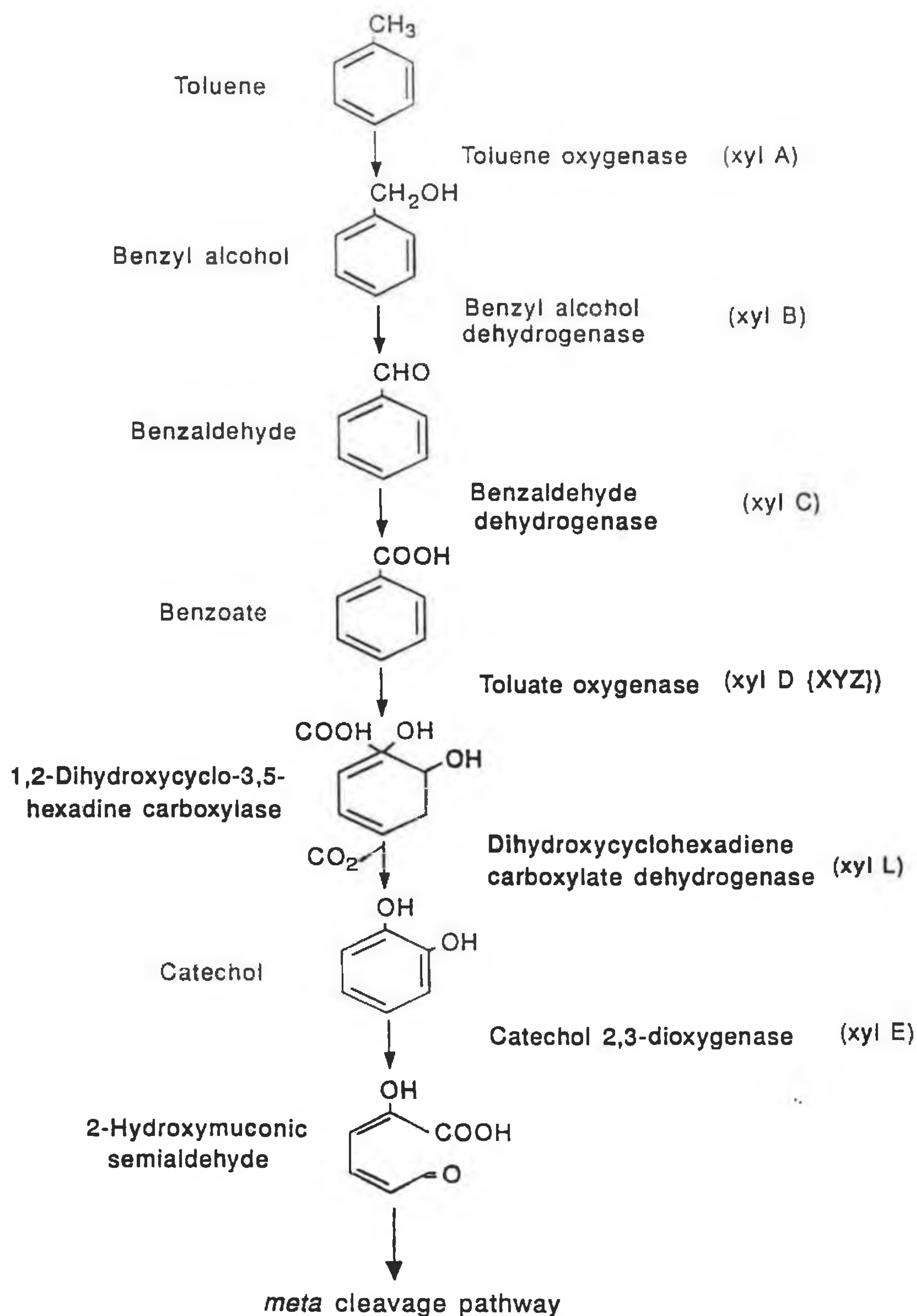


Figure 11: Toluene degradation pathway encoded by the TOL plasmid (Burlage *et al.*, 1989)

substituted toluenes, preventing the accumulation of toxic dead-end products produced by *meta*-cleavage of chlorocatechols (Abril *et al.*, 1989).

At present, the best understood catabolic plasmid is the TOL plasmid, which encodes the enzymes that degrade toluene. The pWWO, the archetypal TOL plasmid, was first described in *Pseudomonas arvilla* mt-2, renamed *Pseudomonas putida* mt-2 (Williams and Murray, 1974). Many other TOL plasmid have since been described, most of which show strong DNA homology to pWWO and encode similar biochemical pathways (Keil *et al.*,1985; Keil *et al.*, 1985; Whitted *et al.*, 1986; Williams and Worsey, 1976). These plasmids are usually found in *Pseudomonas* species, although one TOL-like plasmid has been found in *Alcaligenes eutrophus* (Hughes *et al.*, 1984); gene expression appears to be limited to *Pseudomonas* and related species (Chatterjee and Chatterjee, 1987).

pWWO is a large (117 kb), self-transmissible plasmid belonging to the P-9 incompatibility group. The catabolic and regulatory genes are carried on approximately 40 kb of the plasmid. The spontaneous loss of this region, coupled with the loss of degradative capacity, led Chakrabarty *et al.* (1978) to propose that a transposon may be associated with the TOL plasmid; this loss was later attributed to intraplasmidic recombination between two direct repeat sequences which flank the degradative pathway (Meulien *et al.*, 1981). The transposable element that includes the entire pathway has recently been described (Tsuda and Iino, 1987). Transposition events that have been observed indicate the size of the transposon is approximately 56 kb. The genes involved in the transposition have also been identified, including the transposase gene, tnpA, and two genes that are necessary for resolution, tnpS and tnpT. In addition , a specific DNA sequence required for resolution, the res site, has been identified. The spacial arrangement of these genes is shown in Figure 12.

The catabolic genes are organised into two operons, one encoding enzymes for the upper pathway (7 kb) and the other encoding the *meta* pathway enzymes

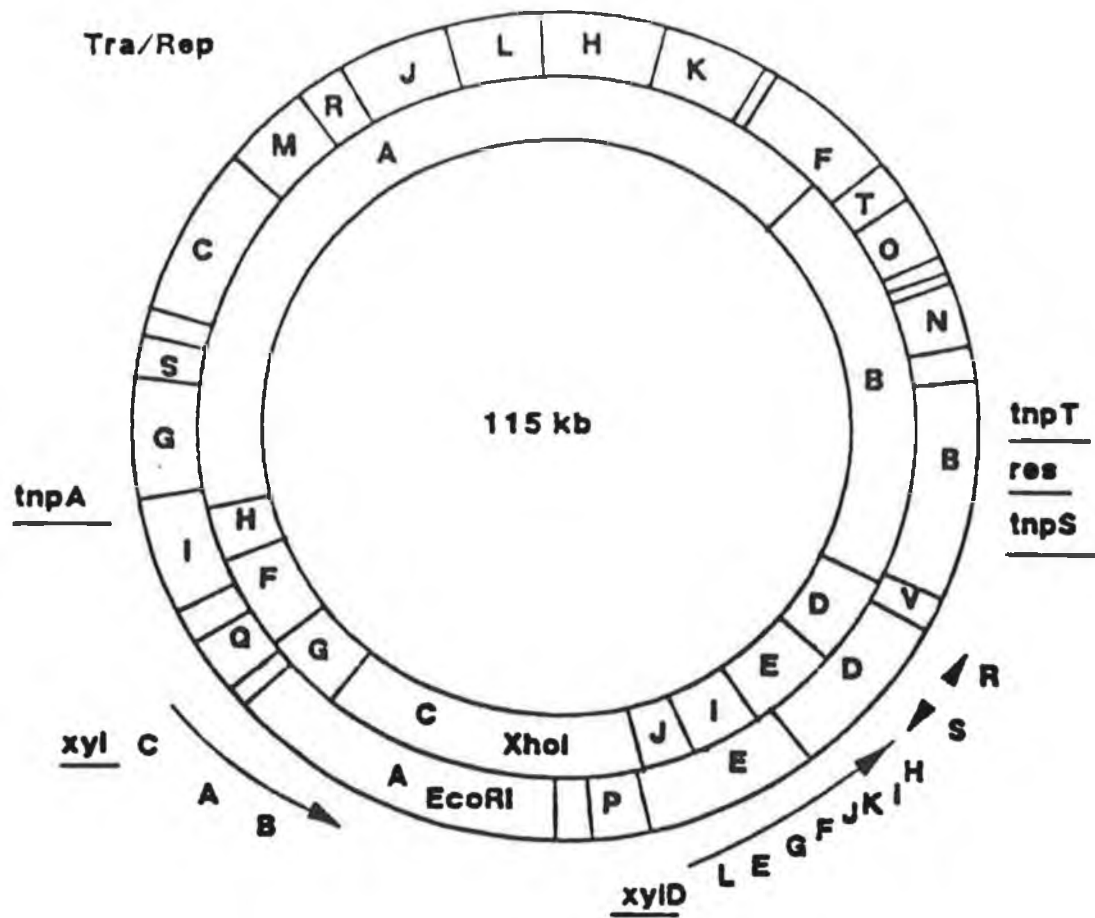


Figure 12: Map of the TOL plasmid pWWO. Restriction fragments for Xho I (inner circle) and Eco RI (outer circle) are noted as are known genes. Arrows indicate the direction of transcription (Burlage *et al.*, 1989)

(10 kb). The two operons are separated by a segment approximately 10 kb in length (Harayama *et al.*, 1986). The genes for replication and conjugal transfer have been located only roughly. The structural genes for the upper pathway enzymes are given in Figure 11. Those of the *meta* pathway enzymes are shown in Figure 4.

Although control of the upper and lower *(meta)* TOL pathways is not fully understood, two regulatory genes have been located on the TOL plasmid, *xyl* R and *xyl* S. Substrates for the upper pathway enzymes eg. toluene, benzyl alcohol are inducers of the pathway when the *xyl* R product is present. The product of *xyl* S combines with the upper pathway can induce both lower and upper pathways when the products of both *xyl* R and *xyl* S are present (Worsey *et al.*, 1978). This prevents the build-up of toxic intermediates and prevents the escape of metabolic pools that might act as a chemoattractant for competing organisms (Burlage *et al.*, 1989). Research indicates that the TOL degradative genes are under positive control at the level of transcription initiation (Harayama *et al.*, 1986).

The TOL plasmid has been shown to be stably maintained in the host, even during growth on non-selective media. Under carbon and energy-limiting conditions, the presence of the TOL plasmid places a strain on the host resulting in loss or deletion of the plasmid (Duetz and van Andle, 1991). The TOL plasmid can be lost or undergo large, plasmid-specific delations when grown on benzoate. Cells carrying the TOL plasmid metabolize benzoate via the *meta*-cleavage pathway. The *ortho*-cleavage pathway is not normally expressed in such cells due to differences in the regulation of the two pathways. The chromosomally encoded *ortho* pathway appears to permit faster growth on benzoate. Therefore, a positive selection exists for cells that have lost the plasmid (Williams and Worsey, 1976). Benzoate is also thought to selectively inhibit the growth of cells containing wild-type or deleted TOL plasmids (Stephens and Dalton, 1988).

The 40 kb fragment which encodes the entire degradative pathway on the

TOL plasmid has been found on the chromosome of some strains that have lost the TOL plasmid and these strains are still capable of toluene and xylene degradation (Broda *et al.*, 1988). In studies of continuous cultures by Keshavarz *et al* (1985) approximately 1% of the total population retained the TOL plasmid after 600 hr growth under benzoate-limiting conditions. However, the TOL^{+} population recovered to 100% when the medium was returned to *m*-toluate. Plasmid stability was greater in TOL^{+} cells isolated from benzoate cultures. The plasmid loss may be due to a failure in the control of partitioning during cell division. This failure is not total and a residual low level of plasmid-containing bacteria persist in the population, which proliferate when toluene is reintroduced to the culture.

Chromosomal toluene degradative pathway

An alternative chrosomally-encoded pathway for toluene degradation has been described in *Pseudomonas putida* F1 (Zylstra and Gibson, 1989). Toluene is metabolized to 3-methylcatechol via *cis*-toluene dihydrodiol. 3-methylcatechol is further metabolized via the *meta*-cleavage pathway . Toluene degradation by *P. putida* F1 is outlined in Figure 13 (Zylstra *et al.*, 1988). The structural genes for this pathway are designated *tod* A - F.

Toluene dioxygenases catalyzes the initial reaction by incorporating both atoms of molecular oxygen into the aromatic nucleus to form *cis*-toluene dihydrodiol ((+) - *cis*-1,2-dihydroxy-3-methylcyclohexa-3,5-diene). Toluene dioxygenase is a multicomponent, membrane associated enzyme system comprising $ferredoxin_{TOL}$ reductase, $ferredoxin_{TOL}$ and ISP_{TOL}. $Ferredoxin_{TOL}$ reductase is a flavoprotein which accepts electrons from NADH and transfers them to a small iron-sulphur protein, $ferredoxin_{TOL}$. $Ferredoxin_{TOL}$ then reduces ISP_{TOL}, the terminal oxygenase component, which is a large iron-sulphur protein. The reduced oxygenase catalyzes the oxidation of toluene to *cis*-toluene dihydrodiol. The action of toluene dioxygenase is summarised in Figure 14 (Gibson and Subramanian, 1984).

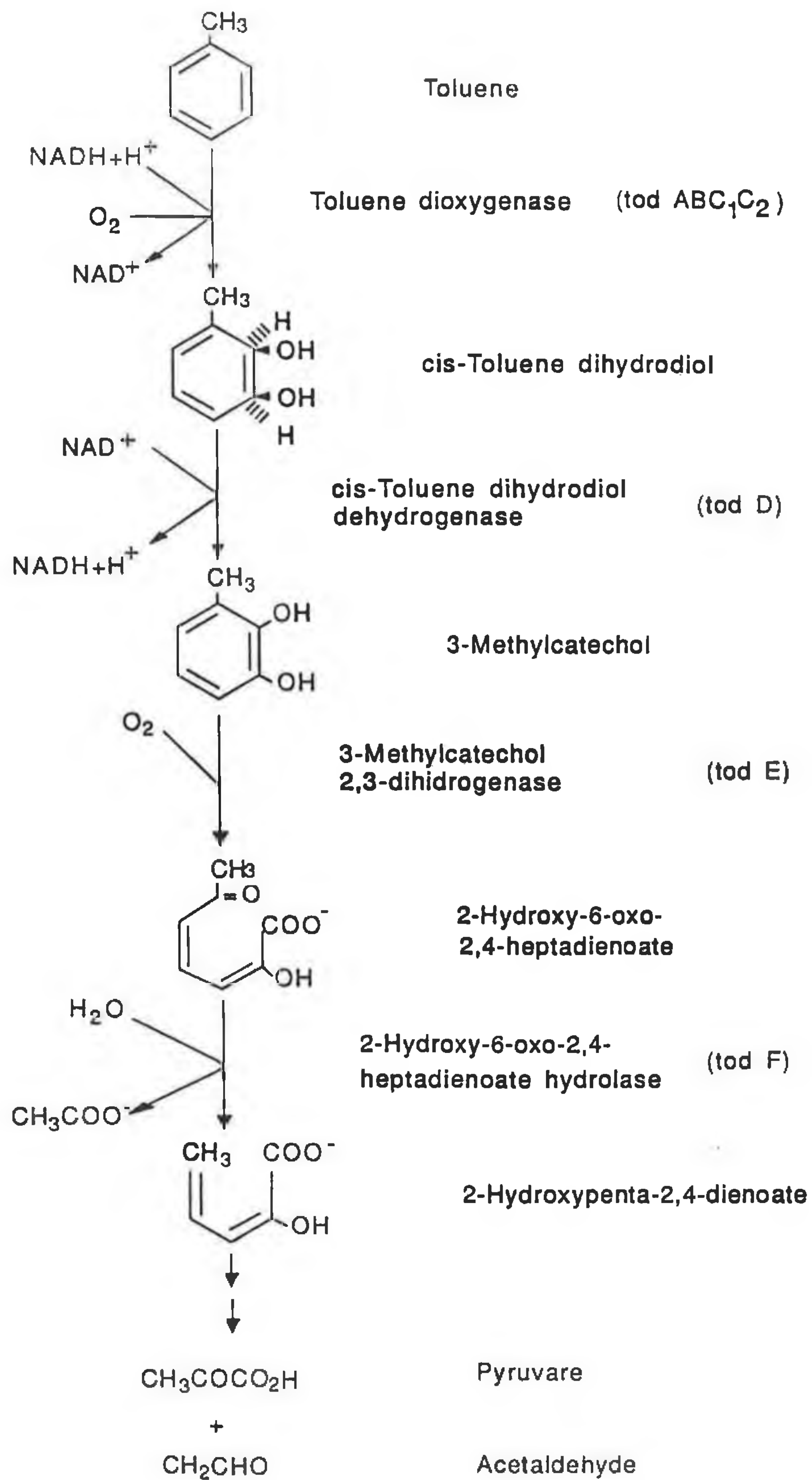
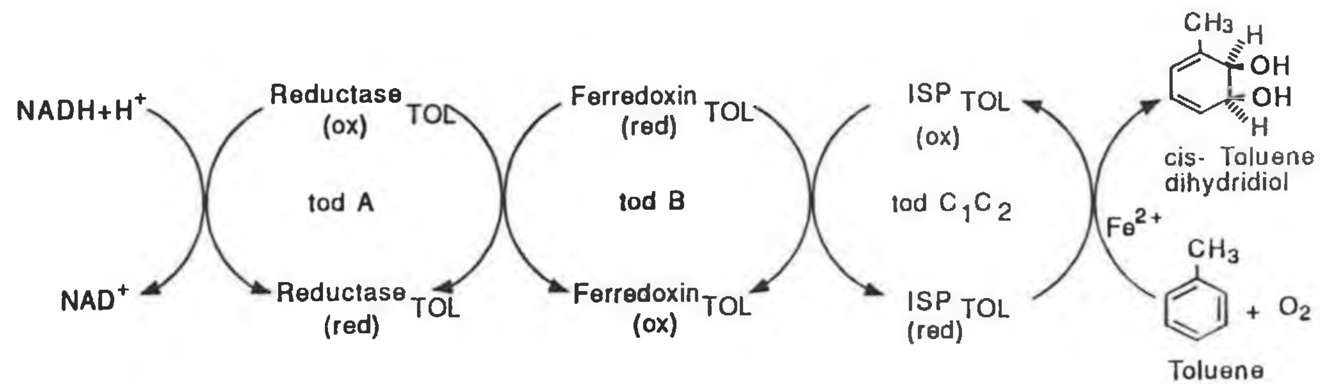


Figure 13: **The degradation of toluene by the chromosomal pathway of *P. putida* F1**



NAD - Nicotinamide adenine dinucleotide
red - reduced
ox - oxidized

Figure 14: Proposed electron transport scheme for toluene dioxygenase

Cis-toluene dihydrodiol is further oxidized to 3-methylcatechol by an NAD^+-dependent dehydrogenase (Figure 14) (Rogers and Gibson, 1977). *Meta*-cleavage of 3-methylcatechol by the dioxygenase yields 2-hydroxy-6-oxo-2,4-hepta-dienoate, which is further metabolized to 2-hydroxypenta-2,4-dienoate and acetate by a hydrolase. A detailed description of the *meta*-cleavage of 3-methylcatechol to the TCA intermediates, pyruvate and acetaldehyde, is described in section 1.5.1

The structural genes encoding the above mentioned enzymes have been designated *tod* A, B, C_1, C_2, D, E, F as shown in Figure 13. These genes form part of the *tod* operon for toluene degradation and are carried on the chromosome of *Pseudomonas putida* F1. A series of mutations and complementation analysis performed by Zylstra *et al.* (1988) revealed the order of transcription of the genes in the *tod* operon to be : *tod* F, *tod* C_1, *tod* C_2, *tod* B, *tod* A, *tod* D, *tod* E. Further studies indicated that the *tod* genes are under positive control.

Toluene degradative pathway in *Pseudomonas mendocina*

A route for toluene degradation specific to *P. mendocina* has been described and summarised in Figure 15.

Rechardson and Gibson (1984) isolated a strain of *Pseudomonas mendocina* that can grow with toluene as the sole source of carbon and energy. This organism will not oxidize benzyl alcohol, *cis*-toluene dihydrodiol or 3-methyl catechol. This result indicated that *P. mendocina* utilizes a novel pathway for toluene degradation.

The metabolism of toluene proceeds by initial hydroxylation of toluene to form *p*-cresol, further metabolism via oxidation of the methyl group to form *p*-hydroxybenzoate, hydroxylation to form protocatechuate, and then complete dissimilation by an *ortho* cleavage pathway (Whited and Gibson, 1991).

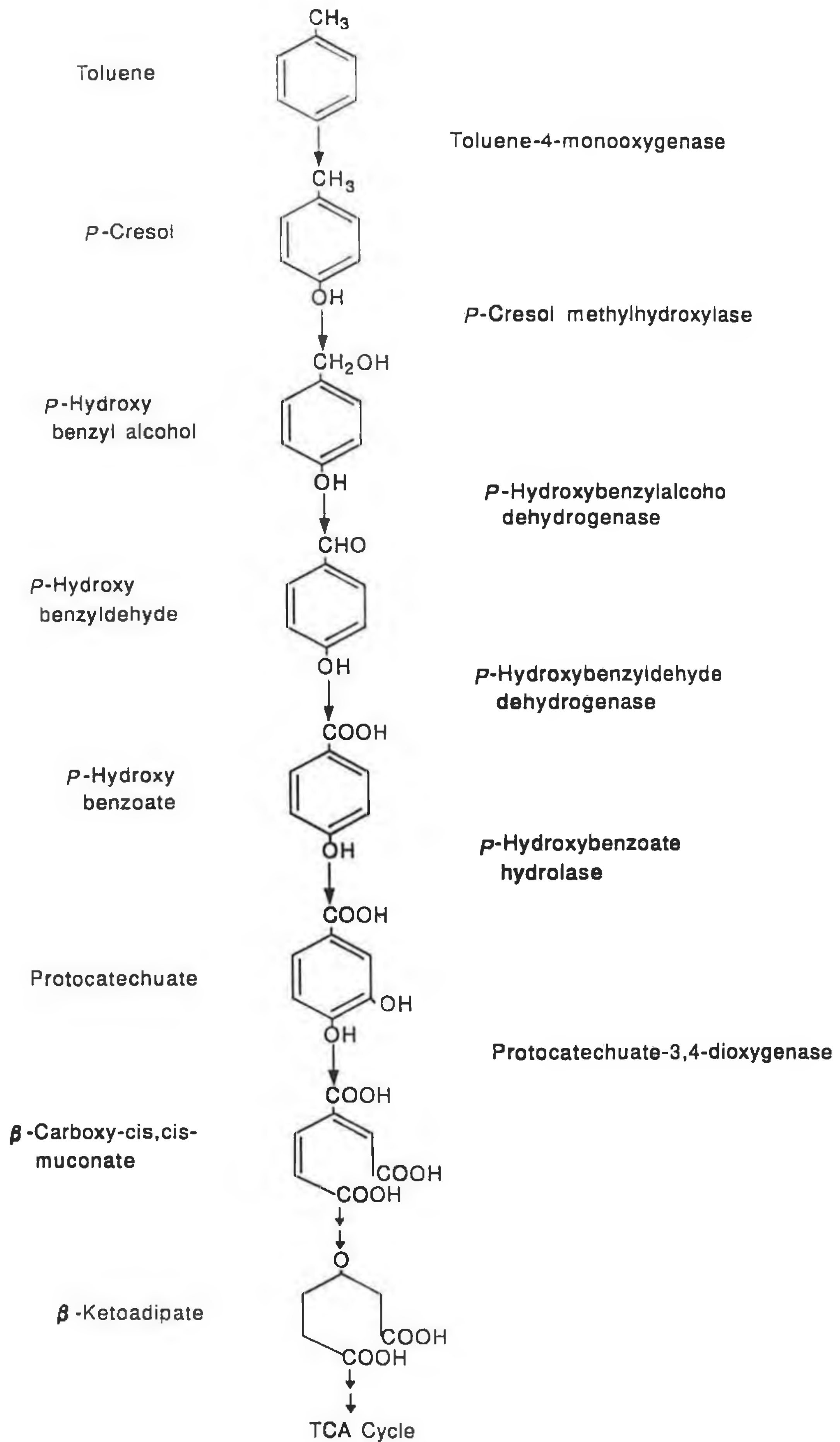


Figure 15: **Toluene degradation in *P. mendocina***

Studies with partially purified cell extracts showed that toluene is oxidized to *p*-cresol by a multicomponent enzyme system. When the reaction was conducted in the presence of $^{18}O_2$ the oxygen in the hydroxyl group of *p*-cresol was shown to be derived from atmospheric oxygen. In addition, incubation of 4-deuterotoluene with the partially purified enzyme components gave *p*-cresol, which retained 68% of the deuterium that was originally present in the deuterated substrate. The results indicate that *P. mendocina* utilizes a monooxygenase to initiate toluene degradation and that toluene-3,4-oxide is an intermediate in the formation of *p*-cresol. The oxidation of *p*-cresol by *P. mendocina* is catalyzed by an enzyme that contains flavin and heme as prosthetic groups. The oxygen in the product, *p*-methylbenzyl alcohol, is derived from water and the reaction presumably proceeds through a quinone methide intermediate (Whitted, 1986). This unique hydroxylation reaction was first described by Hopper and his colleagues (1976 and 1977).The nature of the flavin cofactor in the *p*-cresol methylhydroxylase from *P. mendocina* has not been determined. However, it is probably covalently linked to the enzyme at a tyrosine residue as has been reported for other *p*-cresol methylhydroxylases (Mclntire *et al*., 1981). Other enzymes that are required for toluene degradation by *P. mendocina* are *p*-benzaldehyde dehydrogenase, *p*-benzoic acid hydroxylase and protocatechuate-3,4-dioxygenase (Whitted, 1986).

The importance of the role of microorganisms in metabolizing both natural and xenobiotic compounds has been stressed on many occasions. In recent years many synthetic chemicals were found to be biodegradable under laboratory conditions by especially enriched, adapted, or constructed microorganisms. A particularly attractive possibility is that of adding specialized bacteria with the ability to degrade toxic compounds to the waste treatment system. The development of improved and specialized bacteria for the treatment of hazardous waste requires detailed knowledge of the biochemical and genetic processes involved in microbial degradation.

This thesis is concerned with the isolation and characterization of bacteria

capable of growth on aromatic substrates and in particular toluene. Due to the low water solubility of volatile organic compounds and their toxicity at very low concentrations it was of interest to optimize cultural conditions for isolates capable of growth on toluene. This work studies the degradative capabilities of the isolates at a biochemical and genetic level.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Source of activated sludge

The activated sludge sample was obtained from the oxidation ditch at the waste water treatment plant at Bristol Myers Squibb (Ireland) Ltd. Swords, Dublin.

2.1.2 Bacterial cultures

***Pseudomonas* and *Aeromonas* species**

The *Pseudomonas* and *Aeromonas* spp. studied were isolated from the activated sludge sample described above.

***Pseudomonas putida* NCIB 10432**

Pseudomonas putida NCIB 10432 was obtained from the National Collection of Industrial Bacteria, Aberdeen, U.K.

2.1.3 Media

All media unless otherwise stated were obtained from Oxoid. All media were sterilized by autoclaving at 121°C for 15 minutes.

Gelatine Agar

Gelatine was added to nutrient agar (Oxoid) before sterilization to a final concentration of 0.4% (w/v) as outlined in Harrigan and McCance (1976).

Hugh and Leifsons' Medium

The medium was prepared as outlined in Harrigan and McCance (1976). All the following ingredients except Bromothymol blue were dissolved in distilled water.

	g/l
Peptone	2.0
NaCl	5.0
K_2HPO_4	0.3
Glucose	10.0
Agar	3.0
Bromothymol blue	3.0 ml
(1% (w/v) aqueous solution)	
pH 7.1	

The pH was adjusted to 7.1 with 2 M NaOH. The indicator dye was then added. The medium was dispensed into test tubes in 10 ml aliquots. Following sterilization, the tubes were cooled rapidly in an iced water bath and inoculated immediately.

KCN Broth

The KCN Broth was prepared according to the modified method of Møller (1954). The following ingredients were combined in distilled water.

	g/l
Protease peptone (No.3 Difco)	3.0
Disodium phosphate	5.64
Monopotassium phosphate	0.225
Sodium chloride	5.0
pH 7.6	

After sterilization 0.5% (w/v) solution was added for each 100 ml basal medium. The medium was immediately dispensed into tubes which were tightly capped.

Luria-Bertani Broth & Luria Broth

Luria-Bertani (LB) Broth and Luria Broth were prepared according to the method of Maniatis *et al.*, (1982). The ingredients were combined in distilled water.

LB Broth

	g/l
Tryptone	10.0
Yeast Extract	5.0
Sodium Chloride	10.0
pH 7.0	

Luria Broth

	g/l
Tryptone	10.0
Yeast Extract	5.0
Sodium Chloride	5.0
Glucose	1.0
pH 7.0	

Milk Agar

Skim milk powder (Oxoid) was added to nutrient agar (Oxoid) before sterilization to a final concentration of 10% (w/v) as outlined in Harrigan and McCance (1976).

Nutrient Medium

The nutrient medium (Inoue and Horikoshi, 1989) was prepared by dissolving the following ingredients in *Pseudomonas* minimal medium and adjusting the pH to 7.0 with 2M NaOH.

	g/100 ml
Glucose	0.1
Yeast extract	0.25%
Polypeptone	0.5
pH 7.0	

Pseudomonas Minimal Medium

The *Pseudomonas* minimal medium was prepared as outlined by Goulding *et al.*, (1988). The following ingredients were combined in distilled water and the pH adjusted to 7.0 with 2 M NaOH. The trace salts solution was prepared separately in distilled water and was stored in a dark bottle, without sterilizing, for 6-8 weeks.

	g/l
K_2HPO_4	4.36
NaH_2PO_4	3.45
NH_4Cl	1.0

$MgSO_4.6H_2O$	0.912
Trace Salts Solution	1.0 ml
pH 7.0	

Trace salts solution

	g/100 ml
$CaCl_2.2H_2O$	4.77
$FeSO_4.7H_2O$	0.37
$CoCl_2.6H_2O$	0.37
$MnCl_2$	0.10
$NaMoO_4$	0.02

Tween 80 Agar

The agar was prepared as outlined in Harrigan and McCance (1976). The following ingredients were dissolved in distilled water and the pH adjusted to within 7.0 - 7.4 with 2 M NaOH if required.

	g/l
Peptone	10.0
$CaCl_2$ H_2O	0.1
NaCl	5.0
Tween 80	10.0
Agar	15.0
pH 7.0 - 7.4	

2.1.4 Buffers and Solutions

20 X SSC

A stock solution of 20 X SSC comprised sodium chloride (3 M) and sodium citrate (0.3 M) in distilled water. The pH was adjusted to 7.0 with 2 M sodium hydroxide. This solution was stored at 4°C and diluted as required.

50 X TAE

Tris-acetate buffer was prepared by dissolving Tris (2 M) and

ethylenediaminetetra acetic acid (0.05 M) in distilled water and adjusting the pH to 8.0 with glacial acetic acid. The buffer was stored at room temperature and diluted as required.

TE Buffer

Tris EDTA buffer was prepared by dissolving Tris (10 mM) and EDTA (1 mM) in distilled water and the pH was adjusted to pH 8.0 with 2 M HCl.

Sodium Phosphate Buffer (pH 7.0)

Sodium phosphate buffer (0.01 M) was prepared by dissolving Na_2HPO_4 (0.01 M) and NaH_2PO_4 (0.01 M) in distilled water. The pH of the resulting solution should be 7.0.

Sodium Phosphate Buffer

Sodium phosphate buffer was prepared by adding x ml of NaOH (1 M) to 50 ml of NaH_2PO_4 (1 M) and diluting to a total 100 ml. The pH of the resulting solution will be dependent on the amount of NaOH (1 M) solution. For the pH range 5.8 to 8.0 the following volumes of NaOH were used:

ml NaOH (1 M)	pH
3.5	5.8
18.0	6.6
24.0	6.8
40.0	7.4
45.0	7.8
47.0	8.0

Tris-Maleate Buffer

Tris-maleate buffer was prepared by adding x ml of NaOH (0.2 M) to 50 ml of Tris-acid maleate (0.2 M) and diluting to a total 200 ml. The pH of the resulting solution will be dependent on the amount of x ml of NaOH (0.2 M) solution

ml NaOH (0.2 M)	pH
20.5	5.8
45.0	6.8
69.0	8.0

Tris-HCl Buffer

Tris-HCl buffer (0.033 M) was prepared by dissolving tris (0.033 M) in distilled water and adjusting the pH to 7.6 with 2 M HCl.

50 X Denhardt's Solution

The following reagents were dissolved in distilled water and the solution was stored at -20°C.

1% (w/v) Ficoll

1% (w/v) Polyvinylpyrrolidone

1% (w/v) Bovine serum albumin

Denatured Calf Thymus DNA

Calf thymus DNA was dissolved in water (10 mg/l). The DNA was sheared by passing it through a hypodermic needle several times and was then boiled for 10 minutes. The solution was stored at -20°C in 1 ml aliquots. Just prior to use the DNA was heated to 95°C - 100°C for 5 minutes and cooled immediately on ice.

Ethidium Bromide (10 mg/ml)

One gram of ethedium bromide (warning,a mutagen) was added to 100 ml water and stirred for several hours to ensure that the dye was dissolved. The container was wrapped in tin foil and stored at 4°C. Gloves were worn at all times when handling EtBr containing solutions. EtBr waste was collected, treated with activated charcoal and filtered through 3MM Whatman filter paper. The clear liquid was disposed normally and the solids contained on the filter paper were incinerated.

Hybridization Solution for ^{32}P

6 X SSC

0.01 M EDTA

^{32}P - labelled denatured probe DNA

5 X Denhardt's solution

0.5% SDS

100 µg/ml denatured calf thymus DNA

Hybridization Solution for Dig-oxigenin kit

5 X SSC

2% (w/v) Blocking reagent

0.1% (w/v) N-Laurylsarcosine, sodium salt

0.02% (w/v) SDS

50% (v/v) Formamide

The hybridization solutions could be stored at -20°C and reused several times.

Prehybridization Solution

6 X SSC

0.5% (w/v) SDS

5 X Denhardt's solution

100 µg/ml denatured calf thymus DNA

Prewashing Solution

5 X SSC

0.5% (w/v) SDS

1 mM EDTA (pH 8.0)

Phenol/Chloroform mix (Kirby mix)

100 g of Analar grade phenol was dissolved in 100 ml chloroform. To this, 4 ml of isoamylalcohol and 0.8 g of 8-hydroxyquinoline were added and the mixture was adjusted to pH 7.5 using 100 mM Tris-HCl and then stored in a dark bottle at 4°C.

Reagents for Digoxigenin-labelled kit

Blocking Reagent

Blocking reagent was dissolved in buffer 1 to a final concentration of 10% (w/v) with shaking. This stock solution was autoclaved and stored at 4°C subsequently.

Buffer 1

The following ingredients were dissolved in distilled waster and the pH was adjusted to 7.5 with solid or concentrated NaOH. The solution was autoclaved and stored at 20°C.

Malic acid	100 mM
NaCl	150 mM
pH 7.5	

Buffer 2

Blocking stock solution was diluted to 1:10 in buffer 1 (final concentration 1% blocking reagent).

Buffer 3

The following ingredients were dissolved in distilled water and the pH was adjusted with 2 M NaOH to 9.5. The solution was autoclaved and stored at 20°C.

Tris-HCl	100 mM
NaCl	100 mM
$MgCl_2$	50 mM
pH 9.5	

2.1.5 Source of chemicals

The chemicals used were obtained from a number of sources including Riedel-de-Haen, BDH, Sigma, Aldrich and NBS Biologicals.

2.1.6 Source of genetic materials

Restriction enzymes were supplied by Bethesda Research Laboratories, Life Sciences Inc., Maryland, USA.

λ DNA (0.25µg/µl) and DIG DNA labelling and detection system was supplied by Boehringer Mannheim, Germany.

α-^{32}P ATP was supplied by Du Pont, France.

Prime-a-Gene labelling system was supplied by Promega Corp., Madison, USA.

2.2 Methods

2.2.1 Analysis of the activated sludge sample

Temperature, pH, dissolved oxygen

The temperature and dissolved oxygen of the sample were measured using a WTW Oxi 196 dissolved oxygen meter (WTW, Germany) in accordance with the manufacturers' instructions.

The pH of the system was measured with a Corning pH meter.

Colour

A Nessleriser glass filled to the mark with the supernatant of the sludge sample was placed in the right hand compartment of the Nessleriser (HACH, RATIO/XR, Turbidimeter). The left-hand compartment was left empty. The Nessleriser was placed in the Lovibond white light cabinet and the colour of the sample compared with the colour on a disc corresponding to 5 - 70 on the Hazen Colour Scale.

Sludge Volume Index (SVI)

The sludge volume index (SVI) was calculated according to Standard Methods for the Examination of Water and Wastewater (1985) as follows:

$$SVI\ (ml/g) = \frac{Settled\ sludge\ volume\ (SSV)\ \ (ml/l)}{Suspended\ solids\ (MLSS)\ \ (g/l)}$$

Settled Sludge Volume (SSV)

Mixed liquor (1000 ml) was allowed to settle in a graduated cylinder for 30 minutes. The settled sludge volume was recorded as ml/l after 30 minutes.

Mixed Liquor Suspended Solids (MLSS)

A sample of mixed liquor (10 ml) was filtered through Whatman GF/C, glass fibre filter paper and dried at 103°C for 24 hours. The weight of the dried solids was then determined and recorded as g total solids/l.

Isolation of bacteria from activated sludge

A modification of the method of Banks and Walker (1976) was used to isolate bacteria from activated sludge.

A well mixed sample of sludge was diluted (1:10) with sterile ringers solution and sonicated for 1 minute at an amplitude of 4μ in a MSE Soniprep 150. Appropriate dilutions were then made with ringers solution and spread-plated (0.1 ml) on plate count agar and *Pseudomonas* minimal medium incorporating different aromatic substrates. The aromatic substrates used were toluene, phenol and naphthalene.

Phenol (5 mM) was added to the minimal medium prior to sterilization. Naphthalene crystals (0.15 - 0.25 g) were scattered on the lid of inverted

petridishes containing medium and the organisms were grown in the vapour. Toluene plates were made by adding toluene (107 µl/100 ml MM) to the minimal medium after sterilization. The plates were then incubated in a sealed desiccator containing toluene and water (107 µl/100 ml H_2O). The PCA plates were incubated at 20°C and the aromatic plates at 30°C for 6 - 7 days.

2.2.2 Maintenance of the bacterial cultures

Maintenance of the *Pseudomonas* and *Aeromonas* cultures on nutrient agar

Cultures were maintained on nutrient agar slopes at 4°C and were subcultured every 2-3 months.

Maintenance of the *Pseudomonas* and *Aeromonas* cultures on aromatic compounds

Cultures were maintained on aromatic substrates by plating on *Pseudomonas* minimal medium (section 2.1.3) incorporating Oxoid Bacteriological Agar Number 1 (1%, w/v) together with the appropriate aromatic compounds (section 2.2.1)

2.2.3 Cultural conditions of the *Pseudomonas* and *Aeromonas spp.* on aromatic compounds

The organisms were grown in the presence of aromatic substrates in both solid and liquid culture.

Solid culture

In solid culture the *Pseudomonas* minimal medium was made by incorporating Oxoid bacteriological agar number 1 (1% w/v) together with the appropriate aromatic substrate (section 2.2.1). The condition of incubation of toluene plates is represented in Figure 16.

Liquid culture

Cells from nutrient agar or aromatic plates were used to inoculate nutrient broth (10 ml). Cultures were incubated overnight at 30°C and agitated at 200 rpm. The cells were harvested in Labofuge 6000 bench-top centrifuge (5,000 rpm for 10 minutes). The cultures were washed in sterile 0.033 M Tris-HCl buffer (pH 7.6) (section 2.1.4) and the final pellet resuspended in the same buffer. This cell suspension was then used to inoculate the growth medium, at a concentration of 2% (v/v).

The aromatic substrates was either added directly to the growth medium or were supplied in the vapour phase. Phenol (5 mM) and *m*-toluate (5 mM) were added to the liquid medium before sterilization. Benzoate and Naphthalene were added after sterilization to give a concentration of 5 mM. *p*-, *m*- and *o*-Chlorophenol (1.6 mM) were dissolved in a minimum volume of ethanol and added after sterilization. Ethylbenzene, pseudocumene, p- and m-xylene were supplied in the vapour phase by suspending a small tube containing the substrate inside the culture vessel. Toluene was either added directly to the growth medium or in the vapour phase. All the cultures were incubated at 30°C and agitated at 150 rpm for the required time. The condition of incubation of liquid culture is represented in Figure 17.

Where necessary culture harvested from nutrient broth (subcultured from nutrient agar) was used as toluene uninduced cells and an over night toluene culture was used as induced cells to inoculate the growth medium.

Specific growth rates were calculated using the computer software package Sigma Plot (Verson 4.0), Jandel Corporation. A mathematical transform was used to determine

$$\text{Ln}\,(X/X_0)$$

where X = absorbance at time t

X_0 = initial absorbance

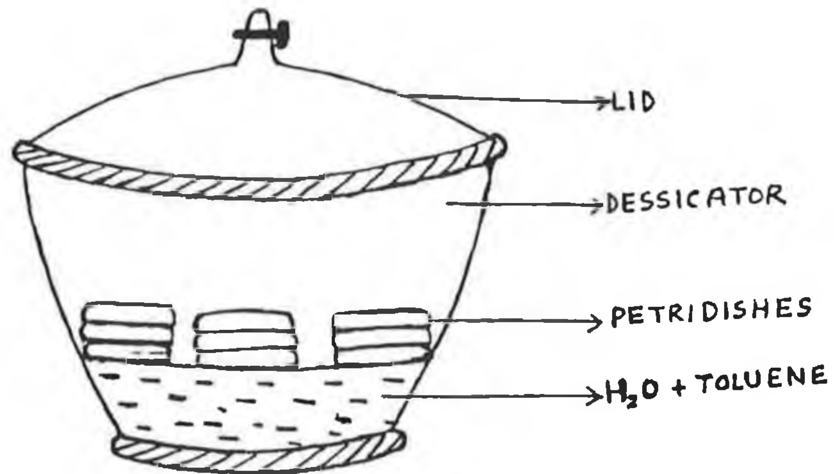


Figure 16: Cultural conditions in petridishes for volatile organic substrates (solid culture)

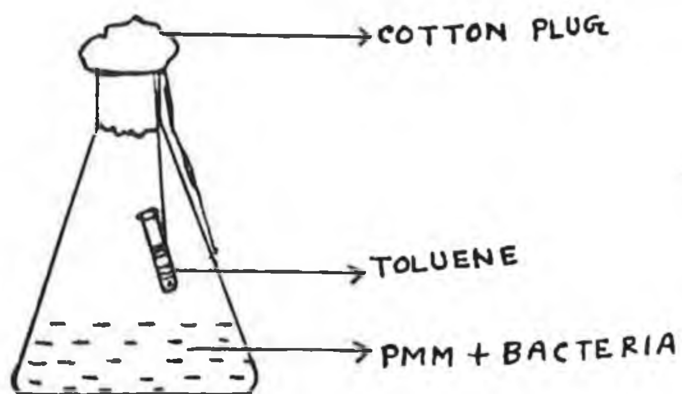


Figure 17: Cultural conditions in flasks for volatile organic substrates (liquid culture)

A plot of $Ln(X/X_0)$ vs t was calculated. Regression analysis was performed on the exponential portion of the curve. The resulting slope being equal to the specific growth rate (μ).

The analysis of variance (ANOVA) technique of the computer MINITAB Statistical Software package (6.1 VAXNMS) was used to check the statistical significance of experimental results. The calculated F-ratio was compared with the standard tabulated value of the F-ratio for specific degrees of freedom and levels of confidence (Drapper and Smith, 1966).

2.2.4 Tests used in the characterization of the *Pseudomonas* and *Aeromonas spp.*

Antibiotic sensitivity

Antibiotic sensitivity was determined by the disk diffusion method (Masten and Barry, 1974)). An overnight nutrient broth culture (0.1 ml) was spread plated onto Oxoid Mueller-Hinton agar. Oxoid Antibiotic Discs were placed on the surface of the agar plates which were incubated at 30°C for 24 hours. The presence of clear zones around the discs indicated sensitivity to the antibiotics.

API tests

A number of API identification systems were used including API 20E, for facultatively anaerobic enterobacteriaceae; API 20NE, for non-enteric Gram negative rods and API 50CH for determining carbohydrate assimilation. The identification systems were used according to the manufacturers' instructions (Bio Merieux, France). The inocula were prepared as follows : an overnight nutrient broth culture (10 ml) was harvested in a Labofuge 6000 bench-top centrifuge (5,000 rpm for 10 minutes) and washed once with sterile 0.1 M sodium phosphate buffer (pH 7.0) (section 2.1.4). The pellet was resuspended in the medium supplied by the manufacturer for API 20E and API 50CH tests. In the case of API 20NE, the pellet was resuspended in 0.85% (w/v) NaCl (10 ml) and used to inoculate a portion of the tests. For the API 20NE

assimilation tests, 200 µl of this suspension was used to inoculate auxiliary medium supplied by the manufacturer and this was then used to inoculate these tests. The following tests were performed as outlined by Harrigan and McCance (1976) unless otherwise stated.

Casein hydrolysis

Milk agar plates were inoculated by streaking once across the surface. Plates were incubated at 30°C for 2-14 days.Clear zones visible after incubation were presumptive evidence of casein hydrolysis. This was confirmed by flooding the plates with 1% (v/v) hydrochloric acid which precipitated unhydrolysed protein.

Catalase

A loopful of growth was emulsified with a loopful of 3% (v/v) hydrogen peroxide on a slide. Effervescence, caused by the liberation of free oxygen as gas bubbles, indicated the presence of catalase.

DNase activity (Collins and Lyne, 1985)

The organisms were streaked onto DNase agar and incubated at 30°C overnight. The plates were flooded with 1 M hydrochloric acid, which precipitated unchanged nucleic acids. Clear zones around the inoculum indicated a positive reaction.

Gelatin hydrolysis

Gelatin agar was inoculated by streaking the test organism once acrose the surface of the medium. Plates were incubated at 30°C for 2-14 days. Plates were flooded with 8-10 ml of mercuric chloride solution 15% (w/v) in 20% (v/v) hydrochloric acid). Unhydrolysed gelatine formed a white opaque precipitate thus a clear zone was recorded as a positive result.

Gram reaction

The Gram stain was carried out on 18-24 hour cultures according to the Hucker method (Collins and Lyne, 1985).

Growth in 6% NaCl

Growth in 6% NaCl was carried out in 1% Oxoid tryptone water containing 6% (w/v) NaCl.

Growth in KCN broth

Two tubes of KCN broth base one without potassium cyanide and the other with added potassium cyanide were inoculated heavily with 1-3 loops of a 24 hour broth culture of the test organisms. The tubes were incubated at 30°C . Observations for growth were made at the end of 24 and 48 hours of incubation. Growth of the organism in both tubes indicated a positive result.

Morphology

The colony morphology of the *Pseudomonas* and *Aeromonas spp.* was examined following growth on nutrient agar at 30°C for 24 hours. The cell morphology was examined following growth in nutrient broth for 24 hours at 30°C with agitation at 200 rpm.

Oxidase activity

a) Filter paper was impregnated with a 1% (w/v) aqueous solution of tetramethyl-p-phenylene-diamine (with 0.1% ascorbic acid to prevent auto-oxidation). Bacterial cultures were gently smeared across the filter paper with a glass rod. The formation of a purple colour within 5-10 seconds indicated oxidase positive cultures.

b) Oxoid oxidase identification sticks were used to take up some bacterial culture. A positive reaction was recorded when purple coloration formed within 30 seconds.

Oxidation - Fermentation test

Two tubes of Hugh and Leifson's medium were stab inoculated with the test culture. One tube was covered with sterile mineral oil and the tubes were incubated at 30°C for up to 14 days.

Acid production was shown by a change in the colour of the medium from blue-green to yellow. Fermentative organisms produced acid in both tubes but oxidative organisms produced acid only in the open tube and usually only at the surface.

Sensitivity to 2,4-diamino-6,7-diisopropyl petridine

An overnight nutrient broth (0.1 ml) culture was spread plated onto Oxoid Mueller-Hinton agar. The vibriostatic agent 0/129 (2,4-diamino-6,7-diisopropyl pteridine) was applied in the form of discs containing 10 µg and 150 µg of 0/129. The plates were incubated for 24-48 hours at 30°C. Zones of inhibition around the discs were indicative of sensitivity to the agent.

Spore stain

A smear of the organism was prepared from a 48 hour nutrient broth culture and heat fixed. The smear was stained with 5% (w/v) aqueous malachite green and kept steaming for 10 minutes (renewing the stain as it evaporated). It was then washed for 30 seconds in tap water and counterstained with 0.25% (w/v) saffarnin for 1 minute.

Starch hydrolysis

Nutrient agar containing 0.8% soluble starch was inoculated by streaking the test organisms once across the surface of the medium. Plates were incubated at 30°c for 3-4 days and flooded with Grams iodine to stain the starch. Hydrolysis was indicated by clear zones around the growth. Unchanged starch gave a blue colour.

Tween 80 hydrolysis

A poured dried plate of Tween 80 agar was inoculated by streaking once across the surface. Plates were incubated at 30°C for 1-7 days. Opaque zones surrounding the inoculum consisted of calcium salts of free fatty acids and were indicative of Tween 80 hydrolysis.

Temperature profiles

The organisms were grown on nutrient agar and incubated as follows :

4°	for 7 - 14 days
20 and 25°C	for 2 - 3 days
30, 37 and 42°C	for 1 - 2 days

2.2.5 Measurement of toluene

Toluene concentrations were measured both in the gas and liquid phases by direct injection of the sample into a Carlo Erba HRGC 5300 Mega Series gas chromatograph equipped with a flame ionization detector. A glass column (2 m length, by 2 mm inside diameter and 6 mm outside diameter; Phase Separation Ltd) packed with 0.2% carbowax 1500 on 80/100 mesh carbopack - C was used to measure the toluene concentration. The carrier gas was nitrogen used at a flow rate of 30 ml/min. The injector port temperature was 150°C and the detector temperature was 225°C. Toluene was measured isothermally at 180°C.

To determine the concentration of dissolved toluene in the liquid phase, culture samples were extracted with methylene chloride (equal volumes) and 1 µl of the sample was injected by a 10 µl syringe (Hamilton) into the injector. The gas phase sample was collected using a 50 µl gas-tight syringe (Hamilton) and 10 µl of the sample was injected directly into the injector. Standards were prepared by dissolving toluene in methanol.

2.2.6 Catechol Dioxygenase assays

Preparation of cell free extracts

Cells were grown on the growth substrates (200 ml) as described previously (section 2.2.3) and harvested in a Sorvall RC-5B high speed centrifuge (5,000 rpm for 10 minutes). The resulting pellet was washed in 0.033 M Tris-HCL buffer (pH 7.6) and the final pellet was resuspended in the same buffer

(3 ml).

The cells were disrupted by sonication for 3 minutes (30 seconds on; 30 seconds off) at 16 amplitude microns in a MSE Soniprep 150. The cell suspension was kept on ice throughout sonication.

Whole cells and cell debris were removed by centrifugation at 20,000 rpm and 0-4°C for 20 minutes in a Sorvall RC-5B centrifuge. The cell-free extract was kept on ice and assayed as soon as possible for catechol dioxygenase activity (Feist and Hegeman, 1969).

Catechol 1,2-dioxygenase activity

The *ortho*-cleavage of catechol by catechol 1,2-dioxygenase was measured by following the formation of the cleavage product *cis, cis*-muconic acid.

The following reagents were added to a quartz cuvette :

2.0 ml	50 mM tris-HCl buffer (pH 8.0)
0.7 ml	distilled water
0.1 ml	100 mM 2-mercaptoethanol
0.1 ml	cell-free extract

The contents of the cuvette were mixed by inversion and 0.1 ml of catechol (1 mM) was then added and the contents mixed again. *cis, cis*-muconic acid formation was followed by an increase in the absorbance at 260nm over a period of 5 minutes.

Catechol 2,3-dioxygenase activity

Catechol 2,3-dioxygenase activity was determined by following the formation of the *meta*-cleavage product, 2-hydroxymuconic semialdehyde.

The following reagents were added to a plastic cuvette :

2.0 ml	50 mM tris-HCl buffer (pH 7.5)
0.6 ml	distilled water
0.2 ml	cell-free extract

The contents were mixed by inversion and 0.2 ml of catechol (100 mM) was added and mixed with the contents. 2-Hydroxymuconic semialdehyde production was followed by an increase in absorbance at 375nm over a period of 5 minutes.

Calculation of enzyme activity

The enzyme activity was calculated using the following equation :

$$Activity\ (\mu\ moles\ product\ formed/\mathrm{min}) = \frac{E \times C \times L}{V} \times \frac{\Delta\, OD}{\mathrm{min}}$$

where

E = Molar extinction co-efficient (1/mol.cm)
C = substrate concentration (moles)
L = path length (cm)
V = reaction volume (L)

Catechol 1,2-dioxygenas0e : E_{260} = 16,800 1/mol/cm

$$Activity = \frac{16{,}800 \times 10^{-7} \times 1}{3 \times 10^{-3}} \times \frac{\Delta\, OD}{\mathrm{min}}$$

$$= 0.56 \times \frac{\Delta\, OD}{\mathrm{min}}$$

$$= \mu\ moles\ product\ formed/\mathrm{min}$$

Catechol 2,3- dioxygenase : E_{375} = 14,700 mol/1/cm

$$Activity = \frac{14,700 \times 2 \times 10^{-7} \times 1}{3 \times 10^{-3}} \times \frac{\Delta OD}{\min}$$

$$= 0.98 \times \frac{\Delta OD}{\min}$$

$$= \mu\, moles\ product\ formed / \min$$

The specific enzyme activity is expressed as µmoles of product formed per minutes per mg of protein and was calculated from :

$$Specific\ activilty\ (\mu moles/\min/mg) = \frac{Activity}{Total\ protein}$$

Protein determination

The protein concentration in the cell-free extracts was determined by the method of Lowry *et al.*, (1959).

Reagent A was added to a 0.5 ml of suitably diluted sample or standard. The tubes were left for 10 minutes in the dark at room temperature. Reagent B (0.125 ml) was then added and the tubes were left for 25 minutes at room temperature in the dark. The tubes were then mixed by vortexing and left for a further 5 minutes. The absorbance was read at 600nm. Standards were prepared with bovine serum albumin (0 - 1 mg/ml). A sample standard curve is presented in Figure 18.

Reagent A :	50 ml	0.2% Na_2CO_3 in 0.1 M NaOH
	0.5 ml	1% $CuSO_4$
	0.5 ml	2% Na K tartarate
Reagent B :	50% (v/v)	Folins - Ciolcalteau reagent in water

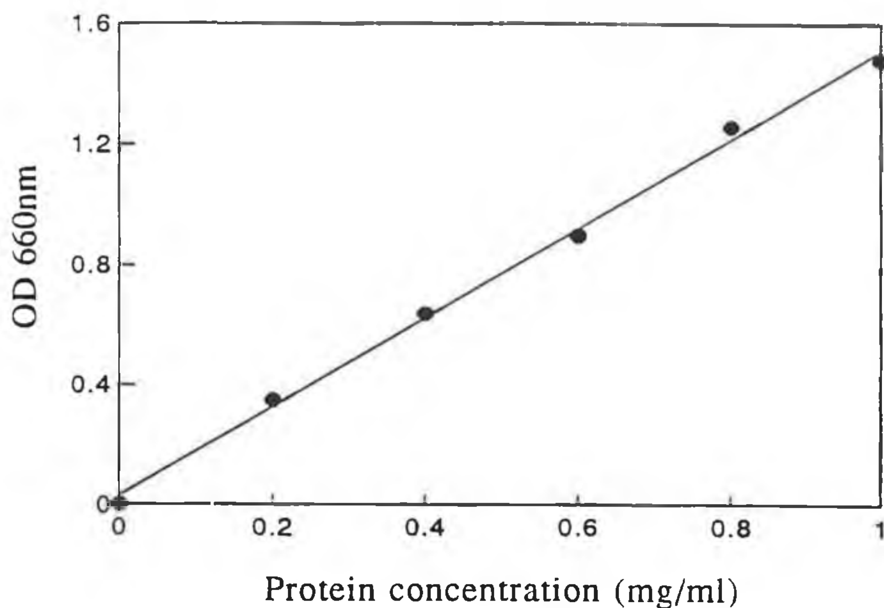


Figure 18: Standard curve for protein using the Lowry assay

2.2.7 Isolation of plasmid DNA from *Pseudomonas* and *Aeromonas* species

A modified method of Hansen and Olsen (1978) was used for the isolation of large plasmids from *Pseudomonas* and *Aeromonas* spp. (Almond *et al.*, 1985).

An overnight culture of 250 ml was harvested at 7,000 rpm for 10 minutes in a Sorvall RC-5B high speed centrifuge. The resulting cell pellet was resuspended in 6 ml of 25% (w/v) sucrose in 0.05 M Tris (pH 8.0). From this point on, all further additions were mixed by gentle inversion of the tube. First, 1 ml of lysozyme (5 mg/ml in 0.25 M Tris, pH 8.0) was added and the tubes were left on ice for 5 minutes. Then, 2.5 ml of EDTA (0.25 M , pH 8.0) was added and the tubes were left on ice for a further 5 minutes. Cell lysis was completed by the addition of 2.5 ml of SDS (20% (w/v) in TE), followed by a brief (1-2 minute) incubation at 55°C.

A freshly prepared 3 M solution of NaOH (0.75 ml) was added and the tubes were mixed by inversion for 3 minutes at room temperature. Then, 6 ml of Tris (2 M,pH 7.0) was added and mixed thoroughly but gently. The addition of 3 ml of SDS (20% (w/v) in TE) was immediately followed by the addition of 6 ml of ice cold NaCl (5 M). The tubes were then mixed gently and left on ice for 5-6 hours or overnight at 4°C.

The precipitated chromosome - membrane complex was removed by centrifugation at 20,000 rpm for 30 minutes (4°C) in the Sorvall RC-5B centrifuge. The supernatant was removed to a fresh centrifuge tube and 1/3 volume (approximately 9.2 ml) of PEG 6000 (42% (w/v) in 0.01 M sodium phosphate buffer, pH 7.0) was added. The tubes were mixed by inversion and chilled overnight at 4°C.

The DNA was harvested by centrifugation at 7,000 rpm for 6 minutes (4°C) in the Sorvall RC-5B and the resulting pellet was resuspended in 6 ml of TE buffer (section 2.1.4). The tubes were kept on ice and gently shaken from time to time to resuspend the DNA. The DNA was visualized using agarose gel electrophoresis or was purified for restriction analysis.

2.2.8 Curing of the TOL plasmid from *Pseudomonas putida* NCIB 10432

Curing was performed using the method of Dunn and Gunsalus (1973). The *Pseudomonas putida* NCIB 10432 was grown in L-broth with 5 - 20 µg/ml of mitomycin C (added after sterilization of the medium) and incubated at 30°C with agitation at 200 rpm for 2 days. Appropriate dilutions were spread - plated onto nutrient agar and grown overnight at 30°C. Colonies were replica plated onto *Pseudomonas* minimal medium incorporating *m*-toluate (5 mM) and incubated at 30°C for 2-4 days. Colonies which failed to grow on *m*-toluate were streaked from the nutrient agar plates onto fresh medium with m-toluate to verify the result.

The DNA was then isolated from presumptive TOL$^-$ cells using the modified Hansen and Olsen procedure (section 2.2.7) and visualized by agarose gel electrophoresis (section 2.2.9). One of the culture which had lost the TOL plasmid was selected and maintained on nutrient agar for use as a chromosomal marker on agarose gels.

2.2.9 Separation of plasmid DNA by agarose gel electrophoresis

Plasmids were separated by horizontal electrophoresis through a 0.7% agarose gel using a Gallenkamp Maxicell submarine gel system. Tris acetate running buffer was used (section 2.1.4). Bromophenol blue solution (0.07% (w/v) in 40% sucrose) was added to the DNA solution (1/5) and 25 µl of the sample was loaded per well. To separate large plasmids, the gel was run at 35 mV overnight using a Gallenkamp Biomed E500 power pack.

Following electrophoresis, the gel was stained in ethidium bromide (0.4 µg/ml in distilled water) for 30-40 minutes, destained in distilled water for 15 minutes and visualized on a UV transilluminator. Successful gels were photographed using Kodak Tri-X-pan, 35 mm film. The negatives were developed with Kodak Universal Developer (1/8 (v/v) in distilled water)and fixed with Kodakfix (1/4 (v/v) in distilled water. The negatives were printed on Kodak F4 photographic paper.

2.2.10 Purification of plasmid DNA

Phenol/chloroform mix (4 ml) was added to total DNA (4 ml). The mixture was shaken by hand for 5 minutes and centrifuged at 7000 rpm, 4°C for 15 minutes. The top layer was removed and if necessary centrifuged again with an equal volume of phenol/chloroform mix. The protein free DNA solution was then purified by a cesium chloride density gradient.

Cesium chloride-ethidium bromide gradients

A modification of the method outlined by Maniatis *et al.*, (1982) was used to

purify plasmid DNA for restriction analysis and probe preparation.

Cesium chloride (6.9 g) was added to 6.5 ml of DNA solution and dissolved by mixing gently. The solution was then centrifuged at 2000 rpm for 5 minutes to remove the excess protein which might block the syringe during extracting of the plasmid band. This solution was transferred to Beckman Quickseal tubes using a pasteur pipette and 0.18 ml of ethidium bromide (10 mg/ml in distilled water) was then added. The total weight of solution (excluding tube) was brought to 14.1 g with Na_2EDTA (10 mM). The tubes were filled with mineral oil and balanced in pairs to within 0.01 g. Air was removed from the tubes which were filled with mineral oil and sealed with a Beckman heat sealer. The density gradient was formed by centrifugation at 50,000 rpm and 18°C in the Ti rotor (Beckman Ultracentrifuge model LH-M).

The lower plasmid band was extracted after the 22-24 hour centrifugation with a syringe in the presence of UV light and transferred to a sterile plastic tube. Extraction involved piercing the tube at the top and under the plasmid band with a lance and withdrawing the band into a syringe. This was extracted 4 times with equal volumes of isopropanol saturated with 20 X SSC (section 2.1.4) to remove the ethidium bromide. Dialysis tubing was boiled in Na_2EDTA (10 mM) for 10 minutes and then boiled in distilled water for a further 10 minutes. The plasmid solution was dialysed against TE buffer (section 2.1.4) twice (including one overnight) to remove the cesium chloride. The dialysed DNA was concentrated by ethanol precipitation where necessary and then stored at -20°C.

Ethanol precipitation of DNA

Two volumes of absolute ethanol and one tenth volume of 3 M sodium acetate were added to the DNA sample (25 µl). The sample was incubated at -20°C overnight and the DNA was then pelleted by centrifugation at 10,000 rpm for 20 minutes. The pellet was then washed once in 70% ethanol, desiccated by vaccuam drayer and resuspended in TE, pH 8.0.

2.2.11 Restriction of plasmid DNA

The purified DNA solutions, were cut with restriction endonucleases according to the suppliers' instructions. λ DNA was cut separately with Hind III and Xho I and the digests were combined to provide appropriate size markers in the range of 1 - 33.5 kb (Gibco-BRL catalogue).

Reaction digests were set up as follows :

	Plasmid DNA	Probe DNA	λ DNA
DNA solution (µl)	20.5	5.0	4.0
Buffer (µl)	2.5	1.0	4.0
Sterile distilled water (µl)	–	3.0	30.0
Enzyme - 10 iu/ml (µl)	2.0	1.0	2.0
	25.0	10.0	40.0

All samples were incubated at 37°C for 2 hours.

2.2.12 Preparation of probe DNA

Determination of DNA concentration

Serial dilutions of linearized probe DNA were prepared on parafilm. First, 5 µl of TE was placed on the parafilm and 5 µl of DNA was added. The dilution was mixed using a micropipette. Then 5 µl of this solution was added to a second 5 µl of TE and so on. Each sample was mixed with 1 µl of bromophenol blue solution and 5 µl was loaded onto a 1% (w/v) agarose mini-gel. λ standards of known concentration were also loaded onto the gel. The gel was run at 100 V for 30 minutes and stained 10 - 15 minutes in ethidium bromide solution. The concentration of probe DNA was estimated by

comparing the intensity of fluorescence of the samples with that of λ DNA of known concentration.

Non-radioactive labelling of TOL probe DNA

Random primer labelling of linearized probe DNA with digoxigenin was carried out using the DIG DNA labelling kit in accordance with the manufacturers' instructions (Boehringer Mannheim, Germany).

First, 5 µl of linearized DNA was added to a sterile eppendorf along with 10 µl of sterile distilled water and denatured by boiling for 10 minutes followed by chilling on ice. The kit reagents were then added to the denatured DNA solution in the following order :

2 µl	hexanucleotide mixture
2 µl	dNTP labelling mixture
1 µl	klenow enzyme

The tube was centrifuged briefly to mix contents and was incubated in a 37°C waterbath for at least 1 hour. The reaction was stopped by adding 2 µl of EDTA (0.2 M, pH 8.0) and the labelled DNA was precipitated with 2.5 µl lithium chloride (4 M) and 75 µl of ethanol (at -20°C). The tube was incubated at -20°C for 2 hours or -70°C for 30 minutes and then centrifuged at 12,000 rpm for 10 minutes in a microfuge. The pellet was washed twice with cold ethanol (70% v/v) and then dried at 55°C for 15 minutes. The pellet was resuspended in 50 µl of TE buffer. The labelled DNA was then used directly for hybridization or stored at -20°C for up to 6 months. The stored probe must be denatured prior to use. This can be done by denaturing the hybridization solution at 95°C for 10 minutes.

Labelling of TOL probe DNA with ^{32}P

Linearized probe DNA was labelled by random primed incorporation of α-^{32}P ATP (Du Pont, France) using the Prime-a-Gene labelling system (Promega Corp., Madison, USA) based on the method developed by Feinburg and

Vogelstein (1983). The kit was stored at -20°C. Before use the components were allowed to thaw on ice except Klenow enzyme, which was kept on at -20°C and returned to the freezer immediately after use. First 25 ng of linearized DNA was added to a sterile eppendorf and the volume was brought to 30 µl with sterile distilled water. The DNA was denatured by heating to 95-100°C for 5 minutes and was then cooled rapidly on ice. Then, 1 µl of each of the non-labelled dNTP's (dCTP, dGTP, dTTP) was mixed to yield a 3 µl solution. The labelling reaction was set by adding the following reagents to the denatured DNA solution, in their stated order :

10 µl 5X labelling buffer
2 µl mixture of non-labelled dNTP's
2 µl nuclease-free BSA
5 µl [α-^{32}P] dATP (50 µCi, 3000 Ci/mMol)
1 µl Klenow enzyme

The tube was centrifuged briefly to mix contents and was incubated for 1 hour at room temperature. Incubation for longer periods resulted in increased specific activity of the probe. The reaction was stopped by heating at 95-100°C for 2 minutes and was subsequently chilled on ice bath. EDTA was added to a concentration of 20 mM and the solution was then used directly for hybridization. The probe may be stored at -20°C depending on the activity of the ^{32}P.

2.2.13 Southern hybridization

Southern transfer and hybridization (Southern, 1975) were performed as detailed in the method of Maniatis *et al.* (1982).

Transfer of DNA from agarose gel to nitrocellulose paper
Following electrophoresis one corner of the gel was cut away in order to mark its orientation on the nitrocellulose.The DNA was partially hydrolysed by acid depurination. The gel was soaked twice for 15 minutes in 0.25 M HCl at room

temperature and then washed very well with water (4-5 minutes). The DNA was denatured by soaking the gel in several volumes of 1.5 M NaCl and 0.5 M NaOH for 1 hour at room temperature with constant shaking. The gel was neutralized by soaking in several volumes of 1 M Tris.HCl (pH 8.0) and 1.5 M NaCl for 1 hour at room temperature with constant shaking. At this stage the pH of the gel was below 8.5. The transfer took place overnight.

For the Southern blot, a piece of nitrocellulose (BA85; Schleicher and Schuell, Germany) was cut about 1-2 mm larger than the gel in both dimensions. Gloves were always used when handling nitrocellulose. The nitrocellulose filter was floated on the surface of 2 X SSC until completely wet from the beneath and then submerged for 5 minutes. This ensured even wetting of the nitrocellulose, eliminating air bubbles. The gel was placed on a 3MM Whatman filter paper wick, the ends of which were in contact with 20 X SSC. The wet nitrocellulose was laid carefully on the gel and the air bubbles removed by rolling a glass test tube over the surface. Two pieces of Whatman filter paper soaked in 2 X SSC were placed on the top of the nitrocellulose and the stack built up with paper towels. A weight was placed on the top to aid the transfer. Parafilm was used to prevent the towels coming in contact with the gel or 20 X SSC, thereby preventing the liquid bypassing the nitrocellulose.

After the overnight transfer of DNA to the nitrocellulose the towels and filter paper were removed and the position of the wells were marked on the nitrocellulose. They were then washed in 6 X SSC for 5 minutes to remove any fragments of agarose and air dried at room temperature. The dried filter was placed between two sheets of 3MM paper and baked at 80°C for two hours.

Transfer was allowed to proceed overnight. The filters were soaked in 6 X SSC for 5 minutes at room temperature and then dried at room temperature. The dried filter was placed between two sheets of 3MM paper and baked at 80°C for 2 hours. The filter could either be stored at room temperature or

used for hybridization.

Hybridization of Southern filters

Hybridization was carried out using either radioactive or non-radioactive labelling.

Hybridization with ^{32}P probe

The baked filter was floated on the surface of 6 X SSC until wet from beneath and then immersed for 2 minutes. The wet filter was placed in a heat-sealable bag. Prehybridization fluid (0.2 ml/cm^2 of filter) was warmed to 68°C and then added to the bag (section 2.1.4)

As much air as possible was squeezed from the bag before it was sealed. The bag was incubated for 2-4 hours at 68°C with occasional agitation. Following incubation the corner of the bag was cut with scissors and the prehybridization solution removed. The hybridization solution (50 µl/cm^2 of filter) was added with the appropriate precautions for the handling of ^{32}P (section 2.1.4).

Air was removed from the bag and it was resealed. The filter was incubated at 68°C overnight with occasional agitation.

The following day the filter was removed from the bag and immediately submerged in a solution of 2 X SSC and 0.5% (w/v) SDS at room temperature. After 15 minutes the filter was transferred to a solution of 2 X SSC and 0.1% (w/v) SDS and incubated at room temperature for 15 minutes. The filter was then transferred to a prewarmed solution of 0.1 X SSC and 0.5% (w/v) SDS and incubated at 68°C for 2 hours with gentle agitation. The buffer was then changed and incubated for a further 30 minutes. The filter was then dried at room temperature on a sheet of 3MM paper. The dry filter was placed in a heat-sealable bag and applied to x-ray film to obtain an autoradiographic image.

Autoradiography

In the dark. a sheet of Kodak x-ray film was taped to an x-ray film holder (shiny-side down). The filter was sealed in a bag was then placed on the top of the film and taped securely in place. a second sheet of x-ray film was then taped on the top of the film (shiny-side up). The x-ray holder was closed and placed in a black plastic bag. The film was exposed for several days at room temperature. When the desired exposure time had elapsed the films were removed, in the dark, and developed as follows :

The film was placed in Kodak DX-80 developer for 4 minutes, washed in water for 30 seconds and the fixed in Kodak FX-40 fixer for about 5 minutes and finally washed under running water.

If necessary another film can be applied to the filter to obtain another autoradiograph.

Hybridization to digoxigenin-labelled probe

Baked filters were floated on the surface of a solution of 2 X SSC until wet from beneath and were then submerged for 5 minutes. The filters were then washed in several volumes of prewashing solutions (section 2.1.4) at 50°C for 30 minutes with occasional agitation.

Bacterial debris was gently scraped from the filters using soft tissue soaked in prewashing solution. This reduced the background hybridization without affecting the intensity or sharpness of positive signals.

The filters were prehybrydized in heat-sealable plastic bags (2 filters/bag) with at least 20 ml of hybridization solution (section 2.1.4) per 100 cm^2 of filter.

They were incubated at 42°C at least 1 hour. The solution was distributed from time to time. The solution was then replaced with 2.5 ml of hybridization solution (section 2.1.4), per 100 cm^2 of filter, containing freshly

denatured labelled DNA (2.2.12). The filters were incubated at 42°C overnight with the solution being redistributed occasionally.

Following incubation, the filters were removed from the plastic bags and washed twice in 2 X SSC and 0.1% (w/v) SDS for 5 minutes at room temperature (501/100 cm^2 filter). The filters were washed twice more 15 minutes at 68°C in 0.1 X SSC and 0.1% (w/v) SDS (50 ml/100 cm^2 filter) The filters were then used directly for detection or stored air-dried for later detection.

All the following incubations were performed at room temperature. All the reactions were carried out with agitation except for the colour development, The volumes were calculated for 100cm^2 filter size.

The filters were washed briefly (1 minute) in several volumes of buffer 1 and then incubated for 30 minutes in 100 ml of buffer 2 (section 2.1.4). The filters were then incubated for 30 minutes in 20 ml of diluted antibody-conjugate solution (150 mU/ml, 1/5000 in buffer 2). Unbound antibody was removed by washing twice for 15 minutes in 100 ml of buffer 1. The filters were equilibrated for 2 minutes with 20 ml of buffer 3 and then incubated in 10 ml of colour solution in the dark. The colour was prepared fresh with NBT-solution (4.5 µl/ml Buffer 3) and X-phosphate solution (3.5µl/ ml Buffer 3). The colour was allowed to develop overnight <u>without</u> shaking or mixing. The reaction was stopped by washing the filters for 5 minutes with 50ml of TE buffer. The filters were stored in sealed plastic bags in TE buffer. The results were documented by photography.

2.2.14 Dot Blot hybridization

Total DNA was prepared and hybridized using the method of Maniatis *et al.*, (1982). The sample and the control DNA were denatured by boiling for 10 minutes and chilling quickly on ice. The DNA was incubated with an equal volume of 1 M NaOH for 20 minutes at room temperature. The DNA was

then neutralized with half the volume of a solution of 1 M NaCl, 0.3 M sodium citrate, 1 M HCl and 0.5 M Tris.HCl (pH 8.0). The sample were mixed well and immediately chilled on ice.

A sheet of nitrocellulose was cut to the desired size and placed on a sheet of clingfilm. First, 5 µl of the DNA solution was spotted onto the filter and allowed to absorb, then another spot of 5 µl was applied. The process was repeated until the desired volume of sample had been applied (30 µl for sample DNA, 10µl for control or probe DNA). The filter was allowed to dry at room temperature for 1 hour and baked at 80°C for 2 hours. The filter was then used directly for hybridization or stored between two sheets of 3MM paper and wrapped in clingfilm. The dot blots were hybridized using non-radioactively labelled probe (section 2.2.12).

For dot blot hybridization, the digoxigenin-labelled protocol (2.2.13) was followed except no prewashing of filters was required, the hybridization solution (section 2.1.4) did not contain formamide and therefore only 1% blocking reagent (section 2.1.4) was added and also prehybridization and hybridization were carried out at 68°C.

3. RESULTS

3.1 The isolation of the bacteria

3.1.1 Source of the isolates

All the bacteria were isolated from activated sludge. The characteristics of this activated sludge sample are outlined in Table 4. The pH was 7.2, temperature 12.2°C and dissolved oxygen concentration 9.2 mg/ml. The colour of the supernatant of the mixed liquor was 175°Hazen. The mixed liquor suspended solids (MLSS) were 3.17 g/l and the settled sludge volume (SSV) was 180 ml/l giving a sludge volume index (SVI) of 56 ml/g. Microscopic examination of the sludge sample showed the presence of flocs and free-swimming bacteria. No protozoa were observed in the sludge. The total viable count on PCA was 1.03 x 10^5 bacteria/g sludge. The number of bacteria capable of growing on the individual aromatics was considerably lower than the total viable count. Twenty five bacteria/g, 16 bacteria/g and 19 bacteria/g sludge were capable of growth on toluene, phenol and naphthalene respectively when these substrates were supplied as the sole carbon source.

3.1.2 Identification of a representative number of bacteria capable of degrading aromatic substrates

The bacteria were isolated and identified based on their ability to grow on either phenol, toluene or naphthalene when supplied as the sole carbon source. There were considerable similarities in the colony morphologies of the isolates grown on the individual aromatics. A representative number of dissimilar colonies were isolated from each substrate for further identification. A total of fifteen bacteria were isolated from the various aromatic plates, five from toluene, four from phenol and six from naphthalene. Strain numbers were given to the isolated bacteria according to

Table 4: Characteristics of the activated sludge sample

Parameter	Sludge sample
pH	7.2
Temperature	12.2°C
Dissolved oxygen	9.2 mg/ml
Colour	175°Hazen
Settle Sludge Volume	180 ml/l
Mixed Liquor Suspended Solid (MLSS)	3.17 g/l
Sludge Volume Index (SVI)	56 ml/g
Microscopic morphology	Presence of flocs, bacteria and absence of protozoa
No. bacteria/g sludge on plate count agar	1.03×10^5
No. bacteria/g sludge on aromatic substrates	Toluene 25; Phenol 16; Naphthalene 19

the aromatic substrates from which they were isolated (To = toluene, Ph = phenol and Na = naphthalene).

Pure cultures of the isolated bacteria were subjected to a variety of tests for their identification. Two genera were identified - the genus *Pseudomonas* and the genus *Aeromonas.* Ten of the isolates were identified as *Pseudomonas* spp. and five were identified as *Aeromonas* spp. Out of ten *Pseudomonas* spp. five were *P. putida,* two were *P. luteola* and the other three were *P. cepacia, P. fluorescens* and *P. pseudomellei.* Among the *Aeromonas* spp. two were *A. caviae,* two were *A. hydrophila*, and one was *A. sobria.*

The cell and colony characteristics of each organism were noted together with their response to a wide range of biochemical tests. All the organisms were Gram negative, motile, non-spore forming rods. With the exception of the colour, the colony characteristics of all the isolates were identical (Table 5).

They were all oxidase and catalase positive. All the *pseudomonas* spp. metabolized glucose oxidatively while all the *Aeromonas* spp. metabolized glucose fermentatively. None of the isolates produce H_2S except strain Na-12. All of the isolates were failed to produce urease or to growth in 6% NaCl and all were sensitive to vibriostatic agent (2,4-Diamino-6,7-diisopropyl-petridine(0/129). All the *Pseudomonas* and *Aeromonas* spp. showed dissimilar reactions for the other biochemical tests (Table 6). All the isolates were capable of utilizing a wide range of carbohydrates as the sole carbon source. Of the thirty six carbohydrates tested, *Pseudomonas* spp. could use fourteen and *Aeromonas* spp. used eighteen substrates (Table 7).

The fifteen isolates were examined for their sensitivity to a number of antibiotics at 30°C (Table 8). All the bacteria with the exception strain Ph-9 were capable of growth in the presence of chloramphenicol (50 µg) and sensitive to polymyxin B (300 iu). With the exception of these two antibiotics all the *Pseudomonas* and *Aeromonas* spp. showed dissimilar responses to the

Table 5: Cell and colony morphology of the isolates

Isolate	Cell				Colony			
	A	B	C	D	E	F	G	H
Pseudomonas putida(To-1)	rod	-	-	+	cream	round	entire	convex
Aeromonas hydrophila(To-2)	rod	-	-	+	buff	round	entire	convex
Pseudomonas putida(To-3)	rod	-	-	+	cream	round	entire	convex
Aeromonas caviae(To-4)	rod	-	-	+	buff	round	entire	convex
Pseudomonas putida(To-5)	rod	-	-	+	cream	round	entire	convex
Pseudomonas cepacia(Ph-6)	rod	-	-	+	cream	round	entire	convex
Pseudomonas luteola(Ph-7)	rod	-	-	+	cream	round	entire	convex
Pseudomonas luteola(Ph-8)	rod	-	-	+	cream	round	entire	convex
Pseudomonas fluorescens(Ph-9)	rod	-	-	+	cream	round	entire	convex
Aeromonas hydrophila(Na-10)	rod	-	-	+	buff	round	entire	convex
Pseudomonas putida(Na-11)	rod	-	-	+	cream	round	entire	convex
Aeromonas sobria(Na-12)	rod	-	-	+	buff	round	entire	convex
Pseudomonas putida(Na-13)	rod	-	-	+	cream	round	entire	convex
Aeromonas caviae(Na-14)	rod	-	-	+	buff	round	entire	convex
Pseudomons pseudomellei(Na-15)	rod	-	-	+	cream	round	entire	convex

A - Cell shape; B - Gram reaction; C - Spore formation; D - Motility; E - Colour; F - Shape; G - Edge; H - Elevation;

+ positive reaction;

- negative reaction.

Table 6: Biochemical properties of the isolates

Isolate	Biochemical test								
	A	B	C	D	E	F	G	H	I
Pseudomonas putida(To-1)	Ox	+	+	-	-	-	-	-	-
Aeromonas hydrophila(To-2)	Fer	+	+	+	+	+	+	+	+
Pseudomonas putida(To-3)	Ox	+	+	-	-	-	-	-	_
Aeromonas caviae(To-4)	Fer	+	+	+	+	+	+	+	+
Pseudomonas putida(To-5)	Ox	+	+	-	-	-	-	-	-
Pseudomonas cepacia(Ph-6)	Ox	+	+	+	-	-	+	+	-
Pseudomonas luteola(Ph-7)	Ox	+	+	-	+	-	-	+	-
Pseudomonas luteola(Ph-8)	Ox	+	+	-	+	-	-	+	-
Pseudomonas fluorescens(Ph-9)	Ox	+	+	-	-	-	-	-	-
Aeromonas hydrophila(Na-10)	Fer	+	+	+	+	+	+	+	+
Pseudomonas putida(Na-11)	Ox	+	+	-	-	-	-	-	-
Aeromonas sobria(Na-12)	Fer	+	+	+	+	+	+	-	+
Pseudomonas putida(Na-13)	Ox	+	+	-	-	-	-	-	-
Aeromonas caviae(Na-14)	Fer	+	+	+	+	+	+	+	+
Pseudomonas Pseudomellei(Na-15)	Ox	+	+	-	+	-	+	-	+

A - Hugh and Leifson test: Ox-Oxidative, Fer-Fermentative; B - Oxidase; C - Catalase; D - β-galactosidase; E - Nitrate reductase; F - DNase; G - Gelatinase; H - Casein hydrolysis; I - Indole production;

+ positive reaction

- negative reaction

Table 6: Cont'd

Isolate	Biochemical test							
	J	K	L	M	N	O	P	Q
Pseudomonas putida(To-1)	-	-	-	-	-	-	-	-
Aeromonas hydrophila(To-2)	-	-	+	+	+	-	-	+
Pseudomonas putida(To-3)	-	-	-	-	-	-	-	-
Aeromonas caviae(To-4)	-	-	-	+	+	-	-	+
Pseudomonas putida(To-5)	-	-	-	-	-	-	-	-
Pseudomonas cepacia(Ph-6)	-	-	+	-	-	-	-	-
Pseudomonas luteola(Ph-7)	-	-	-	-	-	-	-	-
Pseudomonas luteola(Ph-8)	-	-	-	-	-	-	-	-
Pseudomonas fluorescens(Ph-9)	-	-	-	-	-	-	-	-
Aeromonas hydrophila(Na-10)	-	-	+	+	+	-	-	+
Pseudomonas putida(Na-11)	-	-	-	-	-	-	-	-
Aeromonas sobria(Na-12)	+	-	-	+	-	-	-	+
Pseudomonas putida(Na-13)	-	-	-	-	-	-	-	-
Aeromonas caviae(Na-14)	-	-	-	+	+	-	-	+
Pseudomonas pseudomellei(Na-15)	-	-	-	-	-	-	-	-

J - H_2S formation; K - Urease; L - Voges proskauer; M - Tween 80 hydrolysis; N - Growth in KCN broth; O - Sensivity to vivriostatic agent 2,4-diamino-6,7-diisopropyl-petridine; P - Growth in 6% Nacl; Q - Starch hydrolysis;

\+ positive reaction

\- negative reaction

Table 7: Carbohydrate utilization at 30°C by the isolates

Carbohydrate	Strain number of the isolate							
	To-1	To-2	To-3	To-4	To-5	Ph-6	Ph-7	Ph-8
N-acetylglucosamine	-	+	-	+	-	+	-	-
Adipate	-	-	-	-	-	+	-	-
Adonitol	-	-	-	-	-	+	+	+
Amygdalin	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+
Arginine	+	+	+	+	+	-	+	+
Caprate	+	+	+	+	+	+	+	+
Cellobiose	+	-	+	-	+	-	-	-
Citrate	+	-	+	-	+	+	+	+
Dulcitol	-	+	-	+	-	-	-	-
Galactose	+	+	+	+	+	+	+	+
Gluconate	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+
Glycerol	+	-	+	+	+	+	+	+
Inositol	+	-	+	-	+	+	+	+
Inulin	-	-	-	-	-	-	-	-
Lactose	-	+	-	+	-	-	-	-
Lysine	-	+	-	-	-	+	-	-
Malate	+	+	+	+	+	+	+	+
Maltose	-	+	-	+	-	-	+	+

cont'd p. 90

Table 7: Cont'd

Carbohydrate	Strain number of the isolate							
	To-1	To-2	To-3	To-4	To-5	Ph-6	Ph-7	Ph-8
Mannitol	-	+	-	+	-	+	+	+
Mannose	+	+	+	+	+	+	+	+
Meliobiose	+	-	+	+	+	+	+	+
Ornithine	-	-	-	-	-	-	-	-
Phenylacetate	+	-	+	-	+	+	+	+
Rhamnose	-	-	-	-	-	-	-	-
Salicine	-	+	-	+	-	+	-	-
Sorbitol	+	-	+	-	+	+	+	+
Sucrose	-	+	-	+	-	+	+	+
Trehalose	+	+	+	+	+	+	+	+
Xylose	+	-	+	-	+	+	+	+

+ Growth

- No growth

To-1 = *Pseudomonas putida*

To-2 = *Aeromonas hydrophila*

To-3 = *Pseudomonas putida*

To-4 = *Aeromonas caviae*

To-5 = *Pseudomonas putida*

Ph-6 = *Pseudomonas cepacia*

Ph-7 = *Pseudomonas luteola*

Ph-8 = *Pseudomonas luteola*

Table 7: Cont'd

Carbohydrate	Strain number of the isolate						
	Ph-9	Na-10	Na-11	Na-12	Na-13	Na-14	Na-15
N-acetylglucosamine	-	+	-	+	-	+	-
Adipate	-	-	-	-	-	-	+
Adonitol	+	-	-	-	-	-	-
Amygdalin	+	+	+	+	+	+	+
Arabinose	+	+	+	-	+	+	-
Arginine	+	+	+	+	+	+	+
Caprate	+	+	+	+	+	+	+
Cellobiose	+	-	+	-	+	-	-
Citrate	+	-	+	+	+	-	+
Dulcitol	+	+	-	+	-	+	-
Galactose	+	+	+	+	+	+	+
Gluconate	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+
Glycerol	+	-	+	-	+	-	+
Inositol	+	-	+	-	+	-	+
Inulin	-	-	-	-	-	-	-
Lactose	-	+	-	+	-	+	-
Lysine	-	+	-	+	-	-	-
Malate	+	+	+	+	+	+	+
Maltose	-	+	-	+	-	+	-

cont'd p. 92

Table 7: Cont'd.

Carbohydrate	Strain number of the isolate						
	Ph-9	Na-10	Na-11	Na-12	Na-13	Na-14	Na-15
Mannitol	+	+	-	+	-	+	+
Mannose	+	+	+	+	+	+	+
Meliobiose	+	-	+	-	+	-	+
Ornithine	-	-	-	-	-	-	-
Phenylacetate	+	_	+	-	+	-	+
Rhamnose	+	-	-	-	-	-	-
Salicine	-	+	-	+	-	+	-
Sorbitol	+	-	+	-	+	-	+
Sucrose	+	+	-	-	-	+	+
Trehalose	+	+	+	-	+	+	+
Xylose	+	-	+	-	+	-	+

+ Growth

- No growth

Ph-9 = *Pseudomonas fluorescens*

Na-10 = *Aeromonas hydrophila*

Na-11 = *Pseudomonas putida*

Na-12 = *Aeromonas sobria*

Na-13 = *Pseudomonas putida*

Na-14 = *Aeromonas caviae*

Na-15 = *Pseudomonas pseudomellei*

Table 8: Growth of the isolates at 30°C in the presence of antibiotics

Isolate	Antibiotic					
	C-50 µg	NV-30 µg	P-10 µg	S-25 µg	TE-30 µg	PB-30 µg
Pseudomonas putida(To-1)	+	+	+	-	-	-
Aeromonas hydrophila(To-2)	+	-	-	+	+	-
Pseudomonas putida(To-3)	+	+	+	-	-	-
Aeromonas caviae(To-4)	+	-	-	+	+	-
Pseudomonas putida(To-5)	+	+	+	-	-	-
Pseudomonas cepacia(Ph-6)	+	+	+	-	-	-
Pseudomonas luteola(Ph-7)	+	+	+	-	-	-
Pseudomonas luteola(Ph-8)	+	+	+	-	-	-
Pseudomonas fluorescens(Ph-9)	-	+	+	-	-	-
Aeromonas hydrophila(Na-10)	+	-	-	+	+	-
Pseudomonas putida(Na-11)	+	+	+	-	-	-
Aeromonas sobria(Na-12)	+	-	-	+	+	-
Pseudomonas putida(Na-13)	+	+	+	-	-	-
Aeromonas caviae(Na-14)	+	-	-	+	+	-
Pseudomonas pseudomellei(Na-15)	+	+	+	-	-	-

C - Chloramphenical; NV - Novobiocin; P - Penicillin G; S - Streptomycin;
TE - Tetracycline; PB - Polymyxin B

+ growth

- no growth

Table 9: Growth of the isolates on nutrient agar at various temperatures

Isolate	Temperature of incubation					
	4°C	20°C	25°C	30°C	37°C	42°C
Pseudomonas putida (To-1)	-	+++	+++	+++	++	-
Aeromonas hydrophila (To-2)	-	++	+++	+++	+	-
Pseudomonas putida (To-3)	-	++	+++	+++	++	-
Aeromonas caviae (To-4)	+	++	+++	+++	++	++
Pseudomonas putida (To-5)	-	+++	+++	+++	++	-
Pseudomonas cepacia (Ph-6)	-	+++	+++	+++	++	-
Pseudomonas luteola (Ph-7)	-	++	+++	+++	++	-
Pseudomonas luteola (Ph-8)	-	+++	+++	+++	++	-
Pseudomonas fluorescens (Ph-9)	-	++	+++	+++	-	-
Aeromonas hydrophila (Na-10)	-	++	++	+++	++	-
Pseudomonas putida (Na-11)	-	+++	+++	++	++	-
Aeromonas sobria (Na-12)	-	++	+++	+++	++	-
Pseudomonas putida (Na-13)	-	++	++	+++	++	-
Aeromonas caviae (Na-14)	-	++	++	++	+	-
Pseudomonas pseudomellei (Na-15)	-	+	+++	+++	-	-

+++ Good growth

++ Medium growth

+ Less growth

- No growth

other antibiotics tested. All the *Pseudomonas* spp. were capable of growth in the presence of novobiocin (30 µg) and penicillin G (10 iu) and were sensitive to streptomycin (25 µg) and tetracycline (30 µg). While all the *Aeromonas* spp. were capable of growth in the presence of streptomycin (25 µg) and tetracycline (30 µg) and were sensitive to novobiocin (30 µg) and penicillin G (10 iu).

The ability of the organisms to grow on nutrient agar at a range of temperatures (4°C - 42°C) was investigated (Table 9). All the organisms showed an optimum growth temperature of growth between 25°C and 30°C. Good growth was also observed at 20°C and 37°C. *Aeromonas caviae* (To-4) was the only organism capable of growth at 4°C and 42°C.

3.1.3 Stability of the degradative abilities of the isolates

It was noted that when the isolates were maintained for several months on nutrient agar, some lost their ability to grow on the substrates from which they were originally isolated. The isolates were tested for growth on three substrates - toluene, phenol and naphthalene (Table 10). All the toluene grown isolates retained their ability to grow on toluene except strain To-2. However this organism could now grow on phenol and naphthalene. In the case of the phenol isolates only two *Pseudomonas* strains Ph-6 and Ph-9 retained their ability to grow on phenol. None of these isolates were capable of growth on toluene or naphthalene. Of the six naphthalene grown isolates three lost their ability to grow on naphthalene, while three strains Na-10, Na-12 and Na-15 retained this ability. However only one strain, Na-15, showed good growth on naphthalene. Strains Na-10 and Na-12 also acquired the ability to grow on phenol and toluene. One of the naphthalene isolates, strain Na-13, which had lost the ability to grow on naphthalene acquired the ability to grow on toluene. This organism together with the other four organisms (To-1, To-3, To-4 and To-5) capable of good growth on toluene were selected for further study.

Table 10: Capability of the isolates to grow on aromatic substrates following maintainance on nutrient agar

Isolate	Aromatic substrate		
	Toluene	Phenol	Naphthalene
Pseudomonas putida (To-1)	+++	-	-
Aeromonas hydrophila (To-2)	-	+	+
Pseudomonas putida (To-3)	+++	-	-
Aeromonas caviae (To-4)	+++	+	+
Pseudomonas putida (To-5)	+++	-	-
Pseudomonas cepacia (Ph-6)	-	+++	-
Pseudomonas luteola (Ph-7)	-	-	-
Pseudomonas luteola (Ph-8)	-	-	-
Pseudomonas fluorescens (Ph-9)	-	+++	-
Aeromonas hydrophila (Na-10)	+	+	+
Pseudomonas putida (Na-11)	-	-	-
Aeromonas sobria (Na-12)	+	+	+
Pseudomonas putida (Na-13)	+++	-	-
Aeromonas caviae (Na-14)	-	-	-
Pseudomonas pseudomellei (Na-15)	-	-	+++

+++ Good growth

++ Medium growth

+ Less growth

- No growth

3.2 Growth studies of five selected bacteria

3.2.1 Growth on various aromatic substrates

Pseudomonas spp. , strains To-1, To-3, To-5, Na-13 and *Aeromonas* sp. To-4 which were capable of good growth on toluene were selected for further study. They were first tested for their ability to grow on a range of aromatic substrates. The organisms were grown in liquid culture in the presence of various aromatic compounds supplied as the sole carbon source in minimal medium. All the organisms were incubated at 30°C and agitated at 200 rpm (section 2.2.3). The growth was monitored by measuring the O.D at 660nm after 48 hours of incubation.

All five bacteria were capable of good growth on toluene, benzoic acid and *m*-toluic acid. Medium growth were observed on ethylbenzene and pseudocumene. Less growth was observed on *p*- and *m*-xylene and *p*- and *m*-chlorophenol (Table 11). All the *Pseudomonas* spp. failed to grow on phenol, naphthalene and *o*-chlorophenol. Only the *Aeromonas* sp. strain To-4 was capable of growth on phenol and naphthalene. However this organism did not grow on *o*-chlorophenol. The *Aeromonas* sp. grew on a wider range of aromatic substrates than did the *Pseudomonas* spp.

3.2.2 Growth in various concentrations of toluene in solid culture

All five organisms were incubated at 30°C for 5 days in closed desiccators containing different volumes of toluene in 500 ml of water (section 2.2.3). The volumes of toluene ranged from 6.6 µl/100 ml H_2O to 109568 µl/100 ml H_2O. The organisms were also incubated with 500 ml neat toluene without water. The concentration of toluene in the vapour phase of the desiccator was measured after 5 days of incubation.

The concentration of toluene in the vapour phase on day five increased with

Table 11: Growth of *Pseudomonas* and *Aeromonas* spp. on various aromatic substrates at 30°C

Substrate	Conc. or vol. supplied/50 ml PMM	O.D at 660nm at 48 hrs.				
		To-1	To-3	To-4	To-5	Na-13
Toluene	250 µl	1.971	1.934	2.021	2.111	1.859
Benzoic acid	5 mM	1.017	1.016	1.145	1.093	0.905
m-Toluic acid	5 mM	0.990	0.921	1.003	1.085	0.898
Ethylbenzene	250 µl	0.389	0.389	0.455	0.378	0.278
Pseudocumene	250 µl	0.355	0.303	0.411	0.423	0.313
p-Xylene	250 µl	0.202	0.238	0.260	0.239	0.208
m-Xylene	250 µl	0.215	0.203	0.231	0.245	0.211
Phenol	5 mM	0.000	0.000	0.221	0.000	0.000
Naphthalene	5 mM	0.000	0.000	0.189	0.000	0.000
p-Chlorophenol	1.6 mM	0.188	0.175	0.375	0.186	0.166
m-Chlorophenol	1.6 mM	0.182	0.140	0.552	0.150	0.123
o-Chlorophenol	1.6 mM	0.000	0.000	0.000	0.000	0.000

To-1 = *Pseudomonas putida*
To-3 = *Pseudomonas putida*
To-4 = *Aeromonas caviae*
To-5 = *Pseudomonas putida*
Na-13 = *Pseudomonas putida*

Table 12: Growth of the isolates in solid culture in the presence of various concentrations of toluene at 30°C

Vol.toluene(μl)/ 100 ml H_2O	Conc.toluene in vapour phase mg/l	Strain no. of the isolate				
		To-1	To-3	To-4	To-5	Na-13
6.6	0.75	++	++	++	++	++
13.3	1.51	++	++	++	++	++
26.7	3.20	++	++	++	++	++
53.5	6.03	+++	+++	+++	+++	+++
107	12.10	+++	+++	+++	+++	+++
214	24.13	+++	+++	+++	+++	+++
428	48.25	+++	+++	+++	+++	+++
856	96.5	+++	+++	+++	+++	+++
1712	193	+++	+++	+++	+++	+++
3424	386	+++	++	+++	++	++
6848	772	++	++	++	++	++
13696	1544	++	++	++	++	++
27392	3088	++	++	++	++	++
54784	6176	++	+	+	+	+
109568	12352	+	+	+	+	+
Neat 500 ml	12300	+	+	+	+	+

+++ Good growth; ++ Medium growth; + Less growth.

To-1 = *Pseudomonas putida*

To-3 = *Pseudomonas putida*

To-4 = *Aeromonas caviae*

To-5 = *Pseudomonas putida*

Na-13 = *Pseudomonas putida*

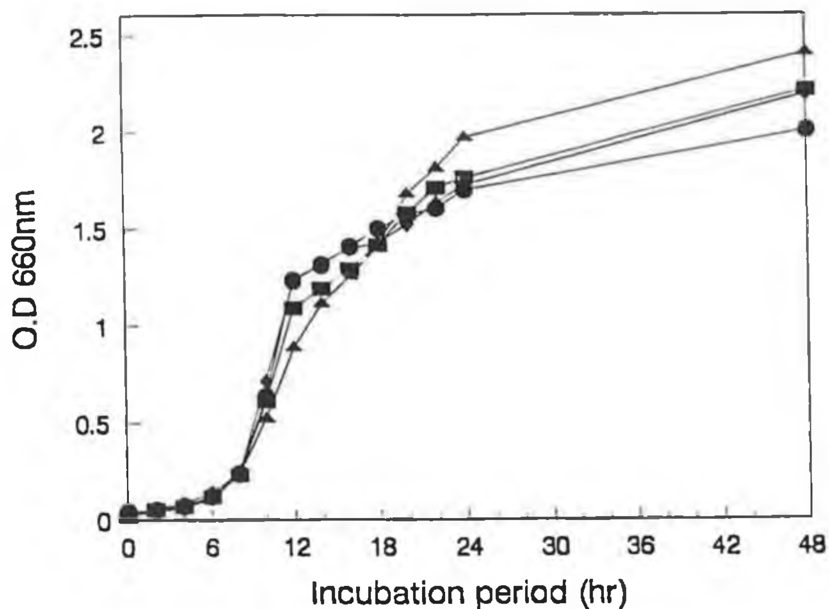
increasing volumes of toluene in the desiccator except when neat toluene was supplied. Here the concentration of toluene in the vapour phase was slightly lower than in the highest diluted system. Growth was observed in all cases. For strains To-3, To-5 and Na-13 optimum growth was observed in the range of 6.25 to 193 mg/l of toluene. Strains To-1 and To-4 grew best in the range of 6.25 to 386 mg/l of toluene. Growth decreased above and below these concentrations. However, the bacteria were still capable of growth in the vapour generated by neat toluene (Table 12).

3.2.3 Growth in liquid culture in the presence of toluene vapour

Comparison of growth rates of the bacteria

The bacteria were grown in liquid culture in flasks containing 50 ml minimal medium and various volumes of toluene ranging from 250 - 2000 µl/50 ml minimal medium, supplied in a small tube hanging inside the flasks. The growth rate (specific growth rate $\mu = h^{-1}$) of the organisms was determined. Changes in pH during growth were also monitored.

All the organisms grew well in all the experimental conditions. The extent of growth increased with increasing volumes of toluene supplied. The maximum O.D values obtained at 2000 µl of toluene for the strains To-1, To-3, To-4, To-5 and Na-13 were 2.416, 2.436, 2.446, 2.449 and 2.411 respectively. At 250 µl of toluene the maximum O.D values were 1.952, 1.927, 2.004, 2.030 and 1.828 respectively. The level of biomass peaked after 48 - 72 hours and was maintained for up to 7 days (Figure 18 - 22). In all cases a lag of 6 hours was experienced. There was no significant difference in the growth rates between the organisms (Appendix 2) There was also no significant difference in the growth rates attained when any one organism was grown in the presence of various volumes of toluene (Appendix 1). The maximum growth rates were 0.14, 0.14, 0.16, 0.15 and 0.14 at 2000 µl of toluene.



250 µl ● O.D ○ pH $\mu = 0.12 \pm 0.0141$

500 µl ✦ O.D ◇ pH $\mu = 0.12 \pm 0.0282$

1000 µl ■ O.D □ pH $\mu = 0.13 \pm 0.0141$

2000 µl ▲ O.D △ pH $\mu = 0.14 \pm 0.0141$

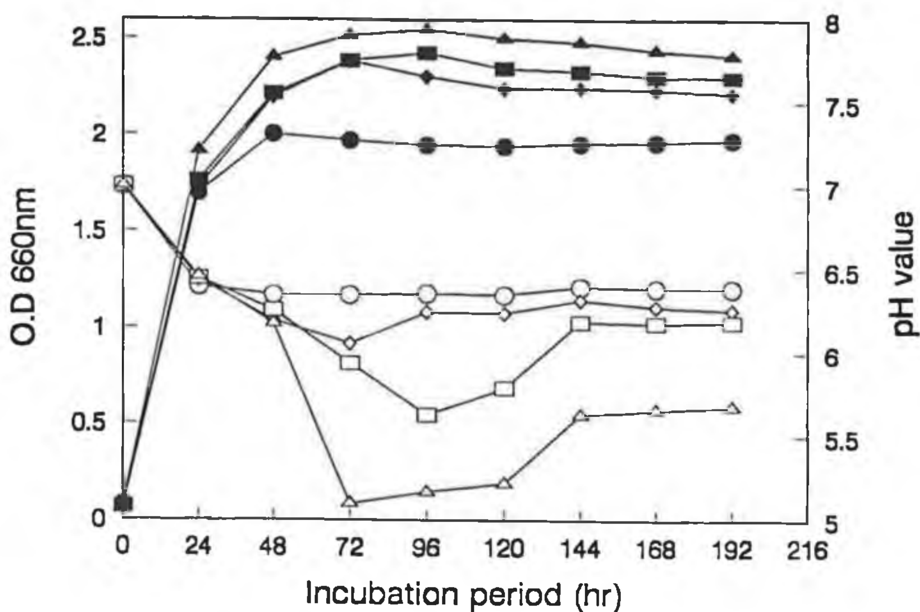
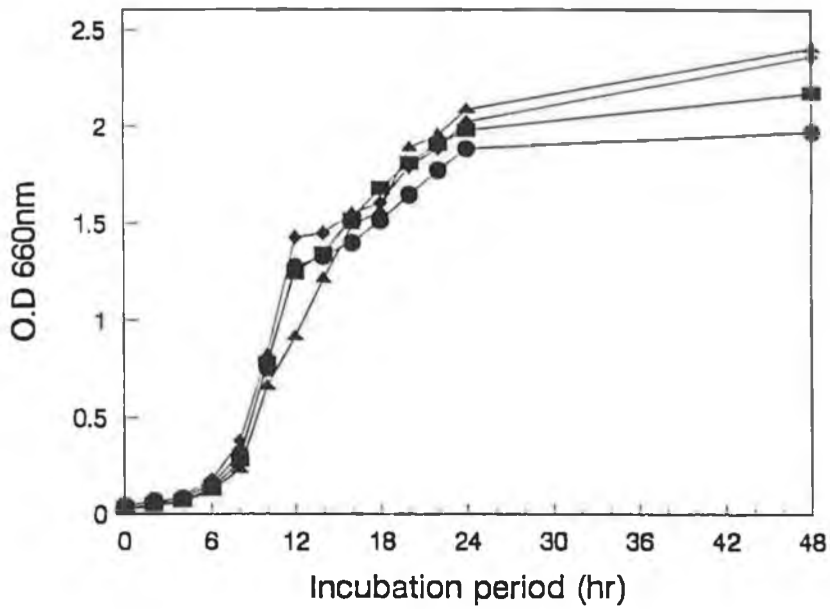


Figure 19: The growth of *P. putida* To-1 in the presence of 250 µl - 2000 µl toluene (1 O.D = 3.0×10^7/ml)



250 µl	-●- O.D	-○- pH	µ = 0.12 ± 0.0141
500 µl	-◆- O.D	-◇- pH	µ = 0.11 ± 0.0141
1000 µl	-■- O.D	-□- pH	µ = 0.13 ± 0.0141
2000 µl	-▲- O.D	-△- pH	µ = 0.14 ± 0.0141

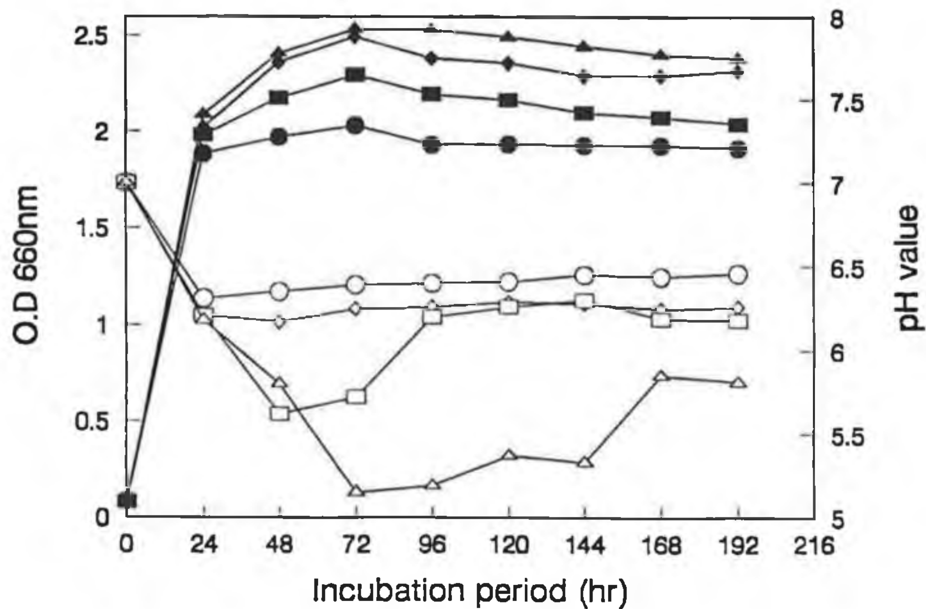
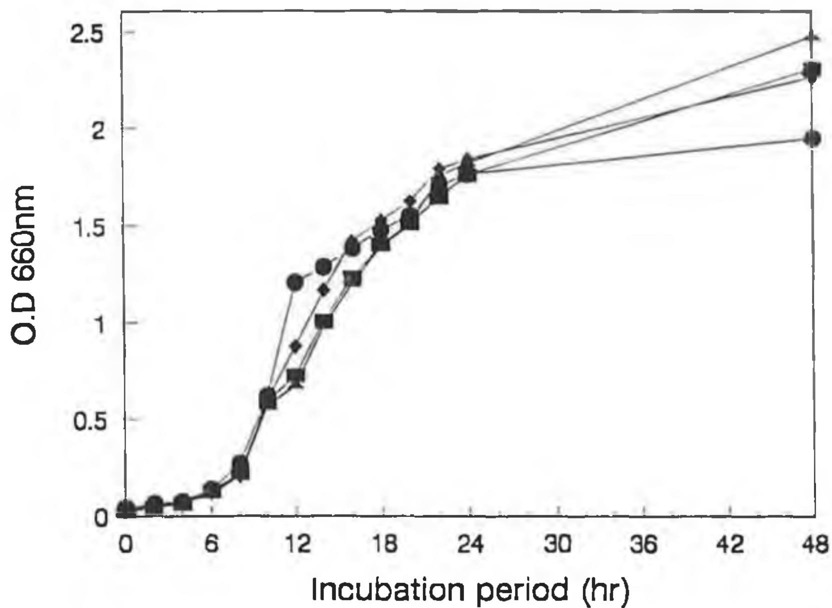


Figure 20: The growth of *P. putida* To-3 in the presence of 250 µl - 2000 µl toluene (1 O.D = 5.2×10^7/ml)



250 µl ● O.D ○ pH $\mu = 0.12 \pm 0.0141$
500 µl ✦ O.D ◇ pH $\mu = 0.14 \pm 0.0141$
1000 µl ■ O.D □ pH $\mu = 0.14 \pm 0.0282$
2000 µl ▲ O.D △ pH $\mu = 0.16 \pm 0.0282$

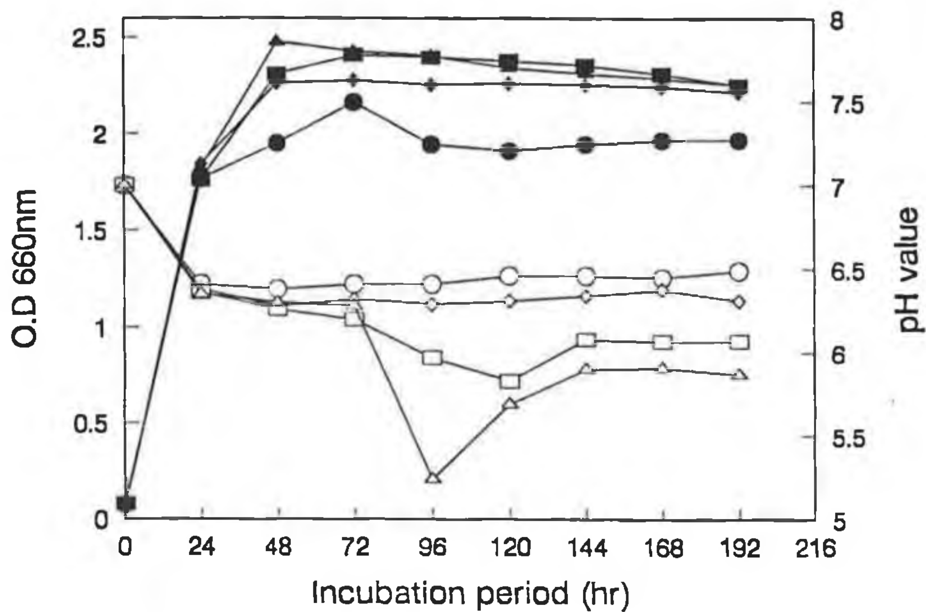
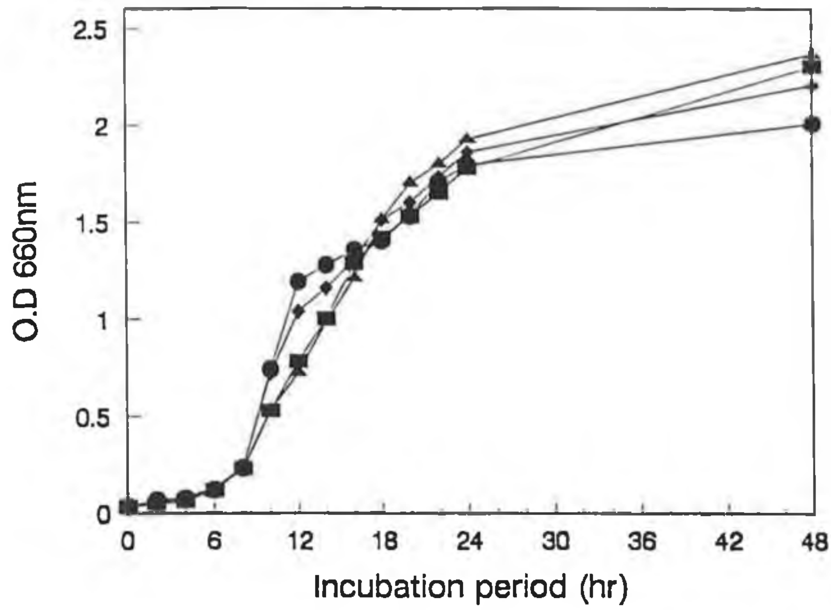


Figure 21: The growth of *A. caviae* To-4 in the presence of 250 µl - 2000 µl toluene (1 O.D = 7.7×10^7/ml)



250 µl -●- O.D -○- pH µ = 0.12 ± 0.0141

500 µl -◆- O.D -◇- pH µ = 0.14 ± 0.0141

1000 µl -■- O.D -□- pH µ = 0.14 ± 0.0141

2000 µl -▲- O.D -△- pH µ = 0.15 ± 0.0141

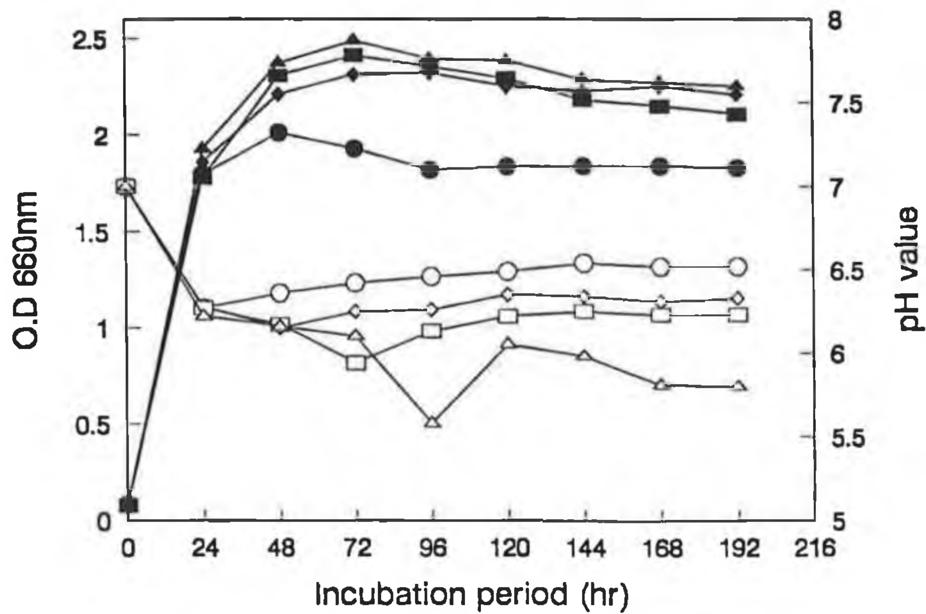
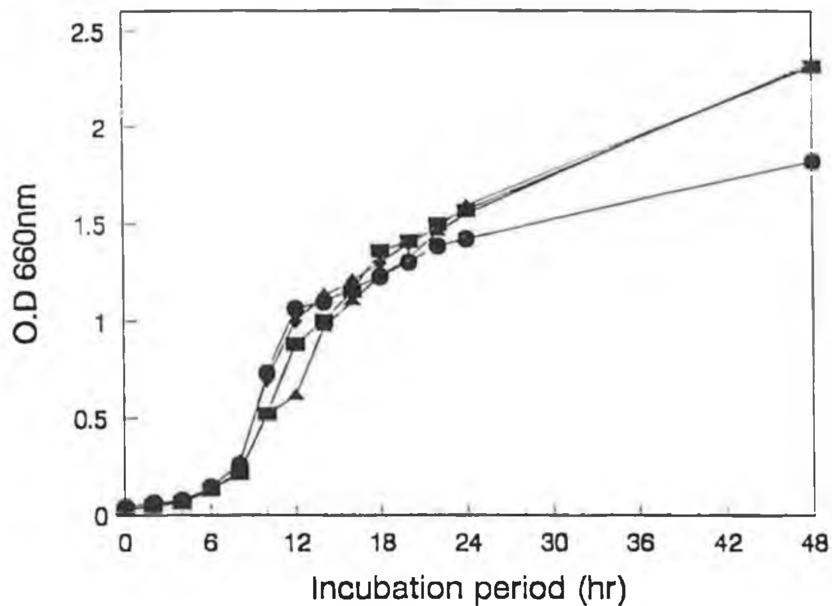


Figure 22: The growth of *P. putida* To-5 in the presence of 250 µl - 2000 µl toluene (1 O.D = 4.1×10^7/ml)



250 µl ● **O.D** ○ pH $\mu = 0.10 \pm 0.0141$

500 µl ✦ **O.D** ◇ pH $\mu = 0.12 \pm 0.0141$

1000 µl ■ **O.D** □ pH $\mu = 0.13 \pm 0.0141$

2000 µl ▲ **O.D** △ pH $\mu = 0.13 \pm 0.0141$

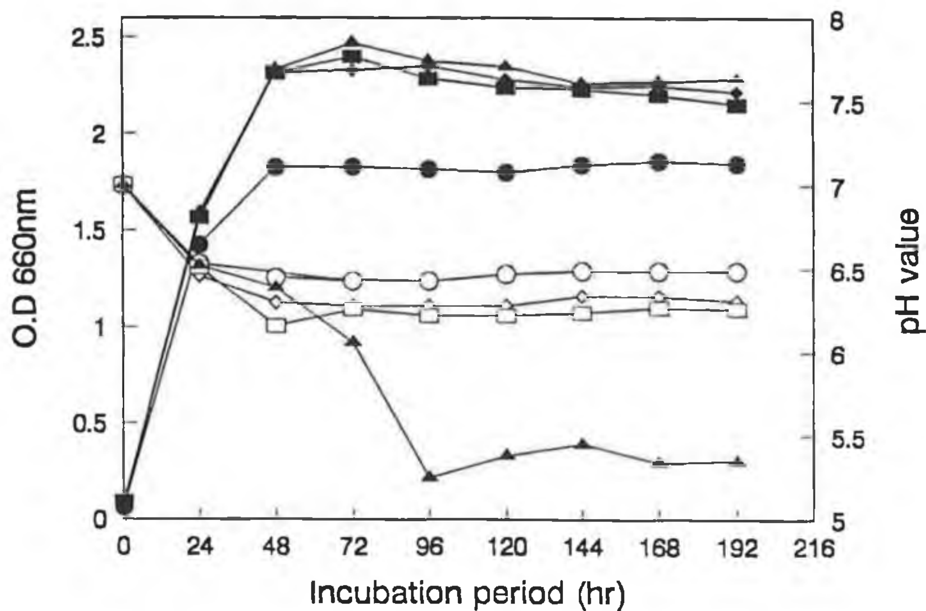


Figure 23: The growth of *P. putida* Na-13 in the presence of 250 µl - 2000 µl toluene (1 O.D = 7.1×10^7/ml)

In all cases the pH of the culture medium decreased during growth from an initial pH 7.0. It was observed that the pH dropped as the toluene was metabolized. The decrease in pH increased with increasing volumes of toluene supplied, decreasing from pH 7.0 to pH 5.24 in the presence of 2000 µl of toluene.

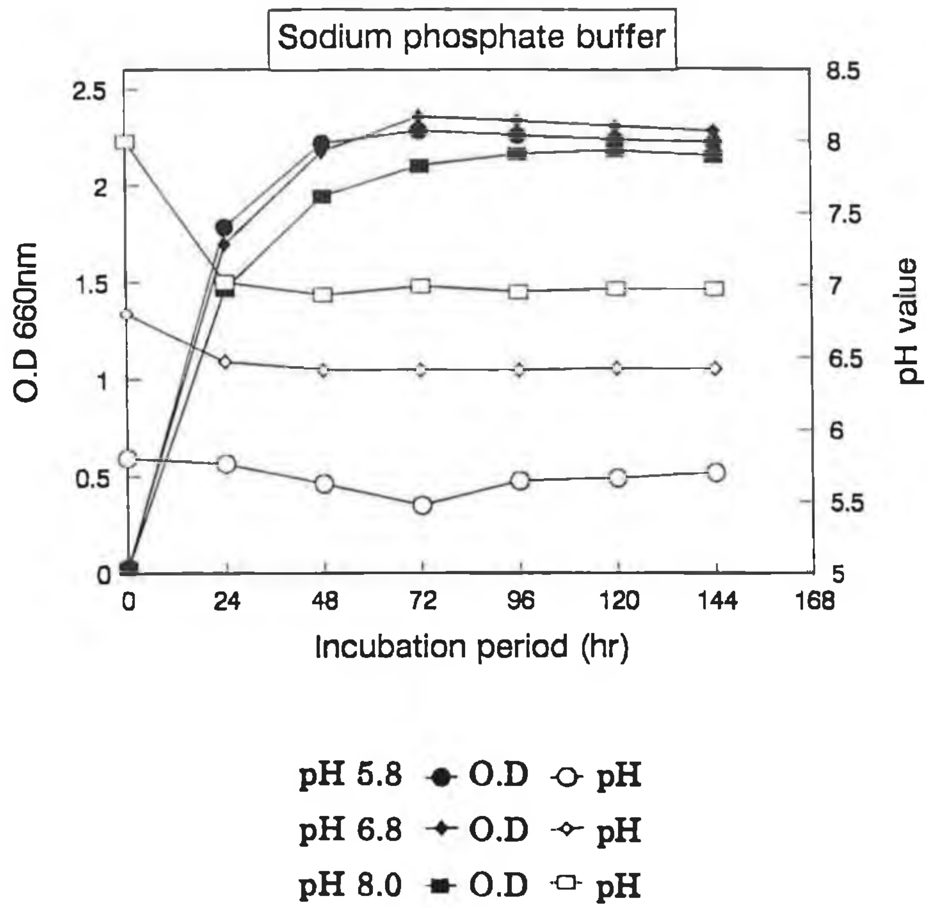
The influence of pH on the growth of the bacteria

Given the decrease in pH with toluene metabolism it was of interest to investigate the influence of pH on the growth of the isolates. Studies were first carried out to investigate a suitable buffering system for the growth medium.

Choice of buffer

Strain To-5 was used to investigate a suitable buffering system. Initially two buffers - sodium phosphate buffer and Tris-maleate buffer were used to grow the organism in the presence of 500 µl of toluene. The bacterium was incubated at 30°C and agitated at 200 rpm. The growth and pH of the culture medium was monitored for up to 7 days. Both of the buffers were used at 0.1 M concentration and pH 5.8, 6.6 and 8.0. The growth was better in the sodium phosphate buffer than the Tris-maleate buffer (Figure 24). The pH of the culture medium fluctuated more during growth in the Tris-maleate buffer than in the sodium phosphate buffer. On the basis of these results the sodium phosphate buffer was chosen for future experiment.

To optimize the concentration of buffer, different molarities of sodium phosphate buffer - 0.1, 0.3. 0.5 and 1 M were used to grow strain To-5 in the presence of 500 µl toluene (Figure 25 - 26). pH values of 5.8, 6.6, 7.4 and 8.0 were tested. The organism grew at all pH values in 0.1 and 0.3 M concentration of buffer, except for pH 8.0 in the 0.5 M buffer. It did not grow at all in 1 M buffer. pH stability was greatest at higher concentrations of



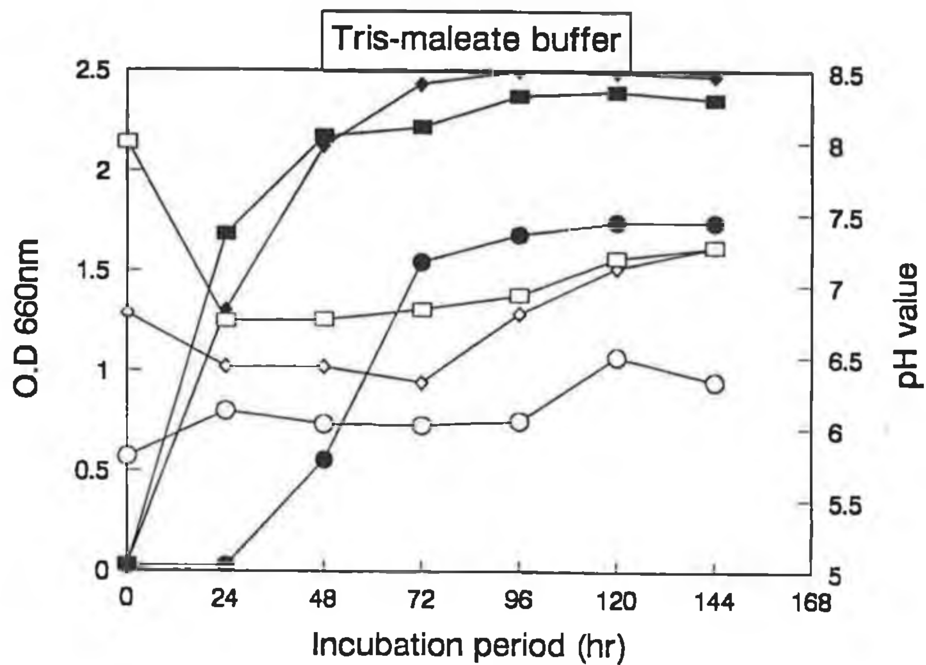
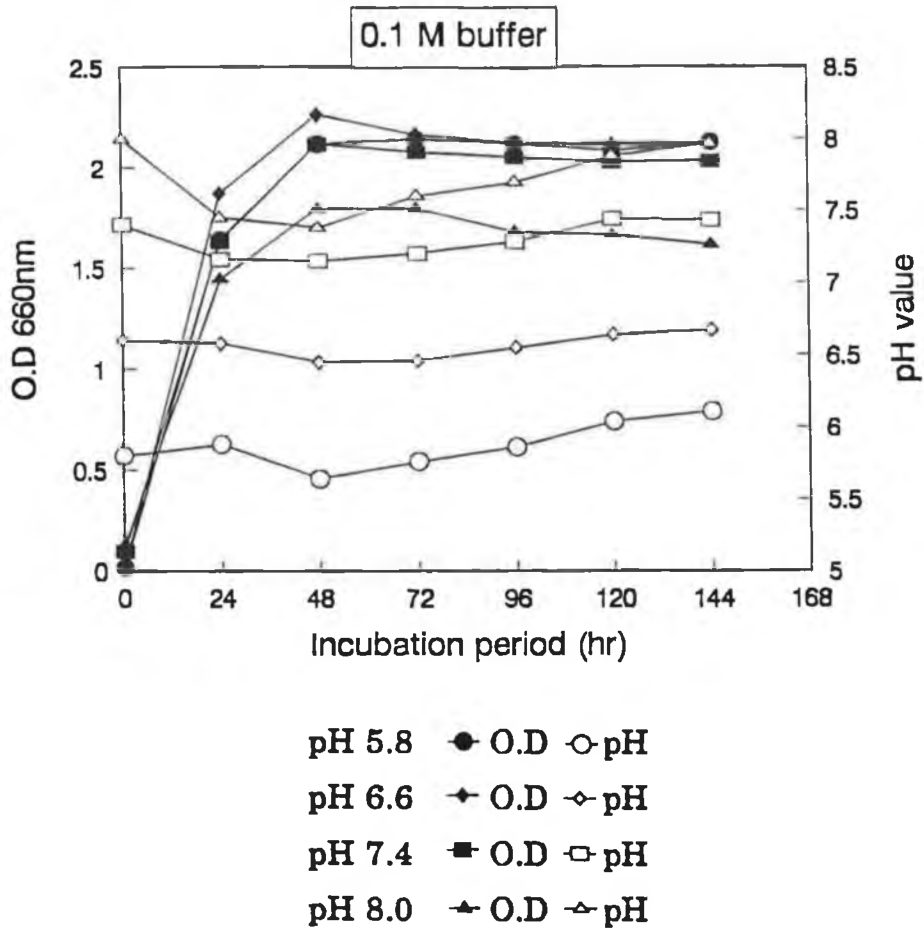


Figure 24: The growth pattern of *P. putida* To-5 in 0.1 M buffer in the presence of 500 µl toluene



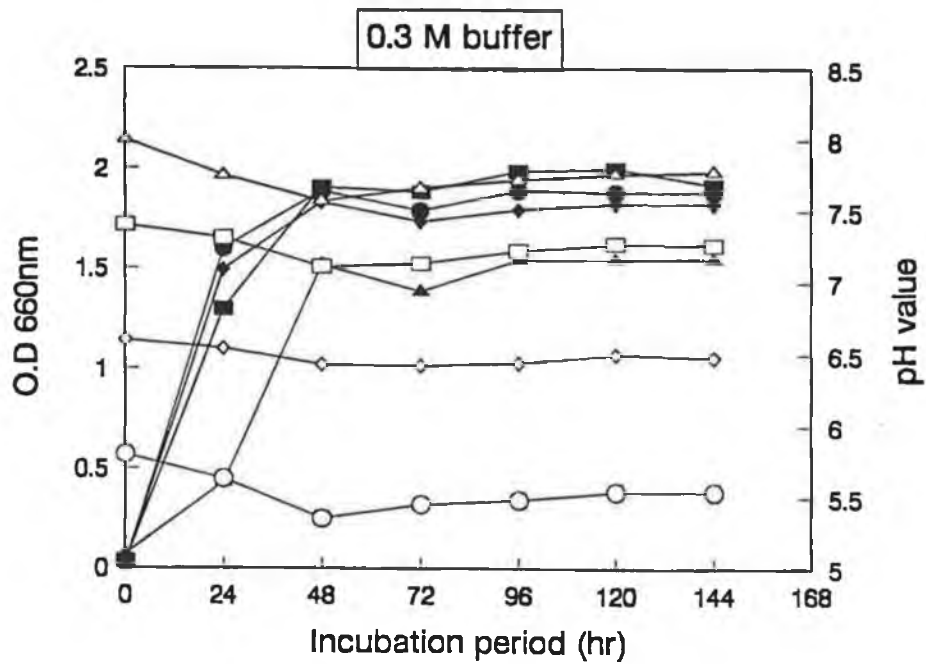
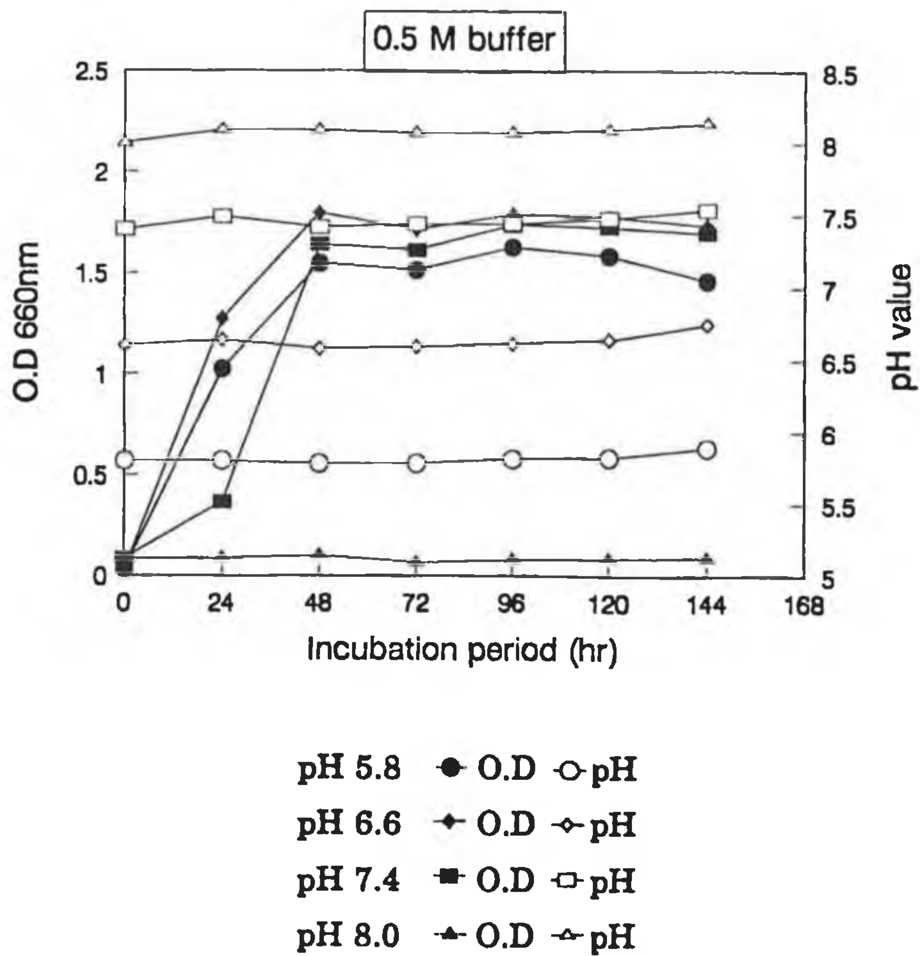


Figure 25: The growth pattern of *P. putida* To-5 in 0.1 M and 0.3 M sodium phosphate buffer in the presence of 500 µl toluene



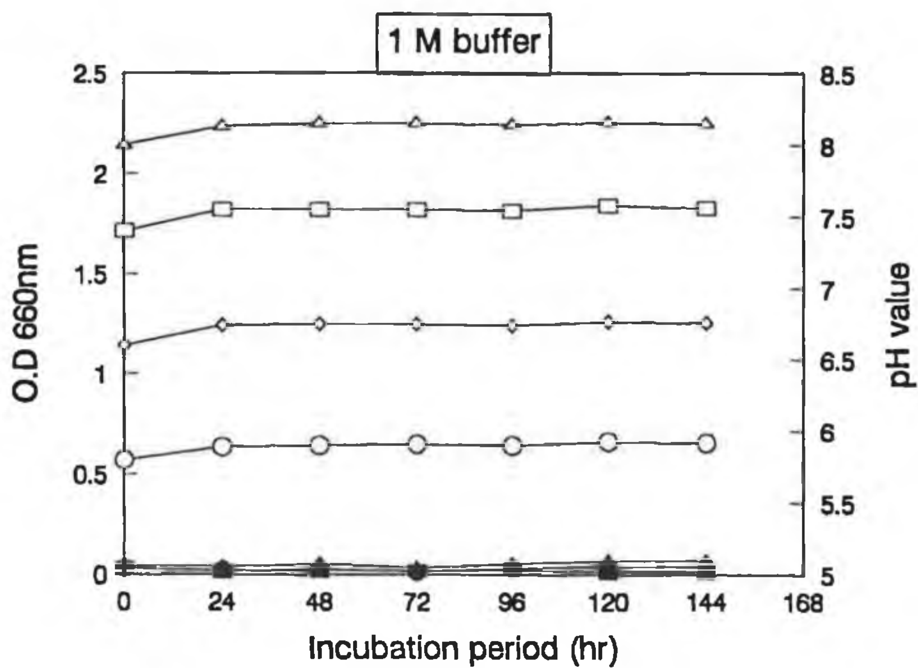
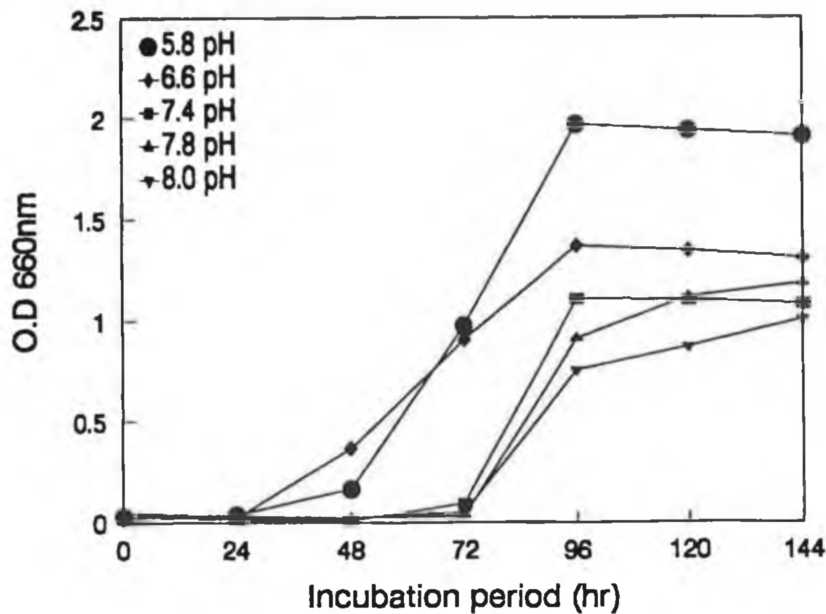
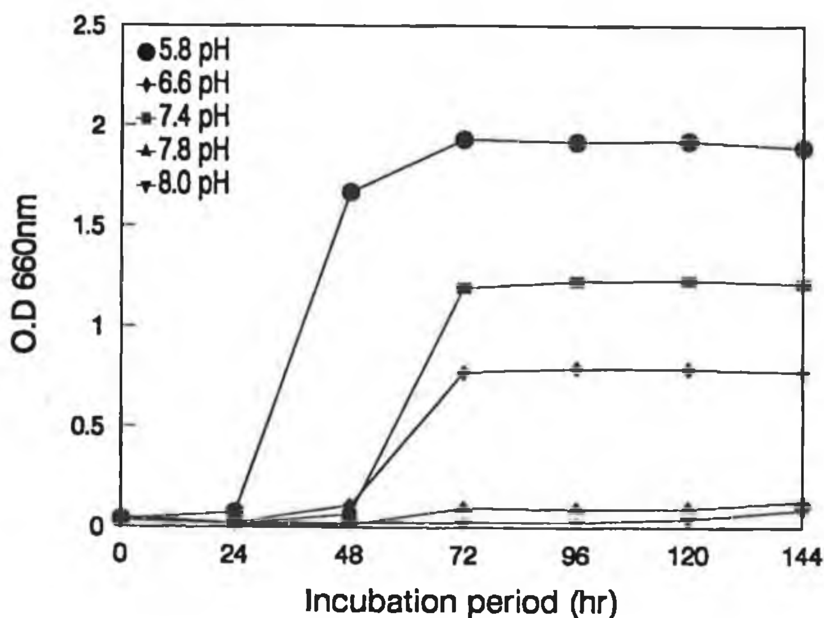


Figure 26: The growth pattern of *P. putida* To-5 in 0.5 M and 1 M sodium phosphate buffer in the presence of 500 µl toluene



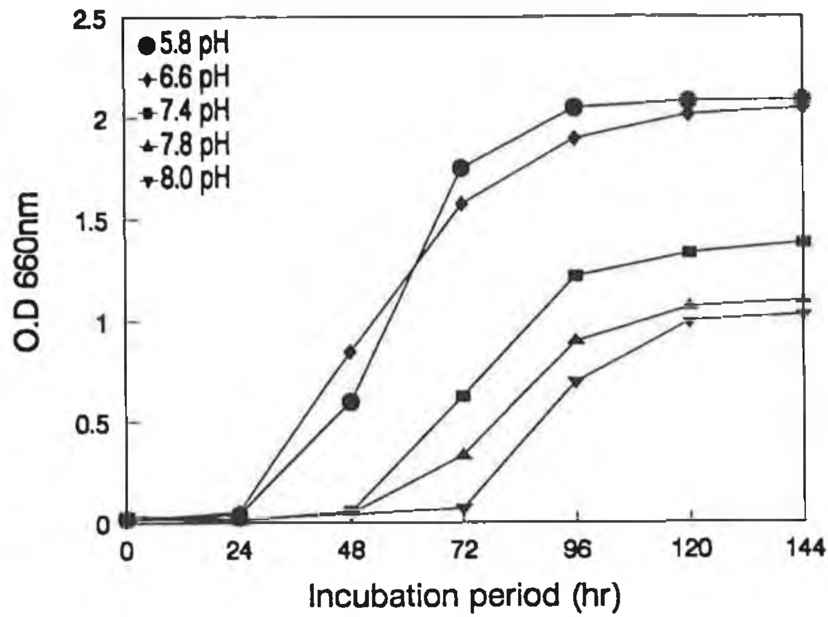
μ = 0.05 (pH 5.8), μ = 0.06 (pH 6.6), μ = 0.10 (pH 7.4),
μ = 0.07 (pH 7.8), μ = 0.04 (pH 8.0)

Figure 27: The growth pattern of *P. putida* To-1 at different pH values at 0.5 M sodium phosphate buffer in the presence of 500 μl toluene



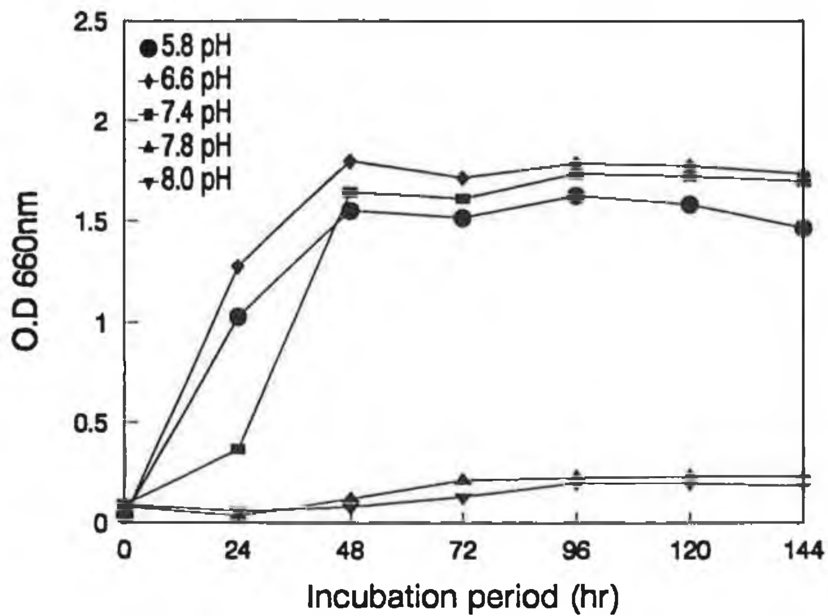
μ = 0.07 (pH 5.8), μ = 0.07 (pH 6.6), μ = 0.06 (pH 7.4),
μ = 0.03 (pH 7.8), μ = 0.02 (pH 8.0)

Figure 28: The growth pattern of *P. putida* To-3 at different pH values in 0.5 M sodium phosphate buffer in the presence of 500 μl toluene



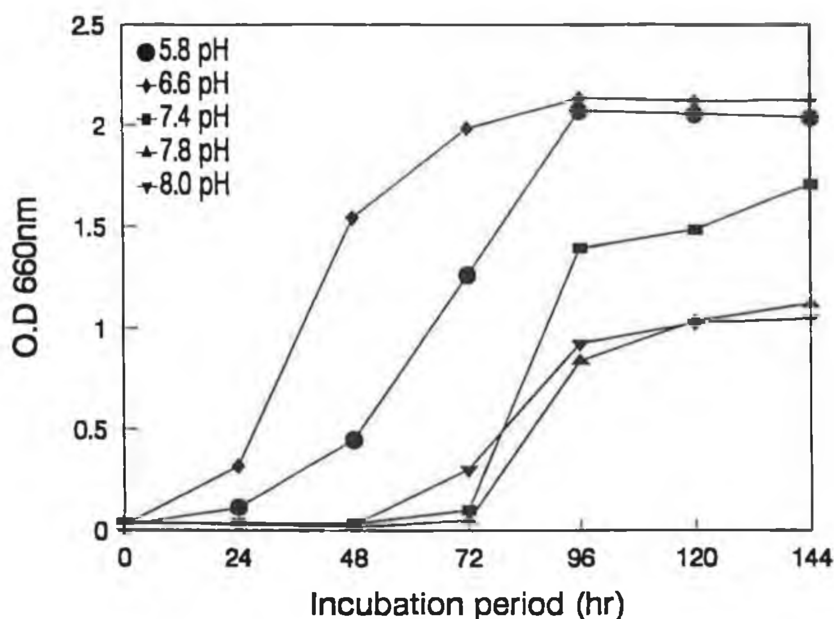
µ = 0.04 (pH 5.8), µ = 0.04 (pH 6.6), µ = 0.07 (pH 7.4),
µ = 0.06 (pH 7.8), µ = 0.03 (pH 8.0)

Figure 29: The growth pattern of *A. caviae* To-4 at different pH values in 0.5 M sodium phosphate buffer in the presence of 500 µl toluene



µ = 0.08 (pH 5.8), µ = 0.07 (pH 6.6), µ = 0.06 (pH 7.4)
µ = 0.01 (pH 7.8), µ = 0.02 (pH 8.0)

Figure 30: The growth pattern of *P. putida* To-5 at different pH values in 0.5 M sodium phosphate buffer in the presence of 500 µl toluene



μ = 0.06 (pH 5.8), μ = 0.07 (pH 6.6), μ = 0.06 (pH 7.4)
μ = 0.04 (pH 7.8), μ = 0.06 (pH 8.0)

Figure 31: The growth pattern of *P. putida* Na-13 at different pH values in 0.5 M sodium phosphate buffer in the presence of 500 µl toluene

buffer. Based on the ability of the organism to grow and the pH control in the system, the 0.5 M buffer was selected for further study.

Optimum pH of growth

The optimum pH for growth for the five bacteria was investigated using sodium phosphate buffer of 0.5 M at pH values of 5.8, 6.6, 7.4, 7.8 and 8.0. The growth of the five organisms was different for various pH values. Strain To-1, To-4 and Na-13 could grow in all pH values. Strain To-3 and To-5 grew at pH 5.8, 6.6 and 7.4 but did not grow at 7.8 and 8.0. A lag was experienced in most cases except in the case of To-4 which grew readily at pH 6.6 and 5.8 and To-5 which grew readily at pH 5.8, 6.6 and 7.4. In all other cases the duration of lag increased with increasing pH values. In general, growth was optimum between pH 5.8 and 7.4 and this was reflected by the growth rates obtained for the organisms at different pH values (Figure 27 - 31).

Toluene utilization by the bacteria

In order to study toluene utilization by the bacteria, the removal of toluene from the culture vessel by strain To-4 and To-5 was monitored. Toluene utilization was expressed in terms of concentration of toluene utilized per unit biomass and was investigated both in the gas and liquid phases. The bacteria were grown in liquid culture in the presence of various volumes of toluene. Two different cultural conditions were used. In one instance (system A) the flasks were sealed with tight cotton bungs to minimize the loss of toluene vapours to the atmosphere and a large headspace was provided to ensure adequate aeration. In the other instance (system B) cotton bungs were covered with two layers of parafilm to further contain the toluene. All the culture flasks were incubated at 30°C and agitated at 200 rpm for 96 hours. Uninoculated control flasks were incubated in parallel. The dissolved oxygen, O.D at 660nm and toluene concentrations both in the gas and liquid phases were monitored. Figures 32 - 35 represents the growth of strain To-4 in system A and B and Figures 36 - 39 represents the growth of strain To-5 in system A and B.

The range of toluene concentrations detected varied with the volumes of toluene supplied in both system A and system B. In general the concentration detected increased with increasing volumes of toluene supplied. Higher concentrations were detected in system B than in system A. A wider range of concentrations were detected in the gas phase than in the liquid phase. The concentration of toluene in the liquid phase ranged from 2 to 12 mg/l in system A and 9 to 15 mg/l in system B. The gas phase concentrations ranged from 8 to 36 mg/l in system A and 15 to 79 mg/l in system B (Figure 32 - 39).

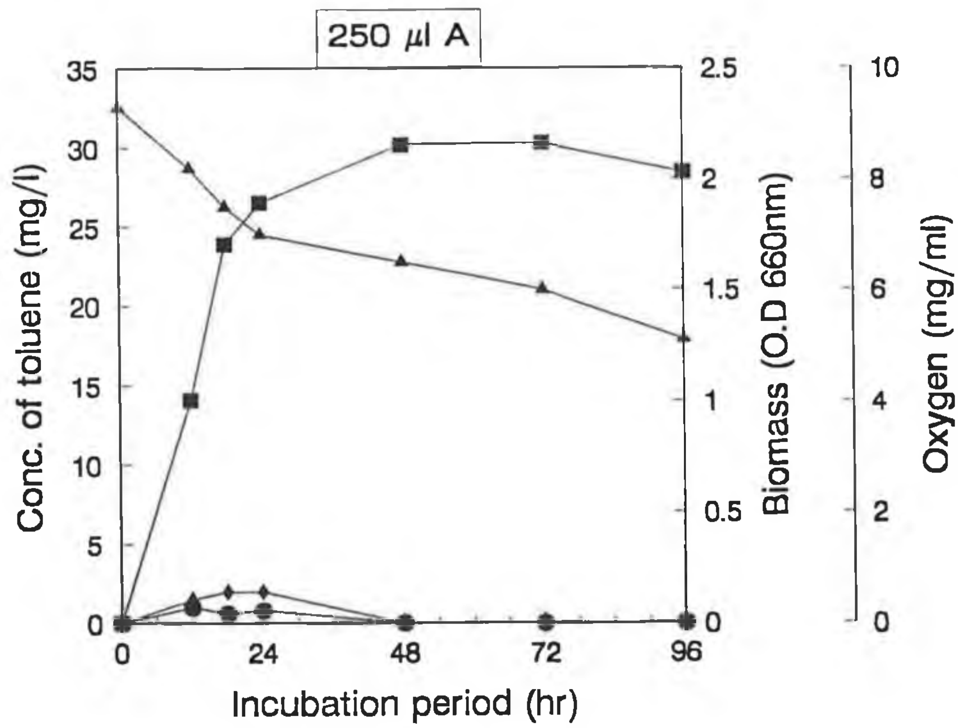
The level of dissolved oxygen in all the systems decreased with an increase in biomass. The rate of oxygen consumption increased with increasing volumes of toluene supplied in system A. The level of biomass also increased

with increasing concentrations of toluene in this system. In system B the decrease in the levels of dissolved oxygen was more marked than in system A and was similar regardless of the volume of toluene supplied. This resulted in a lower level of biomass being produced in system B in spite of the higher levels of substrate detected in both the liquid and gas phases. In system B, the level of dissolved oxygen rose at 72 hours and this again was reflected in an increase in biomass. In system B the level of biomass was similar irrespective of toluene concentrations.

The growth rate ($\mu = h^{-1}$) of the organisms were greater in system A than in system B. However there was no significant differences observed in the growth rates of the organisms in any one system when different volumes of toluene were used or between the organisms (Appendix 3).

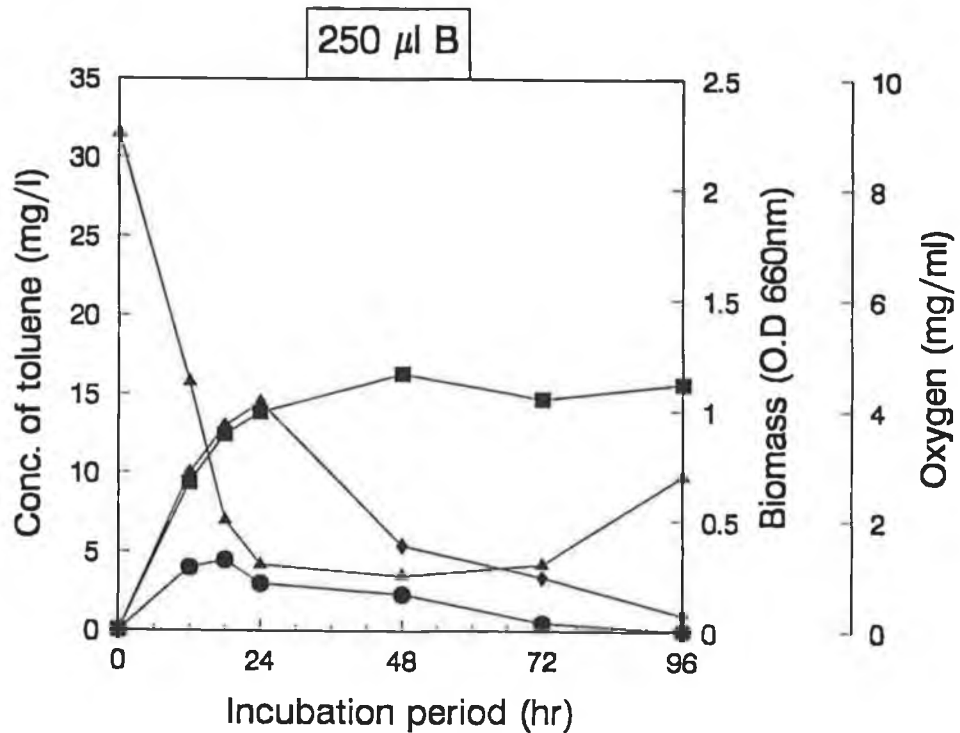
Toluene utilization by strain To-4 is represented in Figure 40 - 41 and that of strain To-5 is represented in Figure 42 - 43. Toluene utilization was higher in system B than in system A. Also the utilization of toluene was higher in the gas phase than the liquid phase for both of the systems. The utilization of toluene increased with increasing concentrations of toluene in both of the systems.

The maximum utilization of toluene in system A for the strain To-4 in liquid and gas phase were 4.20 and 13.33 mg/l/unit biomass. In system B the maximum utilization of toluene of this strain in liquid and gas phase were 11.91 and 49.13 mg/l/unit biomass. Strain To-5 utilized maximum toluene in system A in liquid and gas phases were 3.80 and 12.90 mg/l/unit biomass. While in system B the utilization of toluene for the strain To-5 in liquid and gas phases were 11.33 and 44.75 mg/l/unit biomass.



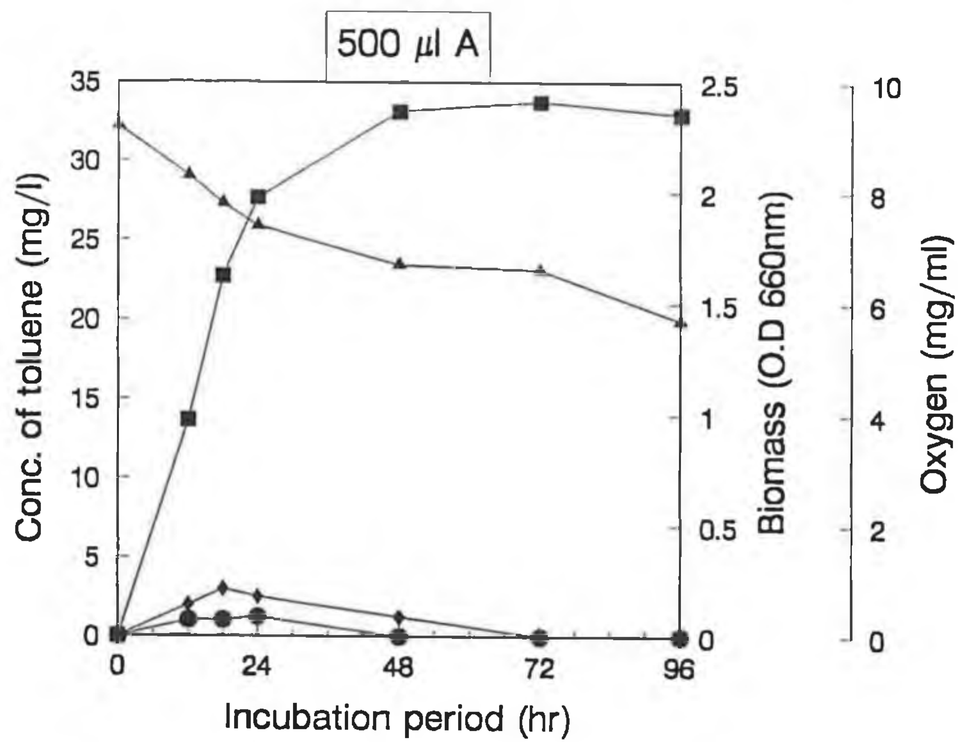
System A μ = 0.070 ± 0.0099

Liquid Gas Biomass Oxygen



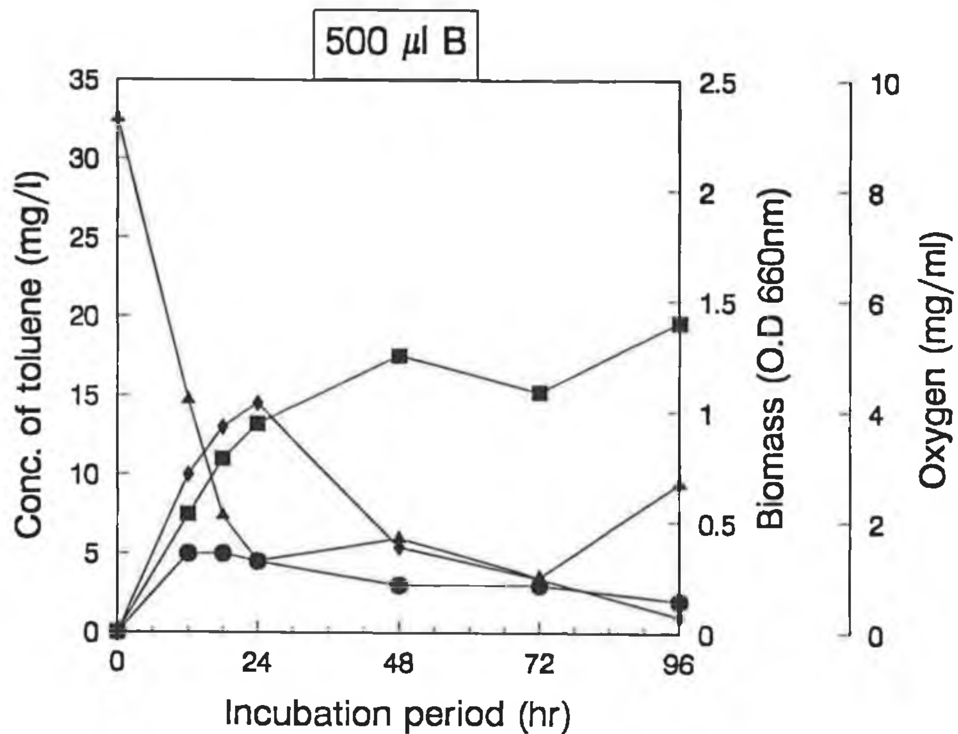
System B μ = 0.060 ± 0.0021

Figure 32: **Removal of toluene by *A. caviae* To-4 in the presence of 250 μl toluene in systems A and B**



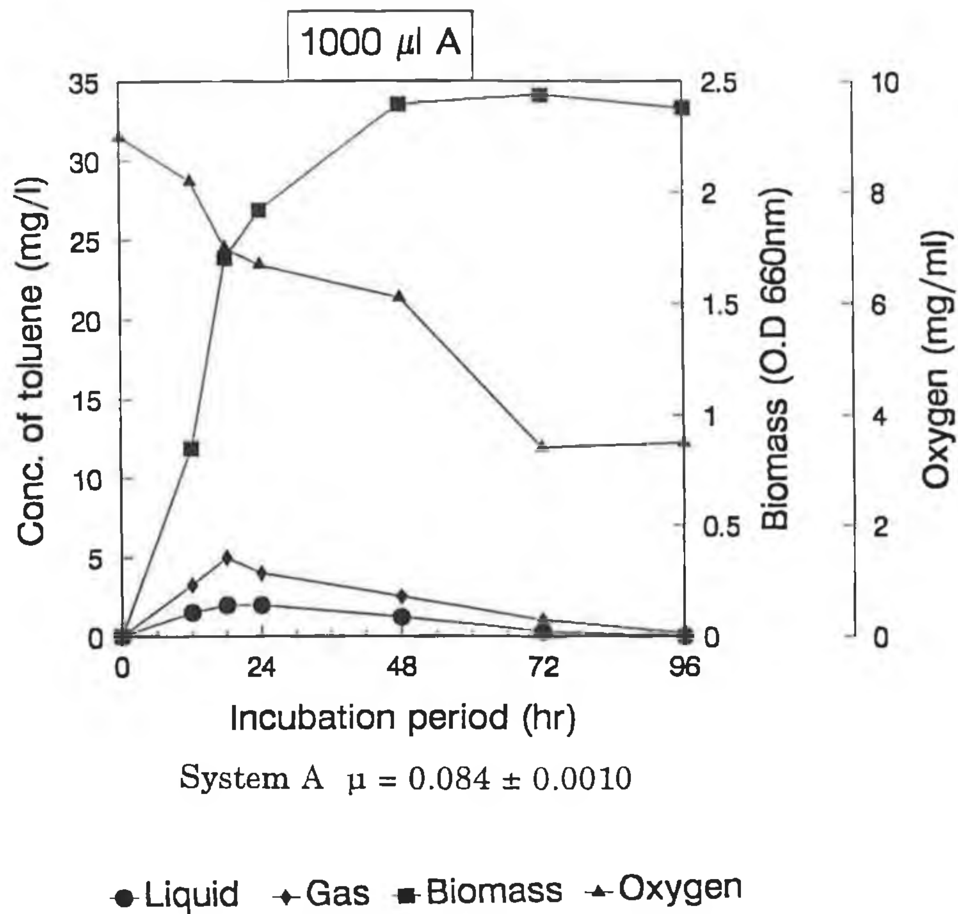
System A µ = 0.080 ± 0.0056

● Liquid ✦ Gas ■ Biomass ▲ Oxygen

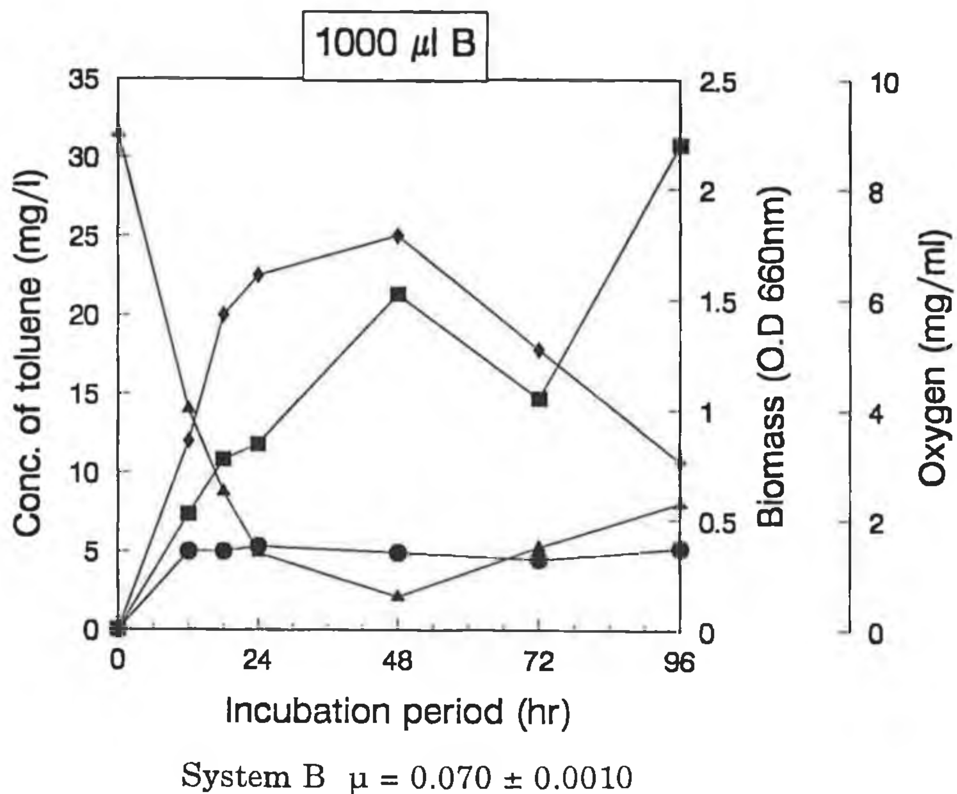


System B µ = 0.063 ± 0.0070

Figure 33: **Removal of toluene by *A. caviae* To-4 in the presence of 500 µl toluene in systems A and B**

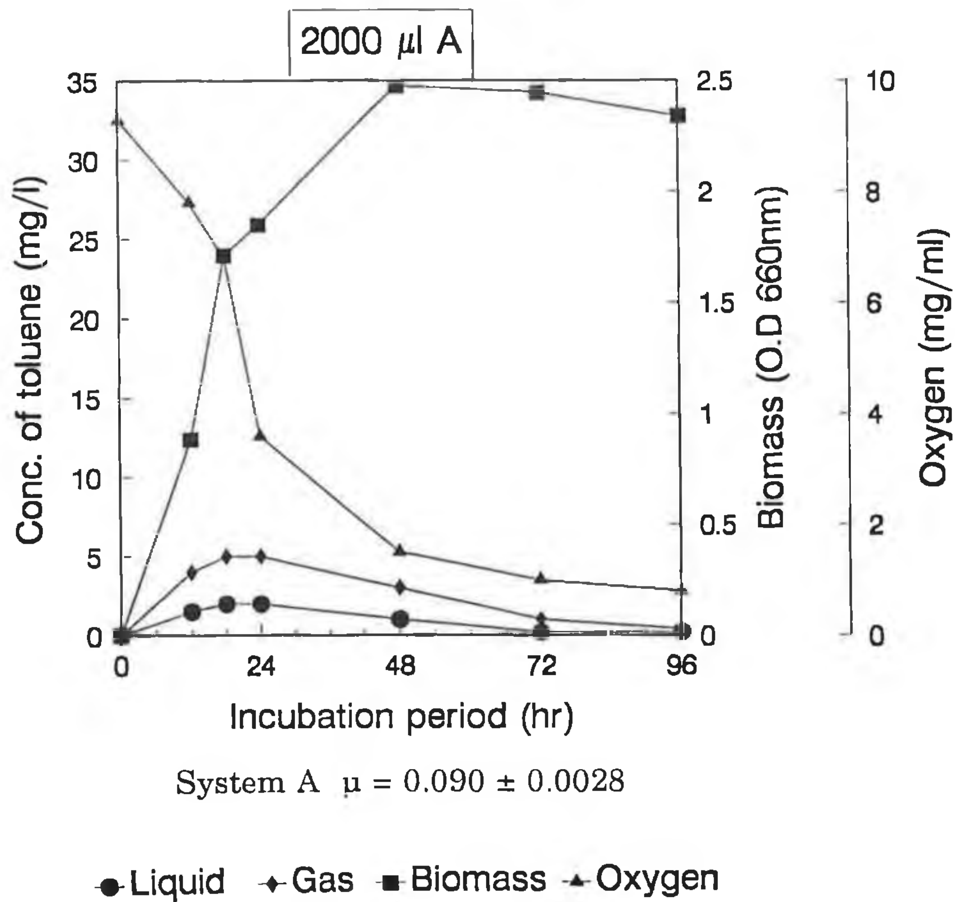


System A μ = 0.084 ± 0.0010

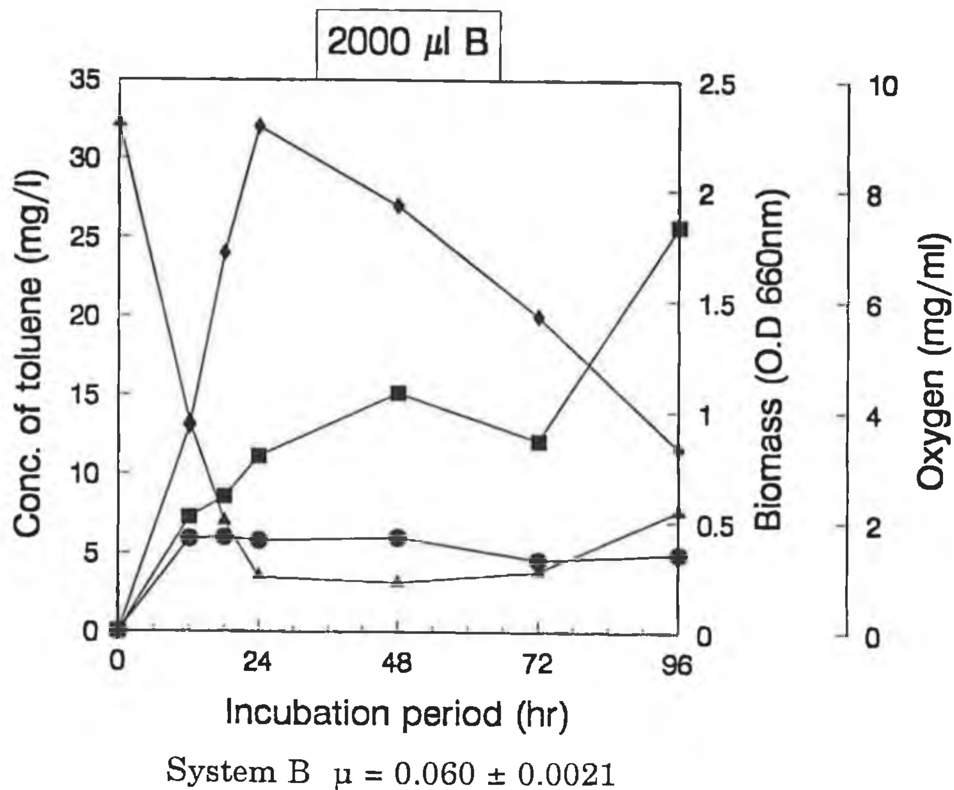


System B μ = 0.070 ± 0.0010

Figure 34: Removal of toluene by *A. caviae* To-4 in the presence of 1000 μl toluene in systems A and B

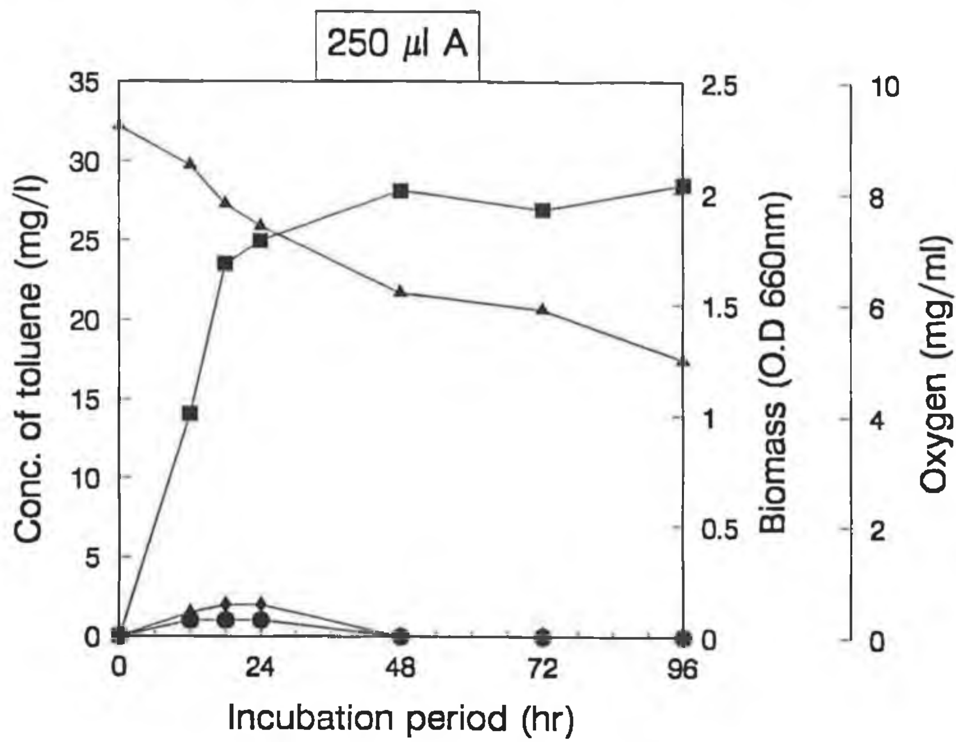


System A μ = 0.090 ± 0.0028

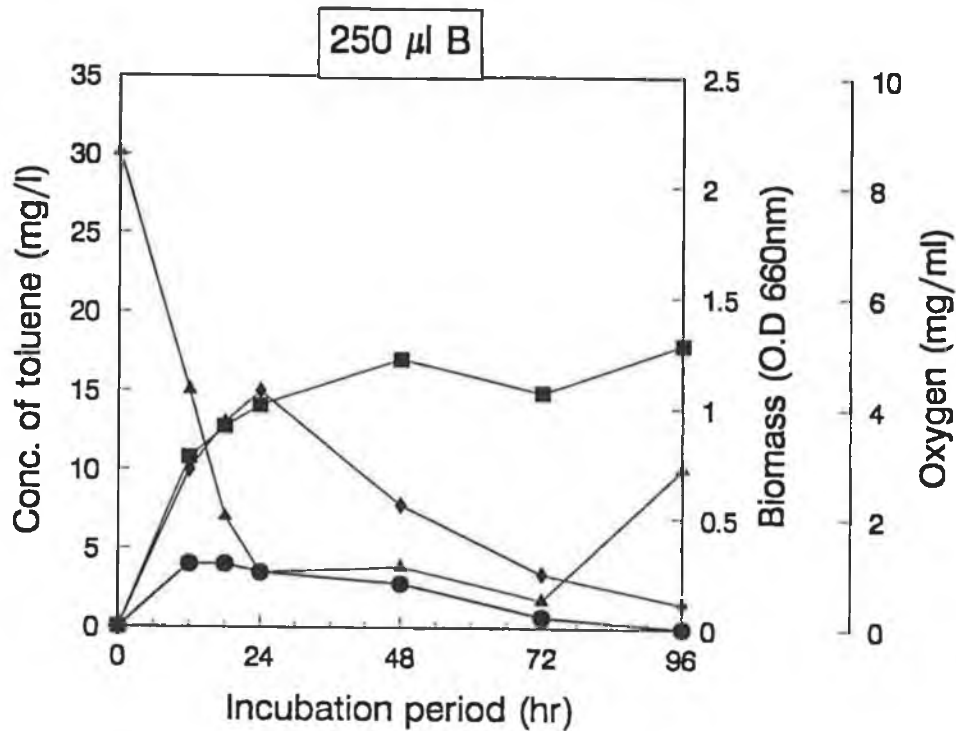


System B μ = 0.060 ± 0.0021

Figure 35: **Removal of toluene by *A. caviae* To-4 in the presence of 2000 μl toluene in systems A and B**

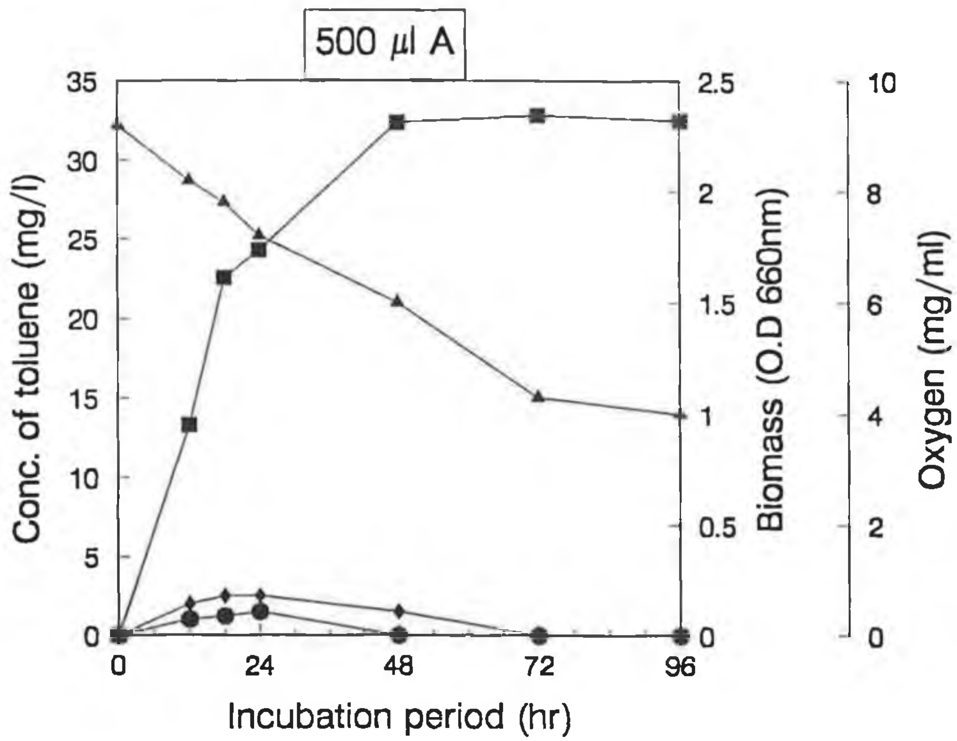


System A µ = 0.070 ± 0.0098



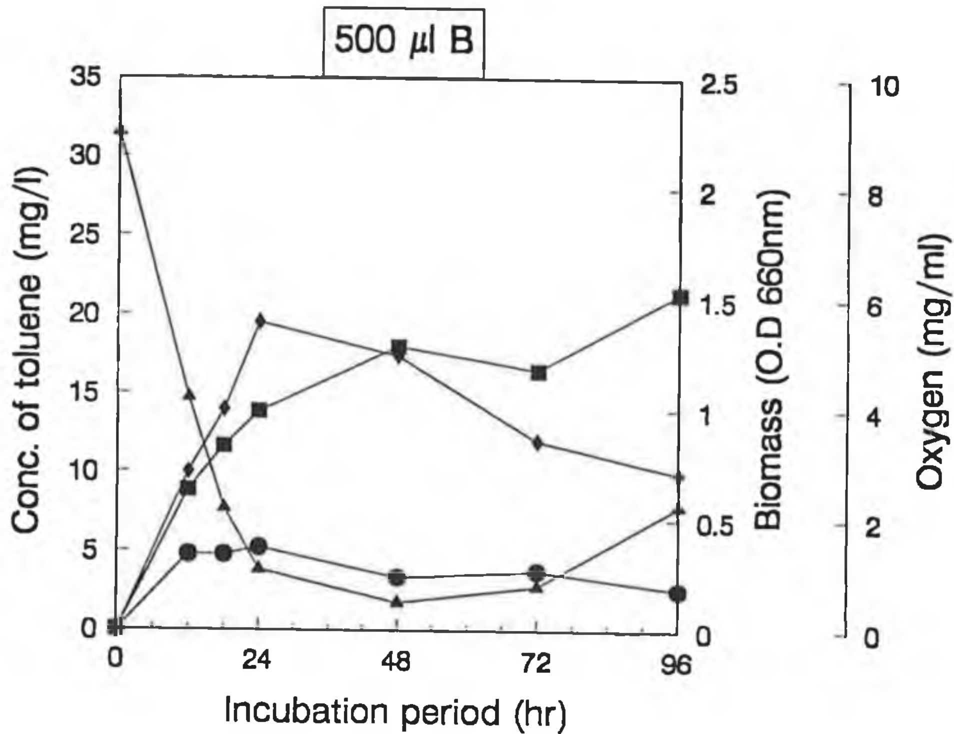
System B µ = 0.060 ± 0.0028

Figure 36: Removal of toluene by *P. putida* To-5 in the presence of 250 µl toluene in systems A and B



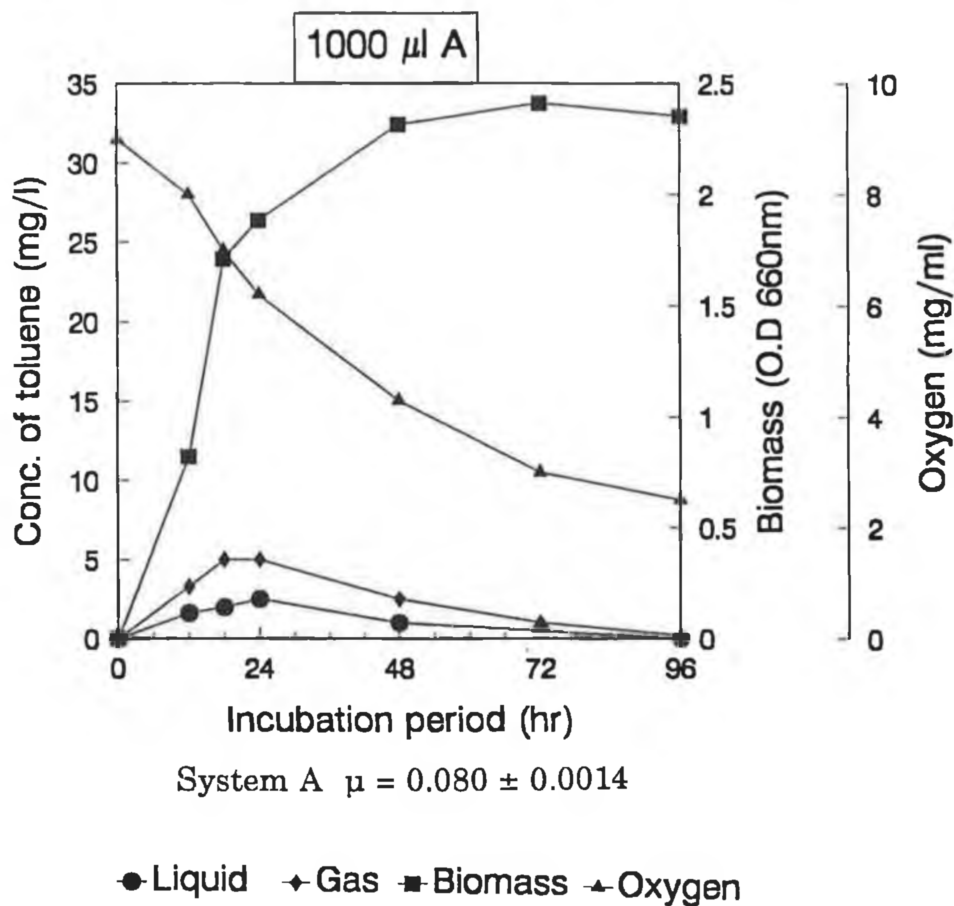
System A μ = 0.073 ± 0.0014

-●-Liquid -♦-Gas -■-Biomass -▲-Oxygen

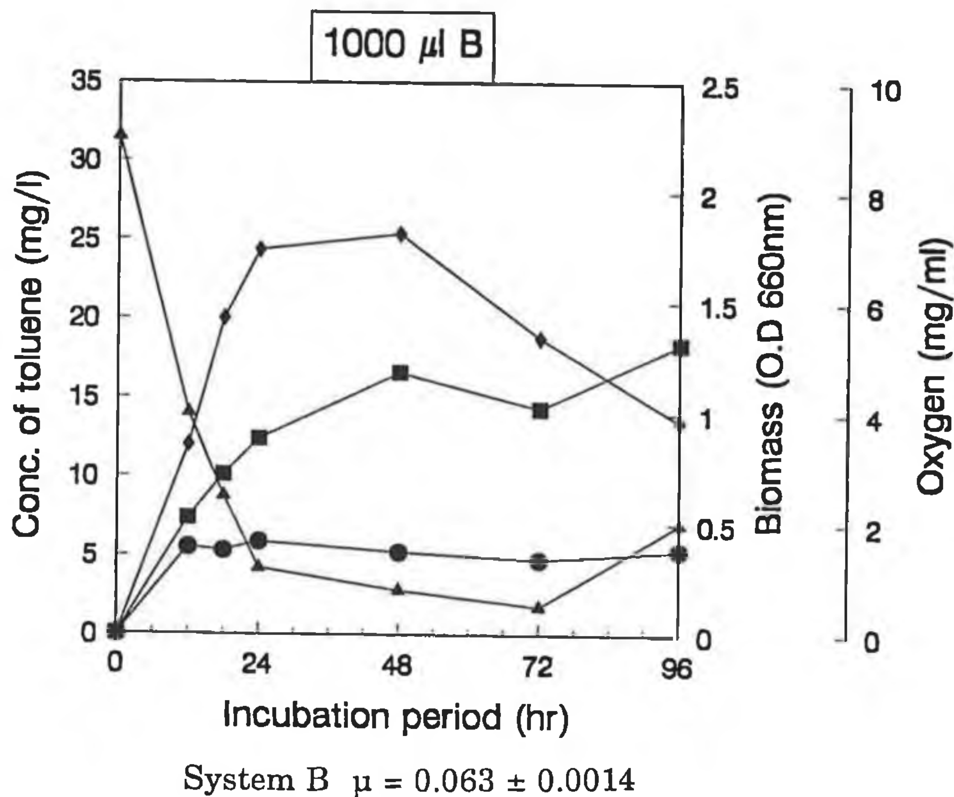


System B μ = 0.062 ± 0.0014

Figure 37: Removal of toluene by *P. putida* To-5 in the presence of 500 μl toluene in systems A and B

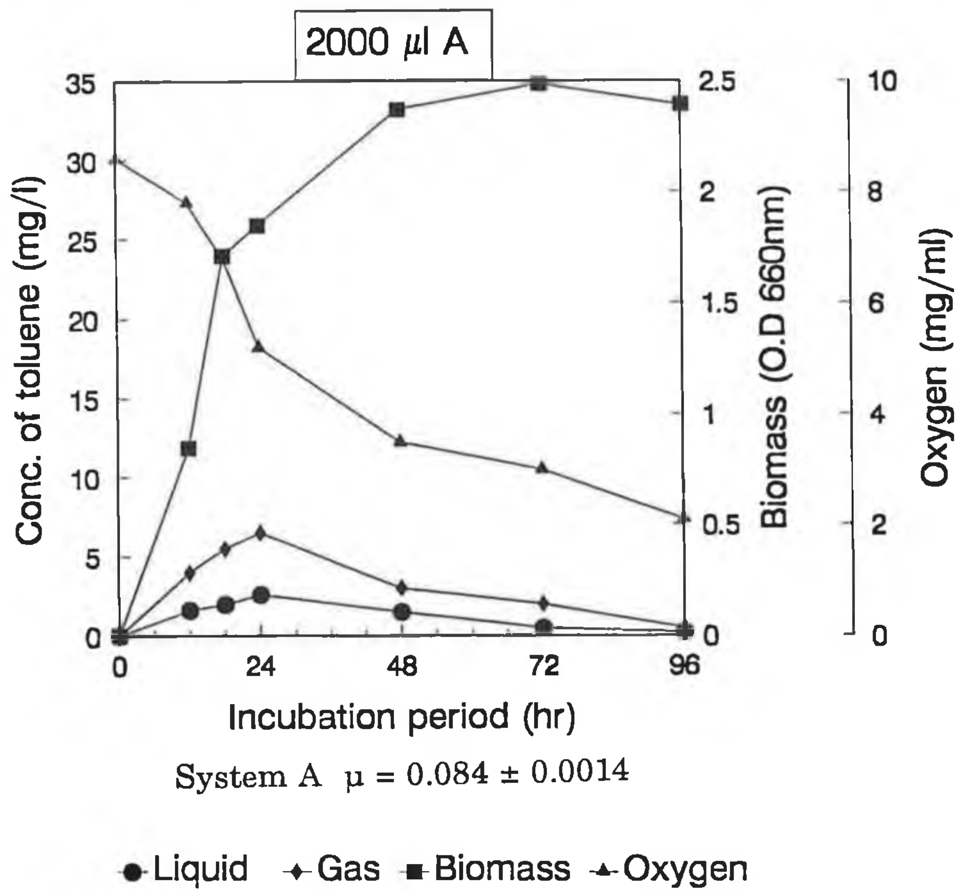


System A µ = 0.080 ± 0.0014



System B µ = 0.063 ± 0.0014

Figure 38: Removal of toluene by *P. putida* To-5 in the presence of 1000 µl toluene in systems A and B



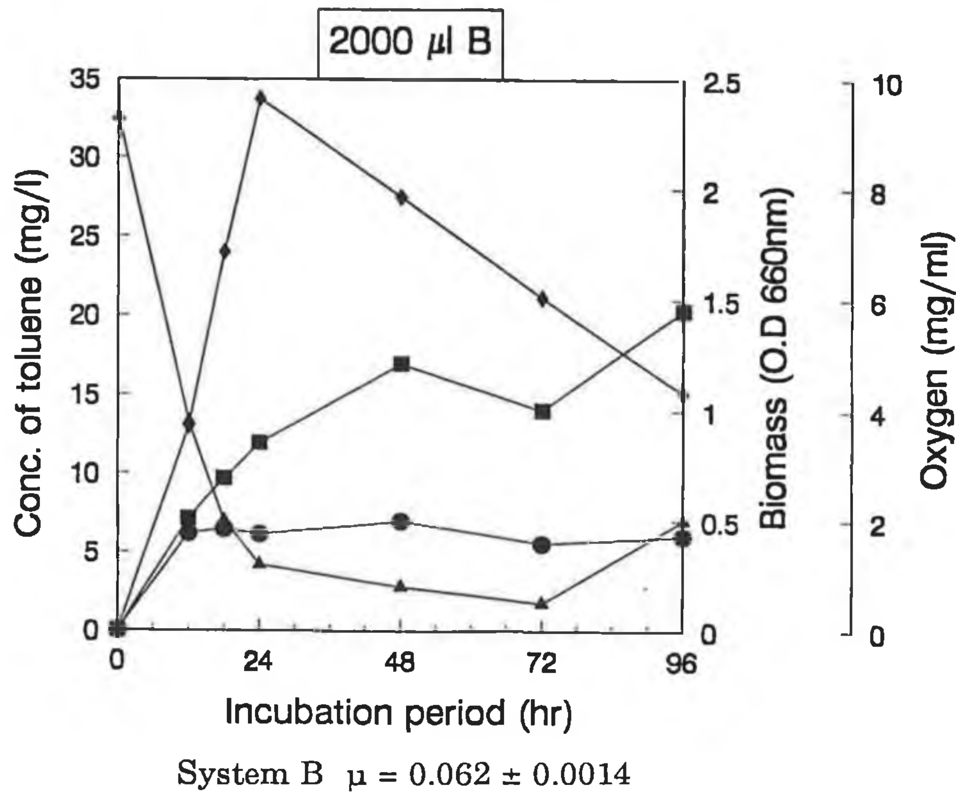
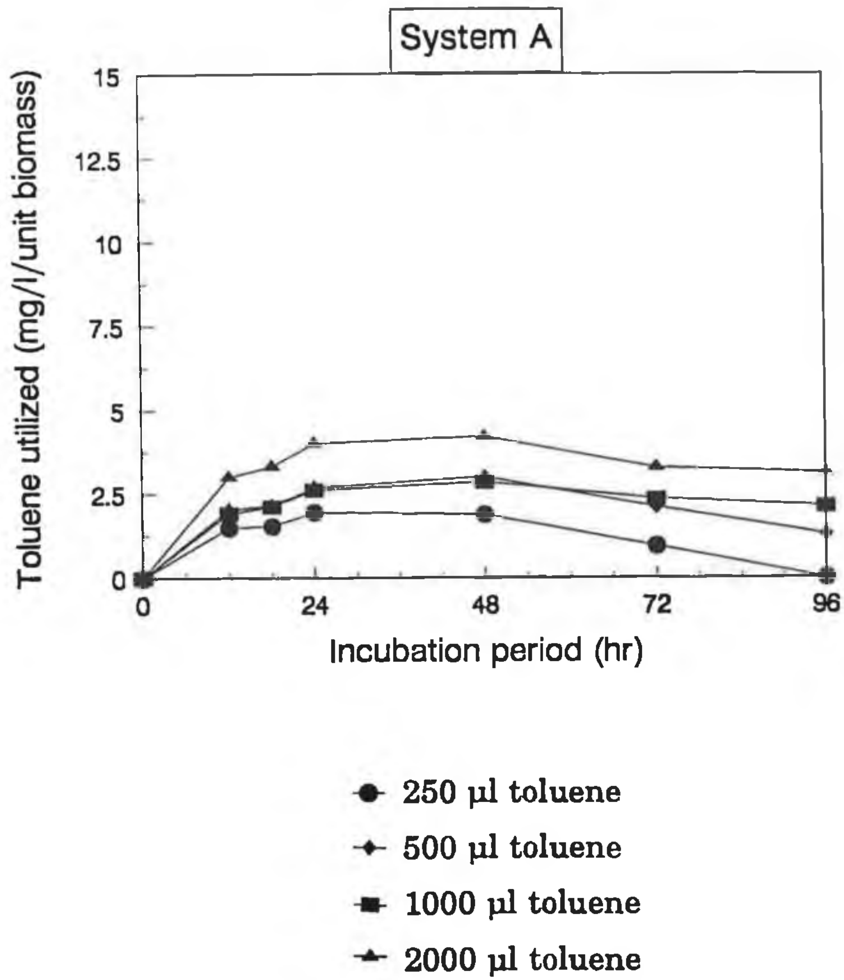


Figure 39: **Removal of toluene by *P. putida* To-5 in the presence of 2000 µl toluene in systems A and B**



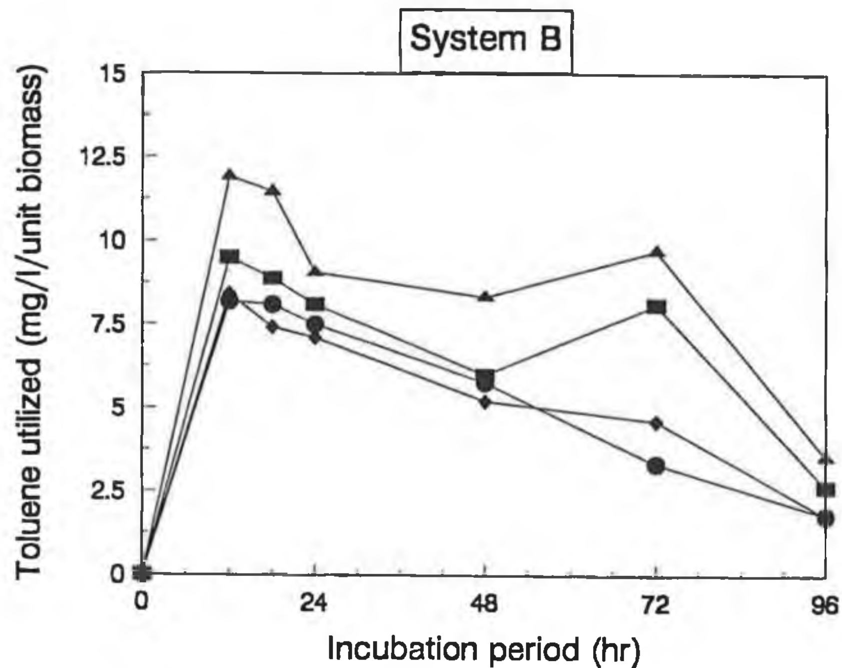
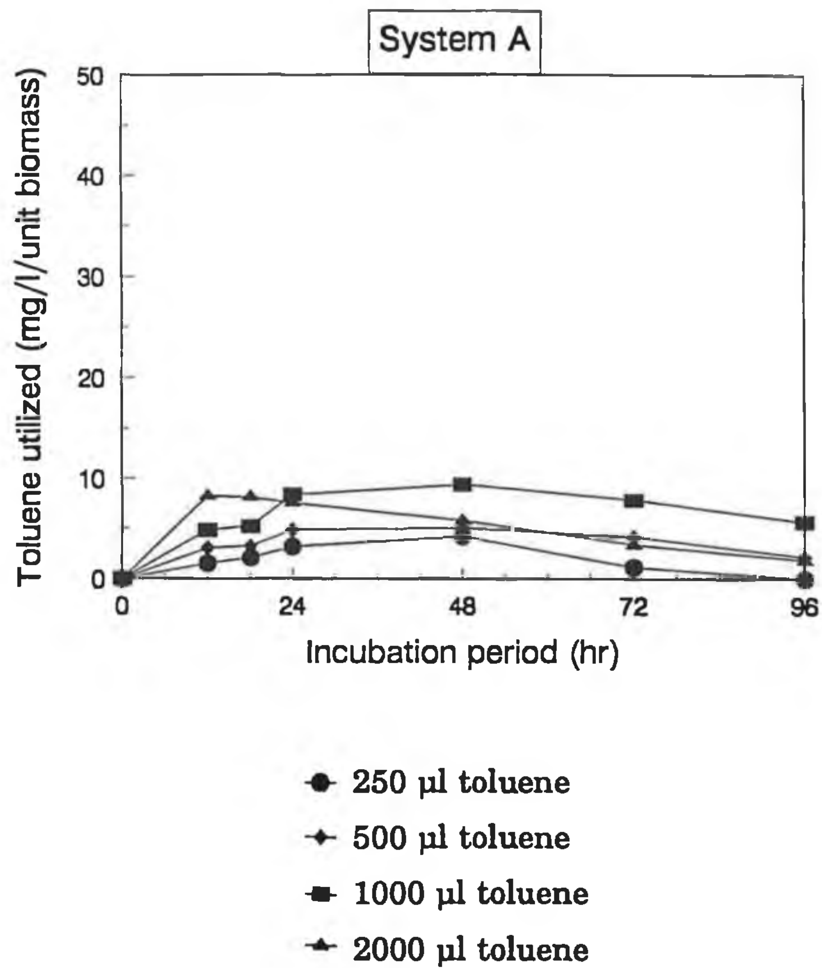


Figure 40: Utilization of toluene by *A. caviae* To-4 in the liquid phase in systems A and B



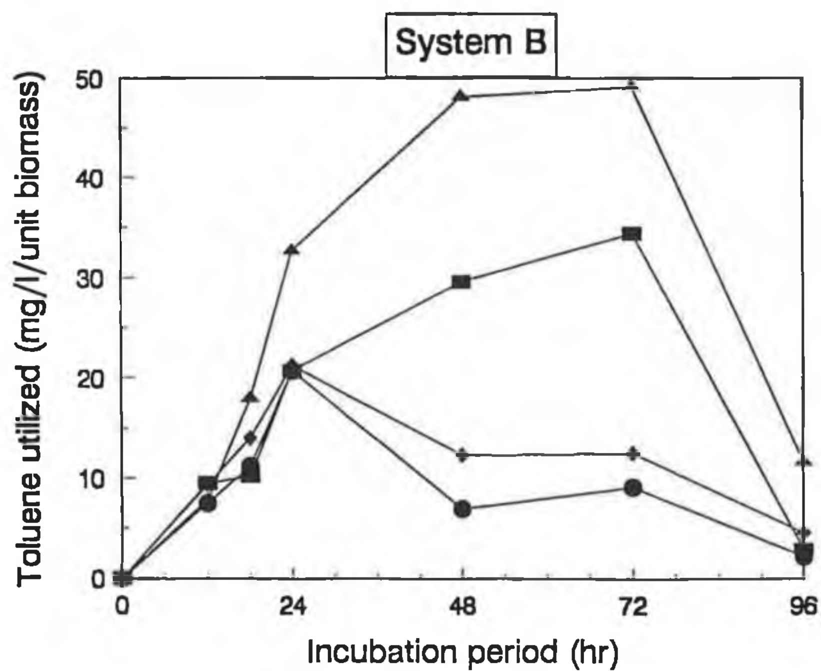
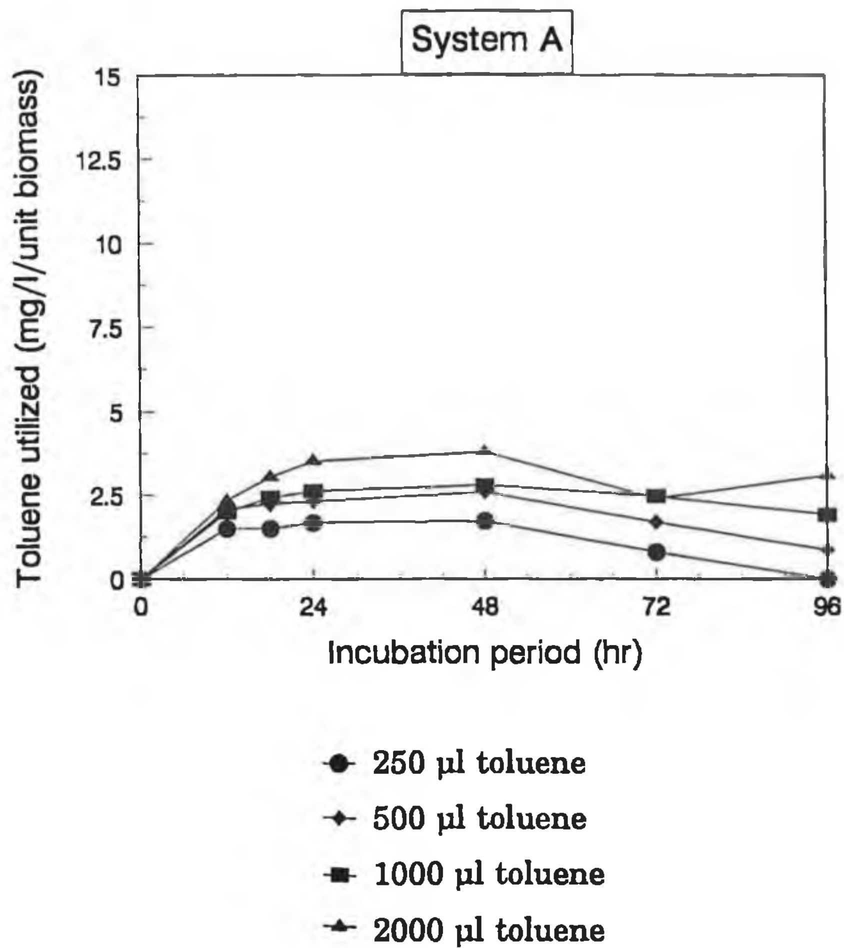


Figure 41: Utilization of toluene by *A. caviae* To-4 in the gas phase in systems A and B



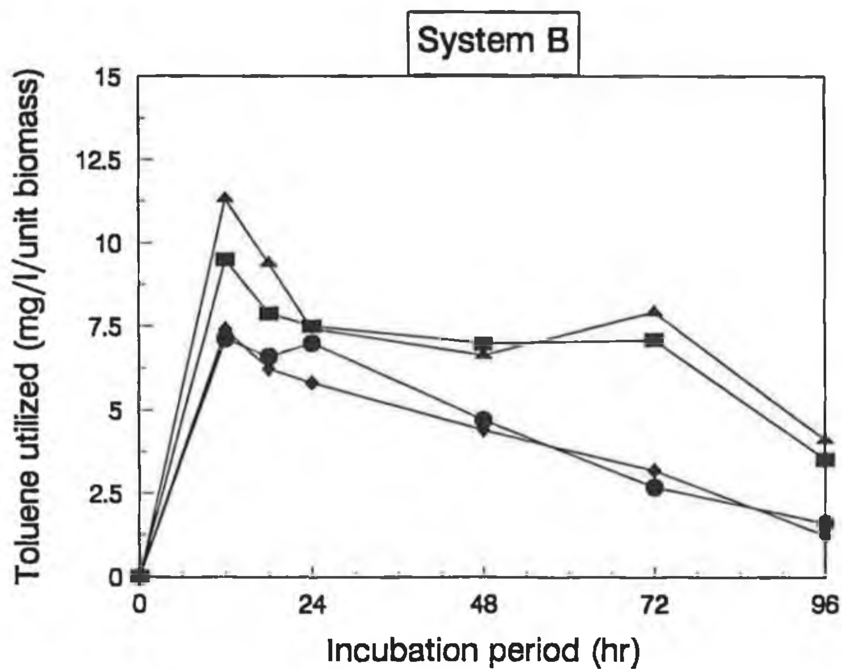
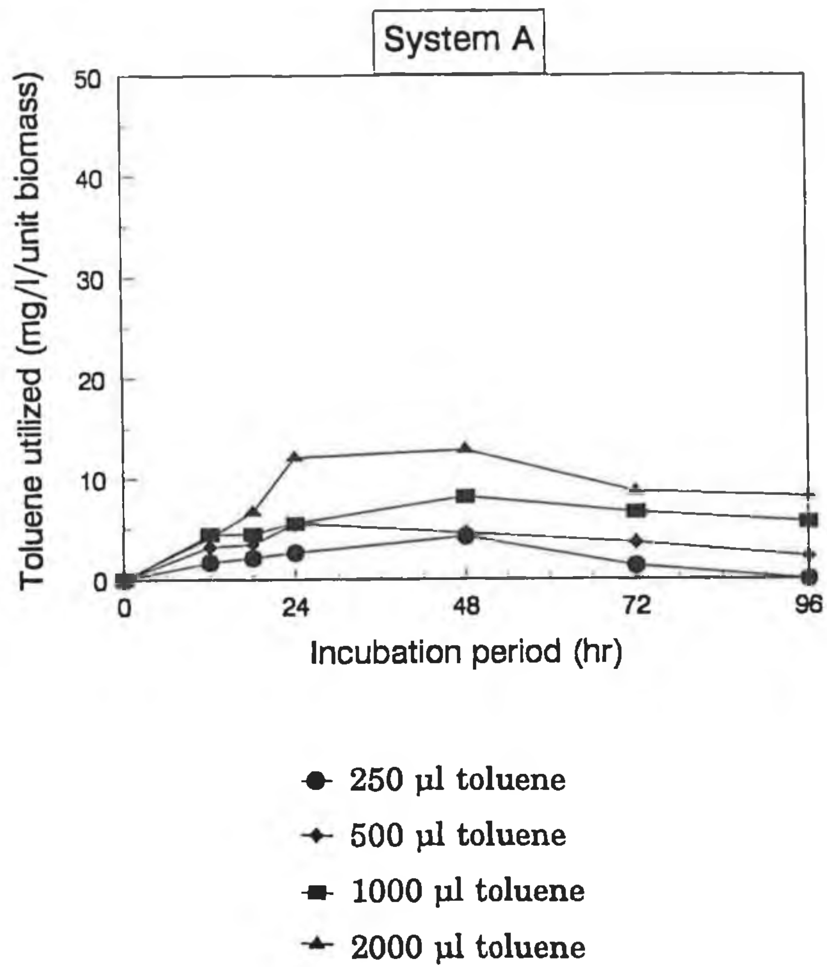


Figure 42: Utilization of toluene by *P. putida* To-5 in the liquid phase in systems A and B



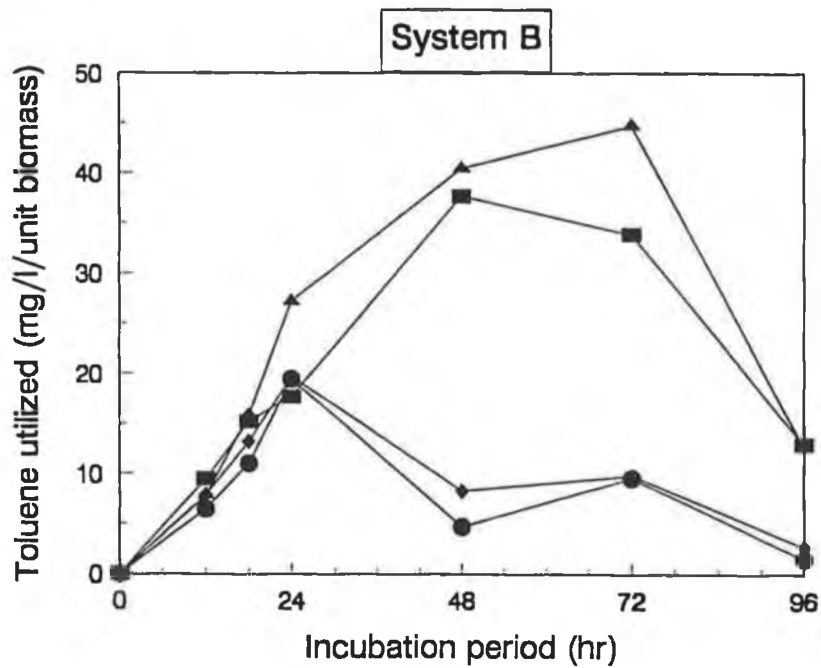


Figure 43: Utilization of toluene by *P. putida* To-5 in the gas phase in systems A and B

3.2.4 Growth in liquid culture with toluene added directly to the growth medium

The influence of acclimation

Growth was investigated to determine any toxic effect which might arise when liquid toluene was added directly to the growth medium. The experiments were conducted in a series of 120 ml serum bottles sealed with mininert valves. Initially the influence of acclimation on the cells was studied. Subsequently the influence of additional carbon source was investigated. Two types of cells - toluene induced and uninduced were used to inoculate the bottles and different volumes of toluene were added to the bottles containing 50 ml of minimal medium. The culture bottles were incubated at 30°C with the agitation of 200 rpm. Control bottles were incubated in parallel containing minimal medium and toluene. Growth was monitored after 48 hours of incubation by measuring the O.D at 660nm and the viability was determined by streaking a loopful of the culture on nutrient agar plates. The concentration of toluene was measured both in the gas and liquid phases (Table 13 - 14).

The concentration of toluene detected in the liquid phase increased with increasing volumes of toluene added. It was observed that when more than 50 µl toluene was added to the growth medium a biphasic layer was formed and above this level the concentration of toluene in the liquid phase detected remained constant. The concentration of toluene in the gas phase increased consistently with increasing volumes of toluene added. The concentration of toluene in the liquid phase ranged from 5.7 to 20 mg/l and in the gas phase from 0.9 to 36 mg/l.

All the toluene uninduced isolates were able to grow in the presence of 25 µl of toluene. Strains To-4 and To-5 were also able to grow in the presence of 35 µl of toluene. All the isolates failed to grow in the presence of more than 35

µl of toluene added directly to the growth medium. In the case of the toluene induced isolates all the bacteria grew following the addition of 50 µl toluene to the medium. However strain To-4 and To-5 also showed good growth following the addition of 100 µl of toluene to the growth medium.

While the organisms could not grow above these levels, the cells did remain viable in higher concentration of toluene. The viability did however decrease with increasing volumes of toluene added. All of the organisms remained viable following the addition of up to 300 µl toluene to the growth medium. None of the isolates were viable in the presence of 400 µl toluene. However strain To-4 and To-5 showed the greatest resistance and remained viable in up to 350 µl toluene.

The influence of an additional carbon source

The organisms were investigated for their ability to grow and survive when toluene was added directly to the growth medium and in the presence of other carbon sources. Again toluene induced and uninduced cells were grown in minimal medium and toluene with the addition of a nutrient medium. The nutrient medium contained glucose, yeast extract and peptone. Different volumes of liquid toluene (up to 20 v/v) were added directly to the growth medium and incubated in a serum bottles sealed with mininert valves at 30°C and agitated at 200 rpm. Control bottles were incubated in parallel containing minimal medium, nutrient medium and inoculum (without toluene). After 48 hours of incubation growth was monitored by measuring the O.D at 660nm (Table 15).

The toluene uninduced cells were unable to grow in any concentration of toluene in the presence of other carbon sources . Only the toluene induced cells were able to grow in all volumes of toluene when other carbon sources were available. The increase in growth of the induced cells in the presence of toluene above that of the control system indicated the utilization of toluene

Table 13: Growth of the toluene uninduced cells when liquid toluene was added directly to the growth medium (PMM + toluene) at 30 °C

Vol. toluene (µl)/50 ml PMM	Conc. of toluene (mg/l)		Growth and viability of the isolate at 48 hr.									
	Liquid phase	Gas phase	*P. putida* To-1		*P. putida* To-3		*A. caviae* To-4		*P. putida* To-5		*P. putida* Na-13	
			Gr	Vi	Gr	Vi	Gr	Vi	Gr	Vi	Gr	Vi
10	5.7	0.9	0.365	+++	0.310	+++	0.355	+++	0.344	+++	0.298	+++
25	13.0	2.25	0.271	+++	0.215	+++	0.385	+++	0.350	+++	0.211	+++
35	17.0	3.15	-	++	-	++	0.275	+++	0.276	+++	-	++
50	20.0	4.5	-	++	-	++	-	++	-	++	-	++
100	20.0	9.0	-	++	-	++	-	++	-	++	-	++
150	20.0	13.5	-	++	-	++	-	++	-	++	-	++
200	20.0	18.0	-	++	-	++	-	++	-	++	-	++
250	20.0	22.5	-	+	-	+	-	++	-	+	-	+
300	20.0	27.0	-	+	-	+	-	++	-	+	-	+
350	20.0	31.5	-	-	-	-	-	+	-	+	-	-
400	20.0	36.0	-	-	-	-	-	-	-	-	-	-

Gr: Growth O.D at 660nm; - no growth

Vi: Viability, +++ good viability; ++ medium viability; + less viability; - no viability.

Table 14: Growth of the toluene induced cells when liquid toluene was added directly to the growth medium (PMM + toluene) at 30 °C

Vol. toluene (µl)/50 ml PMM	Conc. of toluene (mg/l)		Growth and viability of the isolate at 48 hr.									
	Liquid phase	Gas phase	*P. putida* To-1		*P. putida* To-3		*A. caviae* To-4		*P. putida* To-5		*P. putida* Na-13	
			Gr	Vi	Gr	Vi	Gr	Vi	Gr	Vi	Gr	Vi
10	5.7	0.9	0.376	+++	0.389	+++	0.319	+++	0.352	+++	0.288	+++
25	13.0	2.25	0.396	+++	0.384	+++	0.450	+++	0.496	+++	0.315	+++
35	17.0	3.15	0.215	+++	0.381	+++	0.445	+++	0.406	+++	0.305	+++
50	20.0	4.5	-	+++	0.205	+++	0.281	+++	0.256	+++	0.216	+++
100	20.0	9.0	-	+++	-	+++	0.250	+++	0.223	+++	-	+++
150	20.0	13.5	-	+++	-	+++	-	+++	-	+++	-	+++
200	20.0	18.0	-	++	-	++	-	++	-	++	-	++
250	20.0	22.5	-	+	-	++	-	++	-	++	-	+
300	20.0	27.0	-	+	-	+	-	+	-	+	-	+
350	20.0	31.5	-	-	-	-	-	+	-	+	-	-
400	20.0	36.0	-	-	-	-	-	-	-	-	-	-

Gr: Growth O.D at 660nm; - no growth

Vi: Viability, +++ good viability; ++ medium viability; + less viability; - no viability.

Table 15: Growth of the toluene uninduced and induced cells in the presence of high concentrations of toluene when other carbon sources are available (PMM + NM + toluene) at 30 °C

Vol. toluene (µl)/50 ml PMM+NM	Growth (O.D at 660nm) of the isolate at 48 hr.									
	To-1		To-3		To-4		To-5		Na-13	
	A	B	A	B	A	B	A	B	A	B
C	0.928	0	0.885	0	0.915	0	0.930	0	0.870	0
250	1.285	0	1.222	0	1.330	0	1.238	0	1.210	0
500	1.320	0	1.201	0	1.321	0	1.251	0	1.285	0
1000	1.220	0	1.295	0	1.285	0	1.300	0	1.300	0
2000	1.280	0	1.200	0	1.350	0	1.310	0	1.361	0
4000	1.330	0	1.297	0	1.334	0	1.315	0	1.252	0
6000	1.350	0	1.225	0	1.430	0	1.461	0	1.271	0
8000	1.410	0	1.261	0	1.510	0	1.498	0	1.309	0
10000	1.400	0	1.285	0	1.530	0	1.570	0	1.312	0

C: control = PMM + NM + inoculum (without toluene)

A = Toluene induced cells

B = Toluene uninduced cells

To-1 = *Pseudomonas putida*

To-3 = *Pseudomonas putida*

To-4 = *Aeromonas caviae*

To-5 = *Pseudomonas putida*

Na-13 = *Pseudomonas putida*

by the organisms. The growth of strain To-4 and To-5 was greater than the other organisms. Therefore the toxicity effect of the toluene could be alleviated by the addition of another carbon sources.

3.3 Genetic studies of the bacteria

3.3.1 Catechol dioxygenase activity of the organisms

Catechol is the common intermediate in aromatic degradation and can be metabolized via either the *ortho* (Catechol 1,2 dioxygenase) or the *meta* (catechol 2,3-dioxygenase) cleavage pathways. Growth substrates can influence the enzyme produced and so the enzyme activity was determined for the organisms when grown on various substrates.

Enzyme activity following growth on toluene subcultured from nutrient broth

To determine the preferred cleavage route for toluene degradation in each organism, the activities of the two enzymes catechol 1,2-dioxygenase and catechol 2,3-dioxygenase was determined. The organisms were harvested from an overnight grown nutrient broth culture and resuspended in 33 mM Tris-HCl buffer (pH 7.6). This suspension was then used to inoculate toluene flasks. The *ortho* and *meta* activities were measured every 24 hours for interval of up to 96 hours to determine the optimum time for maximum activity. The cultures flasks were incubated at 30°C in the presence of various volumes of toluene (250 -2000 µl) to see the influence of toluene concentration on the enzyme activity,

All the organisms showed greater *meta* activity than *ortho* activity. The specific activity of both the catechol 1,2-dioxygenase and the catechol 2,3-dioxygense increased with increasing volumes of toluene supplied. This demonstrated the inducible nature of the enzyme activity (Figure 44 - 48).

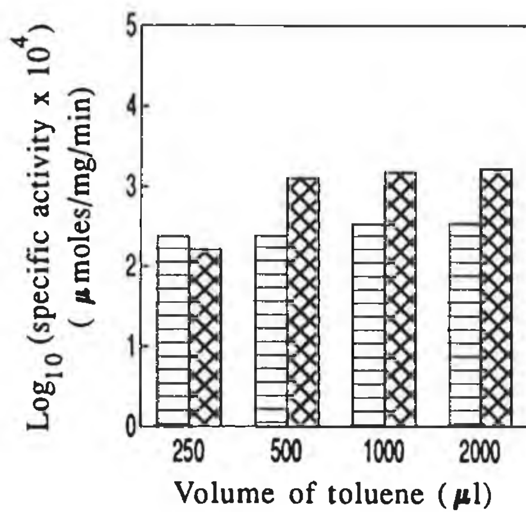
The level of catechol 1,2-dioxygenase activity (*ortho*) was maximum within 24 - 48 hours of incubation and started to decrease after 48 hours. The maximum *ortho* activity for all the organisms was obtained in the presence of 2000 µl toluene. The strongest *ortho* activity was determined for the strains To-1 and Na-13. The catechol 2,3-dioxygenase (*meta*) enzyme showed maximum activity at 48 - 72 hours of incubation and decreased very slowly with incubation time. All the organisms showed their maximum *meta* activity in the presence of 2000 µl toluene. The strongest *meta* activity were determined for the strains To-4 and To-5. Based on these findings all subsequent activities were determined at 48 hours.

Enzyme activity of the isolates following growth on luria broth subcultured from nutrient agar and toluene plates

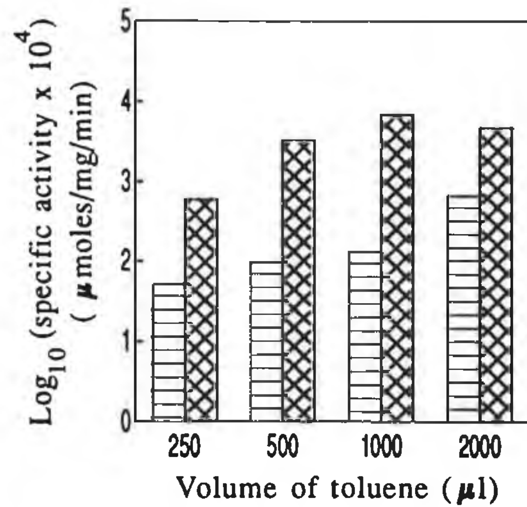
The production of catechol 1,2-dioxygenase and catechol 2,3-dioxygenase was determined following the growth of the bacteria in Luria broth. The organisms had previously been grown either on nutrient agar or toluene plates. The enzyme activity was determined after 48 hours of incubation (section 2.2.6).

The pattern of enzyme activity was similar for all four *Pseudomonas* spp. In all four cases no *ortho* activity was detected. The level of *meta* activity detected was higher for the organisms which had first been grown on toluene plates than for those which had been subcultured from nutrient agar (Table 16).
In the case of the *Aeromonas* sp. To-4 the situation was reversed. No *meta* activity was detected. Again the *ortho* activity was slightly higher when the organism was subcultured from toluene plates rather than nutrient agar plates (Table 16).

However these results indicated that the substrate had a marked influence on the pathway utilized.

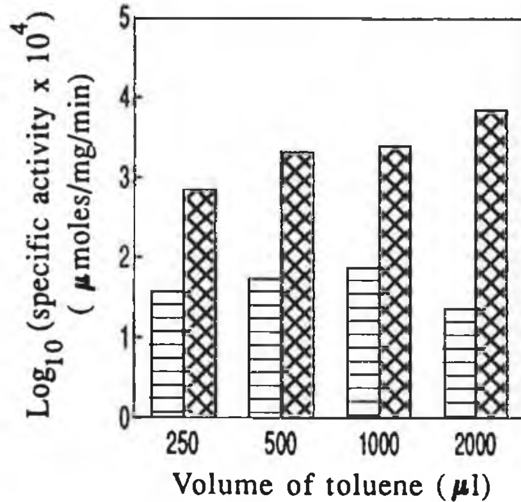


24 hr

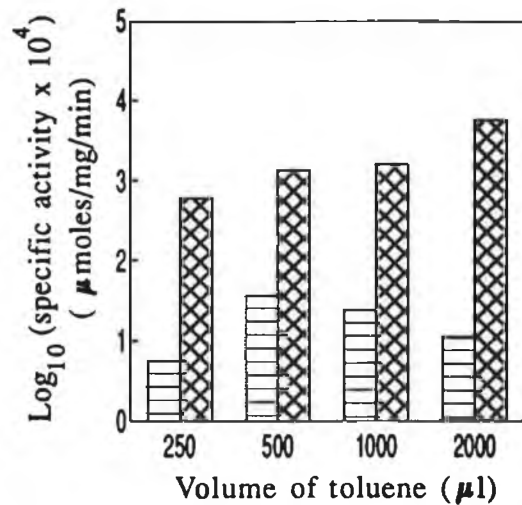


48 hr

catechol 1,2-dioxygenase catechol 2,3-dioxygenase

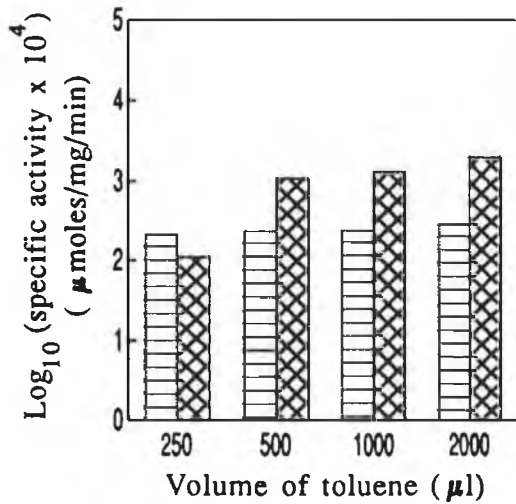


72 hr

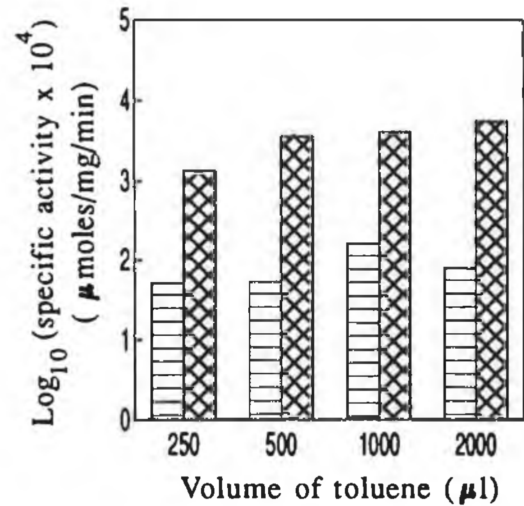


96 hr

Figure 44: Specific enzyme activity of the catechol dioxygenase enzymes of *P. putida* To-1 at different periods of incubation in the presence of toluene

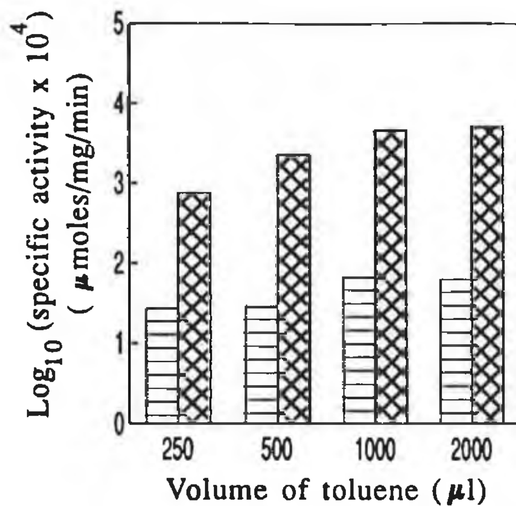


24 hr

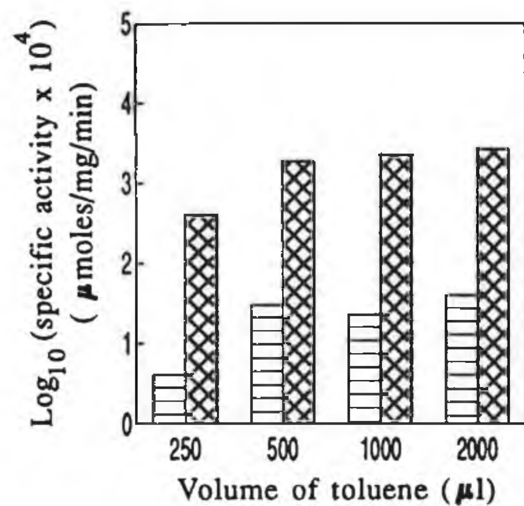


48 hr

catechol 1,2-dioxygenase   catechol 2,3-dioxygenase



72 hr



96 hr

Figure 45: Specific enzyme activity of the catechol dioxygenase enzymes of *P. putida* To-3 at different periods of incubation in the presence of toluene

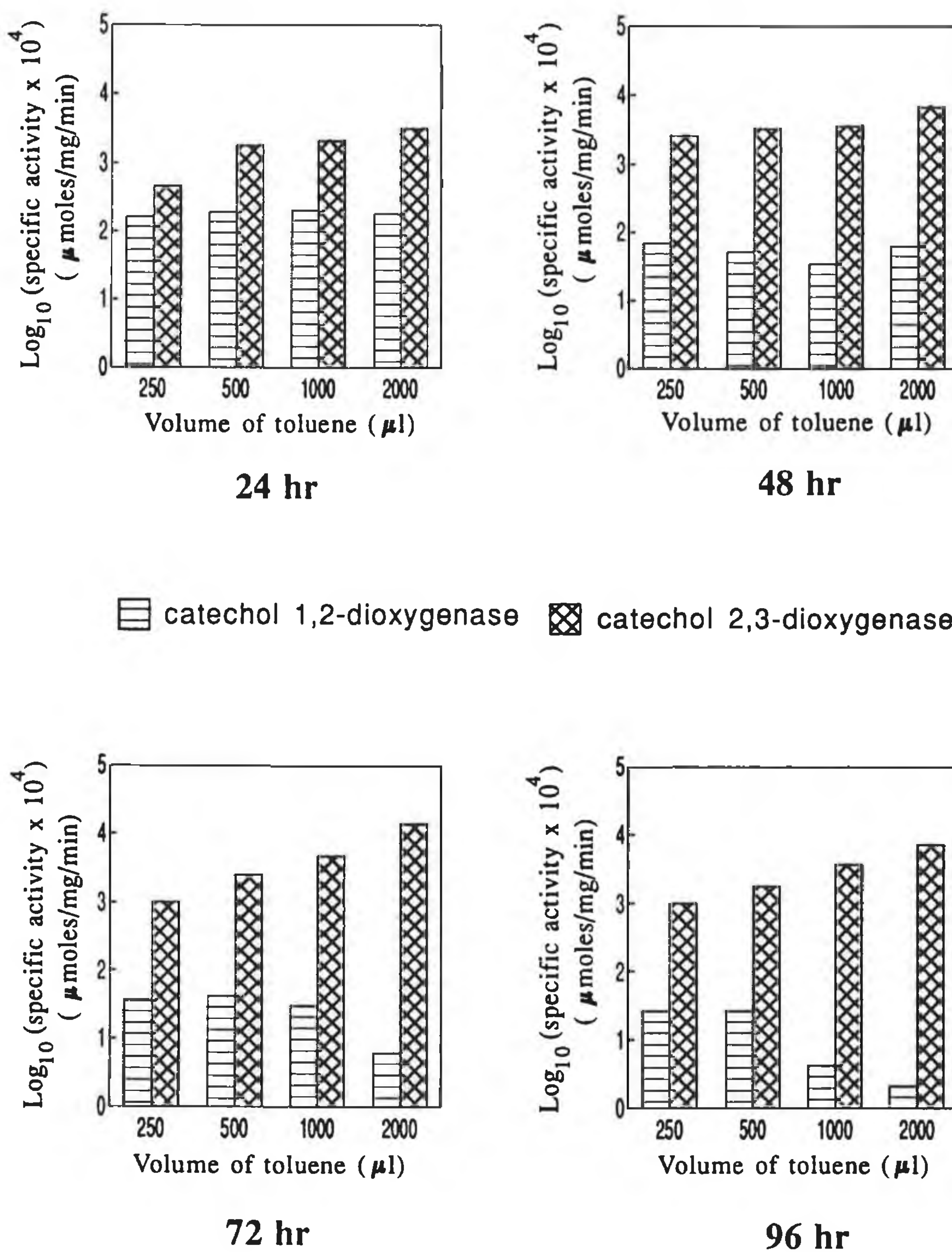


Figure 46: Specific enzyme activity of the catechol dioxygenase enzymes of *A. caviae* To-4 at different periods of incubation in the presence of toluene

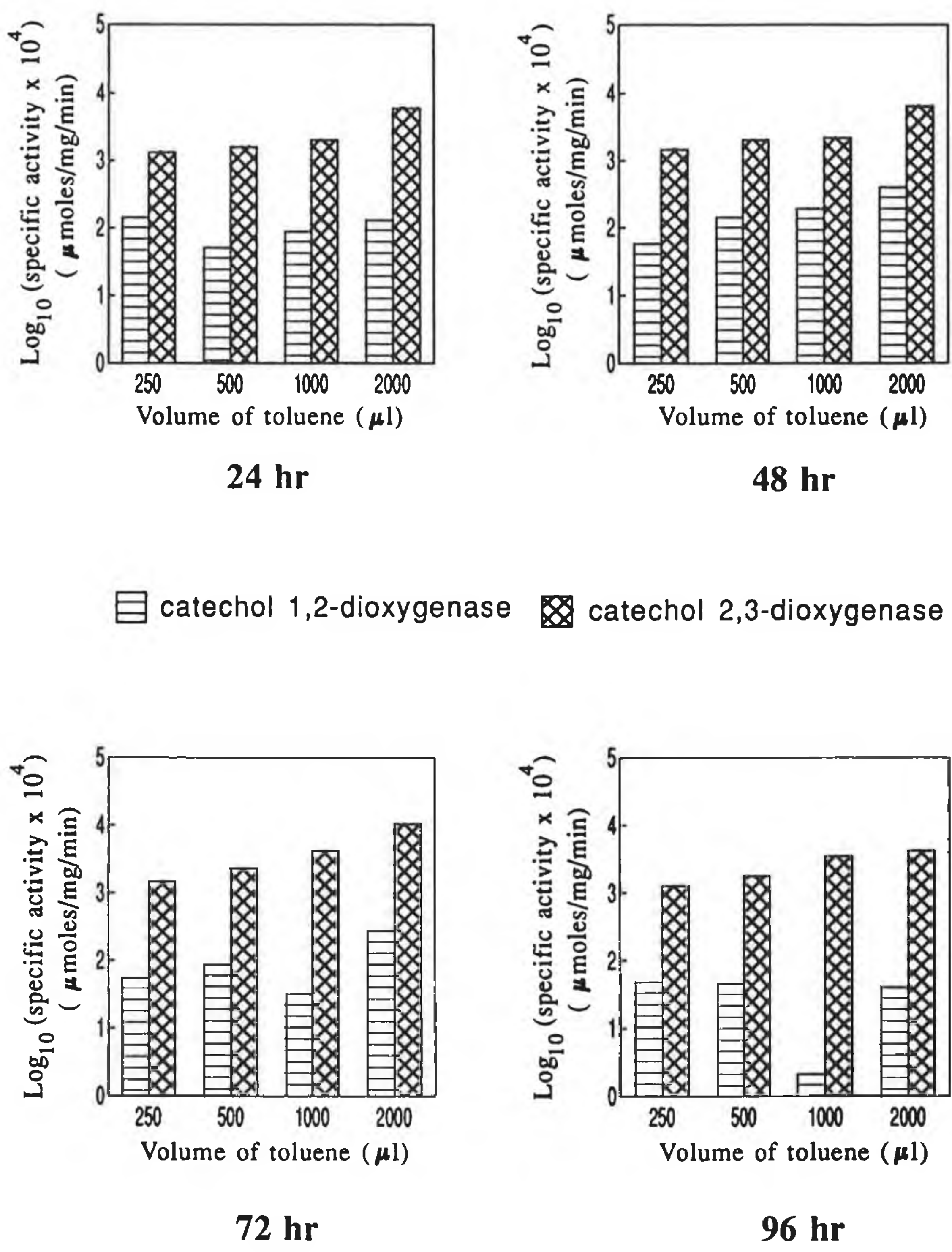


Figure 47: Specific enzyme activity of the catechol dioxygenase enzymes of *P. putida* To-5 at different periods of incubation in the presence of toluene

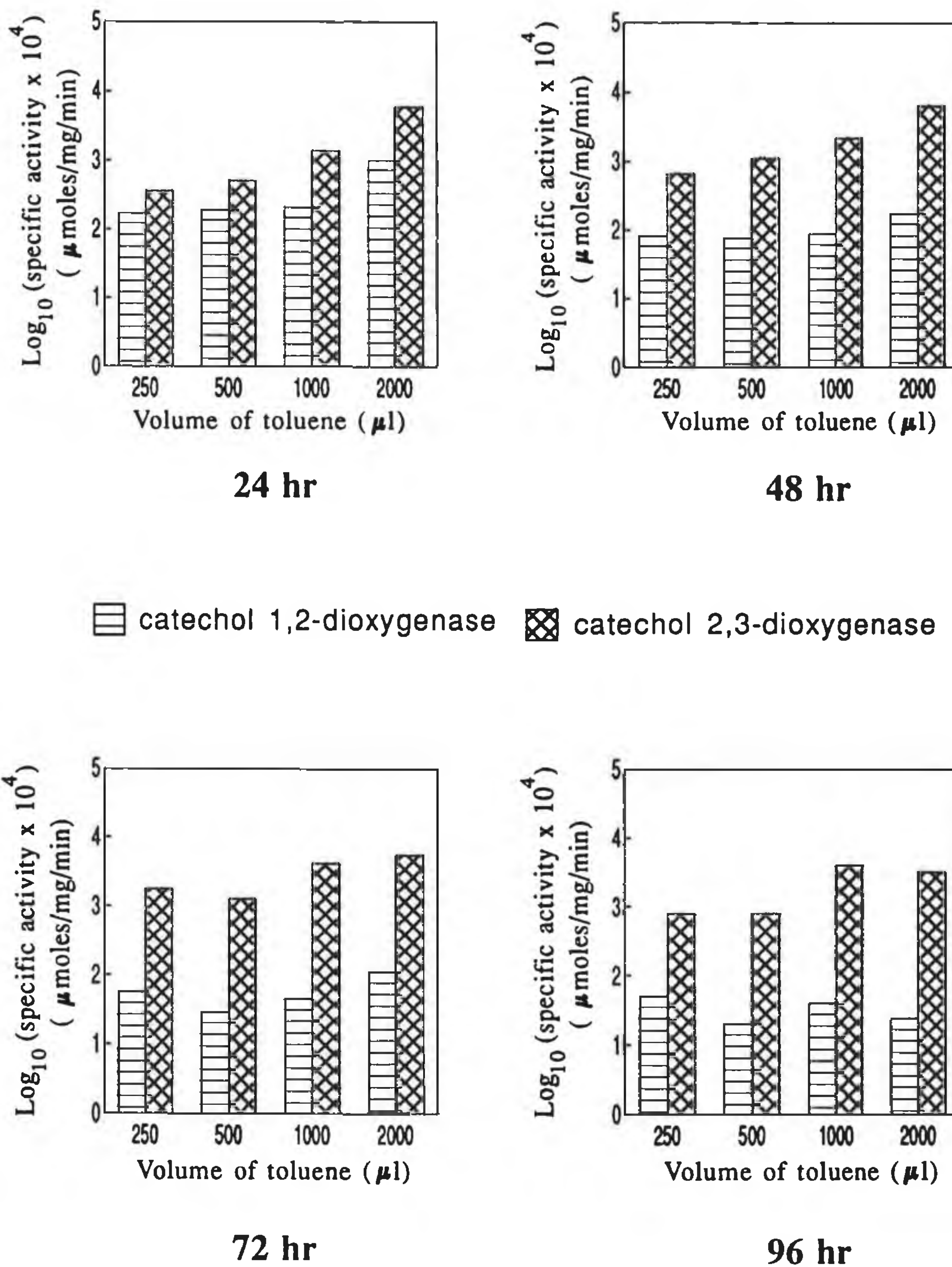


Figure 48: Specific enzyme activity of the catechol dioxygenase enzymes of *P. putida* Na-13 at different periods of incubation in the presence of toluene

Table 16: Catechol dioxygenase activity in cells following growth on luria broth subcultured from nutrient agar and toluene plates

Isolate	Specific activity (μ moles/mg/min)			
	Nutrient agar to luria broth		Tol-plate to luria broth	
	C 1,2 O[a]	C 2,3 O	C 1,2 O	C 2,3 O[b]
Pseudomonas putida (To-1)	0.000	2.4×10^{-3}	0.000	5.5×10^{-2}
Pseudomonas putida (To-3)	0.000	2.15×10^{-3}	0.000	5.8×10^{-2}
Aeromonas caviae (To-4)	3.9×10^{-3}	0.000	9.6×10^{-3}	0.000
Pseudomonas putida (To-5)	0.000	7.8×10^{-3}	0.000	7.0×10^{-2}
Pseudomonas putida (Na-13)	0.000	3.53×10^{-3}	0.000	5.8×10^{-2}

[a] Catechol 1,2-dioxygenase; [b] Catechol 2,3-dioxygenase.

3.3.2 Plasmid profiles of the *Pseudomonas* spp. and *Aeromonas* sp.

The presence of meta activity indicated that the degradation of toluene was plasmid mediated. It was therefore of interest to examine the plasmid profiles of the organisms. Because of the influence of the growth substrates on the enzyme activity, the influence of growth substrates on the plasmid profiles of the organisms was also investigated.

Plasmid profiles of the bacteria following growth on Luria broth subcultured from nutrient agar plates

Plasmid DNA was isolated (section 2.2.7) from cultures grown on Luria broth for 24 hours subcultured from nutrient agar plates. *P. putida* NCIB 10432 contained a transmissible TOL plasmid (117 kb) was used as a marker to established the appropriate size of the plasmids. A cured strain of *P. putida* C-104 was used as a chromosomal marker (section 2.2.9)

The plasmid profiles of the chromosomal marker (Lane 1), *P. putida* NCIB 10432 (Lane 2), *P. putida* To-1 (Lane 3), To-3 (Lane 4), To-5 (Lane 6), Na-13 (Lane 7) and *A. caviae* To-4 (Lane 5) are illustrated in Figure 49.

In all the *Pseudomonas* spp. one large plasmid band was visualized except strain To-1. A faint band was visualized for the strain To-1. No plasmid bands were visualized in the case of *A. caviae* To-4. The large plasmids isolated from all the *Pseudomonas* spp. were in the range of 85 - 120 kb region

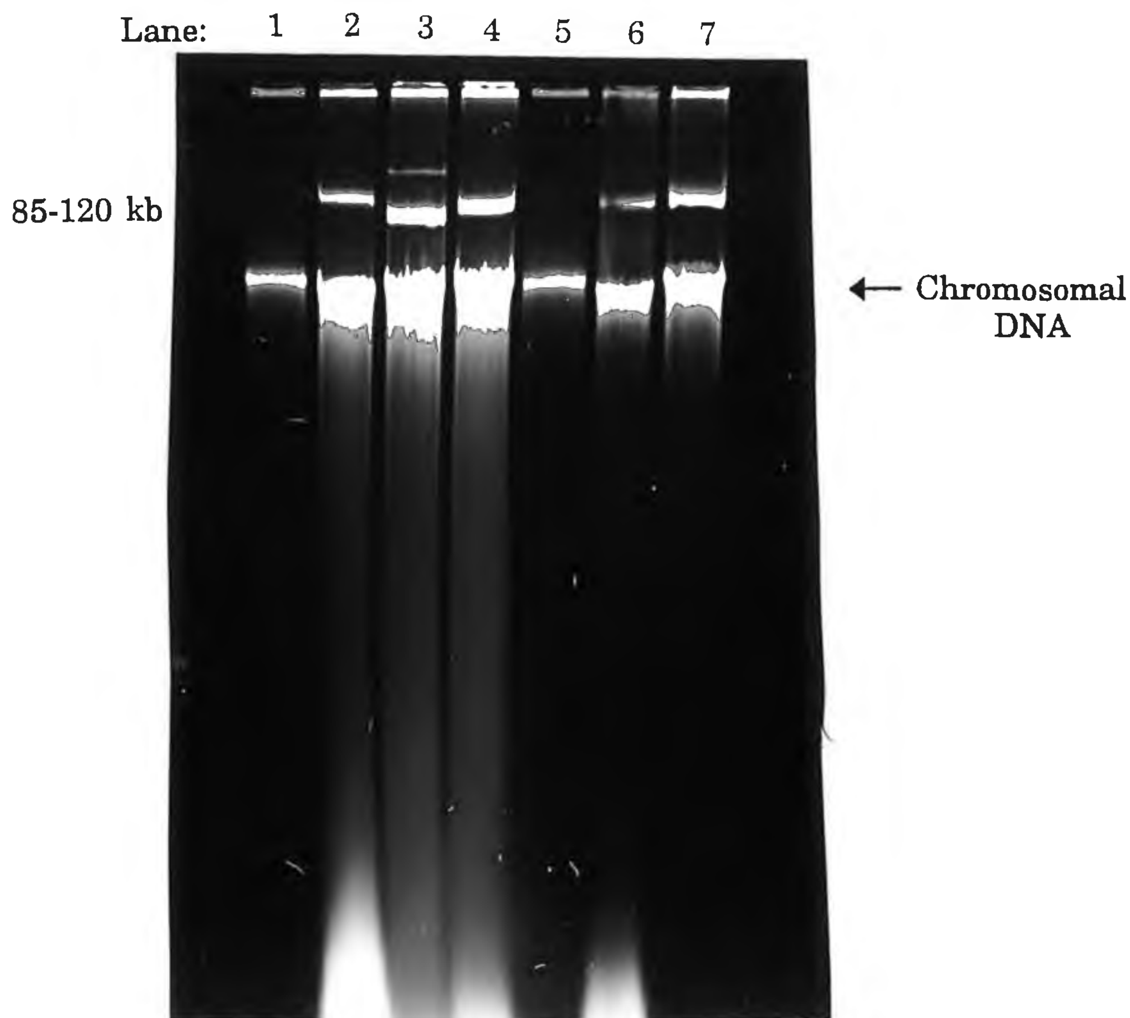


Figure 49: The plasmid profiles of *Pseudomonas* spp. and *Aeromonas* sp. following growth on luria broth subcultured from nutrient agar

Lane 1: *P. putida* C-104

Lane 2: *P. putida* NCIB 10432 (TOL : 117 kb)

Lane 3: *P. putida* To-1

Lane 4: *P. putida* To-3

Lane 5: *A. caviae* To-4

Lane 6: *P. putida* To-5

Lane 7: *P. putida* Na-13

Plasmid profiles of the bacteria following growth on
(i) Luria broth - subcultured from toluene plates
(ii) Toluene culture - subcultured from nutrient broth

The plasmid DNA was isolated from 24 hours cultures following growth on two substrates - luria broth subcultured from toluene plates and toluene culture subcultured from nutrient broth.

The large plasmid bands, previously sized to approximately 85 - 120 kb, visualized in all *Pseudomonas* spp. were stably maintained following growth on all media as when the organisms were grown in luria broth and toluene culture (Figure 50 - 51). No plasmid bands were visualized in *A. caviae* To-4 when grown in luria broth subcultured from toluene plates (Lane 4, Figure 50). A large plasmid band not previously seen was however visualized in *A. caviae* To-4 (Lane 4, Figure 51) when the organism was grown in the presence of toluene. This plasmid band was also sized in the 85 - 120 kb region indicating that *A. caviae* To-4 carried an unstable plasmid which was only visualized when the organism was grown in the presence of toluene.

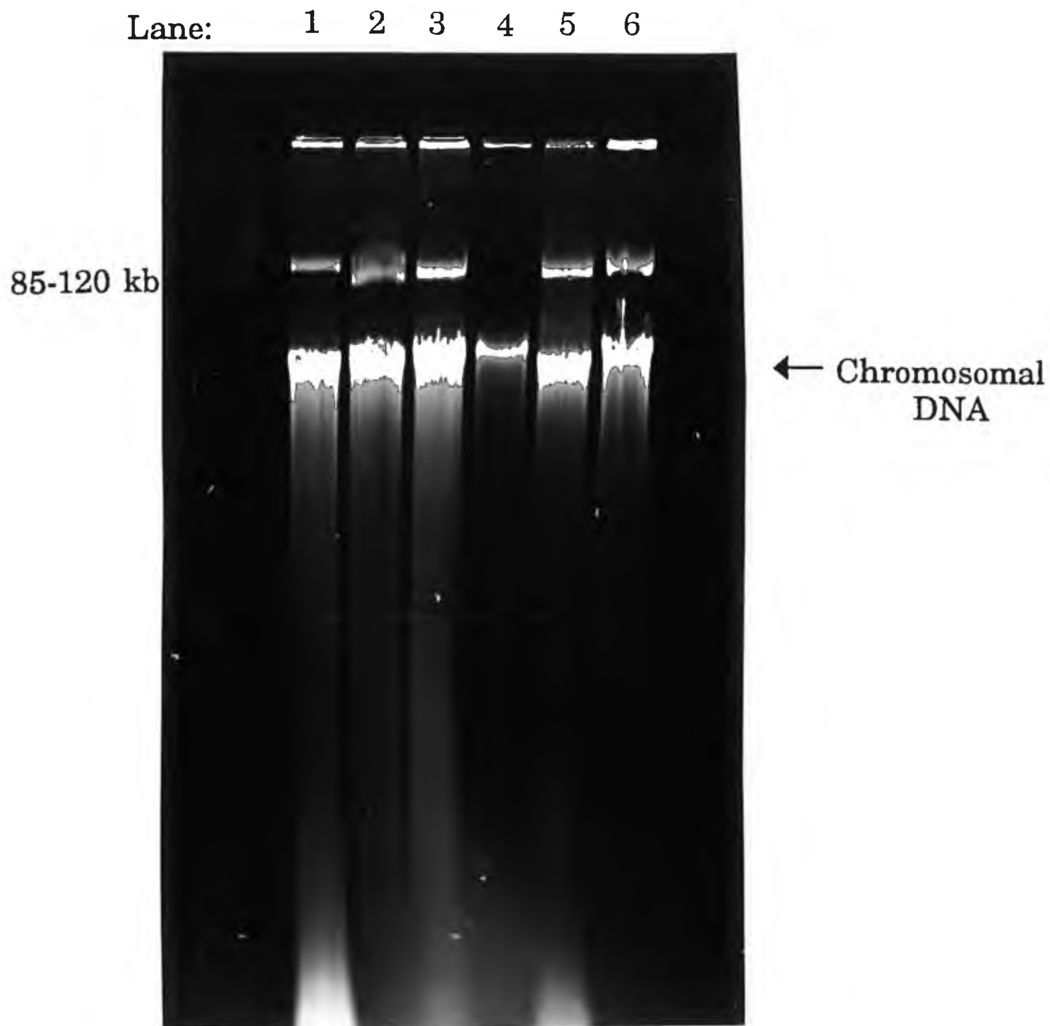


Figure 50: The plasmid profiles of *Pseudomonas* spp. and *Aeromonas* sp. following growth on luria broth subcultured from toluene plates

Lane 1: *P. putida* NCIB 10432 (TOL:117 kb)
Lane 2: *P. putida* To-1
Lane 3: *P. putida* To-3
Lane 4: *A. caviae* To-4
Lane 5: *P. putida* To-5
Lane 6: *P. putida* Na-13

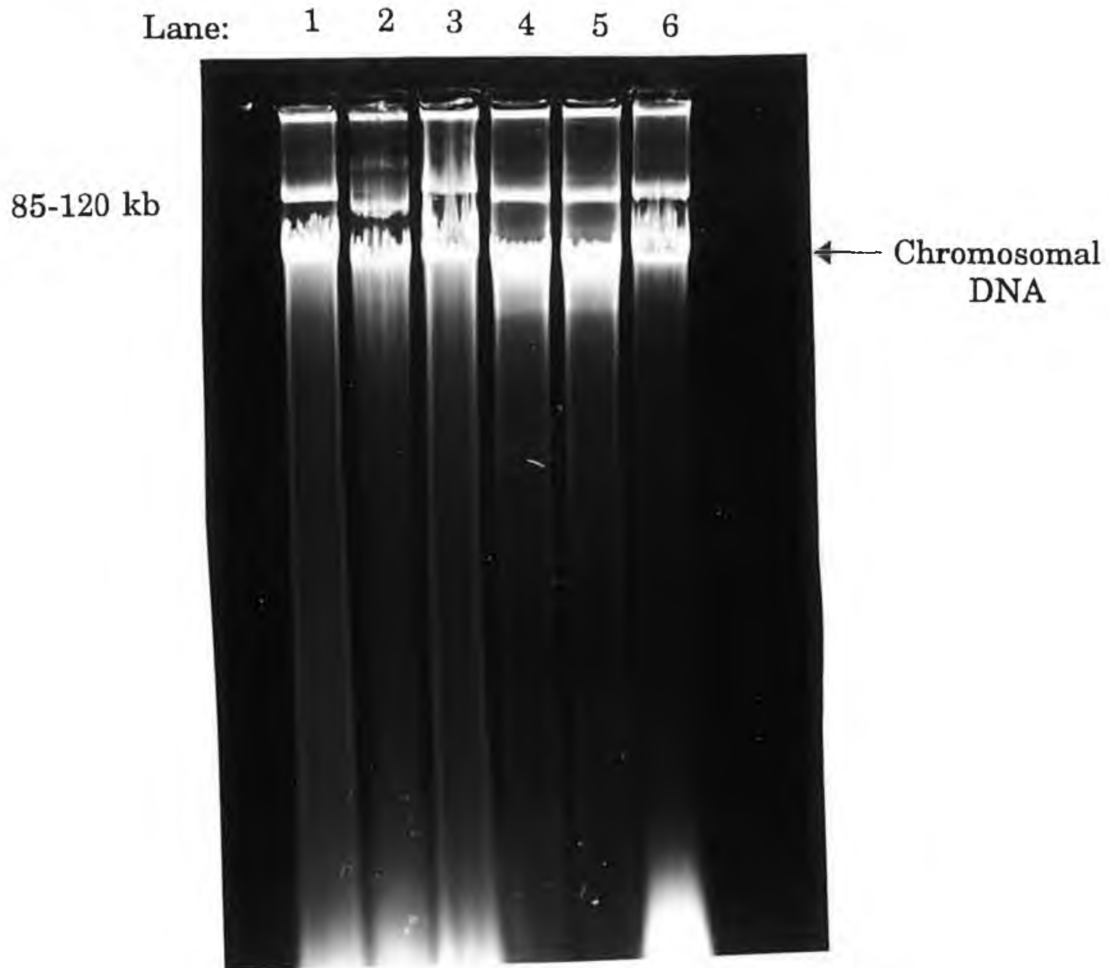


Figure 51: The plasmid profiles of *Pseudomonas* spp. and *Aeromonas* sp. following growth on toluene subcultured from nutrient broth

Lane 1: *P. putida* NCIB 10432 (TOL:117 kb)
Lane 2: *P. putida* To-1
Lane 3: *P. putida* To-3
Lane 4: *A. caviae* To-4
Lane 5: *P. putida* To-5
Lane 6: *P. putida* Na-13

3.3.3 Identification of the TOL plasmid

The presence of a large plasmid band of similar size to the TOL plasmid of *P. putida* NCIB 10432 (117 kb) together with the ability of these organisms to grow on toluene prompted the identification of these plasmids using a TOL probe. The presence of TOL plasmid was investigated using dot blot and Southern hybridization.

Dot blot hybridization

Dot blot hybridization was carried out on the total DNA samples from all five organisms. The plasmid DNA was isolated following growth on two substrates - luria broth subcultured from toluene plates and toluene subcultured from nutrient broth. The hybridization was carried out using a digoxigenin-labelled TOL probe of *P. putida* NCIB 10432 (117 kb).

The TOL probe of *P. putida* NCIB 10432 hybridized to the DNA samples of all the *Pseudomonas* spp. regardless of the growth substrates (Figure 52 - 53). This result confirmed that all the *Pseudomonas* spp. carried a large stable TOL plasmid. The TOL probe of *P. putida* NCIB 10432 hybridized to the DNA of *A. caviae* To-4 following growth on toluene (No. 2, Figure 53). No hybridization was found for this strain when grown on luria broth (No. 2, Figure 52).

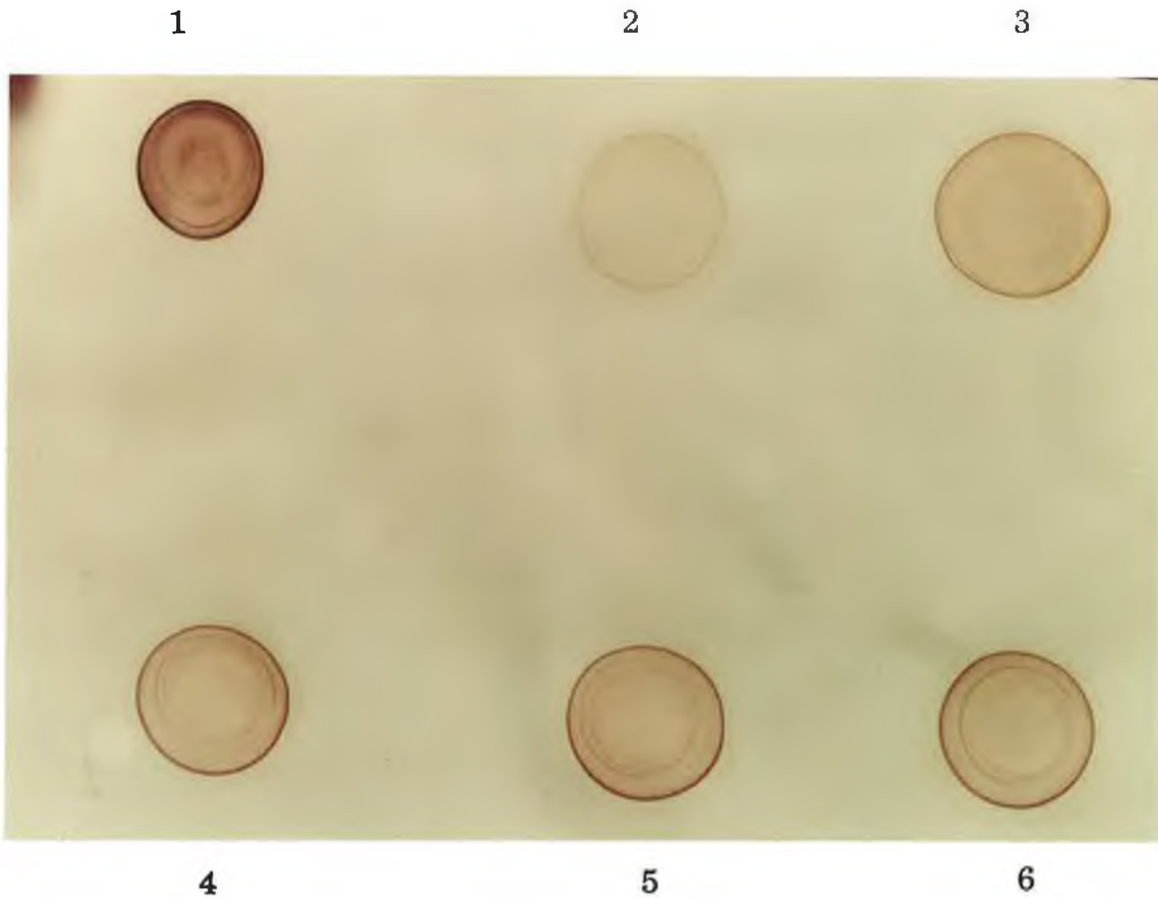


Figure 52: Dot blot hybridization to the TOL probe (NCIB 10432) of the DNA samples of the organisms following growth on luria broth subcultured from toluene plates

1 : *P. putida* NCIB 10432 (TOL: 117 kb) (positive control)
2 : *A. caviae* To-4
3 : *P. putida* To-5
4 : *P. putida* To-1
5 : *P. putida* To-3
6 : *P. putida* Na-13

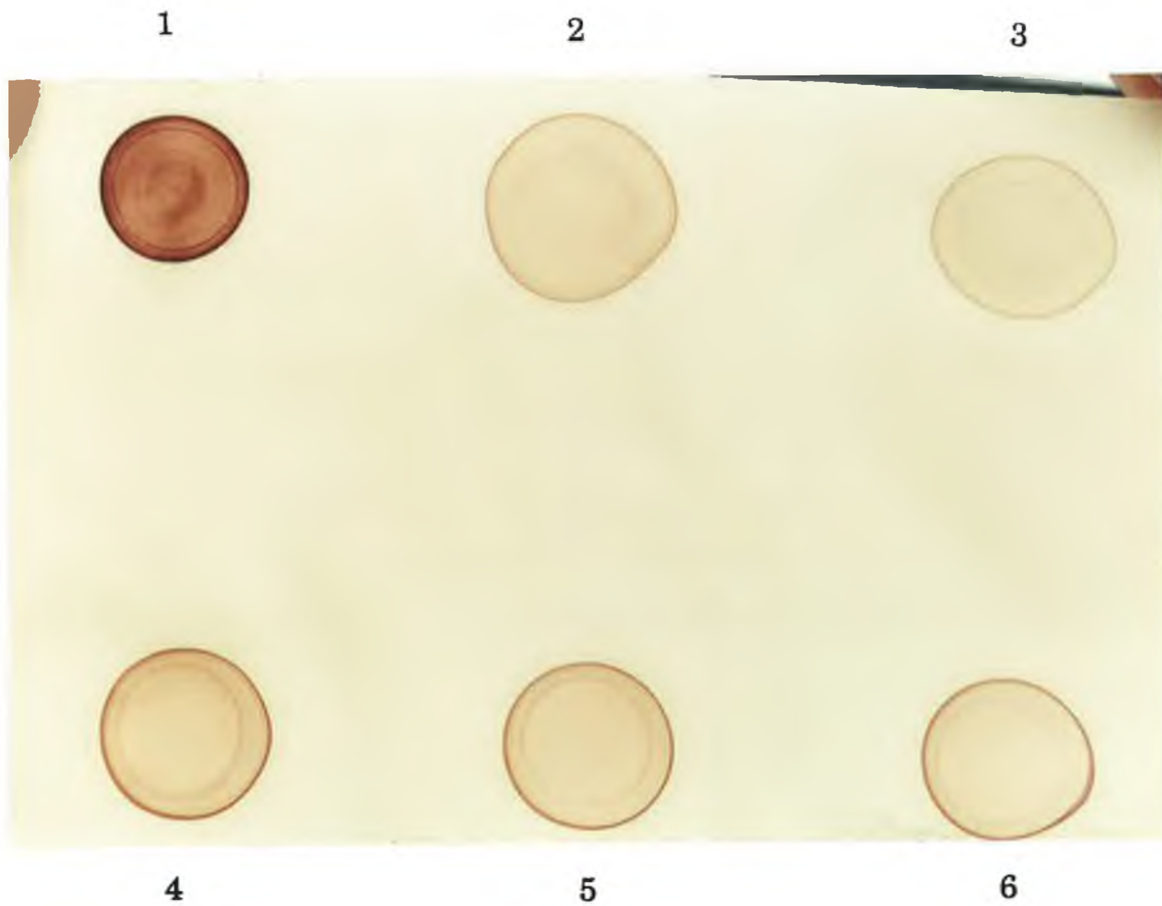


Figure 53: Dot blot hybridization to the TOL probe (NCIB 10432) of the DNA samples of the organisms following growth on toluene subcultured from nutrient broth

1 : *P. putida* NCIB 10432 (TOL: 117 kb) (positive control)
2 : *A. caviae* To-4
3 : *P. putida* To-5
4 : *P. putida* To-1
5 : *P. putida* To-3
6 : *P. putida* Na-13

Southern hybridization

With a view to studying the plasmid in greater detail the plasmid bands from the two organisms strain To-4 and To-5 were identified using Southern hybridization. The DNA was initially restricted and transferred to the nitrocellulose filter and hybridization was carried out using the TOL probe. The hybridization was carried out using digoxigenin-labelled and radioactively labelled methods.

Restriction profiles of plasmid DNA

Restriction analyses were carried out to compare the homology between the restriction fragments of the TOL plasmid of *P. putida* NCIB 10432 and the fragments of the DNA of *P. putida* To-5 and *A. caviae* To-4. The purified plasmid DNA was cleaved with restriction endonucleases Hind III and Eco RI and the resulting fragments were separated by 0.7% agarose gel electrophoresis along with λ size markers in the range of 2 kb to 33.5 kb (Figure 54).

A number of restriction fragments ranging in size from less than 2 kb to 33 kb were visualized following incubation with the restriction endonucleases. The restriction enzyme Hind III cleaved the TOL plasmid of *P. putida* 10432 into 9 fragments (Lane 2) ranging in size from 2 kb 33 kb. The Eco RI restriction enzyme cleaved the TOL plasmid of *P. putida* into 15 fragments (Lane 3) ranging in size from 1 kb to 33 kb.

The restriction profiles of the organisms To-4 and To-5 were identical. The Hind III restriction enzyme cleaved both of the plasmids into 14 fragments ranging in size from 1 kb to 19 kb (To-4: Lane 4, To-5: Lane 6). The restriction enzyme Eco RI cleaved the plasmids into 11 fragments ranging in size from 0.5 kb to 24.5 kb. However only 3 of the Hind III and 5 of the Eco RI fragments of the plasmid DNA of To-4 and To-5 were similar in size to

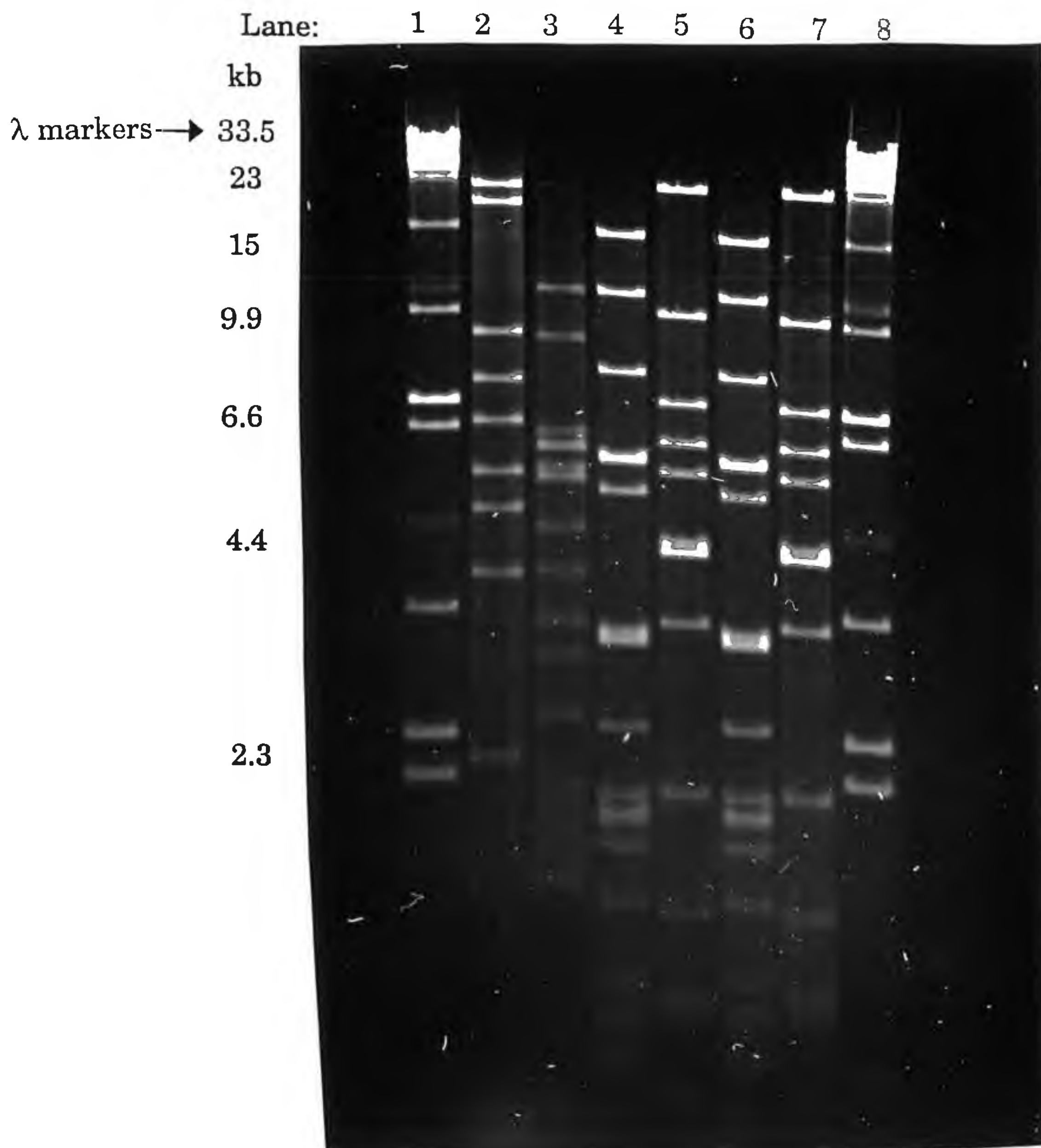


Figure 54: Restriction profiles of *P. putida* NCIB 10432, *A. caviae* To-4 and *P. putida* To-5

Lane 1,8 : λ markers (Hind III + Xho I)
Lane 2 : *P. putida* NCIB 10432 (TOL: 117 kb) (Hind III)
Lane 3 : " " (Eco RI)
Lane 4 : *A .caviae* To-4 (Hind III)
Lane 5 : " " (Eco RI)
Lane 6 : *P. putida* To-5 (Hind III)
Lane 7 : " " (Hind III)
Lane 7 : λ markers (Hind III + Xho I)

that of the fragments of the TOL plasmid of *P. putida* NCIB 10432.

Southern hybridization was carried out to identify similarities between the fragments of the TOL plasmid of *P. putida* NCIB 10432 and that of strains of TO-4 and To-5. Southern hybridization was carried out on the restriction fragments using the DNA of *P. putida* NCIB 10432 (TOL) as a probe. The probe was labelled using both ^{32}P and the digoxigenin-labelled kit. λ probe was used to hybridize the λ fragments for calibrating the size of the hybridized fragments.

The hybridization pattern obtained using the digoxygenin-labelled kit is illustrated in Figure 55. This figure shows clear hybridization in the case of *P. putida* NCIB 10432 (Lane 2: Hind III, Lane 3: Eco RI) and the λ marker (Lane 1 and 8). While the same hybridizing DNA from To-4 (Lane 4 and 5) and To-5 (Lane 6 and 7) occurred the result was not as clear as that obtained when the radioactively labelled probe was used. The hybridization of ^{32}P are illustrated in Figure 56.

The TOL probe of *P. putida* 10432 hybridized to four of the Hind III fragments of both of the strains To-4 and To-5 (Lane 3, 5). Of the four hybridized fragments three hybridized strongly and one weakly. The size of the strongly hybridized fragments were 19, 6.1 and 2.6 kb and the weakly hybridized band was 15.5 kb.

The TOL probe of *P. putida* 10432 hybridized to eight of the Eco RI fragments of both of the strains (Lane 5, 7). Of the eight fragments three were hybridized strongly and five weakly. The size of the strongly hybridized bands were 24.5, 5.0, and 1.0 kb. The weakly hybridized bands were 18.0, 16.0, 13.1, 3.3 and 0.5 kb respectively.

Only one of the hybridized bands 5.0 kb, in To-4 and To-5 was of similar size

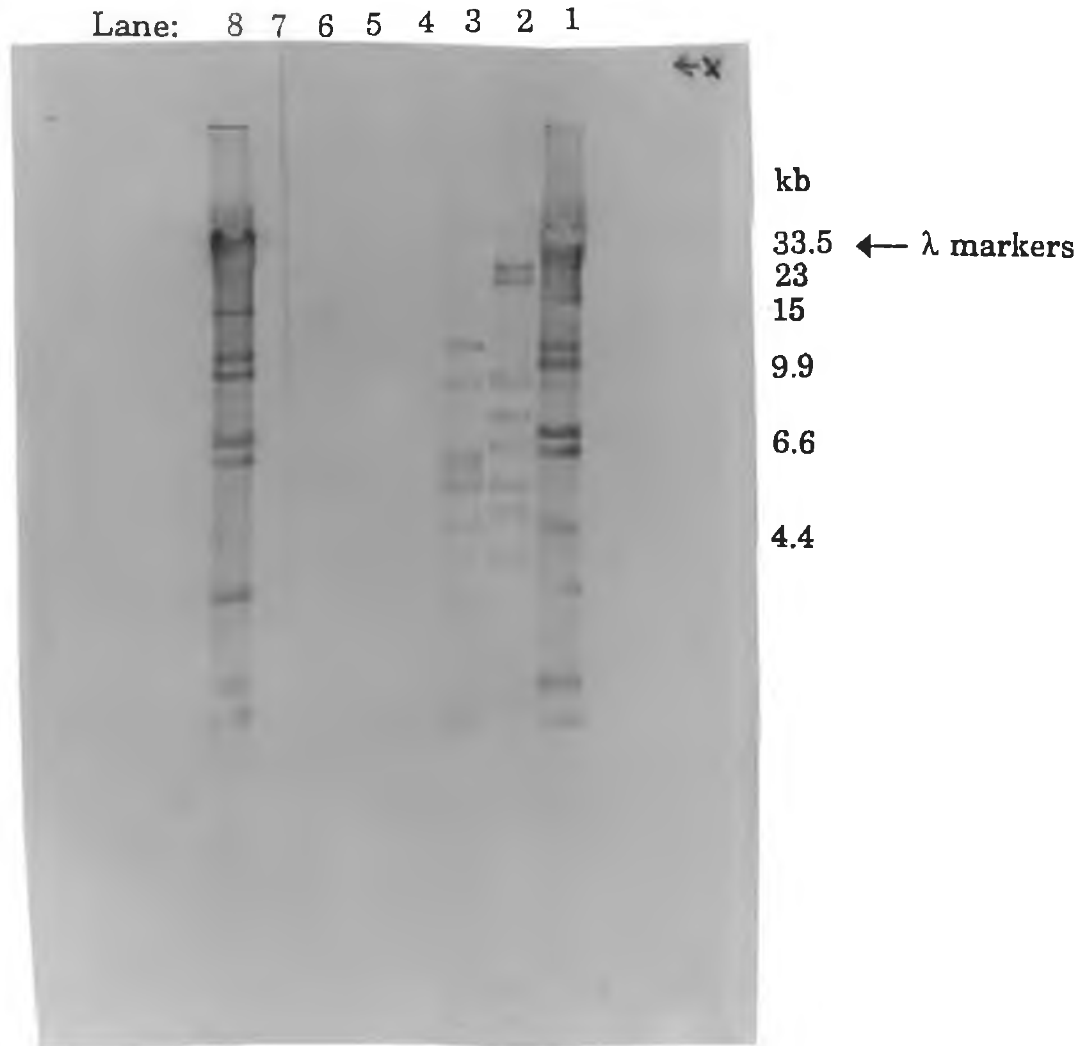


Figure 55: Digoxigenin -labelled hybridization of the DNA from *A. caviae* To-4 and *P. putida* To-5 with the TOL probe

Lanes : as in Figure 54:

Lane 1 : λ markers (Hind III + Xho I)

Lane 2 : *P. putida* NCIB 10432 (TOL: 117 kb) (Hind III)

Lane 3 : " " (Eco RI)

Lane 4 : *A .caviae* To-4 (Hind III)

Lane 5 : " " (Eco RI)

Lane 6 : *P. putida* To-5 (Hind III)

Lane 7 : " " (Hind III)

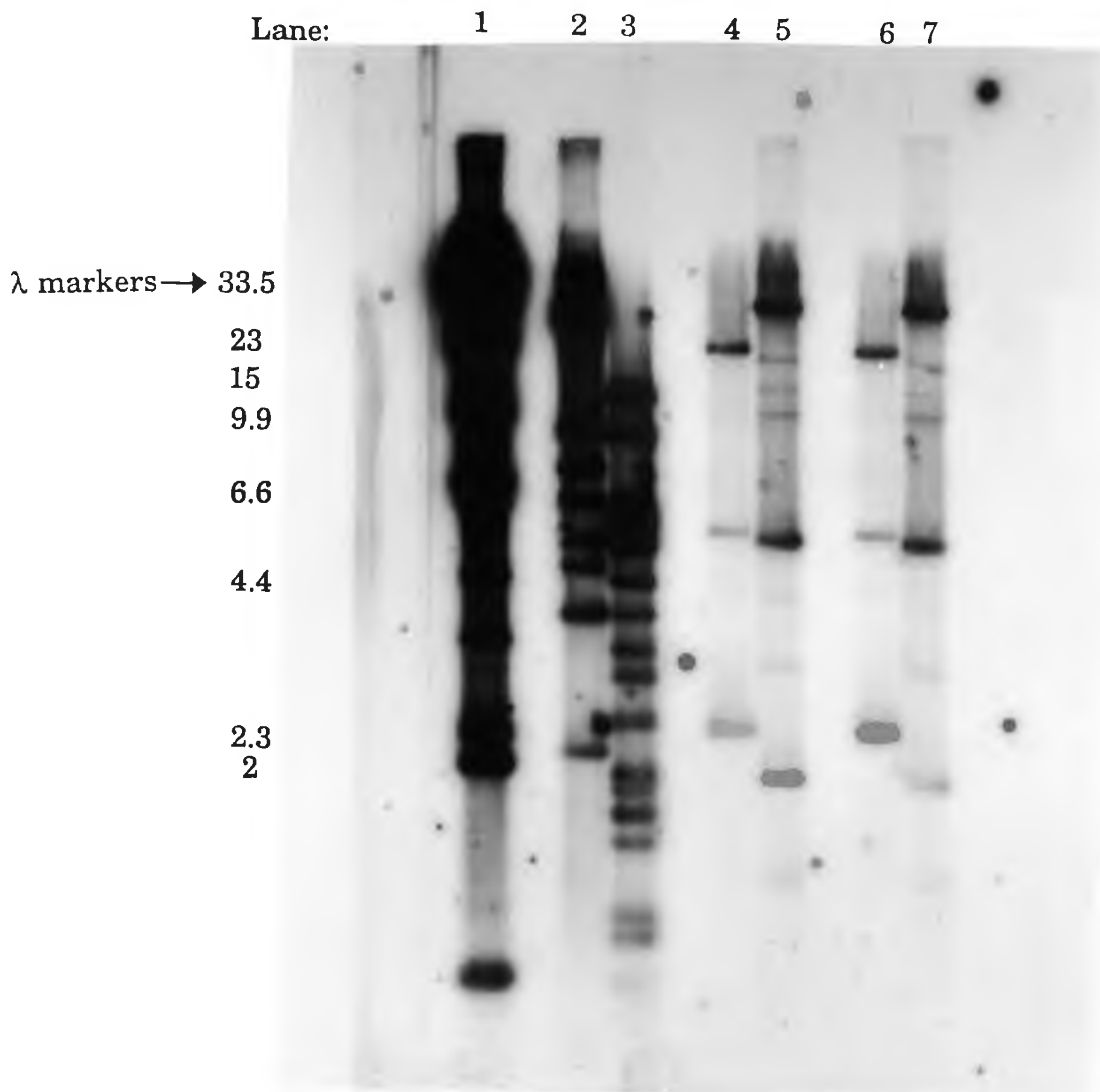


Figure 56: Radioactively labelled hybridization of the DNA from *A. caviae* To-4 and *P. putida* To-5 with the TOL probe

Lanes : as in Figure 54:

Lane 1 : λ markers (Hind III + Xho I)

Lane 2 : *P. putida* NCIB 10432 (TOL: 117 kb) (Hind III)

Lane 3 : " " (Eco RI)

Lane 4 : *A .caviae* To-4 (Hind III)

Lane 5 : " " (Eco RI)

Lane 6 : *P. putida* To-5 (Hind III)

Lane 7 : " " (Hind III)

to that in *P. putida* NCIB 10432. These results indicated that both of the spp. *A. caviae* To-4 and *P. putida* To-5 carried an identical plasmid and that this plasmid contained some of the sequences of the TOL plasmid of *P. putida* NCIB 10432.

4. DISCUSSION

The activated sludge sample used to isolate the bacteria was obtained from the waste treatment plant at a local pharmaceutical company. The characteristics of the activated sludge showed that the treatment plant was operated at ambient temperature and neutral pH. The Hazen colour reading of the sludge sample indicated that the sample was turbid and coloured. The settle sludge volume (SSV) and the sludge volume index (SVI) readings of the activated sludge indicated a healthy non-bulking sludge. The total viable count of 1.03 X 10^5 bacteria /g, (3.2 X 10^5/ml) was not however as high as might be expected. Shimizu and Odawara (1985) and Sayler *et al.*, (1985) detected counts ranging 10^6 - 10^9 cells/ ml from activated sludge and soil sediments exposed to synthetic oil. A significantly lower number of bacteria were capable of growth on the aromatic substrates compared to the total viable count. Similar results had been reported by Sayler *et al.*, (1985). They found that 98.6% of the ^{32}P-TOL$^+$ cells from a total viable count were incapable of growth on toluene vapour plates. Blackburn *et al.*, (1987) found that up to ten times the number of naphthalene degrading bacteria could be detected using a NAH probe compared to the enumeration of bacteria using vapour plates.

The lack of study connecting microbial structure and function is, at least in part, due to the limitations of traditional identification and enumeration techniques, such as selective enrichment, pure-culture isolation, most probable number estimates and determinative identification schemes. These limitations are further magnified in the analysis of these populations comprising the heterotrophic bacteria that are responsible for the catabolism of environmental contaminants (Malloru and Sayler, 1983). Although it is assumed that organisms capable of growth on agar medium containing an environmental pollutant as a sole carbon source must be capable of metabolism of that substrate, a number of potential factors affect the

reliability and utility of this approach. Firstly, the presence of dormant, non-culturable bacteria in populations is likely to distort results (Saunders,1990). Other factors include the temperature of incubation, pH of the medium, possible toxic constituents in the medium and most importantly the release and dispersion of the cells from the sludge flocs. While sonication followed by recovery on PCA has been used successfully to enumerate bacteria in activated sludge (Banks and Walker, 1977) more recently DNA-DNA hybridization techniques have shown very promising results which eliminated the need for selective cultivation. Nucleic acid hybridization techniques can be useful for studying community structure of natural microbial communities at the level of populations and even single cells containing specific DNA molecules (Pickup, 1991; Sayler and Layton, 1990). It should also be possible to track the survival and maintenance of specific native or recombinant DNA within indigenous natural populations.

The isolated bacteria were identified as belonging to the genera *Pseudomonas* and *Aeromonas*. The many biochemical properties of the ten *Pseudomonas* and five *Aeromonas* spp. were typical for their species as outlined in Bergeys Manual for Systematic Bacteriology (Palleroni, 1984; Popoff,1984). The organisms shared many similar characteristics. They were all non-spore forming, Gram negative rods, oxidase and catalase positive and were capable of utilizing a wide range of carbohydrates as the sole carbon source. All the organisms failed to produce H_2S, urease or to grow on 6% NaCl. However while *Pseudomonas* spp. are aerobic rods which metabolise glucose oxidatively, *Aeromonas* spp. are facultatively anaerobic rods and metabolize glucose fermentatively. All the *Aeromonas* spp. showed positive reactions for DNase, tween 80 hydrolysis and starch hydrolysis while all the *Pseudomonas* spp. showed negative reactions for these tests. All the *Aeromonas* spp. were resistant to the vibriostatic agent (2,4-Diamino 6,7-diisopropyl petridine) which is one of the distinguishing characteristics between *Vibrionaceae* and *Aeromonadaceae* (Bergey, 1984). *Pseudomonas* spp. were also resistant to this vibriostatic agent.

Pseudomonas spp. are well known for their metabolic versatility (Palleroni, 1986) and this was borne out by the ability of all the *Pseudomonas* spp. to use a large number of carbohydrates tested for growth. However while the *Pseudomonas* spp. used fifty-eight percent of the carbohydrates tested the *Aeromonas* spp. were able to use sixty-one percent suggesting that the *Aeromonas* spp. isolated were in fact as much if not more versatile than the *Pseudomonas* spp.

The optimum growth temperature for most organisms was between 25°C and 30°C but all were capable of growth in the range 20°C to 37°C. The isolated bacteria were capable of growth at ambient temperature and most waste treatment processes are operated at mesophyllic temperatures. Nozawa *et al.*, (1987) reported that the optimum growth temperature of nine isolates were also in the range 25°C to 30°C. These bacteria were isolated from activated sludge used in the treatment of industrial wastewater. *Aeromonas* sp. strain To-4 was capable of growth on a wider range of temperatures. Growth was observed at 4°C and this had also been reported by many investigators (Bergey's, 1984; Majeed *et al.*, 1990). Temperature is a physical factor which affects the interior of the cell. The mechanism of thermal adaptation must be different from the resistance to salinity, pH changes or chemicals which involves the barrier of the cell membrane. The ability of microorganisms to grow at low temperatures depends on adaptive changes (qualitative/or quantitative) in cellular protein and lipids (Gounot, 1991).

All the *Pseudomonas* spp. were resistant to chloramphenicol, novobiocin, and penicillin and sensitive to streptomycin, tetracycline, polymyxin. The resistance of the *Pseudomonas* spp. to penicillin is a typical feature among *Pseudomonas* spp. (Bergey, 1984). All the *Aeromonas* spp. were resistant to chloramphenicol, streptomycin and tetracycline and sensitive to novobiocin, penicillin and polymyxin. Connerton *et al.*, (1991) reported the resistance pattern of *Aeromonas* spp. to chloramphenicol, streptomycin and tetracycline isolated from faeces, food and water. Transferable drug resistance factors have been detected in *Aeromonas* spp. In motile *Aeromonas* species, the

presence of R factors has also been detected (Aoki and Egusa, 1971). The R factors in *Aeromonas* spp. have markers of resistance to sulfonamide, tetracycline, streptomycin and chloramphenicol (Bergey, 1984).

Many genera of bacteria have been identified in activated sludge samples and these tend to be dominated by *Pseudomonas* spp. (Baker *et al.*, 1983; Nomura *et al.*, 1989; Leonova and Karpukhin, 1974). As far as effective carbonaceous pollutant biodegradation in aerobic treatment processes is concerned, the predominant microbes are true bacteria of the family *Pseudomonadaceae* and the genera *Flavobacterium* and *Alcaligenes. Pseudomonas* spp. are able to use a wide diversity of carbon energy substrates, and this together with their ability to compete effectively with other bacteria, is undoubtedly responsible for their dominance (Hamer, 1985). This accounts for the fact that ten of the fifteen isolates were identified as *Pseudomonas* spp.

The association between *Pseudomonas* spp. and *Aeromonas* spp. was reported by Jimenez *et al.*, (1991). The sample which they took from the activated sludge of a municipal wastewater treatment plant was also dominated by *Pseudomonas* spp.. A total of four isolates were identified - three *Pseudomonas* spp. and one *Aeromonas* sp. capable of biodegradation of LAS (linear alkylbenzene sulfonate). *Pseudomonas* spp. were isolated from phenol contaminated wastewater capable of growth on phenol, benzoic acid and 4-hydroxybenzoic acid by Karzhenevich (1992) and Bettmann and Rehms (1984). They compared the degradation of these substrates by free and immobilized cells and found that the immobilized cells were able to degrade higher concentrations of phenol. Manukovoski *et al.*,(1991) investigated phenol and naphthalene degradation in coke industry wastewater. *Pseudomonas* and *Nocardia* spp. were isolated on selective media containing phenol, guiacol and naphthalene. Two *Pseudomonas* spp. were isolated from phenol production industry wastewater able to remove 80 -95% of phenol from the waste stream (Feodorov *et al.*, 1991).

Aeromonas spp. have been associated with pollutant degradation on many

occassions. They too have been isolated from activated sludge. Hu, (1992) reported the isolation of *Aeromonas* spp. from activated sludge of a textile wastewater treatment plant capable of growth on eleven reactive dyes and their colour removal efficiency was investigated. The removal of zinc by *Aeromonas* spp. isolated from an activated sludge process treating coal gasification effluent was reported by Kasan and Baecker in 1989. *Aeromonas* spp. resistant to high concentrations of methanol, butanol, glycol, cyclohexanone and cyclohexylamine were also isolated from petrochemical wastewater (Bieszkiewicz and Szymanska, 1987). *Aeromonas* spp. capable of degrading iron were isolated from iron-bearing industrial wastewater (Gopalan *et al.*, 1993), from crude oils in marine environments (Ajisebutu, 1988) from naphthalene in soil (Kiyohara *et al.*, 1992)) and petroleum hydrocarbons isolated from soil (Ghosh and Banerjee, 1983). In this study, five *Aeromonas* spp. were isolated and one strain To-4 was able to utilize toluene as the sole source of carbon and energy. An *Aeromonas* sp. capable of growth on toluene has not been reported elsewhere to date.

The ability of bacteria particularly those of the genus *Pseudomonas* to utilize aromatic hydrocarbons has been widely documented (Atlas, 1984; Gibson, 1984). The list of compounds that can be degraded by pseudomonads includes an increasing number of xenobiotic chemicals. All four *Pseudomonas* spp. (To-1, To-3, To-5, Na-13) and the *Aeromonas* sp. (To-4) were able to grow on a range of aromatic compounds. All five organisms showed good growth on toluene, benzoate and *m*-toluate. All the *Pseudomonas* spp. failed to grow on phenol, naphthalene and *o*-chlorophenol while the *Aeromonas* sp. (To-4) was able to utilize phenol, naphthalene, *p*- and *m*-chlorophenol but not *o*-chlorophenol. A *Pseudomonas* sp. JS150 capable of growth on benzene, toluene, ethylbenzene, naphthalene, phenol and *m*-toluate not chlorophenol or xylene was isolated by Haigler *et al.*, (1992). Hughes *et al.*, (1984) reported that *Alcaligens eutrophus* metabolized phenol, *m*- and *p*-toluate via the *meta* cleavage pathway and identified a TOL-like plasmid that encoded *m*- and *p*-toluate degradation. In another study Cruden *et al.*, (1992) reported that *P. putida* indaho was capable of growth on toluene, *m*- and *p*-xylene,

1,2,4-trimethylbenzene and 3-ethyltoluene and contained a TOL plasmid.

Growth on these substrates may be controlled by plasmids. When the degradative pathways of aromatic compounds, such as benzene, toluene, xylene and toluic acid, were elucidated, it was found that the genetic information necessary for their degradation was carried partly on a transmissible plasmid, known as the TOL plasmid and partly on the chromosome (Wheelis, 1975; Wlliams, 1981). The most widely studied of the plasmids involved in aromatic degradation are those that encode the degradation of toluene/xylene (TOL), naphthalene (NAH), and salicylate (SAL) (Gibson. 1984). The enzymes encoded by the TOL plasmids have a relaxed specificity which accounts for the observation that a single organism can grow with more than one aromatic substrate (Whitted *et al.*,1986). This might be why all five isolates were capable of growth on more than one aromatic substrate. At present, the best understood catabolic plasmid is the TOL plasmid, which encodes the enzymes that degrade toluene. The archetypal TOL plasmid, pWWO, was first described in 1974 by Williams and Murray. Besides toluene degradation, this plasmid has been shown to mediate the degradation of *m*- and *p*-toluate, *m*- and *p*-xylene (Worsey and Williams, 1975), and related compounds (Engesser *et al.*, 1988; Kunz and Chapman, 1981) to acetaldehyde and pyruvate through a bifurcating pathway. Many other TOL plasmids have been detected in other strains of *Pseudomonas*, most of which have similar biochemical pathways and regions of strong DNA homology to pWWO (Keil *et al.*, 1985; Keil *et al.*, 1985; Whitted *et al.*, 1986; Williams and Worsey 1976)). These plasmids are usually found in *Pseudomonas* species, although one TOL-like plasmid has been found in *Alcaligenes eutrophus* (Hughes *et al.*, 1984).

All the organisms were capable of growth on benzoate. Benzoate has been shown to support the growth of a variety of microorganisms (Reiner, 1971). The enzymes for benzoate degradation are chromosome encoded and are ubiquitous in *Pseudomonas* spp. (Harayama and Rekik, 1990). The pathway for benzoate metabolism is induced by benzoate itself (Cuskey and Sprenkle,

1988). Catechol, an intermediate in benzoate metabolism, is usually degraded via the *ortho* cleavage pathway (Williams and Murray, 1974). *m*-Toluate is an intermediate in the degradation of *m*-xylene by the TOL plasmid encoded pathway (Davey and Gibson, 1974). All the organisms were capable of growth on *m*-toluate suggesting the involvement of a TOL plasmid.

Some phenol-utilizing bacteria harbour TOL, NAH/SAL, or related plasmids. For example *Pseudomonas* sp. strain NCIB 9816 capable of growth on phenol carries a NAH plasmid pWWO601 (Cane and Williams, 1982; Feist and Hegeman , 1969), and *Alcaligens eutrophus* 345 capable of growth on phenol carries a TOL plasmid, pRA1000 (Hughes *et al.*, 1984). The introduction of the TOL plasmid pWWO into a 3-chlorobenzoate strain, *Pseudomonas* sp. strain B13, has been used to expand the substrate range of the host pathway for the catabolism of halogen-substituted phenolic compounds (Chaterjee and Chakrabarty, 1982). It has also been reported that the presence of the TOL plasmid pWWO facilitates the survival of phenol-utilizing bacteria at higher concentrations of phenol (Wong *et al.*, 1978). However, until now no metabolic plasmid has been reported which allows phenol degradation (Kivisaar *et al.*, 1989)

When the stability of the degradative ability of the isolates have been studied it was found that only the toluene grown organisms did not loose their ability to grow on toluene following maintainance on nutrient agar indicating the presence of a stable plasmid. In the case of phenol and naphathalene grown organisms most of the isolates lost their ability to grow on the aromatic substrates from which they were isolated, which might be due to the instability of the plasmids following growth on non-selective media (Clarke and Laverack, 1984).

The ability of the four *Pseudomonas* spp. (To-1, To-3, To-5, Na-13) and one *Aeromonas* sp. (To-4) to grow at the expense of a range of aromatic compounds was examined in the basal salts medium of Goulding *et al.*, (1988), which provided a source of nitrogen, phosphate and trace salts,

incorporating the aromatic compounds as the sole source of carbon for energy and growth. The solubility of aromatic compounds in the aqueous medium can be important for their metabolism. Phenol, benzoate and m-toluate dissolved readily in the medium. Naphthalene and *p*-, *m*- and *o*-chlorophenol did not dissolve completely. It was necessary to dissolve the chlorophenol in a minimum volume of ethanol. Toluene which has a solubility in water of 0.067% (w/w) at 23.5°C (Merck Index) was supplied in most cases as a vapour. A number of bacteria have been found to be capable of metabolizing aromatic compounds with low water solubilities. Various mechanisms have been suggested for this. Some bacteria may facilitate the uptake of poorly soluble compounds by producing emulsifiers or by the possession of a hydrophobic cell surface. It is also possible that the organisms grow only at the expense of the compound in solution and that the rate of dissolution of such compounds might govern the rate of biodegradation (Stucki and Alexander, 1987).

The growth of the four *Pseudomonas* spp.(To-1, To-3, To-5 Na-13) and one *Aeromonas* sp. (To-4) was investigated in the presence of toluene using different cultural conditions - solid culture, liquid culture, directly added toluene and toluene in the presence of other carbon sources. Many researchers have investigated the ability of bacteria, particularly Pseudomonads to degrade toluene (Claus and Walker, 1964; Gerben *et al.*, 1991 Pettigrew *et al.*, 1991).

The concentrations of toluene was different for different cultural conditions and dependent on the form of supply. In the case of solid culture the concentrations of toluene increased in the vapour phase with increasing volumes of toluene in water and the maximum concentration obtained was 13 g/l. In liquid culture the organisms were grown in both open and closed systems. A variety of open systems were used where toluene was contained to a greater or lesser degree. Up to 50% of toluene was lost from certain systems due to the volatile nature of the substrate. The concentration of toluene increased both in the gas and liquid phases of these systems with increasing volumes of toluene supplied. The concentration of toluene in the

gas phase was higher than in the liquid phase indicating that the availability of toluene in the biolayer (liquid phase) was mass transfer limited due to the low water solubility of toluene (Hartmans *et al.*, 1990). The maximum concentration of toluene detected in the liquid phase of these systems was 2 - 15 mg/l while in the gas phase it was 8 - 79 mg/l.

When toluene was added directly to the growth medium in a closed system the maximum concentration of toluene in the liquid phase in 50 ml minimal medium was found to be 20 mg/l. This was attained following the addition of 50 µl of toluene to the medium. At this point the minimal medium was saturated with toluene. The solubility of toluene in 50 ml water is 26 mg/l (515 mg/l) at 23°C (U.S. Public Health Service, 1989). The difference in solubility could be due to the temperature difference because the minimal medium was incubated at 30°C. So the maximum solubility of toluene at 30°C is 20 mg/l. Up to 100 µl, toluene was completely soluble and above this volume a biphasic-layer was formed. The concentrations of toluene in the gas phase increased with increasing volumes of toluene occurred in the case of solid culture.

Growth of all the organisms was also dependent on the manner in which toluene was supplied. A number of workers have grown bacteria in solid culture in the presence of toluene (Claus and walker, 1964; Finette *et al.*, 1984; Harayama *et al.*, 1986; Carney and Leary, 1989). Though the optimum growth of all the organisms in this study in solid culture was between 6.25 to 400 mg/l of toluene they were also capable of growth at higher concentrations of toluene (13 g/l) in this environment. Above 400 mg/l concentration of toluene growth started to decrease suggesting inhibition of growth at higher concentrations of toluene.

In liquid culture the growth rates were similar for all the organisms regardless of the toluene concentrations. Nor was there any significant difference between the growth rates obtained for To-4 and To-5 when grown under aerobic and oxygen limited conditions. The best growth rates were

obtained for *A. caviae* To-4 and *P. putida* To-5. Values of 0.16 h^{-1} and 0.15 h^{-1} were calculated when the organisms were grown in liquid culture with 2000 µl of toluene. These values compared favourably with other values obtained in the literature. Vecht *et al.*, (1988) found that the growth rate of *P. putida* (ATCC 33015) was 0.12 h^{-1} and Ottengraff *et al.*, (1984) calculated the growth rate of an unidentified isolate 0.025 h^{-1} from soil. Both of these organisms had been cultivated in a similar manner to strains To-4 and To-5 by growing them in shaken flasks with toluene supplied in the vapour phase.

When the bacteria were cultivated in liquid culture in an open system the maximum concentration of toluene in the liquid phase was 15 mg/l and in the gas phase 79mg/l. Thus these values were within the concentration range tested in solid culture. When toluene was added directly to the growth medium the tolerance of the organisms to the substrate was not so great. The organisms were able to grow only up to 20 mg/l in directly added toluene but in the case of solid culture growth was observed up to 400 mg/l of toluene. These results demonstrated that growth was better when toluene was supplied in the vapour phase as opposed to the liquid phase.

The level of biomass increased with increasing level of toluene supplied. However growth was clearly inhibited by oxygen limited in the more highly containment system. The utilization of the substrate was similar for strains To-4 and To-5. In open systems the utilization was calculated at 0.18 mg/l/hr and 0.17 mg/l/hr while in the more highly contained systems the values were 2.54 mg/l/hr and 2.34 mg/l/hr. The utilization of toluene by bacteria has been reported by a number of workers. Haighler *et al.*, (1992) reported the utilization of toluene by *Pseudomonas* sp. JS150 at 1.6 mg/l/hr which was comparable to these values obtained in this study. Alvarez *et al.*, (1991) reported the utilization of toluene at 2 mg/l/hr by *Pseudomonas* sp. strain CFS-215. Utilization of toluene in this study was more effective in more highly contained conditions. To optimize the removal capability of these

organisms, the use of contained systems with an adequate supply of oxygen should therefore be considered.

The pH profiles of the isolates during growth on toluene was monitored. The pH dropped as the toluene was metabolised. This drop was greater at higher concentrations of toluene. The nature of the activities of microorganisms is such that the pH of the environment of a metabolizing culture will not remain constant for long. Hydroxylated compounds might be expected in the early stages of toluene metabolism. The lowering of the pH values of growing cultures must be caused by acidic substances. These were detected by Claus and Walker (1964) as acetic and pyruvic acid when *Pseudomonas* and *Achromobacter* isolates were grown in medium where toluene was the only carbon source. When naphthalene was metabolised by *Pseudomonas* spp. the pH of the culture medium dropped and the drop in pH increased with increasing concentrations of naphthalene (Mulcahy, 1993).

Claus and Walker (1964) reported that the gradual acidification of a culture caused a decrease in the enzymatic activity of the organisms and also a slight loss of benzene or toluene from the aqueous solutions occurring through volatilization. The maintenance of a constant pH during growth of a culture is especially important for those organisms that produce acid but are not acid tolerant. The influence of pH on the growth of the organisms in the presence of toluene was investigated. Initially two buffers were selected - sodium phosphate buffer and Tris-maleate buffer to control the pH of the culture. With respect to growth and pH stability the sodium phosphate buffer was better than the Tris-maleate buffer. Sodium phosphate buffer was selected to investigate the influence of pH on growth in the presence of toluene. Phosphate buffers are widely used in the preparation of media because they are the only inorganic agents that buffer in the physiologicaly important range around neutrality and are relatively non-toxic to microorganisms. In addition they provide a source of phosphorus, which is essential for growth (Stainer *et al.*, 1988)

The growth of the organisms was different for different pH values. Strain To-1, To-3 and To-4 were able to grow in all pH values 5.8 to 8.0 while the strains To-5 and Na-13 were able to grow only up to pH 7.4. The duration of lag increased with increasing pH of the culture medium which is probably due to the time taken to adapt to a higher pH environment. The duration of lag also increased with increasing pH of the culture medium during phenol degradation by a *Pseudomonas* sp. reported by Bettman and Rehm (1984). The growth rate was maximum for all the organisms in buffered media between pH 6.6 and pH 7.4 suggesting that the organisms favoured neutral pH during growth in the presence of toluene. The maximum growth rates observed for a phenol degradation by a mixed culture (*Achromobacter, Aeromonas, Flavobacterium, Pseudomonas*) by Lallai *et al.*, (1987) were between pH 6.0 and pH 7.0.

A toxic effect was observed when more than 100 µl of toluene was added directly to the growth medium. The concentration of toluene detected in the liquid phase in this system was 20 mg/l and the concentration of toluene in the gas phase ranged from 4.5 mg/l to 36 mg/l. In all such systems the concentration of toluene detected in the liquid phase was 20 mg/l. However the organisms did remain viable in volumes of toluene greater than 100 µl. Thus the tolerance of strains To-4 and To-5 to toluene was not as great as that of a number of strains reported in the literature. *Pseudomonas* sp. strain T1 was able to grow in up to 276 mg/l of toluene (Evans *et al.*, 1991). A recently described, *P. putida* indaho was able to grow with toluene as the sole carbon and energy source when the solvent was provided at 5 to 50% (v/v) (43 to 430 g/l) in the culture medium. This organism was also able to survive in solid agar plates flooded with 5 ml of toluene. Cruden *et al.*, (1992) have explained that the resistance of organisms such as *P. putida* indaho to solvents may be due to an ability to synthesize membranes rapidly to compensate for those damaged by the solvent or to some biochemical difference in the cytoplasmic membrane which makes its more stable in the presence of solvent.

The toxic effect of toluene was alleviated by the addition of other carbon source. In the presence of other carbon sources such as glucose all the organisms were able to grow in more than 20% (v/v) of toluene when added directly to the growth medium. Comparing the growth with the control (organisms + PMM + glucose) it was observed that growth was higher in the presence of toluene suggesting that the growth occurred not only at the expense of glucose but also at the expense of toluene. Papanastasiou and Maier (1982) carried out a study on the degradation of 2,4-dichlorophenoxyacetate (2,4-D) in the presence of glucose. Glucose was found to improve 2,4-D metabolism due to a faster production of biomass.

Recent reports have shown that certain strains *P. putida* (Inoue and Horikoshi, 1989) and mutants of *P. putida* (Shima *et al.*, 1991) can grow in the presence of more than 50% (v/v) of toluene (added directly) when other carbon and energy sources are available for growth. In addition, a mutant strain *E. coli* K-12 has been isolated in complex media in the presence of 10% (v/v) *p*-xylene (Aono *et al.*, 1991). However while these organisms could grow in the presence of high concentration of toxic substrate none of these organisms could utilize the toxic substrate while all the organisms in this study were able to utilize toluene in the presence of glucose.

The toxic effect of the direct addition of toluene was further alleviated following acclimation of the cells to toluene. Results demonstrated that acclimation was an important step concerning substrate metabolism. The mineralization of many organic compounds that are introduced into treatment systems or into the natural environment is often proceeded by an acclimation period because the concentrations of many chemicals in industrial waste streams and waste disposal sites are probably sufficiently high to suppress the microorganisms having the capacity to metabolize those compounds. Lappin *et al.*, (1985) reported that the acclimation period for decomposition of mecoprop by a five-member microbial consortium became longer with increasing concentrations of this herbicide.

There are two main pathways for the degradation of aromatic substrates in *Pseudomonas* spp., the *meta* pathway and the *ortho* pathway. *Meta* cleavage activity is plasmid encoded and is known to be carried on a number of different catabolic plasmids (Harayama and Rekik, 1990). The chromosomal pathway is normally only used in the absence of the plasmid since its, introduction requires an accumulation of catechol which does not occur when the *meta* pathway is present (Williams and Worsey, 1976). The *meta* cleavage pathway is the most versatile and is found as the terminal metabolic sequence in the dissimilation of a wide range of polycyclic and substituted aromatics in many different genera (Finette *et al.*, 1984). In several strains of *Pseudomonas* spp. Williams and his colleagues have shown that toluene, and *m*- and *p*-xylene are degraded via the catechol *meta*-cleavage pathway, all the structural genes encoding degradative pathway are located on a plasmid which was designated as the TOL plasmid (1976).

Degradative plasmids tend to be large in size and closely associated with the chromosome, these factors make them difficult to isolate (Hansen and Olsen, 1978). The rapid isolation procedure of Birnboim and Doly (1979) and Eckhardt (1978) for the extraction of plasmid DNA were examined but no plasmids were visualized. The triton lytic procedures of Crosa and Falkow (1981) for the isolation of plasmids from Gram-negative organisms including *Pseudomonas* spp. was also investigated without success. A rapid method for the isolation of large, stable and unstable plasmids involved in the degradation of aromatic compounds in *Pseudomonas* spp. was also investigated (Wheatcroft and Williams, 1981). The main problem encountered with this technique was the handling of a highly viscous solution which resulted after cell lysis.

The procedure of Hansen and Olsen (1978) was designed for the isolation of large plasmids from *Pseudomonas* spp. It involved cell lysis with lysozyme and SDS followed by alkaline denaturation. This procedure was not successful initially with the main problem being a failure to reduce the pH following alkaline denaturation. Alkaline denaturation is an important step in the

isolation of plasmid DNA as it irreversibly denatures chromosomal DNA thus allowing plasmid DNA to be isolated. A number of modifications as outlined by Almond *et al.*, (1985) were incorporated. The chief modification was in the proportions of NaOH and Tris added during the alkaline denaturation step. The volume of Tris used in the modified method was eight times the volume of NaOH used compared to twice the volume as used in the original method. This successfully reduced the pH of the DNA solution. The isolation procedure was also simplified by eliminating the washing step which removed residual medium at the beginning of the procedure. The heating step was also changed to 55°C for 2 minutes compared to eight cycles of heat pulse at 55°C and mixing. A number of other slight modifications to the volumes of reagents added were also incorporated. This modified method proved to be very reliable and reproducible for the isolation of large plasmids from *Pseudomonas* spp.

Due to the growth characteristics of the organisms it was thought that they might carry a TOL plasmid. To investigate this further a strain carrying a TOL plasmid was obtained from the National Collection of Industrial Bacteria (NCIB), Aberdeen, U.K, for comparison. NCIB 10432 was *Pseudomonas putida* (mt-2, PaM1, PaW1), originally isolated from soil, carrying a transmissible TOL plasmid of approximately 117 kb in size. A chromosomal marker was obtained by curing *P. putida* NCIB 10432 of its TOL plasmid using mitomycin C (5 - 20 µg/ml) in a method developed for the curing of large degradative plasmids (Dunn and Gunsalus, 1973).

Toluene can serve as a growth substrate for different *Pseudomonas* species. *P. putida* is well known for its ability to utilize aromatic hydrocarbons such as toluene , xylene and methylbenzoates as the sole carbon source by the *meta* cleavage pathway which is mediated by the TOL plasmid (Harayama., *et al.*, 1986).

All the *Pseudomonas* spp. showed a dominance of *meta* activity regardless of the growth substrate. However the growth substrate was found to influence wheather the *meta* activity or the *ortho* activity dominated in the case of the

Aeromonas sp. This result suggested that the growth substrate might influence the plasmid profile of the *Aeromonas* sp. This was in fact found to be the case when the plasmid profiles of the organisms were examined.

All the organisms showed greater catechol 2,3-dioxygenase activity (*meta* cleavage enzyme) than the catechol 1,2-dioxygenase activity (*ortho* cleavage enzyme) to degrade toluene. This was as expected as the majority of aromatics including toluene are cleaved via the *meta* pathway (Williams and Murray, 1974). The levels of both the *ortho* (catechol 1,2-dioxygenase) and *meta* cleavage (catechol 2,3-dioxygenase) enzymes increased at higher volumes of toluene indicating the inducible nature of the enzyme activities (Claus and Walker, 1964). The *meta* and *ortho* cleavage activity of the strains To-4 were 1.37 and 0.0198 µ moles/min/mg of protein respectively and was higher than the *Pseudomonas* sp. strain JS150 (Haigler et al., 1992). The catechol 2,3-dioxygenase activity for the strain *Pseudomonas* sp. JS150 was 0.289 and catechol 1,2-dioxygenase was 0.009 µ moles/min/mg of protein.

The plasmid profiles of the five organisms following growth on the non-aromatic media - luria broth revealed the presence of a large plasmid band in all the *Pseudomonas* spp. (To-1, To-3, To-5 and Na-13) but not in the *Aeromonas* spp. (To-4). However when DNA isolated from strain To-4 grown on toluene was examined, a large plasmid band was visualized. Due to its large size the plasmid band was resolved near to the wells of the gel. The large plasmids visualized in all the organisms were of similar size range, approximately 85 - 120 kb. These results demonstrated that all the *Pseudomonas* spp. carried a large stable plasmid while the *Aeromonas* sp. carried a large unstable plasmid.

Most naturally occurring plasmids are stably maintained by the host, even during growth on non-selective media, that is in the absence of any selective pressure (Stephens and Dalton, 1988). However reports in the literature have indicated that some large degradative plasmids undergo modification, such as excision of segments of DNA or even complete loss of the plasmid, when

grown on benzoate or a non-selective substrate or other lyophilic weak acids (Carney and Leary, 1989). The studies by Carney and Leary (1989) also found that prolonged growth on these substrates resulted in the maintenance of the altered plasmid profile or a greater degree of alteration.

The stability of degradative plasmids is of particular importance for organisms that from part of a bioaugmentation product for use in the treatment of wastewater from the chemical industry where they may be exposed to a large number of compounds. The stable maintenance of catabolic plasmids provides a means by which gene pools are maintained and disseminated throughout the microbial population (Trevors *et al.*, 1989). The potential for genetic interaction in the environment is high if the organisms can survive the dynamic biological, physical and chemical factors that occur. Obviously both the survival and genetic stability of added organisms are important parameters (Trevors *et al.*, 1989).

To identify and compare the homology of the large plasmids of the organisms dot blot and Southern hybridization were carried out to the TOL probe of *P. putida* NCIB 10432.

The dot blot hybridization was carried out to identify the plasmids of all the organisms to the TOL probe of *P. putida* NCIB 10432 using the digoxigenin-labelled method. The TOL probe of *P. putida* 10432 hybridized to all the DNA from the *Pseudomonas* spp. regardless of the growth substrate. In the case of *A. caviae* To-4 the TOL probe hybridized only the DNA isolated from toluene culture. These results confirmed the presence of a stable TOL plasmid in all the *Pseudomonas* spp. and an unstable TOL plasmid in *A. caviae.*

Southern hybridization was carried out for the plasmids of strains To-4 and To-5. Difficulties can be encountered in transferring large DNA fragments to nitrocellulose (Maniatis *et al.*, 1982). Therefore, the plasmid DNA was first cut with restriction endonucleases. Due to the high salt concentration and the

presence of PEG in the DNA preparations from the Hansen and Olsen method (1978), samples were purified by cesium chloride gradient and dialysis. The DNA from *P. putida* NCIB 10432, *A. caviae* To-4 and *P. putida* To-5 was cut successfully with Hind III and Eco RI restriction enzymes.

The restriction analysis with Hind III and Eco RI was carried out to compare the homology between the TOL plasmid of *P. putida* and the plasmids of *A. caviae* To-4 and *P. putida* To-5. A number of restriction fragments ranging from 2 kb to 33 kb were visualized from all three plasmids. The presence of fragments of the same size in both of the strains To-4 and To-5 indicated the presence of an identical plasmid in both of the strains. However compared with the TOL plasmid of *P. putida* NCIB only three fragments of the Hind III digests and five fragments of the Eco RI digests of To-4 and To-5 were of similar size. Several TOL plasmids have been shown to contain significant homology to the archetype TOL plasmid pWWO (Harayama *et al.*, 1986) but for the plasmids of strain To-4 and To-5 this was not the case.

To confirm the presence of any specific fragments of the TOL plasmid *P. putida* 10432 in the plasmids of To-4 and To-5 Southern hybridizations were carried out using digoxigenin-labelled and radioactively labelled methods. The radioactively labelled hybridization was more sensitive than the digoxigenin -labelled hybridization method. Only one or two of the hybridized bands were found in the case of the dioxigenin-labelled method. While in the radioactively labelled method four of the Hind III and seven of the Eco RI fragments of To-4 and To-5 hybridized. In both of the methods the same and similar size fragments of both of the strains hybridized to the TOL probe of *P. putida* NCIB proving the presence of an identical plasmid in both of these species. The radioactively labelled method is the most widely used and sensitive method for the hybridization of specific DNA sequences while the digoxigenin-labelled method is suitable for the hybridization of dot and colony blot.

Among the four Hind III hybridized bands of both of the strains to the TOL

probe three hybridized strongly and one weakly. The strongly hybridized bands included bands of 19 kb and 6.1 kb. These fragments are known to contain the *xyl* C and the *xyl* G gene of the pWWO plasmid. The *xyl* C gene is responsible for the benzaldehyde dehydrogenase (BZDH) enzyme of the upper pathway and the *xyl* G gene is responsible for the enzyme 2-hydroxymuconic semialdehyde dehydrogenase (HMSD) of the lower pathway of the TOL (pWWO) degradative plasmid. Two other Hind III hybridized fragments detected were of comparable size to those identified by Downing and Broda (1979) in *P. putida* mt-2 but their function was not specified.

The TOL plasmid of *P. putida* 10432 hybridized eight Eco RI fragments of both of the strains To-4 and To-5. Among the eight bands three hybridized strongly and five weakly. Of the eight hybridized bands the 24.5 kb, 16 kb and 13 Kb hybridized fragments are known to contain the *xyl* A, *xyl* B and *xyl* E genes respectively. The *xyl* A and *xyl* B genes are known to responsible for the enzymes toluene oxygenase (TO) and benzyl alcohol dehydrogenase (BADH) of the upper pathway of the TOL degradative plasmid. The *xyl* E is gene known to contain the enzyme catechol 2,3-dioxygenase (C2,3O) of the lower pathway of the TOL (pWWO) degradative pathway. The other Eco RI hybridized fragments were also detected in the *P. putida* mt-2. Eco RI fragments (Burlage *et al.*, 1989).

So it was confirmed that both of the strains To-4 and To-5 carried an identical plasmid though they were different species. Both of the plasmids contained some of the specific sequences of the TOL plasmid of *P. putida* NCIB 10432.

Several degradative plamids encoding genes for utilization of a variety of aromatic compounds are generally present in *Pseudomonas* spp. The degradative plasmids are generally functionally transmissible only between bacteria of the genus *Pseudomonas* and gene expression appears to be limited to *Pseudomonas* and related species (Chaterjee and Chaterjee, 1987). However exceptions have been noted. A plasmid has been found in *Alcaligens*

eutrophus and has the genetic potential to degrade *p*-cresol via a chromosomally encoded *meta*-pathway that is regulated by a TOL-like plasmid (Hughes *et al.*, 1984). In another study, the TOL plasmid of *P. putida* was transferred to *Caulobacter crescentus* and the results clearly suggested that not only could *C. crescentus* accept and maintain the RP4-TOL plasmid, but it could efficiently express the TOL gene. The recipients were able to grow on various TOL-specified aromatic compounds which were normally not substrates that supported growth. From these observations it can be concluded that it could be possible for *Aeromonas caviae* to carry a TOL-like plasmid. The identical characteristics of the plasmid in *Aeromonas* sp. suggested possible transfer from *Pseudomonas* sp.

The archetypal pWWO plasmids (TOL) (Williams and Worsey, 1976), are isofunctionally identical but they differ in several respects including transmissibility (Friello *et al.*, 1976; Williams and Worsey, 1976), the ability to form spontaneous deletion mutants in cells growing on benzoate (Williams and Worsey, 1976; Worsey and Williams, 1977), the capacity to facilitate the rapid degradation of the *p*-methyl-substituted compounds (Williams and Worsey, 1976), and molecular structure as evidenced by molecular weights and fragmentation patterns of restriction endonuclease digests (Duggleby *et al.*, 1977). Though the TOL plasmids identified in the strains To-4 and To-5 was not identical to that of the TOL plasmid of *P. putida* NCIB 10432, they were similar to it.

5. CONCLUSIONS

The main conclusions drawn from this work are :

(i) Two genera of bacteria the genus *Pseudomonas* and the genus *Aeromonas* were isolated from activated sludge and were capable of growth on aromatic substrates including toluene. An *Aeromonas* sp. capable of growth on toluene had not previously been reported.

(ii) The growth of the isolates in the presence of toluene depended on the manner in which toluene was supplied. In general growth was better when toluene was supplied in the vapour phase.

(iii) The addition of toluene directly to the growth medium was found to be toxic to the organisms but the toxic effect could be alleviated in the presence of other carbon sources and by acclimation of the cells.

(iv) All the bacteria contained a TOL plasmid. The *Pseudomonas* spp. carried a large stable TOL plasmid and while the *Aeromonas* sp. carried an unstable TOL plasmid.

(v) The TOL plasmid of *P. putida* To-5 and *A. caviae* To-4 contained some of the sequences of that of the TOL plasmid of *P. putida* NCIB 10432 but was not identical to it. However the TOL plasmids of To-4 and To-5 were identical.

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7. APPENDICES

APPENDIX 1

THE RESULTS OF ANALYSIS OF VARIANCE (ANOVA) FOR SIGNIFICANCE TESTING FOR THE GROWTH RATES IN DIFFERENT VOLUMES OF TOLUENE

Table 1.1: Test of significance for the growth rates of *P. putida* To-1 in the presence of 250 - 2000 µl toluene

Source	Degree of Freedom(DF)	Sum of Square(SS)	Mean Square(MS)	$F_{rat(cal)}$	$F_{rat(tab)}$	Remarks
Treatment	3	0.000550	0.000183	0.52	6.59*	Insignificant
Error	4	0.001400	0.000350			
Total	7	0.001930				

* - 95% confidence limit for $F_{3,4}$

Table 1.2: Test of significance for the growth rates of *P. putida* To-3 in the presence of 250 - 2000 µl toluene

Source	Degree of Freedom(DF)	Sum of Square(SS)	Mean Square(MS)	$F_{rat(cal)}$	$F_{rat(tab)}$	Remarks
Treatment	3	0.001000	0.000333	1.67	6.59*	Insignificant
Error	4	0.000800	0.000200			
Total	7	0.001800				

* - 95% confidence limit for $F_{3,4}$

Table 1.3: Test of significance for the growth rates of *A. caviae* To-4 in the presence of 250 - 2000 µl toluene

Source	Degree of Freedom(DF)	Sum of Square(SS)	Mean Square(MS)	$F_{rat(cal)}$	$F_{rat(tab)}$	Remarks
Treatment	3	0.001600	0.000333	1.07	6.59*	Insignificant
Error	4	0.002000	0.000500			
Total	7	0.003600				

* - 95% confidence limit for $F_{3,4}$

Table 1.4: Test of significance for the growth rates of *P. putida* To-5 in the presence of 250 - 2000 µl toluene

Source	Degree of Freedom(DF)	Sum of Square(SS)	Mean Square(MS)	$F_{rat(cal)}$	$F_{rat(tab)}$	Remarks
Treatment	3	0.000930	0.000317	1.38	6.59*	Insignificant
Error	4	0.000800	0.000200			
Total	7	0.001750				

* - 95% confidence limit for $F_{3,4}$

Table 1.5: Test of significance for the growth rates of *P. putida* Na-13 in the presence of 250 - 2000 µl toluene

Source	Degree of Freedom(DF)	Sum of Square(SS)	Mean Square(MS)	$F_{rat(cal)}$	$F_{rat(tab)}$	Remarks
Treatment	3	0.001200	0.000400	2.00	6.59*	Insignificant
Error	4	0.000800	0.000200			
Total	7	0.002000				

* - 95% confidence limit for $F_{3,4}$

APPENDIX 2

THE RESULTS OF ANALYSIS OF VARIANCE (ANOVA) FOR SIGNIFICANCE TESTING FOR THE GROWTH RATES AMONG THE ORGANISMS

Table 2.1: Test of significance for the growth rates at 250 µl toluene among the strains To-1, To-3, To-4, To-5 and Na-13

Source	Degree of Freedom(DF)	Sum of Square(SS)	Mean Square(MS)	$F_{rat(cal)}$	$F_{rat(tab)}$	Remarks
Treatment	4	0.000640	0.000160	0.80	5.19*	Insignificant
Error	5	0.001000	0.000200			
Total	9	0.001640				

* - 95% confidence limit for $F_{4,5}$

Table 2.2: Test of significance for the growth rates at 500 µl toluene among the strains To-1, To-3, To-4, To-5 and Na-13

Source	Degree of Freedom(DF)	Sum of Square(SS)	Mean Square(MS)	$F_{rat(cal)}$	$F_{rat(tab)}$	Remarks
Treatment	4	0.001440	0.000360	1.12	5.19*	Insignificant
Error	5	0.001600	0.000320			
Total	9	0.003040				

* - 95% confidence limit for $F_{4,5}$

Table 2.3: Test of significance for the growth rates at 1000 µl toluene among the strains To-1, To-3, To-4, To-5 and Na-13

Source	Degree of Freedom(DF)	Sum of Square(SS)	Mean Square(MS)	$F_{rat(cal)}$	$F_{rat(tab)}$	Remarks
Treatment	4	0.000240	0.000060	0.19	5.19*	Insignificant
Error	5	0.001600	0.000320			
Total	9	0.001840				

* - 95% confidence limit for $F_{4,5}$

Table 2.4: Test of significance for the growth rates at 2000 µl toluene among the strains To-1, To-3, To-4 To-5 and Na-13

Source	Degree of Freedom(DF)	Sum of Square(SS)	Mean Square(MS)	$F_{rat(cal)}$	$F_{rat(tab)}$	Remarks
Treatment	4	0.000550	0.000183	0.52	5.19*	Insignificant
Error	5	0.001400	0.000350			
Total	9	0.001950				

* - 95% confidence limit for $F_{4,5}$

APPENDIX 3

THE RESULTS OF ANALYSIS OF VARIANCE (ANOVA) FOR SIGNIFICANCE TESTING FOR THE GROWTH RATES IN SYSTEM A AND SYSTEM B.

Table 3.1: Test of significance for the growth rates of *A. caviae* To-4 in system A in the presence of 250 - 2000 µl toluene

Source	Degree of Freedom(DF)	Sum of Square(SS)	Mean Square(MS)	$F_{rat(cal)}$	$F_{rat(tab)}$	Remarks
Treatment	3	0.0004135	0.0001378	4.00	6.59*	Insignificant
Error	4	0.0001380	0.0000345			
Total	7	0.0005515				

* - 95% confidence limit for $F_{3,4}$

Table 3.2: Test of significance for the growth rates of A. caviae To-4 in system B in the presence of 250 - 2000 µl toluene

Source	Degree of Freedom(DF)	Sum of Square(SS)	Mean Square(MS)	$F_{rat(cal)}$	$F_{rat(tab)}$	Remarks
Treatment	3	0.0001224	0.0000408	1.31	6.59*	Insignificant
Error	4	0.0000195	0.0000345			
Total	7	0.0001319				

* - 95% confidence limit for $F_{3,4}$

Table 3.3: Test of significance for the growth rates of *P. putida* To-5 in system A in the presence of 250 - 2000 µl toluene

Source	Degree of Freedom(DF)	Sum of Square(SS)	Mean Square(MS)	$F_{rat(cal)}$	$F_{rat(tab)}$	Remarks
Treatment	3	0.0002433	0.0000818	3.15	6.59*	Insignificant
Error	4	0.0001040	0.0000260			
Total	7	0.003495				

* - 95% confidence limit for $F_{3.4}$

Table 3.4: Test of significance for the growth rates of *P. putida* To-5 in system B in the presence of 250 - 2000 µl toluene

Source	Degree of Freedom(DF)	Sum of Square(SS)	Mean Square(MS)	$F_{rat(cal)}$	$F_{rat(tab)}$	Remarks
Treatment	3	0.0000093	0.0000032	0.90	6.59*	Insignificant
Error	4	0.0000140	0.0000035			
Total	7	0.0000235				

* - 95% confidence limit for $F_{3.4}$

Table 3.5: Test of significance for the growth rates of A. caviae To-4 in system A and B

Source	Degree of Freedom(DF)	Sum of Square(SS)	Mean Square(MS)	$F_{rat(cal)}$	$F_{rat(tab)}$	Remarks
Treatment	3	0.000166	0.000055	0.30	6.59*	Insignificant
Error	4	0.000743	0.000186			
Total	7	0.000909				

* - 95% confidence limit for $F_{3,4}$

Table 3.6: Test of significance for the growth rates of *P. putida* To-5 in system A and B

Source	Degree of Freedom(DF)	Sum of Square(SS)	Mean Square(MS)	$F_{rat(cal)}$	$F_{rat(tab)}$	Remarks
Treatment	3	0.000081	0.000027	0.22	6.59*	Insignificant
Error	4	0.000497	0.000124			
Total	7	0.000578				

* - 95% confidence limit for $F_{3,4}$

Table 3.7: Test of significance of the growth rates of *A. caviae* To-4 and *P. putida* To-5 in system A

Source	Degree of Freedom(DF)	Sum of Square(SS)	Mean Square(MS)	$F_{rat(cal)}$	$F_{rat(tab)}$	Remarks
Treatment	3	0.0003204	0.0001068	6.05	6.59*	Insignificant
Error	4	0.0000505	0.000126			
Total	7	0.0003709				

* - 95% confidence limit for $F_{3,4}$

Table 3.8: Test of significance of the growth rates of *A. caviae* To-4 and *P. putida* To-5 in system B

Source	Degree of Freedom(DF)	Sum of Square(SS)	Mean Square(MS)	$F_{rat(cal)}$	$F_{rat(tab)}$	Remarks
Treatment	3	0.000049	0.000163	2.42	6.59*	Insignificant
Error	4	0.000027	0.000068			
Total	7	0.000760				

* - 95% confidence limit for $F_{3,4}$

—— X ——