

A Study of the Growth of *Pseudomonas putida* CP1 on Mono-chlorophenols.

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by

A.N.M. Fakhruddin, B.Sc.(Honours), M.Sc.
School of Biotechnology,
Dublin City University,
Dublin 9,
Ireland.

Research Supervisor: Dr. Bríd Quilty

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: A.N.M. Fakhruddin

ID No.: 98970496

Date: 07/08/03

Dedicated to
the Memory of my Father

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Abstract

Pseudomonas putida CP1 grew on all three mono-chlorophenol isomers when supplied as the sole source of carbon and energy. The biodegradability of the mono-chlorophenols followed the order: 4-chlorophenol > 2-chlorophenol > 3-chlorophenol. *P. putida* CP1 was able to degrade 300 ppm 4-chlorophenol, 250 ppm 2-chlorophenol and 200 ppm of 3-chlorophenol. In the presence of fructose (1%, w/v) the organism could degrade 400 ppm 4-chlorophenol, 500 ppm 2-chlorophenol and 300 ppm 3-chlorophenol.

Chlorophenol removal was stimulated in the presence of low concentrations of glucose (0.05% - 0.5%, w/v). Substrate removal was inhibited and there was a significant fall in pH with concentrations of glucose greater than 1.0% (w/v). When the pH was controlled at pH 7.0 inhibition of substrate removal was alleviated. The rate of removal of mono-chlorophenols was greater in the presence of fructose than in the presence of glucose, yeast extract or a combination of fructose and yeast extract.

P. putida CP1 formed clumps of cells when grown on all three mono-chlorophenol isomers and fructose but not when grown on glucose, yeast extract or phenol. When the organism was grown on a combination of chlorophenols and an additional carbon source clumping was present but to a lesser degree. Monitoring growth of the organism by a direct microscopic count technique was found to be more representative than other methods including optical density measurements, dry weight measurements and the plate count technique.

A change in shape of the bacterium from rod shape to a coccus shape coupled with a reduction in cell size was noted when the organism was grown under nutritional stress. Isomerization of *cis* to *trans* forms of the unsaturated fatty acids in *P. putida* CP1 occurred under conditions of environmental stress. Trace amounts of the polyunsaturated fatty acid linoleic acid (*cis*-9, *cis*-12-octadecadienoic acid) rarely found in bacterial membranes, was detected in the membrane of *P. putida* CP1.

Abbreviations

12:0	Dodecanoic acid (lauric acid)
14:0	Tetradecanoic acid (myristic acid)
14:1c	<i>cis</i> -9-tetradecanoic acid (myristoleic acid)
16:0	Hexadecanoic acid (palmitic acid)
16:1c	<i>cis</i> -9-hexadecanoic acid (palmitoleic acid)
16:1t	<i>trans</i> -9-hexadecanoic acid (palmitelaidic acid)
17:0cyc	<i>cis</i> -9,10-methylenehexadecanoic acid
18:0	Octadecanoic acid (stearic acid)
18:1co	Oleic acid (<i>cis</i> -9-octadecenoic acid)
18:1cv	<i>cis</i> -vaccenic acid (<i>cis</i> -11-octadecenoic acid)
18:1tv	<i>trans</i> -vaccenic acid (<i>trans</i> -11-octadecenoic acid)
18:2cc	Linoleic acid (<i>cis</i> -9, <i>cis</i> -12, octadecadienoic acid)
19:0cyc	<i>cis</i> -11,12-methyleneoctadecanoic acid
22:0	Docosanoic acid (behenic acid)
2-cp	2-chlorophenol
3-cp	3-chlorophenol
3-OH 10:0	3-hydroxy decanoic acid (β -hydroxycapric acid)
3-OH 12:0	3-hydroxy dodecanoic acid (β -hydroxylauric acid)
4-cp	4-chlorophenol
AO	Acridine orange
APHA	American Public Health Association
ATCC	American Type Culture Collection
cfu	Colony forming unit
COD	Chemical oxygen demand
DAPI	4,6-diamidino-2-phenylindole
DEFT	Direct epifluorescence filtration technique
DNS	3,5-dinitrosalicylic acid
EDP	Entner-Doudoroff pathway
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immune sorbent assay
EMP	Embden-Mayerhoff-Parnas pathway

EPA	Environmental protection agency
FAME	Fatty acid methyl ester
FAS	Ferrous ammonium sulphate
G6PDH	Glucose-6-phosphate dehydrogenase
GC	Gas chromatography
g l ⁻¹	Gram per litre
h	Hour
K _s	Monod constant
LD ₅₀	Lethal dose, that causes 50% growth inhibition
Log P _{ow}	Logarithm of octanol/water partition coefficient
M	Molar
mg l ⁻¹ h ⁻¹	Milligram per litre per hour
mg O ₂ l ⁻¹ h ⁻¹	Milligram oxygen per litre per hour
ml	Millilitre
μl	Micro litre
mM	Milli molar
μ _{max}	Maximum growth rate
mmol l ⁻¹	Milli molar per litre
MUSFA	Monounsaturated fatty acid
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
OD	Optical density
PEP	Phosphoenol pyruvate
pl	Phospholipid
ppm	Parts per million
(S)	Substrate concentration
SFA	Saturated fatty acid
SPB	Sodium phosphate buffer
SPC	Standard plate count technique
TCA	Tricarboxylic acid
Tris	Tris-(hydroxy-methyl)-methylamine(2-amino hydroxymethyl) propane-1,3-diol
W	Watt

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Chapter 1

Introduction

1. Introduction

1.1 Xenobiotics

Xenobiotics are chemicals having structural moieties or groups, which are not found in natural products. They are foreign to the biosphere. Thus xenobiotic compounds are substances which are normally absent from the ecosphere and are introduced by man, often by industrial processes involving synthetic chemicals. According to the figures currently available, more than 400 million tonnes of chemicals are now being produced annually throughout the world. Among the millions of known chemical substances, around 100,000 varieties are available on the market. Some ten thousand of these substances are hazardous, including about 200 to 300 carcinogenic agents. In addition, thousands of new chemicals are produced and utilized every year (Luong, 1997). Many xenobiotic compounds appear to be perfectly harmless after entering the biosphere, but others are quite the reverse (Hutzinger and Veerkamp, 1981). Portions of these chemical products enter the environment and become pollutants where they exert undesirable toxic effects. The type and magnitude of the undesirable toxic effects depends chiefly on the intrinsic toxicity of the compound on the one hand and on the available concentration on the other. The chemicals, depending on their fate in air, water, soil or sediment, may thus become available in different concentrations to the organisms in different environmental sub-compartments (Paxeus *et al.*, 1992).

Microorganisms play a key role in the biogeochemical cycles that occur in the environment. In these cycles, molecules of biological origin are being recycled by the respiratory process of microorganisms. Microorganisms also play an important part in the degradation of many man-made molecules. Some xenobiotic molecules, however appear to be resistant to microbial attack (Hutzinger and Veerkamp, 1981). Of particular interest are xenobiotic compounds, which have unnatural structural features. For many environmental scientists halogenated, particularly polychlorinated organic chemicals, are the most readily recognizable xenobiotic compounds. Yet several hundred naturally occurring organic chlorine compounds are known. During

the last few years it has become evident that even some chemicals which are considered typical industrial organochlorine compounds also have significant natural sources. Examples are carbon tetrachloride, methyl chloride, chloroform and bromoform. A natural process can even form typical persistent chlorinated aromatic compounds. Some synthetic compounds such as certain sugars and amino acids, antimetabolites, nucleic acid derivatives and analogues which resemble natural products closely, are truly xenobiotic (Tate and Heiny, 1996).

The entry of xenobiotic chemicals into the environment depends on the production methods, shipment, use patterns and ultimate disposal of industrial compounds. The use pattern, in particular, often determines the total amount of compound released into the environment. Pesticides are usually directly and deliberately applied in the environment whereas some industrial chemicals are locked for tens of years in closed systems. Most xenobiotic compounds are intermediate in this respect: solvents are regenerated, yet evaporate; hydraulic systems leak, plasticizers, flame retardants and other additives leach out and surface coatings are exposed to the environment (Hutzinger and Veerkamp, 1981).

1.1.1 Relationship between structure, toxicity and biodegradation of xenobiotics

The closer the structure of a contaminant compound is to the structure of a natural compound, the easier it will be to degrade by the organisms capable of degrading the natural compounds. If the contaminants are added in excess of the population's ability to deal with it, it will not be degraded completely even though a capable group of organisms is present (Knackmuss, 1996). Microbial activity against a compound depends on several physical and chemical properties of the contaminant. These include:

Genetic potential: Genetic potential is the presence or expression of genes capable of the degradation of the compound. The more a contaminant of concern resembles a

natural compound, the more likely there will be an enzyme that can degrade it. If the compound does not have a natural analog, it may take a genetic change to degrade it. That is, there will be development of new genes. This may take a long time (Davies, 1994; Tsuda *et al.*, 1999; Top *et al.*, 2000; Top *et al.*, 2002).

Bioavailability of the compound: Compounds that are not in the water phase are not viewed as being bioavailable and are degraded only slowly. This includes compounds that are present as a separate phase themselves, and those that are sorbed onto a separate phase (Timmis and Pieper, 1999). Organisms can change the bioavailability of the compounds by producing surfactants (Ron and Rosenberg, 2002; Cameotra and Bollag, 2003).

Contaminant structure: Both steric and electronic effects will determine the degradability of a compound. Steric effects include whether the structure of a molecule will fit inside an enzyme active site and whether the bonds that must be broken are sterically accessible. The electronic effects include whether substituent groups on the compounds effect the interaction of the enzyme and the compound as well as in some cases directing the conditions under which the compound can be degraded (Dorn and Knackmuss, 1978; Guanghua *et al.*, 2001).

Toxicity: If the compound has specific toxic properties, it can slowly or completely prevent the degradation process. For nonionic contaminants the toxicity is usually due to partitioning of the chemical into the membrane and disruption of the membrane. There are also numerous direct inhibitions of specific enzymes or respiratory proteins that organic pollutants are responsible for (Timmis and Pieper, 1999).

1.1.2 Xenobiotic compounds and pollution

The pollution potential of xenobiotic compounds has increased for a number of reasons.

1. An increase in both population density and industrial activity has led to a greater release of xenobiotic compounds into the environment.

2. Laboratory studies and analysis of environmental samples have shown several xenobiotic compounds to be persistent. They do not just disappear by natural mechanisms. They remain in the environment and thus dilution is no solution for pollution.
3. Certain lipophilic xenobiotic compounds accumulate (concentrate) in biological organisms, some of them reaching very high and dangerous concentrations (Hutzinger and Veerkamp, 1981).

Thus a xenobiotic may be difficult to handle by nature. Such a compound becomes a pollutant if it has undesirable toxic effects, which are compounded if the chemical is persistent, and bioaccumulates. The majority of potentially toxic known chemicals in the environment, however are xenobiotic compounds. Such chemicals become environmental pollutants when they have an effect on the environment or on man via the environment. The effect on the environment can be on organisms in the environment or on non-living entities. In most cases the undesirable effect is a toxic effect, that is detrimental to life processes (Neilson *et al.*, 1985).

1.1.3 Xenobiotic compounds: effects and toxicity

Xenobiotic compounds can be considered pollutants only if they have undesirable effects. Many different effects of chemicals on man and his environment have been recognized. Effects on the non-living environment include damage to structures by acidic air pollutants, effect of fluorocarbons and nitrogen oxides on the ozone layer, effect of carbon dioxide, aerosols and some trace gases on the earth's heat balance (e.g. greenhouse effect) and reduced visibility. These effects have a secondary biological effect on living organisms (Hutzinger and Veerkamp, 1981).

Health effects on man have always been the most-studied undesirable property of chemicals. Some halogenated organics are potentially hazardous to public health. They are among the Environmental Protection Agency (EPA) in the United States list

of 129 water-related priority organic pollutants (EPA-US, 2002). The pollutants are described under three headings in Table 1.

Special emphasis in recent years has been on the long-term effect from exposure to small concentrations of chemicals, e.g. carcinogenicity, mutagenicity, and teratogenicity. The effect on the nervous system as well as on reproductive capacity is also being studied. Examples of effects on man which are mainly based on psychological or psycho-physiological reactions are e.g. the taste of drinking water, the smell of air, and the general dirtiness of the environment.

Table 1: List of priority organic pollutants (EPA-US, 2002)

Base Neutrals and Acids:

1,2,4-Trichlorobenzene	2-Nitrophenol	Benzo(b)fluoranthene	Di-n-Butylphthalate
1,2-Dichlorobenzene	3&4-Methylphenol	Benzo(g,h,i)perylene	Di-n-Octylphthalate
1,3-Dichlorobenzene	3,3'-Dichlorobenzidine	Benzo(k)fluoranthene	Fluoranthene
2,4,5-Trichlorophenol	3-Nitroaniline	Benzoic Acid	Fluorene
2,4,6-Tribromophenol	4,6-Dinitro-2-Methylphenol	Benzyl Alcohol	Hexachlorobenzene
2,4,6-Trichlorophenol	4-Bromophenyl-Phenylether	Bis (2-Chloroethoxy) Methane	Hexachlorobutadiene
2,4-Dichlorophenol	4-Chloro-3-Methylphenol	Bis (2-chloroisopropyl) ether	Hexachlorocyclopentadi-ene
2,4-Dimethylphenol	4-Chloroaniline	Bis(2-Chloroethyl) Ether	Hexachloroethane
2,4-Dinitrophenol	4-Chlorophenyl methylsulfone	Bis(2-Ethylhexyl)Phthalate	Indeno(1,2,3-cd)pyrene
2,4-Dinitrotoluene	4-Chlorophenyl-Phenylether	Butylbenzylphthalate	Isophorone
2,6-Dinitrotoluene	4-Nitroaniline	Chrysene	Naphthalene
2-Chloronaphthalene	4-Nitrophenol	D14-Terphenyl	Nitrobenzene
2-Chlorophenol	Acenaphthene	D5-Nitrobenzene	N-Nitroso-Di-N-Propylamine
2-Fluorobiphenyl	Acenaphthylene	D6-Phenol	N-Nitrosodiphenylamine
2-Fluorophenol	Anthracene	Dibenzo(a,h)anthracene	Pentachlorophenol
2-Methylnaphthalene	Benzidine	Dibenzofuran	Phenanthrene
2-Methylphenol	Benzo(a)anthracene	Diethylphthalate	Phenol
2-Nitroaniline	Benzo(a)pyrene	Dimethylphthalate	Pyrene

PCBs (Organochlorine):

Aroclor-1016	Aroclor-1242	Aroclor-1254	Decachlorobiphenyl
Aroclor-1221	Aroclor-1248	Aroclor-1260	Tetrachlorometaxylene
Aroclor-1232			

Volatile Organic Compounds (VOCs):

1,1,1,2-Tetrachloroethane	2-Hexanone	Hexachlorobutadiene
1,1,1-Trichloroethane	4-Chlorotoluene	Iodomethane
1,1,2,2-Tetrachloroethane	4-Isopropyltoluene	Isopropylbenzene
1,1,2-Trichloroethane	4-Methyl-2-Pentanone	m&p Xylenes
1,1-Dichloroethane	Acetone	Methylene Chloride
1,1-Dichloroethene	Acrylonitrile	Naphthalene
1,1-Dichloropropene	Benzene	n-Butylbenzene
1,2,3-Trichlorobenzene	Bromobenzene	n-Propylbenzene
1,2,3-Trichloropropane	Bromochloromethane	O-Xylene
1,2,4-Trichlorobenzene	Bromodichloromethane	p-Bromofluorobenzene
1,2,4-Trimethylbenzene	Bromoform	sec-Butylbenzene
1,2-Dibromo-3-Chloropropane	Bromomethane	Styrene
1,2-Dibromoethane	Carbon Disulfide	tert-Butyl Methyl Ether
1,2-Dichlorobenzene	Carbon Tetrachloride	tert-Butylbenzene
1,2-Dichloroethane	Chlorobenzene	Tetrachloroethene
1,2-Dichloroethane-d4	Chloroethane	Toluene
1,2-Dichloropropane	Chloroform	Toluene D-8
1,3,5-Trimethylbenzene	Chloromethane	trans-1,2-Dichloroethene
1,3-Dichlorobenzene	Cis-1,2-Dichloroethene	trans-1,3-Dichloropropene
1,3-Dichloropropane	Cis-1,3-Dichloropropene	trans-1,4-Dichloro-2 Buten
1,4-Dichlorobenzene	Dibromochloromethane	Trichloroethene
2,2-Dichloropropane	Dibromofluoromethane	Trichlorofluoromethane
2-Butanone	Dibromomethane	Trichlorotrifluoroethane
2-Chloroethyl Vinyl Ether	Dichlorodifluoromethane	Vinyl Acetate
2-Chlorotoluene	Ethylbenzene	Vinyl Chloride

1.2 Biodegradation

Biodegradation is the microbiologically-mediated process of chemical breakdown of a substance to smaller products caused by microorganisms or their enzymes. Thus, biodegradation can be defined as the biologically catalyzed reduction in the complexity of chemicals. In the case of organic compounds, biodegradation frequently, although not necessarily, leads to the conversion of much of the carbon, nitrogen, phosphorus, sulfur and other elements in the original compound to inorganic products. Such a conversion of an organic substrate to inorganic products is known as mineralization. Ultimate biodegradation is a term sometimes used as a

synonym for mineralization. Biodegradation of individual compounds has been the subject of active concern for the last few decades. Technologies have been developed that markedly enhance biodegradation or that result in microbial destruction of organic pollutants that otherwise would persist at the sites of contamination (Alexander, 1990 & 1994a). The key to the assessment of the fate of organic chemicals in the environment is a realistic evaluation of their susceptibility to mineralization and biodegradation means the biological transformation of an organic pollutant to another form (Grady Jr, 1985).

Biodegradation is important for natural and industrial cycling of environmental chemicals. The ultimate goal in biodegradation studies is the mineralization of the substrate. This is the complete conversion of the carbon in the compounds to CO₂, and the conversion of the other components e.g. nitrogen and sulfur to their mineral forms (NO₃, NH₃, SO₄, S⁻² etc.). When anaerobic conditions are used, then CH₄ can be a mineralized product. In some cases the compounds are not mineralized completely, when this occurs, it is referred to as biotransformation. Biotransformation produces end products that may be non-toxic and may also be more toxic than the parent compounds. In some cases the compound of interest is not degraded as a substrate, but is metabolized by co-metabolism (Alexander, 1981 & 1994a). Various biotransformation reactions, with only minor alteration of the parent compound, are an alternative to biodegradation, and are often misinterpreted as biodegradation. The term biotransformation should be used in cases where the major structure of the compound remains unaltered and only a few reactions such as methylation, demethylation, hydroxylation, or reduction take place (Nelson *et al*, 1985).

Many synthetic chemicals discharged into the environments are directly toxic or become hazardous following biomagnification. Because mineralization results in the total destruction of the parent molecule and its conversion to inorganic products, such processes are beneficial. In contrast non-biological and many biological processes, although degrading organic compounds, convert them to other organic products.

Some of these products are toxic, but others evoke no untoward response. Thus mineralization is especially important in ridding natural environments of actual or possible hazards to humans, animals and plants (Alexander, 1994a).

Microorganisms carry out biodegradation in many different types of environments. Of particular relevance for pollutants or potential pollutants are sewage treatment systems, soils, and underground sites for the disposal of chemical wastes, ground water, surface waters, oceans, sediments, and estuaries. Microbial processes in various kinds of aerobic and anaerobic systems for treating industrial, agricultural, and municipal wastes are extremely important because these treatment systems represent the first point of discharge of many chemicals into the environment. Microbial processes have long been known to be important in sewage and wastewater for the destruction of a large number of synthetic chemicals. Soils also receive countless synthetic chemicals from farming operations, land spreading of industrial wastes, accidental spills, or sludge disposal, and the degradation of natural materials in soils. Ground water adjacent to waste disposal sites, lakes and rivers that receive inadvertent or deliberate discharge of chemicals, and the oceans and estuaries similarly contain highly diverse and often very active communities of bacteria, fungi, and protozoa that directly or indirectly, destroy many natural products as well as various synthetics. In addition, the sediments below fresh and marine waters retain a variety of pollutants, and these sediments also contain large and metabolically active communities of heterotrophic microorganisms. Natural communities of microorganisms in this various habitats have an amazing physiological versatility. They are able to metabolize and often mineralize an enormous number of organic molecules (Alexander, 1994a & 1994b). Several conditions must be satisfied for biodegradation to take place in an environment. These include the following:

- (a) An organism must exist that has the necessary enzymes to bring about the biodegradation.
- (b) That organism must be present in the environment containing the chemical.
- (c) The chemical must be accessible to the organism having the requisite enzymes.

- (d) If the initial enzyme bringing about the degradation is extracellular, the bonds acted upon by the enzymes must be exposed for the catalyst to function.
- (e) The products of an extracellular reaction must penetrate the cell for the transformation to proceed further.
- (f) Because the population or biomass of bacteria or fungi acting on many synthetic compounds is initially small, conditions in the environment must be conducive to allow for proliferation of the potentially active microorganism (Alexander, 1994a).

Because microorganisms are frequently the major and occasionally the sole means for degradation of particular compounds, the absence of a microorganism from a particular environment, or its inability to function, frequently means that the compound disappears very slowly. If microorganisms are the sole agents of destruction, the chemical will not be destroyed at all. If any of the conditions mentioned are not met, the chemical similarly will be long-lived. Hence, the frequent finding that organic pollutants are persisting is evidence that microorganisms are not functioning, they are acting very slowly, or no microorganisms exist with the capacity to modify the molecule. A wide variety of factors can influence the biodegradation rates of many organic pollutants in aquatic environments. These include the chemical structure of the pollutant, temperature, salinity, pH, availability of inorganic nutrients and oxygen, and a perexposure of the microbial community to the target chemicals (Shimp and Peaender, 1985; Liu *et al.*, 1991).

1.2.1 Biodegradation of xenobiotic compounds

Biodegradation of most naturally occurring organic compounds is relatively fast. A significant number of man-made chemicals are also biodegradable by microorganisms. Thus microbial degradation has a major impact on the fate of many synthetic chemicals in the environment. Biodegradation of xenobiotics by microorganisms depends on two factors:

1. The ability of microbial enzymes to accept substrate compounds with structures similar to, but not identical with chemicals found in nature, and
2. The ability of novel substrates to induce or depress the synthesis of the necessary degradative enzymes in the microorganisms (Hutzinger and Veerkamp, 1981).

Biodegradation is less likely in a molecule having structural features never encountered in natural products. In addition to the structure of a molecule, two other factors are also responsible for the degradation or lack of degradation in a given environmental situation (Hutzinger and Veerkamp, 1981). These two parameters are:

Available microorganisms: Types and number, available or inducible enzymes.

The environment: Presence or absence of oxygen and nutrients, light, pH, temperature.

The chemical structure influences the biodegradability of a compound in two ways. First, the molecule may contain groups or substituents, which can not react with available or inducible enzymes. Secondly, the structure may determine the compound to be in a physical state where microbial degradation does not easily occur (Hutzinger and Veerkamp, 1981; Knackmuss, 1996).

1.2.1.1 Biodegradation of aromatic hydrocarbons

Certain species of microorganisms can degrade certain aromatic hydrocarbons. Most of the aromatic hydrocarbons in the environment do not have a biosynthetic origin, but they do have a natural pyrolytic origin and must have been in contact with living organisms throughout evolutionary periods of time. Thus, organisms may acquire the knowledge to degrade aromatic compounds. Most of the knowledge of aromatic hydrocarbon degradation has been derived from laboratory studies with simple aromatic hydrocarbon substrates and pure microbial cultures. The ability of bacteria to utilize aromatic hydrocarbons for growth was first demonstrated in the early 1900s (Dagley, 1984). The detailed studies on the degradation of aromatic hydrocarbons

began after the Second World War and over the last decades various aspects of the field have been reviewed. The toxic, and sometimes carcinogenic, properties of aromatic hydrocarbons are related to the oxidation products of the parent molecules. These features have attracted the attention of organic chemists, biochemists, toxicologists, pharmacologists, and enzymologists who have focused mainly on the oxidation of aromatic hydrocarbons by higher organisms (Gibsons and Subramanian, 1984).

1.2.1.1.1 Biodegradation of halogen substituted aromatic compounds

One of the important groups of xenobiotic compounds is the halogenated aromatic compounds. Halogenated aromatics, particularly chlorinated aromatics, are produced in vast quantities due to their numerous applications such as herbicides, insecticides, fungicides, solvents, hydraulic and heat transfer fluids, plasticizers, and intermediates for chemical synthesis. Because of their toxicity, bioconcentration, and persistence, the ubiquitous distribution of the halogenated compounds in the biosphere has caused public concern over the possible effects on the quality of life (Fetzner and Lingens, 1994). They can be removed from the environment by biodegradation or by biotransformation. The mineralization of halogen- substituted aromatic compounds is considerably effected by the position of the halogen substituents. The removal of the halogen substituent is the key step in the degradation of halogenated aromatic compounds. This may occur as an initial step via reductive, hydrolytic or oxygenolytic mechanisms or after cleavage of the aromatic ring at the latter stage of metabolism. In addition to degradation, several biotransformation reactions, such as methylation and polymerization, may take place and produce more toxic or recalcitrant metabolites (Häggbloom, 1992).

Studies with pure bacterial and fungal cultures have given detailed information on the biodegradation pathways of several halogenated aromatic compounds. Much progress has been made in the last few years in understanding the mechanisms of microbial degradation of halogenated aromatics, both under aerobic and anaerobic conditions.

The degree of degradation varies from compound to compound. Some are apparently resistant to microbial attack, while others may be partially broken down to non-degradable intermediates, or transformed to possibly more toxic products. Complete biodegradation will ultimately result in the mineralization of the compound to carbon dioxide or methane, and in the case of haloaromatics, with release of halogen substituent as halide. In aerobic environments, Oxygen is both the terminal electron acceptor and frequently a reactant in the initial reactions. In the absence of oxygen, nitrate, sulfate or carbonate may function as alternate electron acceptors in the microbial degradation of organic material, and the presence or absence of these electron acceptors may affect the biodegradability of a compound (Grady Jr, 1985; Neilson, 1990; Fetzner, 1998). Even though many chlorinated aromatic compounds are biodegradable, they are often recalcitrant and natural purification of contaminated sites seems slow. This may be because microorganisms able to degrade the contaminant are missing or that the environmental conditions (temperature, redox potential, pH, and concentration of contaminant) are such that degradation is not promoted.

1.2.2 Biodegradation of chlorophenols

Chlorophenols are a group of toxic chemicals that constitute a series of 19 compounds consisting of mono-, di-, tri- and tetrachloro- isomers and one pentachlorophenol. Chlorinated phenols have been in use as biocides to control bacteria, fungi, algae, mollusks, insects, slime, and other biota. They have also been used as precursors in the synthesis of other pesticides. The use of polychlorinated phenols has been banned or restricted in several countries since 1980s, but because of past practices, chlorinated phenols are widespread in the environment today (Hale *et al.*, 1994). The inadequate handling of chlorophenol-treated materials, accidental spills, and leaching from dumping sites have resulted in the serious contamination of soil and ground water (Kitunen *et al.*, 1987). Among the halogenated compounds, chlorophenols are a special group of chemicals that have attracted worldwide public attention, mainly due to their environmental persistence and their inherent toxicity to

a broad spectrum of organisms. Mono-chlorophenols can be formed during wastewater chlorination, and as a result of breakdown of pesticides and chlorinated aromatic compound, and since their solubility in water is relatively high, chlorophenols can easily migrate within different aqueous environments and contaminate ground waters (Armenante *et al.*, 1999). Potential environmental sources of chlorinated phenols include:

1. direct soil applications as biocides,
2. leaching or vaporizing from treated wood items,
3. synthesis during routine chlorination process of drinking water and waste water at treatment plants,
4. synthesis during production of bleached pulp in which chlorine is used,
5. release from factories into air and water, and
6. incineration of waste materials and burning of fresh lignocellulosic biomass e.g. forest fires (Hale *et al.*, 1994; Puhakka and Melin, 1996; Fetzner, 1998).

1.2.2.1 Chemical and physical properties of chlorophenols

The mobility and bioavailability of chlorophenols and their bioremediation are affected by their chemical and physical properties. The solubility of chlorophenols in water decreases as the number of chlorine substituent increases. In addition, the increase in the number of chloro-substituents in the phenol ring increases the lipophilicity of chlorophenols and thus their tendency to bioaccumulate. Environmental pH is the most important factor affecting chlorophenol adsorption and mobility. Both protonated and deprotonated chlorophenols may exist in the natural condition, but lower chlorinated phenols are more protonated in natural environments than their polychlorinated congeners. Soil organic content is another important factor affecting chlorophenol mobility (Puhakka and Melin, 1996).

1.2.2.2 Toxicity of chlorophenols

The bactericidal effect of chlorophenols have been known for many years (Sykes, 1965). The potential antibacterial effectiveness generally increases with degree of chlorine substitution, up to trichloro derivatives. The tetrachloro isomers are generally considerably less active than any of the trichloro isomers against aerobic bacteria. Penta-chlorophenol is less effective than tetra-chloro isomers and is about as effective as phenol itself (Sykes, 1965; Baker *et al.*, 1970). The sensitivity of anaerobes against the chlorophenols varies (Ruckdeschel *et al.*, 1987; Zhang and Wiegel, 1990). Some higher chlorophenols, particularly pentachlorophenol, have enhanced fungicidal activity.

1.2.2.3 Degradation rates of chlorophenols

Chlorophenols are much more environmentally stable than the parent unsubstituted phenols. As the number of chlorine substituents increases, the rate of aerobic decomposition decreases, whereas the opposite is generally true for the anaerobic degradation (Tabak, 1964). Compounds containing a *meta*-chlorine are more persistent under aerobic conditions than compounds lacking a chlorine substituent in positions *meta* to hydroxyl group (Alexander and Aleem, 1961; Alexander and Lustigman, 1966). Persistence of chlorophenols in the environment depend on the presence of microbial populations and environmental parameters such as pH, temperature, aeration rate, available nutrients, the absence or presence of inhibitory copollutants, and the absence or presence of substances changing the electron flow in the system. Chlorinated phenols may be removed from a water body via: volatilization, photo-degradation, adsorption onto suspended or bottom sediments, and microbial degradation (Hale *et al.*, 1994).

1.2.2.4 Microbial degradation of chlorophenols

Several microorganisms that degrade chlorophenols have been isolated, and in some cases, the mechanism by which degradation occurs has been elucidated. The main factors influencing chlorophenol remediation include temperature, the properties of the environmental matrix, the toxicity of chlorophenols and the composition of indigenous or added microbial cultures. There are numerous studies reported on the degradation of chlorophenols (Knackmuss and Hellwig, 1978; Knackmuss 1981; Schwein and Schmidt, 1982; Bartels *et al.*, 1984; Spain and Gibson, 1988; Häggblom, 1990; Sáez and Rittman, 1991; Hinteregger *et al.*, 1992; Schlömann, 1994; Arnesdorf and Focht, 1995; Hollender *et al.*, 1997; Seo *et al.*, 1998). Bacteria use different strategies to degrade chlorophenols:

1. Mono- and di-chlorophenols are usually degraded aerobically by hydroxylation to chlorocatechols and by a spontaneous dechlorination after *ortho*-cleavage of the chlorocatechols.
2. Trichloro- and polychlorophenols are degraded aerobically via par-hydroquinones, which are subsequently dechlorinated before ring cleavage.
3. All chlorophenols (mono- to penta-chlorophenol) are degraded under anaerobic conditions by a variety of microbial communities; degradation is initiated by reductive dechlorination followed by ring cleavage (Hale *et al.*, 1994).

1.2.2.4.1 Microbial degradation of mono-chlorophenols

The presence of a chlorine group on phenol makes the compound more persistent in the environment. The biodegradation of a halogenated compound can be considered complete only when its carbon skeleton is converted into intermediary metabolites and its organic halide is converted to its mineral state. For a bacterium to achieve mineralization of chlorophenol, it should be capable of removing the chlorine substituents at an early stage of metabolism or have enzymes with substrate specificities that can cope with the chlorinated substrate. In the latter case, the

chlorine group is removed following ring cleavage (Häggbloom, 1990). Following hydroxylation, ring cleavage may take place via an *ortho* or a *meta*-cleavage pathway.

1.2.2.4.1.1 Hydroxylation

The initial step in the aerobic degradation of mono-chlorophenols is their transformation to the central metabolites, chlorocatechols. This step may be carried out by either a hydroxylase or a dioxygenase enzyme system. Degradation of 2- and 3-chlorophenol generally results in the production of 3-chlorocatechol, while metabolism of 4-chlorophenol leads to the production of 4-chlorocatechol (Knackmuss, 1981).

Pseudomonas sp. B13, an organism shown to be capable of the degradation of 3-chlorobenzoate and 4-chlorophenol as sole carbon source, and also of the co-metabolism of 2- and 3-chlorophenol (Knackmuss and Hellwig, 1978), was shown to carry out hydroxylation to the corresponding chlorocatechols using a broad spectrum phenol hydroxylase (E.C. 1.14.13.7). Conjugation of *Alcaligenes* sp. strain A7, a strain capable of growth on benzoate and phenol, with *Pseudomonas* sp. B13 resulted in a transconjugant of the *Alcaligenes* sp., strain A7-2, capable of growth on all three mono-chlorophenols, a property not possessed by either the donor or recipient strains (Schwein and Schmidt, 1982). The increased ability of the transconjugant strain, *Alcaligenes* A7-2, to grow on mono-chlorophenols over *Pseudomonas* sp. B13 can be attributed to the presence of a more rapidly inducible non-specific phenol hydroxylase, which correlates with the greater level of resistance to the mono-chlorophenols.

Transformation of mono-chlorophenols to chlorocatechols was demonstrated in *P. putida* F1 (Spain and Gibson, 1988). Cells pre-grown in toluene were studied for their ability to degrade many substituted aromatics. Degradation of 2- and 3-chlorophenol resulted in an accumulation of 3-chlorocatechol in the medium, while growth on 4-

chlorophenol led to the transient accumulation of 4-chlorocatechol. It was suggested that a toluene dioxygenase was responsible for the transformation of chlorophenols to chlorocatechols as generation of mutants of *P. putida* F1, lacking toluene dioxygenase activity, failed to transform chlorophenols.

When the degradation of 4-chlorophenol by *P. putida* PpG4 was studied, it was found that transformation of 4-chlorophenol could only occur following growth of the bacterium on phenol (Sáez and Rittman, 1991). This suggested that degradation could only occur following induction of a phenol hydroxylase (E.C. 1.14.13.1) which catalysed the first step in the degradation of 4-chlorophenol. The phenol-degrading strain *P. putida* EKII was found to be capable of the degradation of mono-chlorophenols both by co-metabolism with phenol and as sole carbon source with cells pre-grown on phenol (Hinteregger *et al.*, 1992). Degradation of the chlorophenols was considerably faster in the presence of phenol than in its absence. Although activity of the broad-spectrum phenol hydroxylase was not detectable in cell free extracts, it was detectable in whole cells.

The transformation of chlorophenols to chlorocatechols by a phenol hydroxylase in the yeast *Rhodotorula glutinis* was demonstrated (Katayama-Hirayama *et al.*, 1994). Degradation of mono-chlorophenols was improved following the addition of phenol to the medium, suggesting the induction of phenol hydroxylase by phenol. The order of biodegradability was 4-chlorophenol > 3-chlorophenol > 2-chlorophenol suggesting that the substrate specificity of phenol hydroxylase may be related to the position of the hydroxyl group in the chlorophenol molecule.

1.2.2.4.1.2 Ring cleavage

The critical step in the aerobic degradation of chlorophenols is cleavage of the aromatic ring. As is the case during the degradation of non-halogenated aromatics, ring cleavage may take place using either the *ortho*- or the *meta*- pathway. Aromatic compounds found naturally in the environment, such as phenol and benzene, are

typically broken down via the *meta*- cleavage pathway. While methyl- substituted aromatic compounds are also successfully degraded via the *meta*- cleavage pathway, chlorinated aromatics are generally broken down via the *ortho*- pathway.

1.2.2.4.1.3 The *ortho*- cleavage pathway

Normal 1,2-dioxygenases involved in catechol metabolism exhibit low activities for halogenated substrates (Schmidt and Knackmuss, 1980). Compared with ordinary dioxygenases, enzymes with broad substrate specificities that have a high affinity for chloroaromatic substrates have been identified (Dorn and Knackmuss, 1978).

In *Pseudomonas* sp. B13, catechol and chlorocatechol were assimilated via two separate *ortho*- cleavage pathways. Correspondingly, two types of isofunctional enzymes for ring fission were found. Catechol 1,2-dioxygenase (pyrocatechase Type I, E.C. 1.13.11.1, gene *catA* or *clcA*) was present in cells grown on benzoate and was highly specific for catechol, while chlorocatechol 1,2-dioxygenase (pyrocatechase Type II, E.C. 1.13.11.1, gene *tfdC*) was induced when 3-chlorobenzoate was the growth substrate. Chlorocatechol 1,2-dioxygenase had relaxed specificities and high activities for chlorinated aromatic compounds (Knackmuss and Hellwig, 1978). Chlorocatechols interfere with the oxygen-binding of the normal catechol 1,2-dioxygenase due to the steric and inductive effects of the chloride atom. This results in the accumulation of the chlorocatechols which polymerise due to auto-oxidation causing a brown/black colouration of the culture medium.

Transformation of chlorocatechols via the “modified” *ortho*- cleavage pathway by chlorocatechol 1,2-dioxygenase yields chloromuconates (Fig. 1) (Schlömann, 1994). The chlorocatechol is oxidised at both carbon atoms carrying hydroxyl substituents, yielding two carboxylic acid (-COOH) groups at the end of a chain. Ring cleavage occurs between the hydroxyl-substituted carbons. *Ortho*- cleavage of 3-chlorocatechol yields 2-chloro-*cis,cis*-muconate while cleavage of 4-chlorophenol results in the production of 3-chloro-*cis,cis*-muconate.

Enzyme assays revealed that *Pseudomonas putida* CP1 degraded all three chlorophenol isomers via the modified *ortho*-cleavage pathway (Farrell and Quilty, 2002). The mechanism is same as described by Knackmuss (1981) shown in Fig. 1.

1.2.2.4.1.4 Cycloisomerisation

Following ring cleavage via the modified *ortho*- pathway, the products produced undergo cycloisomeration as described by Schmidt and Knackmuss (1980). The chloro-substituted *cis,cis*-muconates are transformed resulting in the production of dienelactones (4-carboxymethylenebut-2-en-4-olides). This is a critical step in the degradation of chlorophenols as it results in the elimination of the chloro-substituent (Fig. 1). The enzyme involved, chloromuconate cycloisomerase (cycloisomerase Type II, E.C. 5.5.1.7) is homologous to muconate cycloisomerase (cycloisomerase Type I, E.C.5.5.1.1) involved in the degradation of catechol via the *ortho*- pathway.

Both cycloisomerases catalyse similar reactions and differ with respect to their substrate specificities and product formation. Cycloisomerase II has been shown to exhibit higher activities with substituted-muconates than with unsubstituted substrates (Schmidt and Knackmuss, 1980). Cycloisomerase I responsible for the conversion of *cis,cis*-muconate to muconolactone also converts 2-chloro-*cis,cis*-muconate to mixtures of 2- and 5-chloromuconolactone by carrying out both 1,4 and 3,6-cycloisomerisations of the substrate (Vollmer *et al.*, 1994) and 3-chloro-*cis,cis*-muconate to form the bacteriotoxic protoanemonin (Blasco *et al.*, 1995). Cycloisomerase I is unable to further metabolise 2- or 5-chloromuconolactone. Cycloisomerase II however converts 2-chloro-*cis, cis*-muconate to the intermediate 5-chloromuconolactone and then dehalogenates the metabolite to form *trans*-dienelactone, while 3-chloro-*cis, cis*-muconate is converted to *cis*-dienelactone (Vollmer *et al.*, 1998).

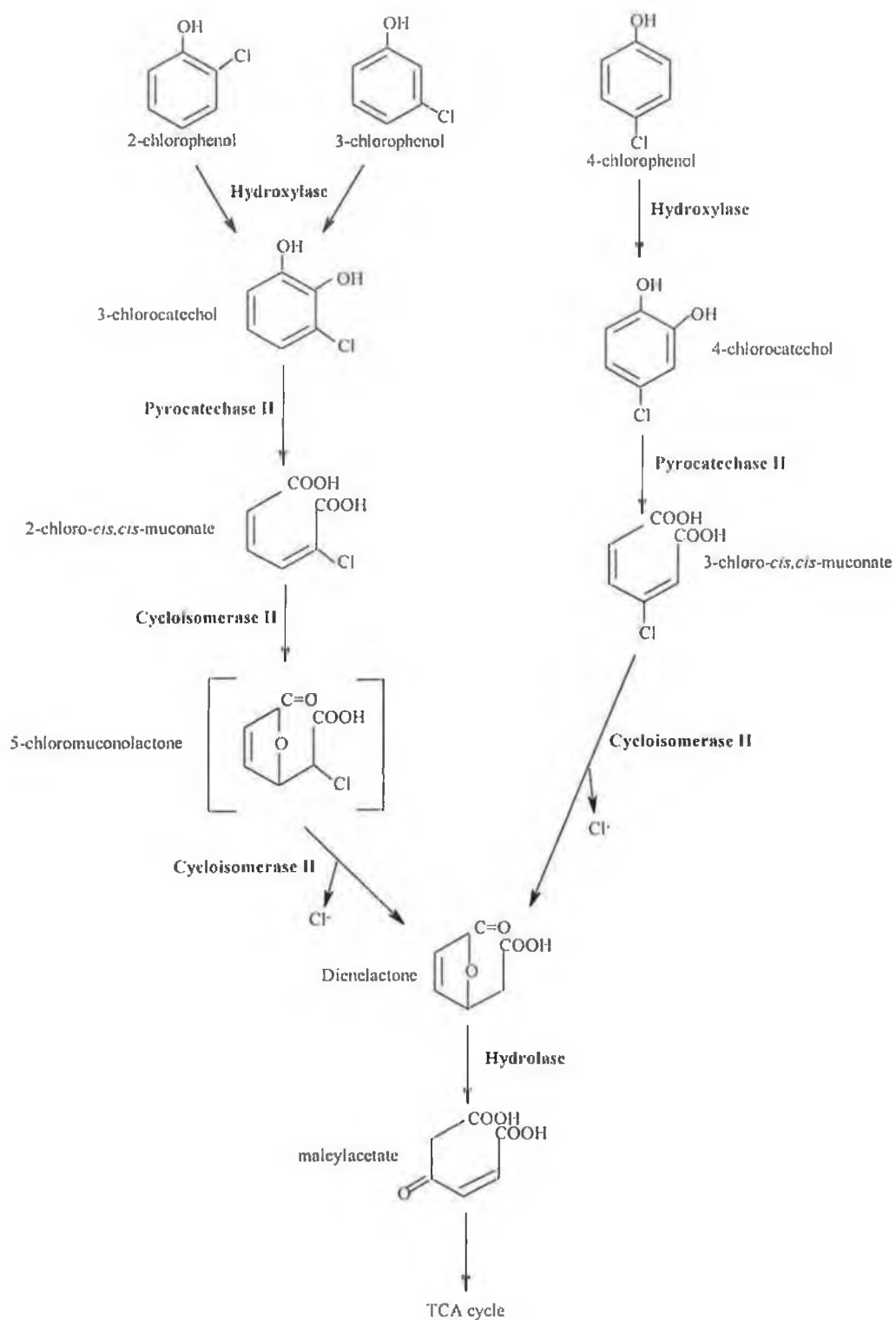


Fig. 1. The degradation of mono-chlorophenols via the modified *ortho*- pathway (Knackmuss, 1981).

The final steps in the degradation of chlorophenols is the conversion of dienelactones produced following cycloisomeration into metabolites of the normal metabolism of the bacterium. This is achieved by the conversion of *cis*- and *trans*-dienelactone, by the enzyme dienelactone hydrolase, to produce maleylacetate which is readily converted to intermediates of the TCA cycle, and therefore may be utilised for the production of biomass, energy, carbon dioxide and water, resulting in complete degradation.

In contrast to the ordinary *ortho*- cleavage pathway, the genes for the modified *ortho*- pathway are generally located on catabolic plasmids (van der Meer *et al.*, 1992). The most extensively studied genes encoding the modified *ortho*- cleavage pathway are the *clcABD* operon of *P. putida* (pAC27), the *tfdCDEF* operon of *Alcaligenes eutrophus* JMP134 (pJP4) and the *tcbCDEF* of *Pseudomonas* sp. strain P51 (p51). It has been shown that the chlorocatechol 1,2-dioxygenase genes appeared to be linked to the genes for the rest of the pathway in a single operon and that the chlorocatechol 1,2-dioxygenase and chlorocycloisomerase genes of these operons are significantly homologous to the counterparts of the ordinary *ortho*- cleavage pathway genes, while the relationship between dienelactone hydrolase and 3-oxoadipate enol-lactone hydrolase is more distant (Schlömann, 1994).

1.2.2.4.1.5 The *meta*- cleavage pathway

The alternative route to *ortho*- cleavage of chlorocatechols, is ring cleavage via the *meta*- cleavage pathway by the enzyme catechol 2,3-dioxygenase (E.C. 1.13.11.2). In general, *ortho*- cleavage is required to bring about complete mineralization of chlorophenols as *meta*- cleavage can result in dead-end pathways. Dead-end pathways result in two ways, generation of a metabolite which may not be degraded further or generation of a metabolite which acts as a specific enzyme inhibitor. The latter is called “lethal synthesis” and can result from competitive inhibition of an essential enzyme, removal of an essential metal co-factor from the enzyme’s active

site by a chelating agent, or from an irreversible form of inhibition (Bartels *et al.*, 1984).

Generally *meta*- cleavage of 3-chlorocatechol, produced following hydroxylation of 2- and 3-chlorophenol, results in the inactivation of catechol 2,3-dioxygenase. Two mechanisms have been proposed by which 3-chlorocatechol may interfere with the activity of catechol 2,3-dioxygenase. The first way is by 3-chlorocatechol itself, acting as a chelating compound (Klecka and Gibson, 1981). 3-chlorocatechol may chelate the iron co-factor required for the catabolic activity of catechol 2,3-dioxygenase causing a large build up of chlorocatechols. Following chelation, enzyme activity may be reactivated by treatment with ferrous iron and a reducing agent. Alternatively, *meta*- cleavage of 3-chlorocatechol can lead to the production of a highly reactive acyl chloride, 5-chloroformyl-2-hydroxypenta-2,4-dienoic acid, which has been proposed to act as a suicide compound, binding irreversibly to catechol 2,3-dioxygenase with a subsequent release of chloride, destruction of metabolic activity and an accumulation of chlorocatechols (Fig. 2) (Bartels *et al.*, 1984). In both cases the accumulated chlorocatechols then polymerise due to auto-oxidation resulting in the production of brown/black coloured chlorocatechol polymers.

The complete degradation of chloro-aromatics via 3-chlorocatechol by a *meta*-cleavage pathway has been demonstrated in the organism *P. putida* GJ31 (Mars *et al.*, 1997) but is extremely rare. The proposed pathway by which *P. putida* GJ31 degrades 3-chlorocatechol is shown in Figure 3. *P. putida* GJ31 appears to possess a catechol 2,3-dioxygenase which is able to convert 3-chlorocatechol to 2-hydroxy-*cis,cis*-muconic acid, an intermediate of the *meta*- cleavage of catechol, avoiding the rapid inactivation of the enzyme by the highly reactive acyl chloride usually associated with the *meta*- cleavage of catechol 2,3-dioxygenase. 2-hydroxy-*cis,cis*-muconic acid can then be degraded by the normal *meta*- cleavage pathway involved in the degradation of catechol.

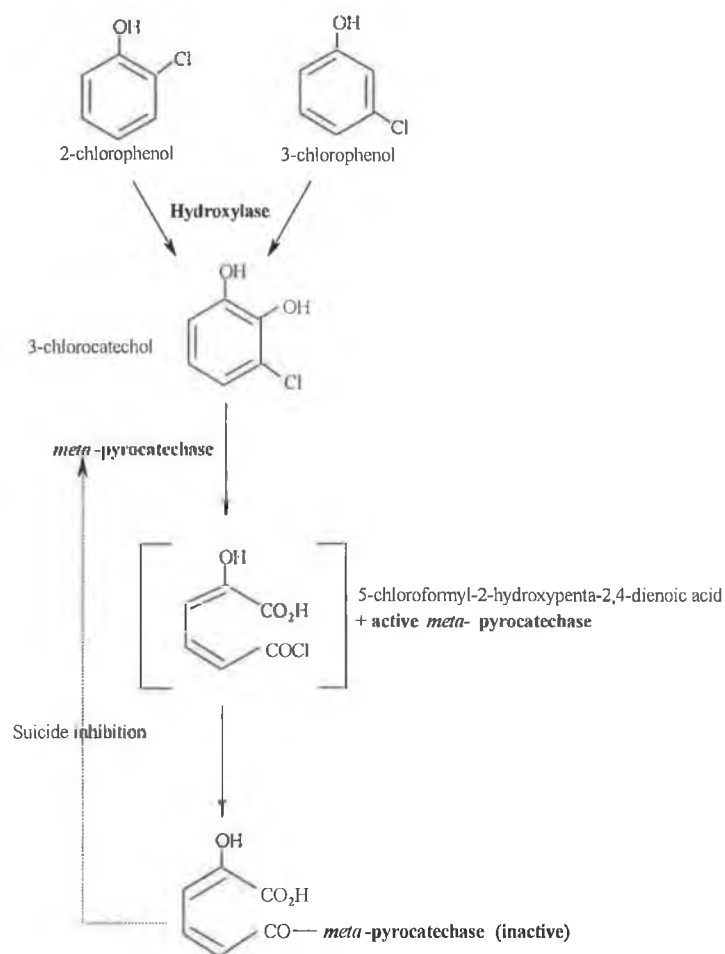


Fig. 2. The *meta*- cleavage of 3-chlorocatechol (Bartels *et al.*, 1984).

The *meta*- cleavage of 4-chlorocatechol, produced following metabolism of 4-chlorophenol, results in the production of a chlorinated aliphatic compound, 5-chloro-2-hydroxy-muconic semialdehyde (Wieser *et al.*, 1994) which has been widely reported as being a dead-end metabolite (Reineke *et al.*, 1982; Westmeier and Rehm, 1987). Accumulation of this compound, resulting from the fact that this product ordinarily cannot be further metabolised, causes an intense lime/yellow coloration of the culture medium (Knackmuss, 1981). However, recent reports have shown that further metabolism of 5-chloro-2-hydroxymuconic semialdehyde may occur, resulting in complete degradation of 4-chlorophenol via a *meta*- cleavage pathway (Bae *et al.*, 1996b; Hollender *et al.*, 1997; Farrell and Quilty, 1999). Although the exact mechanism by which 4-chlorophenol is degraded via 4-chlorocatechol by a *meta*- cleavage pathway is unclear, it is likely to be similar to a proposed mechanism

as described by Arnesdorf and Focht (1995) for the degradation of 4-chlorobenzoate (Fig. 4).

5-chloro-2-hydroxymuconic semialdehyde is transformed to 5-chloro-2-hydroxymuconic acid and 5-chloro-2-hydroxy-pent-2,4-dienoic acid (enol) by the enzymes 2-hydroxymuconic semialdehyde hydrolase, 4-oxalocrotonate isomerase and 4-oxalocrotonate decarboxylase. 5-chloro-2-hydroxy-pent-2,4-dienoic acid is then transformed to 5-chloro-4-hydroxy-2-oxopentanoic acid which is further transformed to chloroacetic acid and pyruvic acid. Chloroacetic acid may then be dehalogenated to form glycolate, which may be utilised along with pyruvic acid in the TCA cycle (McCullar *et al.*, 1994) leading to the production of biomass, energy, carbon dioxide and water.

Similarly, Seo *et al.* (1997 & 1998) reported the complete degradation of 4-chlorobenzoate, via 4-chlorocatechol, using a *meta*- cleavage pathway by a *Pseudomonad*, sp. S-47 (Fig. 4). They described how 4-chlorocatechol was initially transformed to 5-chloro-2-hydroxymuconic semialdehyde and then 5-chloro-2-hydroxymuconic acid. *Pseudomonas* sp. S-47 appeared to dehalogenate at this stage, converting 5-chloro-2-hydroxymuconic acid to 2-hydroxypenta-2,4-dienoic acid via 2,5-dihydroxy-muconic acid. 2-hydroxypenta-2,4-dienoic acid is a normal metabolite following the degradation of catechol via the *meta*- pathway and was easily metabolised to form pyruvate and acetaldehyde which may be utilised by the TCA cycle.

Despite these examples, the *meta*- cleavage of chloroaromatics usually results in dead-end pathways, resulting in large accumulations of polychlorinated catechol polymers. The destruction of *meta*- cleavage activity by the chlorocatechols and their inefficient oxidation by unmodified *ortho*- cleavage enzymes explains the instability and dark coloration of activated sludges suddenly loaded with chloroaromatics (Bartels *et al.*, 1984). Consequently the degradation of chloroaromatics in

wastewaters generally requires the action of the modified *ortho*- cleavage pathway for their efficient removal from wastewaters.

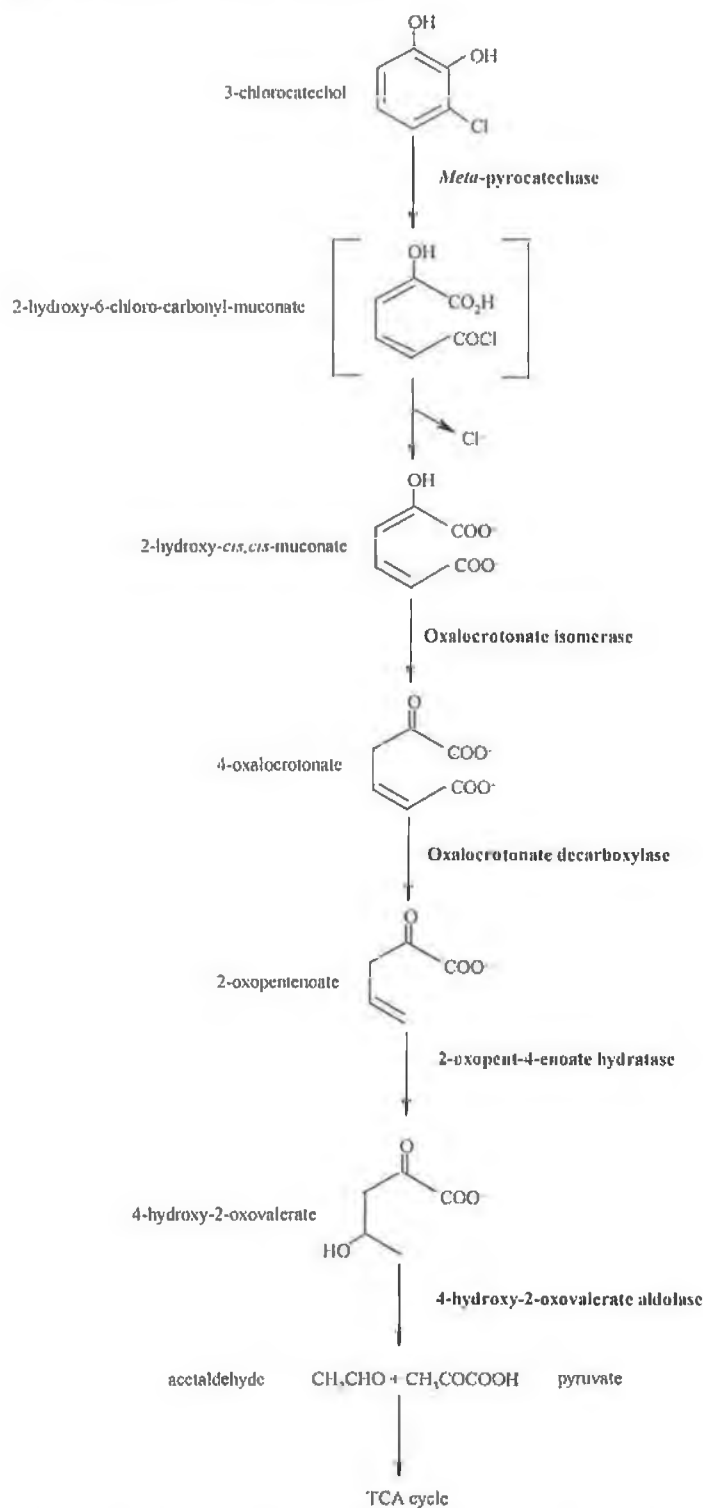


Fig. 3. The *meta*- cleavage of 3-chlorocatechol by *Pseudomonas putida* GJ31 (Mars *et al.*, 1997).

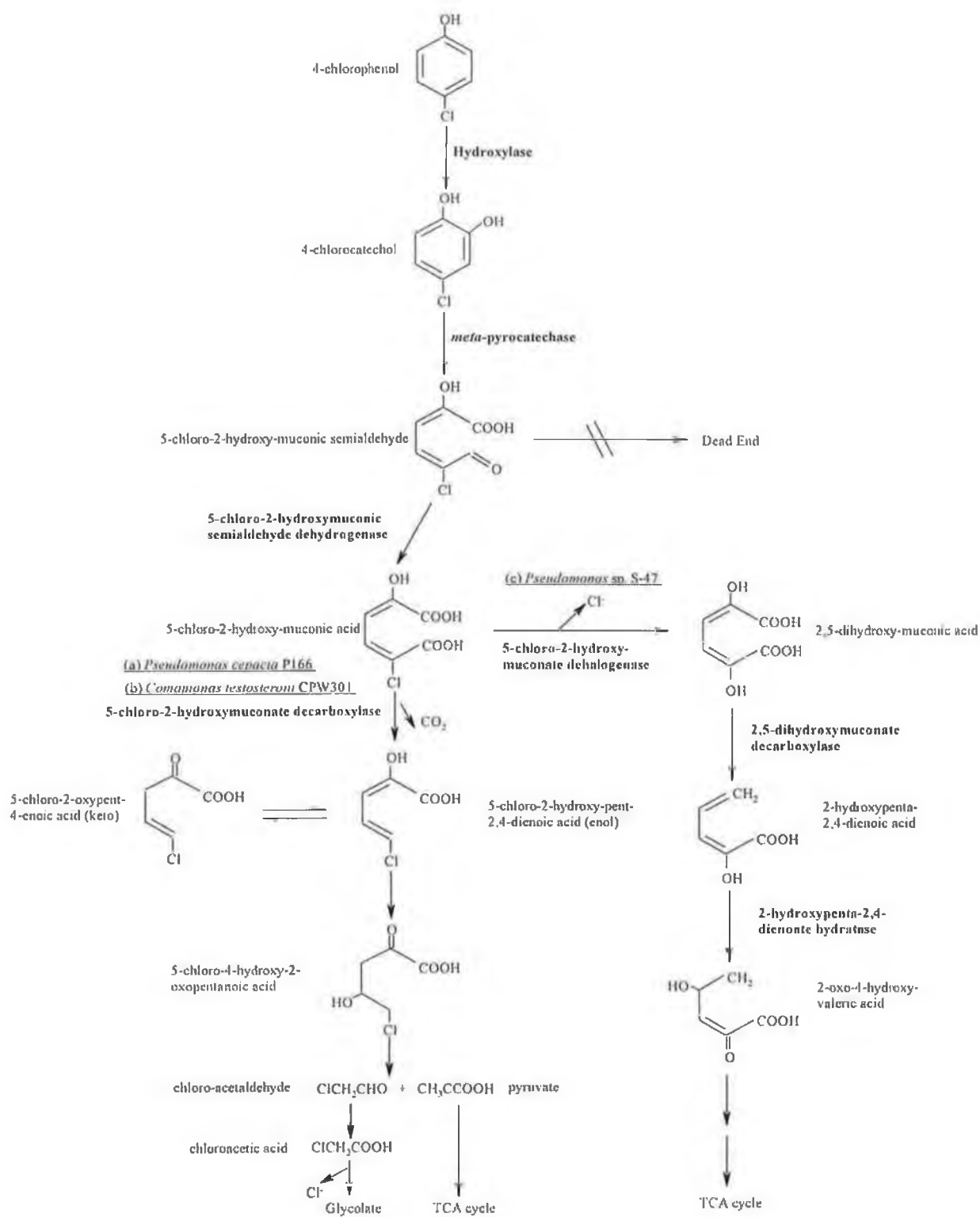


Fig. 4. The *meta*- cleavage of 4-chlorophenol (a) Arnesdorf and Focht, 1995; (b) Hollender *et al.*, 1997; (c) Seo *et al.*, 1998.

1.2.3 Biodegradation of phenol and phenolic compounds in the presence of easily degradable carbon sources

Widespread industrial and agriculture uses of phenol and phenolics have resulted in a variety of water pollution problems. Industrial wastewaters associated with the manufacture of phenolics characteristically have concentrations as high as hundreds of parts per million. Urban or agricultural run-off concentrations, range from a few parts per billion to several parts per billion. Conventional methods of treatment of phenol and phenolic wastes have been largely chemical or physical, but these processes have led to secondary effluent problems. Biological treatment for the bulk removal of these pollutants is generally preferred. Therefore, biological degradation of phenolics has been extensively studied using pure and mixed culture. Various methods have been proposed to overcome substrate inhibition in order to treat high-strength phenolic wastewater. These include adapting the cells to higher concentrations of phenols, immobilization of cells and using genetically engineered microorganisms. Another possible method of increasing the tolerance of the cells to substrate inhibition is to supplement the growth medium with conventional carbon sources (Loh and Tan, 2000).

The effect of the presence of conventional organic substrates on the biodegradation of toxic waste components is of great practical importance. In the natural environment toxic or inhibitory components will be found in mixtures with nontoxic or conventional wastes. When alternative carbon sources are presented to the microbial population, substrate interactions can occur (Rozich and Covin, 1986). The presence of such easily degradable carbon sources may enhance, decrease or have no effect on the biodegradation of target contaminants like chlorophenols. The practical importance of mixed toxic/nontoxic substrate metabolism is perhaps most obvious at biological wastewater treatment facilities that could deal with these multicomponent wastes.

Several investigators showed the facilitation of chlorophenol degradation by using conventional carbon sources such as glucose, sodium glutamate, and yeast extract (Topp *et al.*, 1988; Yu and Ward, 1994; Fava *et al.*, 1995b; Loh and Wang, 1998; Wang and Loh, 1999). Their findings suggested that the addition of some conventional carbon sources might aid in reducing the toxicity and growth inhibition of xenobiotics on cells, thereby increasing the transformation rates of xenobiotics. Conventional carbon sources might also act as an inducing agent for biodegradative enzymes (Chaudhuri and Wiesman, 1995), or provide reducing power for degradation of recalcitrant organic compounds (Perkins *et al.*, 1994). Potentially these have greater significance in the conversion of non-growth substrates since cometabolism generally leads to a slow conversion of the substrate (Alexander, 1994c).

1.2.4 Biodegradation of mono-chlorophenols in the presence of mixed substrates

Mixed substrate systems are characterized by the presence of multiple sources of nutrients that serve a similar physiological function. The term mixed substrates encompasses all types of nutrients; such as the carbon sources, energy sources and nitrogen sources.

Mixed substrates are very relevant in environmental sciences. The great majority of biological waste treatment processes involve the metabolism of mixed substrates. Also, culture media of many industrial fermentations are frequently formulated so that they contain complex mixtures of nutrients. In the environment, growth and metabolism of microorganisms often occurs on low concentrations of diverse mixed substrates. Thus microbial growth on substrate mixtures is commonly encountered in bioremediation, wastewater treatment, and fermentation. Organic chemical mixtures are prevalent in waste waters from industrial and municipal sources as well as in contaminated ground water. In natural environments, mono-chlorophenol waste streams often contain some other higher chlorinated phenols, phenol and other toxic substances as well as easily degrading substrates. The presence of secondary carbon

sources may enhance or retard the biodegradation of recalcitrant compounds. The presence of more easily biodegraded compounds may enhance the biodegradation of chlorinated phenols whereas toxic compounds may retard the degradation. Thus biodegradation of mono-chlorophenols may be synergistically or antagonistically affected by the presence of other compounds (Kim and Maier, 1986; Sáez and Rittmann, 1993). The presence of a secondary carbon source may stimulate the growth of microorganisms, which then accelerates the biodegradation of chlorophenols. The enhancement of biodegradation of recalcitrant compounds is due to the concurrent utilization of the secondary carbon source and the recalcitrant compounds or the introduced production of degradative enzymes or both. However, the increased microbial population may adversely affect the biodegradation of recalcitrant compounds, if the microorganisms shift the major carbon source from the recalcitrant compounds to the relatively easily biodegraded compounds, the secondary carbon sources (Lu and Speitel, 1988; Lu and Tsai, 1993).

1.2.5 Cometabolism

The transformation of an organic compound by a microorganism that is unable to use the substrate as a source of energy or of one of its constituent elements is termed cometabolism (Alexander, 1994c). Active populations derive no nutritional benefit from the substrates they cometabolize. To fully sustain the growth sufficient energy is not acquired even if the conversion is an oxidation and releases energy, and the carbon, nitrogen, sulfur, or phosphorus that may be in the molecule is not used as a source, or at least a significant source, of these elements for biosynthetic purposes. Thus in cometabolism, a separate growth substrate is provided to the organisms, and the resulting non-specific enzymes catalyze the oxidation of cometabolized substrates. A growth substrate is an electron donor that provides reducing power and energy for cell growth and maintenance (Criddle, 1993). Many cometabolic enzymes and cofactors are induced by a growth substrate, although cometabolic agents may also be induced by other factors, or they may be produced constitutively. An energy

substrate is an electron donor that provides reducing power and energy but does not by itself support growth.

Many compounds of environmental and toxicological significance are transformed by cometabolism. That is under normal conditions in the environment a significant proportion of xenobiotic biodegradation involves cometabolism. Cometabolic reactions have impacts in nature that are different from growth-linked biodegradations, and when the transformations take place, it is usually totally unclear whether the microorganisms do or do not have a second substrate available on which they are growing. The majority of cometabolic studies reported appear to be based on the concept of using a simpler substrate as the cometabolite to achieve the degradation of a more complex compound. However, a higher chlorinated but biodegradable chemical could be effectively used as a cometabolite to achieve the enhanced biodegradation of other lower chlorinated recalcitrant analogues (Lu and Speitel, 1988).

As chlorinated solvents, for the most part, can not serve as carbon and energy sources for microbial growth, aerobic cometabolism of chlorinated solvents is a potential treatment technology for contaminated water and air streams. Biological treatment processes based on cometabolism offer a challenge in process design and operation. Appropriate combinations of reactor technologies, microorganisms, and operating strategies are required to develop commercially attractive treatment processes.

1.2.6 Acclimation of microorganisms to organic compounds

Prior to the degradation of many organic compounds, a period is noted in which no destruction of chemical is evident. This time interval is designated an acclimation period or sometimes, an adaptation or lag period. It may be defined as the length of time between the addition and entry of the chemical into the environment and evidence of its detectable loss. During this interval, no change in concentration is noted, but then the disappearance becomes evident and the rate of destruction often

becomes rapid (Alexander, 1994d). The acclimation of microorganisms to organic compounds is a very important phase of the biodegradation process, especially for a compound considered difficult to biodegrade. The duration of the acclimation period ranges from a few hours to several weeks or even months and it depends on the quality and quantity of the inoculum as well as the condition under which acclimation is carried out (Aelion *et al.*, 1989; Buitrón and Capdeville, 1995).

The acclimation phase is considered to end at the onset of the period of detectable biodegradation. After the acclimation, the rate of metabolism of the chemical may be slow or rapid, but if a second addition of the chemical is made during this time of active metabolism, the loss of the second increment characteristically occurs with little or no acclimation.

This acclimation phase may be of considerable public health and ecological significance because the chemical is not destroyed. Hence, the period of exposure of humans, animals, and plants is prolonged, and the possibility of an undesirable effect is increased. Furthermore, if the chemical is present in flowing waters, above and below ground, it may be widely disseminated laterally or vertically because of the absence of detectable biodegradation. In the case of toxicants, such increased disposal may result in exposure of susceptible species at distant sites before the harmful substance is destroyed (Alexander, 1994d).

The capacity of microorganisms to degrade industrial effluents containing toxic organics is well known, but in practice very few systems are highly efficient because of poor control of the organisms involved in the degradation. The degradation capacities of an activated sludge can be enhanced by acclimation of the inoculum. There is selection and a multiplication of specialized microorganisms during this phase (Buitrón *et al.*, 1998). A variety of phenomena have been proposed to explain the acclimation phase. Buitrón and Capdeville (1995) described the following mechanisms that occurs during the period of acclimation:

1. **Enzymatic:** The organism does not possess the inductive enzyme unless the compound is present in the medium. A fortuitous metabolism is produced when an existing enzyme happens to have suitable catalytic activity towards novel substrates.
2. **Multiplication of specialized microorganisms:** Acclimation is the time needed for the multiplication of an initially small population of active microorganisms. In this phase there is a selection of degraders.
3. **Genetic changes:** Genetic changes or mutations have been associated with certain cases of acclimation. Acclimation may be phenotypic or genotypic. In the first case genetic information in the cells remain unchanged; only the degree of expression of genes is modified. In the genotypic process a genetic modification is produced and the modified genes are transmitted to the daughter cells.
4. **Inorganic nutrients limitations:** The absence of carbon dioxide in the culture medium may be responsible of acclimation because this one is used by cells in the biosynthesis of complex molecules (purines, pyrimidines, aminoacids, etc.). Other nutrient needs are the nitrogen and phosphate salts.
5. **Toxicity:** During acclimation degraders are accustomed to toxins or inhibitors in their environment.

1.3. Phenol and chlorophenol degrading bacteria

A number of phenol and chlorophenol degrading bacterial species have been isolated from a variety of environments, like mud samples, garden, forest and humus soils. Bacterial isolates known to degrade phenol and chlorophenols include *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Azotobacter*, *Bacillus*, *Comamonas*, *Flavobacterium*, *Mycobacterium*, *Novospingobium*, *Pseudomonas*, *Rhodococcus*, *Rhizobiaceae*, and *Sphingomonas*. Table 2 shows the different species of bacteria so far isolated and identified having phenol and chlorophenol degrading capabilities. Members of the genus *Pseudomonas* are particularly important.

Table 2: Different species of bacteria isolated and identified having phenol and chlorophenols degrading capabilities.

Compound	Bacterial Isolates	References
Phenol	<i>Acinetobacter</i>	Kim and Hao, 1999
	<i>Alcaligenes eutrophus</i>	Hughes <i>et al.</i> , 1984
	<i>Bacillus</i> sp. A2	Mutzel <i>et al.</i> , 1996
	<i>Bacillus stearothermophilus</i>	Buswell, 1975
	<i>Bacillus stearothermophilus</i>	Gurujeyalakshmi and Oriel, 1989
	<i>Bacillus stearothermophilus</i>	Yanase <i>et al.</i> , 1992
	<i>Pseudomonas putida</i>	Bayly and Wigmore, 1973
	<i>Pseudomonas putida</i>	Kotturi <i>et al.</i> , 1991
	<i>Pseudomonas putida</i> EKII	Hintereger <i>et al.</i> , 1992
	<i>Pseudomonas</i> sp.	Bettman and Rehm, 1984
	<i>Pseudomonas putida</i>	Feist and Hegeman, 1969
	<i>Pseudomonas putida</i> DSM 548	Sá and Boaventura, 2001
	<i>Rhizobiaceae</i>	Yanase <i>et al.</i> , 1992
	<i>Rhodococcus</i> sp. P1	Straube, 1987
Mono-chlorophenols	<i>Alcaligenes</i> sp. A 7-2	Westmeier and Rehm, 1985
	<i>Alcaligenes eutrophus</i>	Hill <i>et al.</i> , 1996
	<i>Arthrobacter chlorophenolicus</i>	Westerberg <i>et al.</i> , 2000
	<i>Arthrobacter ureafaciens</i> CPR706	Bae <i>et al.</i> , 1996a
	<i>Azotobacter</i> sp. GP1	Wieser <i>et al.</i> , 1994
	<i>Comamonas testosteroni</i> CPW301	Bae <i>et al.</i> , 1996b
	<i>Pseudomonas</i> sp. B13	Knackmuss and Hellwig, 1978
	<i>Pseudomonas putida</i> CP1	Farrell and Quilty, 2002
	<i>Pseudomonas solanacearum</i> TCP114	Bae <i>et al.</i> , 1997a
	<i>Pseudomonas putida</i>	Dapaah and Hill, 1992
	<i>Pseudomonas pickettii</i>	Kiyohara <i>et al.</i> , 1992
	<i>Pseudomonas pickettii</i> strain LD1	Fava <i>et al.</i> , 1995a
	<i>Pseudomonas putida</i> ATCC 49451	Loh and Wang, 1998
	<i>Rhodococcus opacus</i> GM-14	Zaitsev <i>et al.</i> , 1995
Dichlorophenol and other polychlorinated phenols	<i>Arthrobacter</i> sp.	Chu and Kirsch, 1972
	<i>Flavobacterium</i> sp.	Topp <i>et al.</i> , 1988
	<i>Flavobacterium</i> sp.	Saber and Crawford, 1985
	<i>Flavobacterium</i> ATCC 39723	Pignatello <i>et al.</i> , 1983
	<i>Mycobacterium</i> sp.	Suzuki, 1983
	<i>Novospingobium</i> sp. strain MT1	Tirola <i>et al.</i> , 2002
	<i>Pseudomonas</i> sp.	Slater <i>et al.</i> , 1979
	<i>Pseudomonas</i> sp. Bu34	Lee <i>et al.</i> , 1998
	<i>Pseudomonas cepacia</i>	Banerji and Bajpai, 1994
	<i>Pseudomonas solanacearum</i> TCP114	Bae <i>et al.</i> , 1997a
	<i>Rhodococcus</i> spp.	Hägglblom <i>et al.</i> , 1988
	<i>Rhodococcus erythropolis</i> 1cp	Gorlatov <i>et al.</i> , 1989
	<i>Sphingomonas</i> sp UG30	Cassidy <i>et al.</i> , 1999

1.3.1 Genus *Pseudomonas*

The genus *Pseudomonas* is a diverse group of bacteria that is well known for its ability to cause diseases in plants and animals, and for its role in biodegradation and bioremediation. Pseudomonads are also known for their metabolic activities that allow them to grow under extreme nutrient limitation, as well as to produce commercially and environmentally important products. The genus contains more than 140 species, most of which are saprophytic in soil or water where they play an important role in decomposition, biodegradation, and the carbon and nitrogen cycles. The name Pseudomonad is commonly used to designate a rod-shaped, Gram-negative, nonsporulating, polarly flagellated bacterium. *Pseudomonas* strains are very common in natural habitats, particularly soil, water, spoiled foods, and diseased plants. The capacity of *Pseudomonas* for growth in very simple media and their widespread occurrence makes them appear as prime participants in the process of mineralization of organic matter in nature. Many strains of *Pseudomonas* were isolated from enrichment cultures by using a great variety of low molecular weight organic compounds as the only sources of carbon and energy (Palleroni, 1986).

1.3.1.1 General properties of *Pseudomonas*

Properties of *Pseudomonas* colonies such as size, shape, colour, edge, and surface ornamentations in some instances give important clues for species identification. Colony features of some species can be very striking. For instance, the colonies of many strains of *Pseudomonas stutzeri* are wrinkled and coherent (van Niel and Allen, 1952; Stainer *et al.*, 1966), but these properties frequently are lost by repeated subculture in the laboratory media. *Pseudomonas aeruginosa* strains normally have flat, creamy colonies, which have a tendency to spread over the agar surface. Strains with very mucoid colonies can be isolated from respiratory infections associated with cystic fibrosis. *Pseudomonas aeruginosa* has the capacity to produce alginate, the same property was also found in carbenicillin-resistant strains of the related species

P. fluorescens, *P. putida* and *P. mendocina*, but not in strains of many other species (Govan *et al.*, 1981).

Pseudomonas strains are typically polar flagellated, usually less than 1 µm in diameter and not more than 4-5 µm in length, although some strains (fluorescent plant pathogen, *P. putida*) may have cells which are considerably longer. The insertion and number of flagella are commonly used in species description. Typically, the insertion is polar, but in some instances subpolar attachment is more common. Pilli or fimbriae can be observed in the cells of many species.

Physiological characteristics that have been frequently used to describe *Pseudomonas* species include the oxidase reaction, growth factors requirements, nitrate reduction, denitrification, hydrolysis of gelatin, starch, poly-β-hydroxybutyrate, lecithin, and Tween 80, the arginine hydroxylase reaction, and ring fission mechanisms (Palleroni, 1986).

Organic compounds readily used by many *Pseudomonas* include hydrocarbons, carbohydrates, aliphatic acids, amines, amides, amino acids, aromatic compounds and alcohols. Of these different chemical families, the aromatic compounds appear particularly interesting due to the biochemical intricacies of the various pathways by which they are metabolized by Pseudomonads.

1.3.1.1.1 *Pseudomonas putida* species

Pseudomonas putida is a Gram-negative rod-shaped bacterium with multitrichous flagella. While this species is not pathogenic, it can make a capsule, like many of its pathogenic cousins, this capsule is made of complex polysaccharides and plays a role in helping the bacterium attach to surfaces, and may provide the cell with protection from desiccation and phagocytosis (Ghiorse and Kachlany, 2003).

Pseudomonas putida is a unique soil microorganism, which can resist the adverse effects of organic solvents. In fact, some strains of *Pseudomonas putida* are capable of decontaminating organic substances including solvents (Dapaah and Hill, 1992; Sá and Boaventura, 2001; Farrell and Quilty, 2002). These bacteria display chemotaxis toward a number of aromatic compounds, including environmental pollutants, therefore *Pseudomonas putida* strains are sometime known as pollutant degrading bacteria. Once they detect low concentrations of the compound (e.g., benzoate), they begin swimming toward the source of that compound (Harwood, 2003). It is an example of an organism that can be used to carry out bioremediation. Polluted soil and groundwater purification technology, which uses the power of microbes, renders many toxic organic substances harmless. *Pseudomonas putida* is listed among microorganisms most commonly found in various environments such as various consumer products including paints and solvents. It is also listed as one of the safe and effective pesticidal microorganisms (Marcus, 2003).

Scientists have sequenced the genome of one of nature's most versatile microbes, *Pseudomonas putida* (Nelson *et al*, 2002; Weinel *et al*, 2002). With an appetite for organic pollutants, the soil microbe has the potential to help clean up the environment. The petroleum industry is investigating *P. putida* as a cheap means of purifying fuel, while crop scientists are studying its ability to protect plants from pests and help them grow (Marcus, 2003). The genome sequencing revealed new information about the organism. It has the most genes of any known species involved in breaking down aromatic hydrocarbons. The microbe is already considered 'safe', but knowing its genome will allow scientists to ensure it remains benign when foreign genes are introduced. That could aid efforts to exploit its talents in degrading pollutants and fostering crops. The sequencing revealed at least 80 genes in one family of enzymes, called oxidative reductases, which are involved in breaking down substances in the environment like decomposed connective matter in plants, or lignin. *P. putida*'s single ring-shaped chromosome contains roughly 6.2 million base pairs, about two to three times the average for sequenced bacteria. Of those, 5,420 appear to be genes that produce proteins (Marcus, 2003).

1.3.1.2 Biodegradative properties of *Pseudomonas* spp.

An interesting group of plasmids that specify degradation of diverse groups of compounds such as aliphatic and aromatic hydrocarbons, terpenes, alkaloids, and chlorinated aliphatic and aromatic compounds is the degradative plasmids (Inouye, 1998). Whereas chromosomal genes encode for all essential functions of cells, plasmids carry information for a wide range of biological functions that give the host cell a survival or growth advantage under particular environmental conditions. A number of pathways for degradation of novel compounds is known to be plasmid encoded (Inouye, 1998). Some encode for fairly complete degradations involving a number of enzymes leading to products of central metabolism whereas the other encodes for only a few enzymes. Plasmids encoding degradation of simple organics as well as hydrocarbons and synthetic compounds have been characterized in *Pseudomonas* species, which play a key role in the environment in the biodegradation of natural and man-made toxic chemicals and the plant-bacteria interactions and their resistance to many antibiotics. Genetic research in *Pseudomonas* has been truly impressive. A fairly detailed chromosomal map of *P. putida* is now available (Dean and Morgan, 1983; Sykes *et al.*, 1985). Some catabolic activities of *Pseudomonas* are specified by genes on degradative plasmids. These plasmids occur naturally and are either transmissible or nontransmissible. The degradative plasmids specify a set of genes involved in the biodegradation of organic compounds, such as aliphatic and aromatic hydrocarbons, alkaloids, and chlorinated aromatics (Chakrabarty, 1976; Hass, 1983; Frantz and Chakrabarty, 1986). The existence of degradative plasmids with relaxed substrate specificity of enzymes and the presence of *Pseudomonas* species widespread in the environment give Pseudomonads a variety of important functions.

Being dispensable elements, plasmids can be lost, and some salient properties of bacterial cells may change, such as the capacity for degradation of aromatic or terpenoid compounds, the production of plant hormones, the sensitivity of physical and chemical agents (including antibiotics), the sensitivity to phages, and the

production of or sensitivity to bacteriocins (Palleroni, 1986). Characteristics such as the resistance to antibiotics or the capacity for degradation of certain compounds and intermediates may be coded for by both chromosomal and plasmid genes in some instances, and the respective mechanism may be different. For example, the degradation of aromatic compounds controlled by chromosomal genes in *P. putida*, follows the β -ketoadipate pathway, with *ortho* cleavage of catechol by a 1,2-dioxygenase. The presence of certain plasmids superimposes second pathways for catechol, where this is cleaved by a 2,3-dioxygenase-a *meta* cleavage pathway (Austin and Dunn, 1980).

Pseudomonas putida CP1 degrades monochlorophenols by the modified *ortho*-cleavage pathway. It possesses a large 110 kb plasmid, having a gene for the key enzyme (chlorocatechol 1,2-dioxygenase, E.C. 1.13.11.1, gene *tfdC*) of the modified *ortho*-cleavage pathway (McLaughlin and Quilty, 2000 & 2001).

1.3.1.3 Carbohydrate catabolism in *Pseudomonas* species

Glycolytic pathways of the most thoroughly investigated member *Pseudomonas aeruginosa* have several unique features. Lacking phosphofructokinase, the organisms metabolize three- and six-carbon sugars via a central cycle which includes the Entner-Doudoroff pathway (EDP) enzymes rather than utilizing the fermentation pathway of Embden-Mayerhoff-Parnas (EMP) pathways (Temple *et al.*, 1998). Glucose, gluconate, mannitol, fructose, and glycerol are transported and metabolized by peripheral pathways which feed into a central cycle including EDP enzymes (Figure 5).

1.3.1.3.1 Glucose utilization

Extracellular glucose can be brought directly into the cell by an active transport system and phosphorylated intracellularly by glucokinase to produce the central metabolite 6-phosphogluconate. This route of metabolism is obligatory during

anaerobic catabolism and predominates when glucose concentrations are low (Whiting *et al.*, 1976a; Hunt and Phibbs, 1981 & 1983). Metabolism of glucose by this route is subject to catabolic repression control.

Extracellular glucose may also be metabolized by one of two oxidative routes and enter the central cycle as 6-phosphogluconate. These pathways are predominant during oxidative growth; in fact, gluconate is reported to cause repression of the glucose transport system leading to the phosphorylated route of metabolism (Whiting *et al.*, 1976a & 1976b).

1.3.1.3.2 Fructose utilization

Fructose is brought into the cell via a PEP-dependent phosphotransferase system producing intracellular fructose 1 phosphate (Baumann and Baumann, 1975; Sawyer *et al.*, 1977; van Dijken and Quayle, 1977; Roehl and Phebbs, 1982). This vectorial transfer is unique among carbohydrate uptake mechanisms in this organism. Other carbon sources are accumulated by active transport. The phosphotransferase system appears to be comprised of only two components, a soluble enzyme of 72,000 M_r (EnzI) and a membrane-associated enzyme II. The Hpr-like low molecular weight phosphate carrier protein found in other phosphotransferase systems (PTS) is lacking in *P. aeruginosa*. Intracellular fructose-1 phosphate is subsequently converted to fructose 1,6-bisphosphate by 1-phosphofructokinase, which is co-induced with both components of the fructose 1-phosphotransferase system during growth on fructose. Fructose 1,6-bisphosphate is metabolized primarily via central cycle to pyruvate and glyceraldehyde 3-phosphate. A secondary route of catabolism produces triose phosphate via the lower EMP reactions.

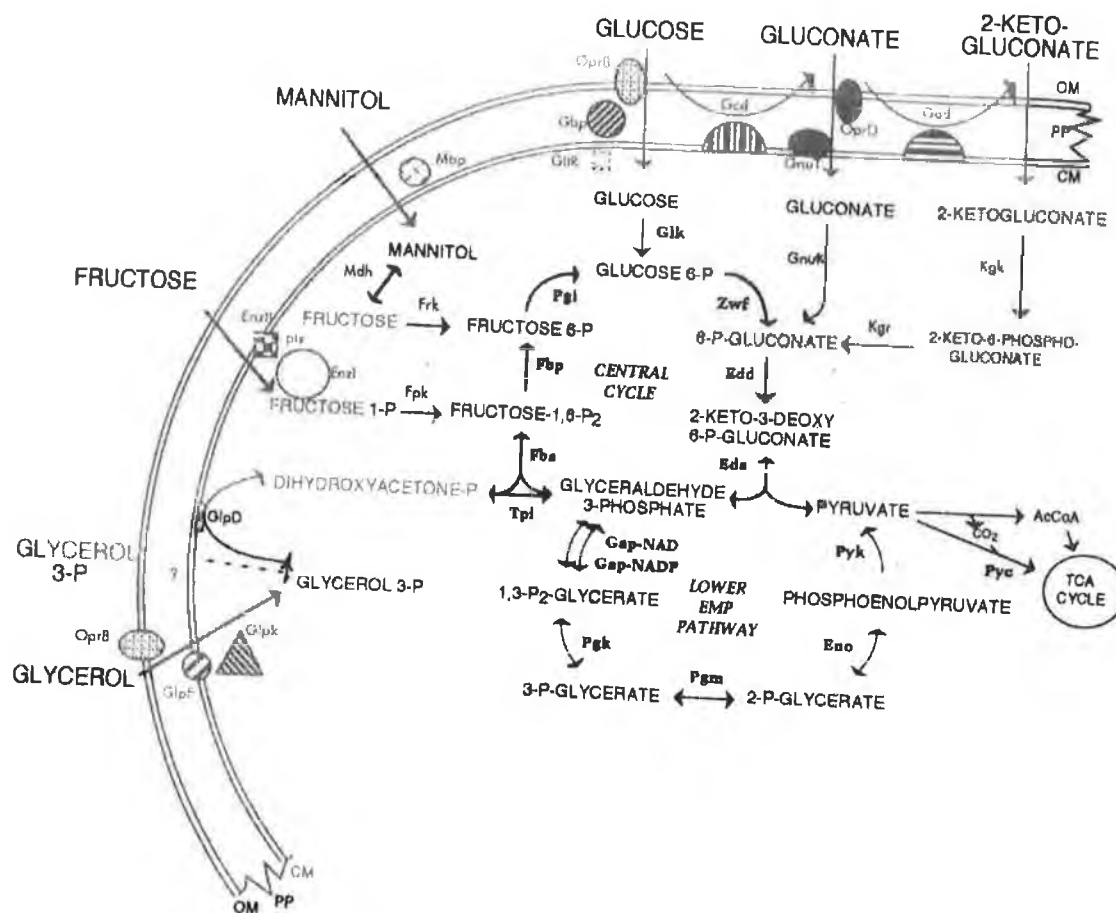


Fig. 5. Pathways of carbohydrate metabolism in *Pseudomonas aeruginosa* (Temple *et al.*, 1998). Abbreviations: OM, outer membrane; CM, cytoplasmic membrane; pp, periplasm; Gad, gluconate dehydrogenase; Gcd, glucose dehydrogenase; Gbp, glucose-binding protein; Mbp, mannitol-binding protein; Kgk, 2-ketogluconate kinase; GnuK, gluconate kinase; GnuT, gluconate permease; Kgr, 2-keto-6-phosphogluconate reductase; Glk, glucokinase; Zwf, glucose-6-phosphate dehydrogenase; Edd, 6-phosphogluconate (Entner-Duodoroff) dehydrogenase; Eda, 2-keto-3-deoxy-6-phosphogluconate aldolase; Pyc, Pyruvate carboxylase; Pyk, pyruvate kinase; Eno, enolase; Pgm, phosphoglucomutase/ phosphoglucoisomerase; Pgk, 3-phosphoglycerate kinase; Gap, glyceraldehyde 3-phosphate dehydrogenase; Tpi, triose phosphate isomerase; Fba, fructose 1,6-bisphosphate aldolase; Fbp, fructose 1,6-bisphosphatase; Pgi, phosphoglucoisomerase; Frk, fructokinase; Mdh, mannitol dehydrogenase; Fpk, fructose 1-phosphate kinase; GlpD, glycerol 3-phosphate dehydrogenase; Glpk, glycerol kinase.

1.4 Morphology of the bacterial cell

The size, shape and arrangement of bacteria, and other microbes, is the result of their genes and thus is a defining characteristic called morphology. Bacteria come in a bewildering and exciting variety of size and shapes, with new ones being discovered all the time. Nature loves variety in its life forms as you can see just looking at your fellow humans. The most common bacterial shapes are rod, cocci and spiral. However, within each of these groups are hundreds of unique variations. Rods may be long, short, thick, thin, have rounded or pointed ends, thicker at one end than the other etc. Cocci may be large, small, or oval shaped to various degrees. Spiral shaped bacteria may be fat, thin, loose spirals or very tight spirals. The group associations of microbes, both in liquid and on solid medium, are also defining. Bacteria may exist mainly as single cells or as common grouping such as chains, uneven clusters, pairs, tetrads, octads and other packets. They may exist as masses embedded within a capsule (Prescott *et al.*, 1993; Anderson, 2003). Figure 6 shows the morphology of a typical bacterial cell.

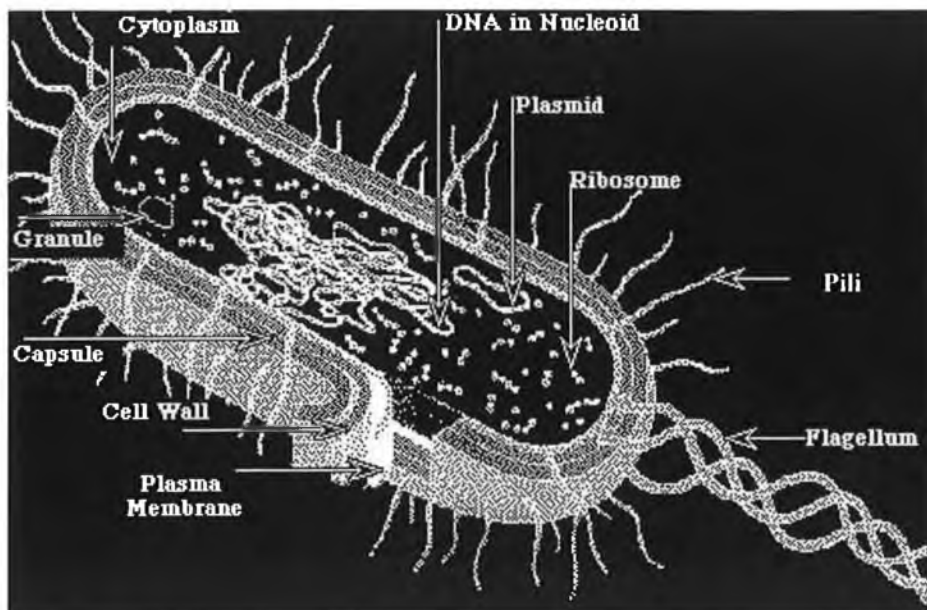


Fig . 6. Morphology of a typical bacterial cell (Anderson, 2003).

A variety of structures are found in bacterial cells. Not all structures are found in every genus. Gram-negative and Gram-positive cells differ with respect to their cell walls in particular.

1.4.1 Overview of a typical bacterial cell wall

The cell wall is one of the most important features of bacterial cells. It is located outside the cell membrane and its main function is to surround and protect the cell, as well as giving the cell its shape and configuration. Some bacteria have long appendages that increase the surface area of the cell and allow it to live in very dilute environments. Any cell that has lost its wall becomes amorphous, or without a defined shape. The cell walls of all bacteria are not identical. In fact, cell wall composition is one of the most important factors in bacterial species analysis and differentiation. There are two major types of walls: Gram-positive and Gram-negative. The wall of Gram positive bacteria are relatively thick (20 to 80 nm), and contain a dense layer of peptidoglycan. The peptidoglycan polymer is composed of an alternating sequence of N-acetylglucosamine and N-acetyl-muraminic acid. Each peptidoglycan layer is connected, or crosslinked, to the other by a bridge made of amino acids and amino acid derivatives. The particular amino acids vary among different species, however. The crosslinked peptidoglycan molecules form a network, which covers the cell like a grid. Also, 90% of the Gram-positive cell wall is comprised of peptidoglycan. The Gram-positive cell wall also contains teichoic acids, which are polymers of glycerol or ribitol joined by phosphate groups. Teichoic acid is covalently linked to muramic acid and links various layer of peptidoglycan mesh together, which comprises of up to 30 to 70% of total dry weight of the cell envelope. On the other hand, Gram negative bacteria have a much thinner cell wall, which is 1-3 nm peptidoglycan layer surrounded by a 7 to 8 nm thick outer membrane and comprises of less than 10% of total dry weight of cell wall. The wall is high in lipid content but low in peptidoglycan. The cell wall of Gram-negative bacteria is much thinner, being comprised of only 20% peptidoglycan. Gram negative cell walls are more complicated than Gram positive because there are two separate areas with an

additional membrane besides the cellular membrane. The outer membrane of Gram negative bacteria is composed of high concentrations of lipids, polysaccharides and proteins. Outside the central membrane is an open area called the periplasmic space. Beyond this a thin layer of peptidoglycan. Finally, external to the peptidoglycan is an additional membrane called the outer membrane. Phospholipids are the predominant lipid component of all biomembranes. They play an important role in the structural and functional organization of the bilayer membrane system. Phospholipids are also necessary for the functioning of many proteins. Phospholipids are also of special importance for the insertion of proteins into the membrane and for their translocation through the membrane (Prescott *et al.*, 1993; Backer, 2002). Figure 7 shows the cross section of two types of bacterial cell walls.

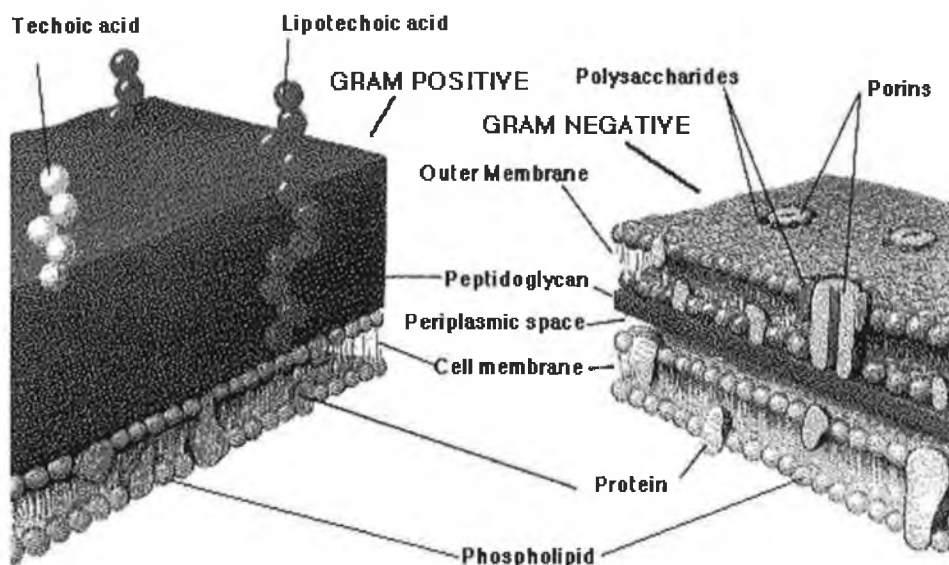


Fig. 7. Cross section of Gram-positive and Gram-negative bacterial cell walls (Backer, 2002).

1.4.2 Toxicity of organic solvents to microorganisms

Organic solvents can be toxic to microorganisms, depending on the inherent toxicity of the solvent and the intrinsic tolerance of the bacterial species and strains (Ramos *et al.*, 2002). Some groups of microorganisms are able to transform organic solvents,

often leading to complete mineralization of these compounds. Although many microorganisms degrade organic solvents at low concentrations, various solvents may become toxic at slightly higher concentrations (Sonja and Heipieper, 2002). Hydrophobic organic solvents are toxic to living organisms because they accumulate in cell membranes and cause disruption. The toxicity of a compound correlates with the logarithm of its octanol/water partition coefficient ($\log P_{ow}$). Substances with a $\log P$ value between 1 and 5 are in general toxic for whole cells (Gibson and Subramanian, 1984; Saito *et al.*, 1993; Sikkema *et al.*, 1995). Such hydrocarbons can only be degraded at low concentrations and, consequently, often stay as persistent pollutants in the environment. Table 3 shows the hydrophobicity of certain solvents, expressed as the $\log P$ value and is a good indicator of toxicity. In addition to hydrophobicity, the molecular structure of a compound also influences its solubility. Many organic solvents are toxic to microorganisms because they partition preferentially in membranes, causing an increase in membrane fluidity and subsequent permeability (Heipieper *et al.*, 1991; Heipieper and de Bont, 1994).

1.4.3 Effect of solvents on microorganisms

The primary site of action of organic solvents is the cell membrane. The cytoplasmic membrane of bacterial cells, a phospholipid bilayer, is a matrix in which various enzymes and transport proteins are embedded. It plays a vital role in solute transport, maintaining the energy status of the cell, regulation of the intracellular environment, turgor pressure, signal transduction and energy transducing processes (Sardesai and Bhosle, 2002).

Most microorganisms tolerate water-miscible solvents such as lower alcohols and acids. In addition very lipophilic natural solvents including some hydrocarbons are not toxic to whole cells. However solvents of intermediate hydrophobicity, such as aromatic solvents are very toxic to cells.

Table 3: Hydrophobicity and toxicity of several solvent classes (Sonja and Heipieper, 2002).

Solvent class	Compound	Log P _{ow} ^a	LD ₅₀ ^b (mmol l ⁻¹)
Short-chain alcohols	Methanol	-0.76	501.1
	Ethanol	-0.28	239.9
	1-butanol	0.9	64.5
Weak acids	Acetic acid	-0.23	60.0
Aromatic alcohols	Phenol	1.5	7.94
	4-chlorophenol	2.4	1.29
	2,4-dichlorophenol	3.2	0.144
	2,4,5-trichlorophenol	4.1	0.016
	Pentachlorophenol	5.1	0.0067
Aromatic solvents	Benzene	2.1	17.38
	Toluene	2.6	3.58
	Styrene	2.9	3.15
	Ethylbenzene	3.2	0.9
	Tetraline	3.9	0.75
Alkenes	Hexadecane	8.8	>1000

Log P_{ow}^a values measured with goldfish GFS cells.

LD₅₀^b values measured with *Pseudomonas putida* cells.

Although no individual analytical technique is able to determine fully the effects of solvents on membranes, several mechanisms of membrane toxicity have been reported.

1. The accumulation of organic solvents leads to a permeabilization of the cell membrane.
2. The energy status of the cell is reduced.

3. Besides the proteins engaged in energy transduction, the accumulation of solvents into a membrane also affects the function of other proteins embedded in the membrane.
4. Organic solvents also affect the fluidity of the membrane, an important aspect of membrane structure.
5. Membrane-active compounds can affect the hydration characteristics of the membrane surface and the thickness of the membrane (Sonja and Heipieper, 2002).

It can be concluded that once a solvent has dissolved in a membrane, it will disturb the integrity of the membrane and hence its function as a barrier, as matrix for enzymes and as energy transducer.

1.4.4 Adaptation of microorganisms to organic solvents

Despite the general toxic effects of organic solvents, some microbial strains can adapt to high concentrations of toxic organic solvents. Organic-solvent tolerant bacteria are a relatively novel group of bacteria. Some of the strains are able to metabolize the solvents as sole carbon sources, whereas others have to use alternate carbon and energy sources. Changes in the membrane composition play a crucial role in the mechanism of solvent tolerance. Several adaptive changes in the structure of the membrane have been observed in the accumulation of organic solvents in the membrane of microorganisms (Sardessai and Bhosle, 2002; Sonja and Heipieper, 2002). Sonja and Heipieper (2002) described the following adaptation mechanisms that protect bacterial cells against toxic effects of organic solvents.

- a) Changes in the structure of the cytoplasmic membrane,
- b) Modification of lipopolysaccharide or porines of the outer membrane,
- c) Reduction of cell wall hydrophobicity,
- d) Active export of the solvents, and
- e) Transformation of solvents.

1.4.4.1 Adaptation at the level of the cytoplasmic membrane

In the cytoplasmic membrane, changes in the level of the lipids and protein have been observed. These adaptations reestablish the stability and fluidity of the membrane once it is disturbed by solvents (Weber and de Bont, 1996). Several mechanisms have been described, which may even vary from strain to strain. Mechanisms described so far are: a) alterations in the length of the fatty acids chains, b) changes in the degree of the saturation of fatty acids, c) *cis/trans* isomerization of unsaturated fatty acids, d) composition of phospholipid head groups, and e) dynamics of the phospholipid turnover.

Generally microbial cells react to the presence of organic solvents by increasing the amount of saturated fatty acids in the membrane (Ingram, 1977). Alterations in the degree of saturation of fatty acids change the fluidity of the membrane and in this way compensate for the effects caused by solvents. Most bacteria are only able to change their membrane fluidity by *de novo* synthesis of membrane lipids with a different ratio of saturated to *cis*-unsaturated fatty acids during growth. Bacteria are not able to perform a postbiosynthetic modification of membrane fluidity. However, some representative genera *Pseudomonas* and *Vibrio* are able to change their membrane fluidity by isomerization of *cis*-unsaturated fatty acid to *trans*-unsaturated isomers (Heipieper *et al.*, 1992; Heipieper *et al.*, 1995; Keweloh and Heipieper, 1996). This conversion also takes place in non-growing cells. The isomerization of the double bond is a special mechanism of these bacteria to adapt to high concentrations of toxic substances under conditions not allowing growth and *de novo* synthesis of lipids. The benefit of this reaction lies in the steric differences between *cis* and *trans*-unsaturated fatty acids. Unsaturated fatty acids in the *cis*-configuration with their bent steric structures (a nick in the acyl-chain) result in a membrane with a relatively high fluidity. In contrast, the long extended steric structure of the *trans*-configuration is able to insert into the membrane structures similar to saturated fatty acids, which also possess a long extended conformation allowing denser packing of the membrane.

Apart from the fatty acid composition, the headgroups of lipids are also altered during solvent adaptation (Weber and de Bont, 1996; Fang *et al.*, 2000a). Next to changes in membrane composition, the dynamics of biosynthesis of membranes may play an important role in solvent tolerance (Pinkart and White, 1997). Apart from changes in the composition of the cytoplasmic membrane and in the dynamics of the formation of phospholipids, alterations in the protein content have been observed as a response of solvents.

1.4.4.2 Adaptation at the level of the outer membrane

The Gram-negative bacteria have an additional outer membrane, compared to the single cytoplasmic membrane of Gram-positive bacteria. Therefore Gram negative bacteria are better equipped to cope with the solvent shock (Inoue *et al.*, 1991). The outer membrane was shown to be engaged in promoting solvent tolerance (Weber and de Bont, 1996). In some instances, solvent adaptation was related to less hydrophobicity (Kobayashi *et al.*, 1999) and it was also shown that a reduction of the cell hydrophobicity correlates with the change in the lipopolysaccharide content (Pinkart *et al.*, 1996; Aono and Kobayashi, 1997). In addition to changes in lipopolysaccharide, the outer membrane protein, porins have also been related to the tolerance of solvent (Li *et al.*, 1995; Ramos *et al.*, 2002). The organic solvent molecules are able to pass through the porins. Therefore the mutant lacking these porins have a higher tolerance to solvents.

1.4.4.3 Adaptation at the level of the cell wall

Bacteria with hydrophobic cell walls are shown to have a higher affinity for hydrophobic compounds. Thus the alteration of the cell wall may lead to changes in the sensitivity to solvents. Cell wall modifications that lower the hydrophobicity of the cell may provide a higher tolerance to solvents (van Loosdrecht *et al.*, 1990; Jarlier and Nikaido, 1994). van Loosdrecht *et al.* (1990) and Jarlier and Nikaido

(1994) showed that bacteria with hydrophobic cell walls have a higher affinity for hydrophobic compounds than bacteria with more hydrophilic cell walls. Thus, the changes of cell walls from hydrophobic to hydrophilic could provide a means of protecting the organism with a shield against toxic solvents.

1.4.4.4 Adaptation caused by active excretion of solvents

Microorganisms have evolved different devices to detoxify and extrude toxic compounds in order to protect themselves. One group of such devices comprises the multi-drug resistance (MDR) efflux systems, which catalyze the active extrusion of many structurally and functionally unrelated compounds from the bacterial cytoplasm (or internal membrane) to the external medium. Some of the substances of these MDR pumps are xenobiotics that do not resemble any of the known natural substrates. The physical characteristics of the compounds (e.g. charge, hydrophobicity, or amphipathicity), the van der Waal's interactions they established with the active sites and effector pockets, and the flexibility of these sites in the target proteins determine the specificity of these multidrug efflux systems (Ramos *et al.*, 2002).

Four main families of multi-drug resistance transporters have been identified. All efflux pumps for organic solvents identified so far in Gram-negative bacteria belong to the resistance-nodulation-cell division (RND) family (Paulsen *et al.*, 2001). The RND pump of Gram-negative bacteria exports toxic substances across both membranes of the cell envelope in a single energy-coupled step. These efflux pumps are made of three components: a cytoplasmic membrane export system that acts as an energy-dependent extrusion pump, a membrane fusion protein (MFP), and an outer membrane factor (OMP) (Zgurskaya and Nikaido, 1999; Koronakis *et al.*, 2000). Active efflux as a mechanism of solvent tolerance has been observed for a number of *Pseudomonas* strains (Isken and de Bont, 1996 & 2000; Ramos *et al.*, 1997; Kim *et al.*, 1998).

1.4.4.5 Adaptation caused by biodegradation of solvents

Many toxic solvents studied can be degraded by microorganism, therefore tolerance could be mediated by degradation of the solvents. Many of the solvent tolerant strains are able to cope with a broad range of solvents, which often can not be biodegraded by these strains. *Pseudomonas putida* S12 is tolerant of both styrene and toluene. Of these solvents only styrene can be metabolized (Weber *et al.*, 1993). Hence, degradation may mediate the resistance of some strains to specific solvents, but it can not be the main mechanism contributing to the tolerance of a broad range of solvents (Sonja and Heipieper, 2002).

1.5 Flocculation or Aggregation

Flocculation or aggregation is the gathering together of units to make a larger unit. In biology cell flocculation is defined as the gathering of cells to form a fairly stable, contiguous, multicellular association under physiological conditions. Thus aggregation or flocculation contains two principal units: physical movement and stable multicellular contact. Microbial metabolic activity can change after the formation of a floc, as adhered microorganisms sometimes display a higher metabolic activity (McFeters *et al.*, 1990), and flocculated microorganisms also display a high degree of resistance to biocidal compounds (Costerton *et al.*, 1987; Giwerzman *et al.*, 1991; Anwer *et al.*, 1992). Microbial flocculation can be brought about by the production of complex mixture of macromolecules including exopolysaccharides, protein, DNA and RNA (Sutherland, 2001).

Wingender and Flemming (1999) listed the following components in the floc or biofilm:

1. Microorganisms: heterotrophic and autotrophic bacteria, algae, protozoa, and fungi.
2. Extracellular polymeric substance: polysaccharides, proteins, glycoproteins, nucleic acids, lipids, and humic substances.

3. Biogenic particles: cellular debris, detritus.
4. Inorganic particles: clay, sand, slit, corrosion products.
5. Inorganic ions: multivalent ions (e.g. Ca^{2+} , Mg^{2+} , SO_4^{2-} , Fe^{3+}).
6. Dissolve organic molecule: amino acids, sugars, benzene, toluene, xylene.
7. Water: free, bound.

Cell aggregation in the microbial world can be classified into homotypic and heterotypic systems. A homotypic system is made up of similar units, whereas a heterotypic system is made up dissimilar units. Flocculation of brewer's yeast during fermentation is homotypic. Co-flocculation of yeast with bacteria is heterotypic. Microbial aggregation systems are distributed among many microbial taxa. They are found in both prokaryotes and eukaryotes (Calleja, 1984).

Bacterial adhesion was defined as a two-step event by Zita and Hermanson (1994): reversible adhesion, due to long-distance forces and irreversible adhesion mediated by direct contact between the surfaces, such as hydrophobic interactions due to bacterial surface structures. Bivalent cations, like calcium play an important role for the formation of bridges between the negatively charged bacteria and exopolymers (Zita and Hermanson, 1994).

Bossier and Verstraete (1996) described the following conditions that can trigger microbial aggregation.

1. Aggregation by substrate acquisition: In oligotrophic environments like activated sludge, where bacteria are fed at near starvation rate. There an aggregate might be a nutrient rich place. Exopolysaccharides, primary constituents of aggregate (Urbain *et al.*, 1993; Jorand *et al.*, 1994), can trap nutrients from the bulk liquid by loose binding. Cell death and, or lysis can also form a source of nutrients (Costerton *et al.*, 1987).
2. Aggregation by slow growth or starvation: Depletion of substrates, leading to slow growth or starvation has been associated with aggregation. Bacteria become

more hydrophobic during starvation which facilitates aggregation (Sanin *et al.*, 2003).

3. Aggregation by physical and chemical stress: Microorganisms growing in aggregates are sheltered from environmental assaults, like chemical or physical stresses (Bossier and Verstraete, 1996; Farrell and Quilty, 2002).
4. Aggregation to protect against predation: Bacterial cells living in aggregates are physically better protected against protozoan predation (Martz *et al.*, 2002).

1.5.1 Importance of microbial flocculation in bioremediation

The ecological importance of microbial aggregation appears to lie in its contribution to the microbiota of a habitat. As an alternative state of a microbial population, cell aggregation may add to the viability of a biotic community beleaguered by unpredictable stresses. Flocculation by natural and synthetic polymers is a large part of waste treatment by the activated sludge process. It is of minor significance in the trickling filter process (Calleja, 1984).

The ability of microbes to aggregate is one of great importance in the bioremediation of toxic chemicals. In the activated sludge process, microbes capable of flocculation have distinct advantages over nonflocculating organisms as they remain within the sludge units for longer periods of time through biomass recycling (Farrell and Quilty, 2002).

Aerobic wastewater treatment relies heavily on the ability of microorganisms to aggregate, allowing a straightforward separation of the formed microbial biomass and the effluent in the final settling tank (van Limbergen *et al.*, 1998).

1.5.2 Induction of microbial aggregation

Microbial aggregation is a surface phenomenon (Zita and Hermanson, 1994). The cell surfaces, be it membrane or wall or mural appendages, must undergo some change to allow and promote cell to cell interaction. Whether or not the change is in fact for the purpose of bringing the cells together. There are four major factors that induce microbial flocculation (Calleja, 1984). The first two categories comprise conditions inherent to the cell, the other two external to it, these are:

Genetic conditions: The information for aggregation must reside in the genetic makeup of the organism involved. Despite its widespread distribution, the capacity for aggregation is not found in all microbes. Not all species are aggregative. Within a species, strains differ in their capacity for aggregation. Some are more aggregative and some are less. Even among members of a large clone, there are deviants. Mutations followed by selection may convert a genetically competent strain to one that is not, an incompetent strain to one that is.

Physiological conditions: Microbial aggregation is inducible, and present only when the cells are physiologically competent, only at a specific portion of the population growth curve or only at a particular stage of the life cycle. Genetically competent organisms may be physiologically competent only during a stretch of time in the cell cycle or the life cycle. Conditions that favour cell proliferation do not make for good aggregation. Another aspect of the physiological state of the cell is the development of some other function upon which aggregations depends.

Environmental conditions: Aggregation occurs at a given time in the population growth curve. The environment in which the cells find themselves must allow aggregation to occur, and the environment must allow the development of competence among the cells physiology poised to be induced. The environmental conditions include physical, mechanical, and chemical effectors. Principal among them are light, temperature, agitation, cell population density, aeration, ionic strength,

pH, nutrition, energy source, pheromones, metabolic inhibitors, and stimulators. Their effect may be either direct or indirect.

Manipulative conditions: The manipulative condition is not trivial, because in many systems, it constitutes the zero hour of a time-course experiment. By manipulation, one may bring about aggregation of already induced cells. This must be distinguished from induction of cells to competence for aggregation. Failure to do so has brought much confusion in the literature. The mixing of already competent mating types is simply induction by manipulation, and so reflocculation of mechanically dispersed flocs or of heat-killed cells.

1.5.3 Flocculation of *Pseudomonas* species

Floc formation has been observed in several *Pseudomonas* strains (Sakka *et al.*, 1981; Sakka and Takahashi, 1981 & 1982; Jahn *et al.*, 1999; Farrell and Quilty, 2002; Matz *et al.*, 2002; Sanin *et al.*, 2003).

DNA as a flocculation factor for *Pseudomonas* sp. C-120 was reported (Sakka and Takahashi, 1981 & 1982). They worked with the deoxyribonuclease (DNase)-susceptible floc forming bacterium *Pseudomonas* sp. strain C-120 and concluded that the component involved in flocculation of that organism was highly polymerized double stranded DNA.

In the presence of a flagellate predator a sub-population of *Pseudomonas* sp. CM10 was able to form cell linkages by means of exopolysaccharides (Matz *et al.*, 2002). As cells aggregates exceeded the size of the flagellate predator they became inedible. Sanin *et al.* (2003) reported the aggregation of *Pseudomonas* sp. A (isolated from municipal sewage) and *Pseudomonas* sp. D (isolated from soil) during starvation. They described the aggregation as due to the production of exopolysaccharides.

The biofilm of a *Pseudomonas putida* was analyzed for the composition of water-soluble extracellular polymeric substances (Jahn *et al.*, 1999). The macromolecular composition of the biofilm matrix was heterogenous. Protein was found to be the largest fraction of the extracted polymers, polysaccharides and DNA were found to be the other major components.

Substrate dependent autoaggregation in *Pseudomonas putida* CP1 was reported by Farrell and Quilty (2002). The organisms formed clumps during growth on higher concentrations of chlorophenols but no clump was noted when grown on phenol. They described that *Pseudomonas putida* CP1 flocculated as a result of chemical stress.

A clump forming bacterium *Pseudomonas* sp. KEWA-1 was isolated from a water treatment plant, which was hydrophobic and formed an extensive capsule while clumping in a dilute medium (Singh and Vincent, 1987).

1.6 Aims of the Project

Earlier studies in the laboratory had indicated that *Pseudomonas putida* CP1 had the capability to grow on all three mono-chlorophenol isomers. The aims of this project were to study;

- The ability of the bacterium to grow on various concentrations of mono-chlorophenols when supplied as the sole carbon source
- The influence of additional sources of carbon on the degradation of mono-chlorophenols and
- The response of the organism to environmental stress at a morphological and cellular level.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Source of *Pseudomonas putida* CP1

The isolate *Pseudomonas putida* CP1 was obtained from Dr. Favio Fava, University of Bologna, Italy. It was originally isolated from nature in the United States and identified later in our laboratory by Dr. Alan Farrell (Farrell, 2000).

2.1.2 Chemicals

Chemicals were obtained from a number of sources including Reidel-de-Haen, BDH, Sigma-Aldrich, Lab-Scan, BioTrend. For further details of the chemicals see Appendix A.

2.1.3 Media

All media unless otherwise stated were obtained from Oxoid. All media were sterilized by autoclaving at 121⁰C for 15 minutes.

***Pseudomonas* Minimal Medium:**

The ingredients of the minimal medium (Goulding *et al.*, 1988) were combined in distilled water and the pH was adjusted to 7.0 with 2M NaOH. The trace salts solution was prepared separately in distilled water and was stored in a dark bottle for 6-8 weeks. The mono-chlorophenols/ phenol were added to the minimal medium after sterilization. The ingredients of the minimal medium are as follows:

	g ^l ⁻¹

K ₂ HPO ₄	4.360
NaH ₂ PO ₄	3.450
NH ₄ Cl*	1.000
MgSO ₄ .6H ₂ O	0.912
pH	7.000

Trace salts solution was added at a concentration of 1ml⁻¹

* 1.26 g^l⁻¹ (NH₄)₂SO₄ was used instead of 1.0 g^l⁻¹ NH₄Cl whenever chloride release was monitored.

The composition of the trace salts solution was as follows:

	g/100ml

CaCl ₂ .2H ₂ O	4.77
FeSO ₄ .7H ₂ O	0.37
CoCl ₂ .6H ₂ O	0.37
MnCl ₂ .4H ₂ O	0.10
Na ₂ MoO ₄ .2H ₂ O	0.02

Chlorophenol / phenol agar:

Pseudomonas minimal medium was prepared as described above. Bacteriological agar at a concentration of 1% (w/v), was added to the minimal medium. Following sterilization by autoclaving at 121⁰C for 15 minutes, the agar was allowed to cool. Immediately prior to pouring, chlorophenol / phenol was added to the agar to give the appropriate concentrations.

Media for Biodegradation Studies:

Chlorophenol/ phenol broth was used for biodegradation studies, the composition of which was exactly similar to the chlorophenol / phenol agar except that no agar was added to it. Separately sterilized (by autoclaving at 121⁰C for 15 minutes) additional carbon and nitrogen were added to the sterilized minimal media to give the appropriate final concentration of the added substrates, where appropriate.

2.1.4 Buffers

Potassium Phosphate Buffer:

Potassium phosphate buffer was prepared as outlined in Standard Methods for the Examination of Water and Wastewater (1998). The following ingredients were combined in distilled water. The pH of the resulting solution should be 6.8.

	g l ⁻¹
K ₂ HPO ₄	104.5
KH ₂ PO ₄	72.3

Sodium Phosphate Buffer:

Sodium phosphate buffer (0.01 M) was prepared by dissolving Na₂HPO₄ (0.01 M) and NaH₂PO₄ (0.01 M) in distilled water. The pH of the resulting solution should be 7.0.

Tris-HCl Buffer:

50 mM Tris-HCl buffer (50 mM) was prepared by dissolving tris (50 mM) in distilled water and adjusting the pH to 8.0 with 2 M HCl.

Citric acid – Na₂HPO₄, (for pH Studies):

This wide range buffer (pH 2.6 to 8.0) was prepared by combining X ml of 0.1M citric acid and (100 - X) ml of 0.2M Na₂HPO₄. The pH of the resulting solution will be dependent on the amount of citric acid added, for example:

X (ml)	81.1	49.0	17.8	2.8
pH	3.0	5.0	7.0	8.0

Sodium Phosphate Buffer, NaH₂PO₄ – NaOH (for pH studies):

Sodium phosphate buffer (pH 5.8 to 8.0) was prepared by adding X ml of NaOH (0.2 M) to 50 ml of NaH₂PO₄ (0.2 M) and diluting to 100 ml distilled water. The pH of the resulting solution will be dependent on the amount of NaOH added, for example:

X (ml)	3.5	13	30	47
pH	5.8	6.4	7.0	8.0

Phosphate Buffer, Na₂HPO₄ -NaH₂PO₄ (for pH studies):

Phosphate buffer (pH 5.8 to 8.0) was prepared by adding x ml 0.2 M Na₂HPO₄ and (50 - X) ml 0.2 M NaH₂PO₄ and diluting to 100 ml. The pH of the resulting solution will depend on the amount of Na₂HPO₄ added, for example:

X (ml)	4.0	13.25	30.5	47.35
pH	5.8	6.4	7.0	8.0

Phosphate Buffer (for epifluorescence microscopic studies):

13.6 g KH_2PO_4 was dissolved in MilliQ water and diluted to 1 L. The pH was adjusted to 7.2 if necessary and filtered through a 0.2 μm membrane filter.

2.2 Methods

2.2.1 Maintenance of *Pseudomonas putida* CP1

The bacterium was maintained on chlorophenol agar by plating on *Pseudomonas* minimal medium incorporating Oxiod Bacteriological Agar Number 1 (1%, w/v) together with different isomers of mono-chlorophenols. The organism was kept at 4°C for around 1 month and then sub-cultured.

2.2.2 Cultural conditions for the biodegradation studies

Pseudomonas putida CP1 was grown overnight in nutrient broth, centrifuged at 5000 rpm for 10 minutes and washed twice with 0.01M sodium phosphate buffer. Five ml was used to inoculate 95 ml sterile minimal medium (Goulding *et al.*, 1988) containing 2-chlorophenol, 3-chlorophenol and 4-chlorophenol in 250 ml conical flasks. Where added carbon and nitrogen effects were being studied, the concentrated stock solution of the added substrates were separately sterilized by autoclaving prior to their addition to the sterilized minimal medium to give the appropriate final concentration of the added substrates. After inoculation, flasks were incubated in an orbital shaker at 150 rpm at 30°C. Control flasks were run in parallel. Samples were aseptically removed at regular intervals and analyzed for growth and pH. Samples were then centrifuged at 5000 rpm for 10 minutes, the supernatants were then analyzed for phenol/ chlorophenol removal, chloride release, COD, and for reducing sugar where appropriate.

2.2.3 Measurement of growth of *Pseudomonas putida* CP1

Growth of *Pseudomonas putida* CP1 was monitored by using a number of methods including optical density measurement at 660 nm, dry weight measurement, measurement of bacterial number by the pour plate method and the direct epifluorescence microscopic method for the determination of both viable and nonviable cells.

Deflocculation of *Pseudomonas putida* CP1 flocs

P. putida CP1 flocs were disrupted by sonication prior to measurement of cell numbers. The procedure was as follows: 100 ml of minimal medium containing flocculated *P. putida* CP1 samples was taken in a glass beaker and dispersed with an ultra sound probe (Labsonic 2000 U, Standard 19 mm probe) by using low power (50 W output) for 30 sec. Beaker containing suspensions were kept in crushed ice for 15 min before and through out the treatment. Deflocculation was observed microscopically.

Cell lysis was investigated using the method of Biggs and Lant (2000). Activity of the intracellular enzyme glucose-6 phosphate dehydrogenase (G6PDH) was used to identify the cell lysis. If the enzyme is detected in the supernatant of culture fluid, cell lysis is assumed to have occurred. The technique involved adding 1 ml of 0.002 M NAD (Sigma Chemicals) and 1 ml of 0.002 M NADP (Sigma Chemicals) to 2 ml of 50 mM Tris-HCl buffer (pH 8.0) and incubating at 37°C for 5 min. 1 ml of supernatant was added to the mixture and incubated for further 5 min. Finally, 1 ml of 0.04 M glucose-6-phosphate was added and incubated at 37°C. The absorbance was measured every 10 min for 1 hour. A standard solution (5 ppm) of glucose-6-phosphate dehydrogenase was prepared and absorbance at 340 nm was read identical manner.

2.2.3.1 Measurement of bacterial numbers by the pour plate method

Samples were serially diluted and poured in triplicate using plate count agar. Plates were incubated in 30⁰C for 2 days and counted. The bacterial count was expressed as colony forming units per ml (cfu/ml).

2.2.3.2 Dry weight measurement

A specific volume of suspended culture was filtered through two tarred filters (Whatman GF/C and Gelman 0.2 μ m, 47 mm membrane filters), dried at 85⁰C for 2 hours and then reweighed.

2.2.3.3 Optical density measurement

Growth was monitored turbidimetrically by noting the optical density (OD) at 660 nm using a Unicam UV/VIS spectrophotometer. OD values could be expressed as dry weight or as cell number, where appropriate as described in Figure 8.

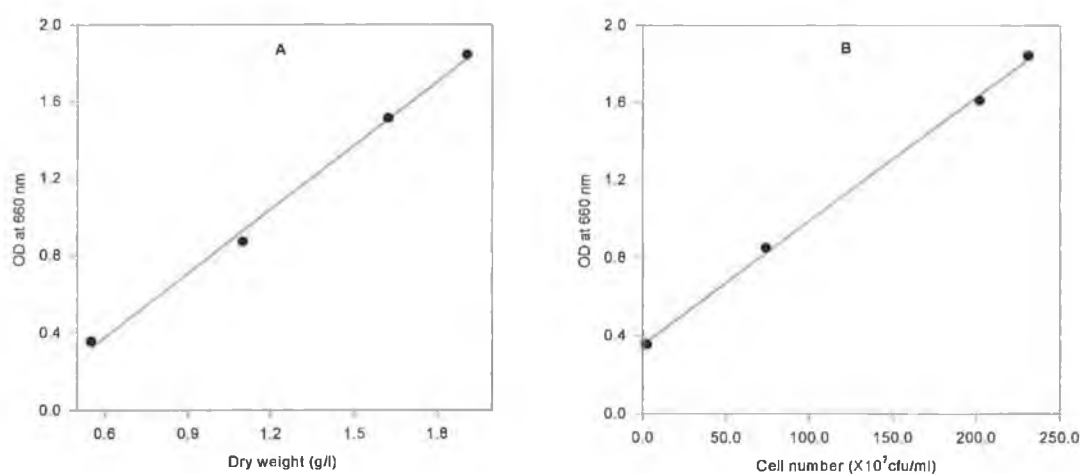


Fig. 8. Standard curves for the conversion of optical density at 660 nm to dry weight (A) and optical density to cell number (B) for *P. putida* CP1 grown on 0.5% (w/v) glucose in minimal medium for 18 hours at 30⁰C.

2.2.3.4 The determination of *Pseudomonas putida* CP1 cell number using Epifluorescence Microscopy

Procedure:

The samples were collected and immediately diluted ten fold with quarter strength Ringer Solution and the viable and nonviable bacterial count was determined according to the method described by Bitton *et al.* (1993).

One ml of the diluted suspension was mixed with 1 ml of fluorochrome (0.1%, w/v acridine orange solution) in sterile test tubes. The fluorochrome was sterilized by using a 0.2 µm pore size disposable sterile syringe filter. After a 2 minute contact time, the bacteria were recovered by vacuum filtration through 0.2 µm black polycarbonate membrane filters (Millipore GTBP 04700) which were covered with a small volume of filtered MilliQ water. The filter was washed with 5 ml phosphate buffer Phosphate Buffer (buffer for epifluorescence microscopic studies) and then filtered again. The filter was removed with a forceps and air dried for 10 to 15 minutes. The filter was placed on a drop of immersion oil (non/low fluorescing) on a clean glass microscopic slide. A small drop of immersion oil was added to the filter surface and the filter was covered gently with a clean glass cover slip. As acridine orange is a carcinogenic agent, gloves were used throughout the experiment.

At least 10 randomly selected fields containing 10-50 cells were examined on the filter using the 100X oil immersion lens. Live cells fluorescence orange-red whereas dead cells fluorescence green.

The number of bacteria per ml of sample was calculated using the formula (Boulos *et al.*, 1999):

$$T = N \times A / a \div V$$

Where, T = number of bacteria / ml,

N = average number of bacteria / field,
A = area of the filter (mm²),
a = area of the microscopic field (mm²),
V = volume of the sample filtered (ml).

Pictures for documenting cell shape and size were taken by using a JVC KY-F55B colour video camera (Vistor Company of Japan Ltd., Japan) attached to a Zeiss Axioplan Epifluorescence Microscope equipped with a Zeiss filter 09 at 1000X magnification. The images were stored as RGB files (red, green and blue bands with intensity ranges of 0-225) and processed with software Optimas 6.5 (Media Cybernetics, WA) using a Dell Dimension XPS D300.

2.2.4 Measurement of pH

The pH was measured by using an Orion 420A pH meter.

2.2.5 Assay of mono-chlorophenols and phenol

Mono-chlorophenols and phenol concentrations were determined by using the 4-aminoantipyrene colorimetric method based on the procedure detailed in Standard Methods for the Examination of Water and Wastewater (1998). Samples were taken from the inoculated flasks at different time intervals. Cells were removed by centrifugation at 5,000 rpm for 10 minutes and the supernatant was diluted if necessary. A range of standards was prepared containing 0.0 to 0.05 mg of chlorophenols in 10 ml of distilled water. The samples and standards were placed in test tubes and 0.25 ml of 0.5N NH₄OH was added to each tube. The pH was adjusted to 7.9 ± 0.1 by using potassium phosphate buffer (pH 6.8). 2% (w/v) 4-aminoantipyrene solution (100 µl) was added and mixed well. Finally 100 µl of 8% (w/v) potassium ferricyanide was added to the tubes and mixed well. The tubes were allowed to stand at room temperature for 15 minutes and the absorbance was read at

500 nm. The concentrations of mono-chlorophenols and phenol were calculated from the standard curves of the corresponding substrates (Fig. 9).

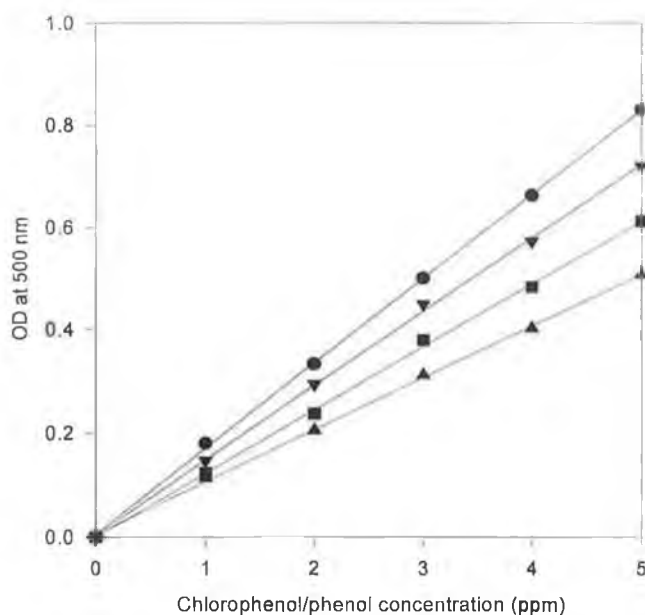


Fig. 9. Standard curves for mono-chlorophenols and phenol using the 4-aminoantipyrine colorimetric assay. Symbols: ●, 2-chlorophenol; ■, 3-chlorophenol; ▲, 4-chlorophenol and ▼, phenol.

Some loss of 2-chlorophenol was detected during incubation due to the volatilization, which was corrected accordingly. No loss of 3-chlorophenol, 4-chlorophenol or phenol was found.

2.2.6 Reducing Sugar Estimation

Bacterial cells were removed from samples by centrifugation (13,000 rpm for 4 minutes) before assaying. The glucose concentrations were determined by the dinitrosalicylate (DNS) colorimetric method (Miller, 1959). 1 ml of standard and suitably diluted samples and 1 ml of water was placed in a test tube. 2 ml of D.N.S. reagent was added. The tubes were capped and placed in boiling water bath for 10

minutes. The tubes were then readily cooled and 10 ml of water added to each. Absorbance was read at 540 nm. The glucose concentrations were determined from the calibration curve of corresponding known standard concentrations. A sample standard curve is presented in Figure 10.

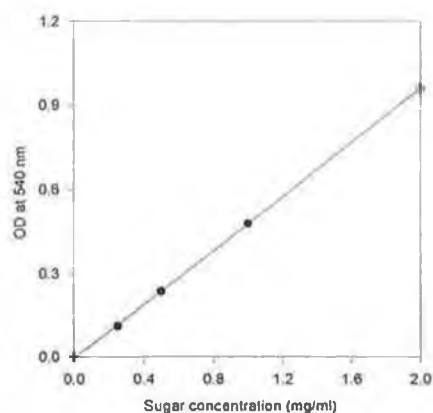


Fig. 10. Standard curve for reducing sugar estimation using the dinitrosalicylate colorimetric method.

DNS Reagents:

1 g 3,5-dinitrosalicylic acid

30 g potassium sodium tartarate (for long time storage)

50 ml distilled water

16 ml 2.5 M sodium hydroxide

Warm to dissolve (do not boil). When dissolved, cool and make volume up to 100 ml with distilled water.

Reducing sugar stock solution: 2.0 g reducing sugar was dissolved in 100 ml distilled water and stored at 4°C.

Reducing sugar standard solution: Dilute 10 ml of stock reducing sugar solution to 100 ml. This gives a final concentration of 2 mg/ml.

2.2.7 Chloride assay

Chloride release was analyzed with an Orion chloride specific electrode (model 9417). The electrode was calibrated with standard NaCl solutions (0.1M and 0.01M) and chloride concentrations were calculated using the direct readout capability of an Orion benchtop pH/ISE meter (model 920A). Samples and standards were diluted with 2% (w/v) 5M NaNO₃ ionic strength adjusting solution.

2.2.8 Chemical Oxygen Demand (C.O.D.)

The chemical oxygen demand (C.O.D.) was determined using a modification of the method outlined in Standard Methods for the Examination of Water and Wastewater (1998).

Reagents:

Digestion solution (Potassium dichromate solution):

K₂Cr₂O₇ (4.913 g), previously dried at 85⁰C for 2 hours, was dissolved in distilled water. Concentrated H₂SO₄ (167 ml) and HgSO₄ (33.3 g) were added, dissolved and the reagent was allowed to cool. The reagent was then diluted to 1000 ml.

Sulphuric acid reagent:

AgSO₄ (5.5 g) was added to H₂SO₄ (545 ml) and allowed to stand for 2-3 hours.

FAS reagent (Ferrous Ammonium Sulphate reagent):

Fe(NH₄)₂(SO₄)₂.6H₂O (0.02 M) was dissolved in distilled water. Concentrated H₂SO₄ (20 ml) was added and the solution was allowed to cool. The reagent was then diluted to 0.02 M.

Potassium Hydrogen Phthalate Standard:

Potassium Hydrogen Phthalate (425 mg), previously dried at 103⁰C for 2 hours, was dissolved in 1 litre of distilled water. This solution should give a C.O.D. of 500 mgO₂l⁻¹.

Procedure:

C.O.D. tubes and caps were washed with 20% (v/v) H₂SO₄. Suitably diluted sample and standard (2.5 ml) was added to the tube. Digestion solution 1.5 ml was carefully added. Sulphuric acid reagent (3.5 ml) was then carefully added down the side of the tube. The caps were screwed on tightly and the tubes were inverted with care to mix the contents. The tubes were placed in preheated digestion block at 150⁰C for 2 hours. After the elapsed time, the tubes were removed and cooled. The entire contents of each tube were transferred to a 100ml flask and Ferroin indicator (50 µl) was added. The contents were titrated against the FAS reagents. The end point was a sharp colour change from blue/green to reddish brown.

The sensitivity of the assay was in the range of 0-500 mgO₂l⁻¹.

The C.O.D. was calculated as follows:

$$\text{C.O.D. (mgO}_2\text{l}^{-1}) = \frac{(A - B) \times M \times 8000}{\text{Sample volume}}$$

Where, A = volume of FAS used for blank (ml),

B = volume of FAS used for sample (ml), and

M = molarity of FAS.

$$\text{Molarity of FAS (M)} = \frac{\text{Volume in digestion tube}}{\text{Volume of FAS used for blank}} \times 0.02$$

2.2.9 Gas chromatographic analysis of bacterial fatty acids

2.2.9.1 Extraction and esterification of whole-cell fatty acids

Whole cell fatty acids were extracted and esterified according to the method described by Smirnova (2001). 10 ml samples were taken in universals when cells reached a certain optical density (OD at 660 nm) in the range of 1.5–4.0. Cells were spun down at 5000 rpm for 10 minutes, resuspended in 2 ml reagent (15%, w/v NaOH in CH₃OH/H₂O, 50:50 v/v), and then saponified for 30 minutes at 95–100°C. After cooling at room temperature, 4 ml of reagent (6 N HCl in CH₃OH/H₂O, 50:50 v/v) was added. Fatty acids were converted into methyl esters following incubation at 80°C for 10 minutes. The extraction of the methylated fatty acids was performed in 3 ml of reagent (n-hexane/diethylether 50:50, v/v). After subsequent centrifugation at 5000 rpm for 20 minutes, the lower phase was discarded and the upper phase was washed with 3 ml of reagent (1.2%, w/v NaOH in H₂O). 2/3 of the upper phase containing fatty acid methyl esters (FAME) was transferred to a GC- tube using a glass made Pasteur pipette. 0.5 g anhydrous Na₂SO₄ was added to the GC tubes in order to dry the sample. After 2–3 hours, fatty acid methyl esters (FAME) was separated from anhydrous Na₂SO₄ and transferred to GC- tubes using a glass made Pasteur pipette. In order to preserve the samples before analysis the solution was purged with nitrogen gas by gently bubbling with nitrogen for approximately 2–3 minutes. As soon as the solution became viscous, it was kept at –20°C until analysis.

2.2.9.2 Isolation of phospholipids

Bacterial phospholipids were extracted using the so-called Folch method (Folch *et al.*, 1957). 10 ml samples were taken when cells reached a certain optical density (OD at 660 nm) in the range of 1.5–4.0. Cells were spun down at 5000 rpm for 10 minutes and resuspended in 8 ml of chloroform-methanol 2:1 (v/v) in a glass tube. Following stirring at room temperature for 20 minutes and a centrifugation for 10 minutes at

5000 rpm, 1/5 volume (1.6 ml) of a 0.05 M CaCl_2 solution was added and mixed well to form an emulsion. After phase separation by subsequent centrifugation at 5000 rpm for 20 minutes, the upper phase were discarded and the lower (chloroform) phase containing phospholipids was washed twice with methanol-water 1:1 (v/v). The lower phase was concentrated to near dryness under a flow of nitrogen gas in a fume hood. The nitrogen flow was set to the lowest possible pressure and the evaporation was stopped when the solution became viscous or ~1ml of sample left. Phospholipids were esterified and extracted according to the procedure described above.

2.2.9.3 Analysis of fatty acids

The methyl esters of fatty acids were analysed with a VARIAN CP-3800 gas chromatograph equipped with a flame ionization detector and a WCOT Fused Silica CP-Sil 88 column- 60m X 0.25 mm ID, 0.2 μ m film thickness (Chrompack, Middleburg, The Netherlands). The operating conditions were as follows: 1 μ l aliquots of the samples were injected and the sample was split in a 1:20 ratio. The injector temperature was maintained at 250°C and the detector at 280°C. The column temperature was operated isothermally at 180°C for 10 minutes after injection of samples and then programmed from 180°C to 225°C at 3°C /min with a final hold of 10 min at 225°C. Nitrogen was used as carrier gas with column flow rate 0.7 ml/min. Total flow was 19.4 ml/min with linear velocity 20.3 cm/sec and actual flow pressure 24.5 psi. Chromatograms were recorded as data file, which facilitated peak integration for quantitative analysis.

Fatty acids were identified by comparison of the relative retention times of their methyl esters with those of known standards. The relative retention times were relative to the internal standard. Behenic acid methyl ester (Methyl docosanoate, 22:0) was used as the internal standard. Peak areas, retention times were automatically determined by the integrator, the percentage of each fatty acid was calculated. Table 4 shows the chemical name, abbreviation, retention time and origin

of different known standard fatty acid methyl esters used for identification of bacterial fatty acids.

Table 4: Chemical name, abbreviation, retention time and source of standard fatty acid methyl esters used for the identification of bacterial fatty acids.

Chemical Name of standard fatty acid methyl ester	Abbreviation	Retention time (min.)	Source
Methyl dodecanoate	12:0	7.50	Sigma
Methyl tetradecanoate	14:0	8.80	Sigma
Methyl <i>trans</i> -9-tetradecanoate	14:1t	9.43	Sigma
Methyl <i>cis</i> -9-tetradecanoate	14:1c	9.78	Sigma
Methyl 2-hydroxydecanoate	2-OH 10:0	10.55	Sigma
Methyl hexadecanoate	16:0	11.00	Sigma
Methyl <i>trans</i> -9-hexadecanoate	16:1t	11.78	Sigma
Methyl <i>cis</i> -9-hexadecanoate	16:1c	12.16	Sigma
Methyl 3-hydroxydecanoate	3-OH 10:0	12.75	Biotrend
Methyl 2-hydroxydodecanoate	2-OH 12:0	13.40	Biotrend
Methyl 9,10-methylenehexadecanoate	17:0cyc	13.55	Biotrend
Methyl octadecanoate	18:0	14.09	Sigma
Methyl <i>trans</i> -9-octadecanoate	18:1to	14.82	Sigma
Methyl <i>trans</i> -11-octadecenoate	18:1tv	14.97	Sigma
Methyl <i>cis</i> -9-octadecenoate	18:1co	15.33	Sigma
Methyl <i>cis</i> -11-octadecenoate	18:1cv	15.56	Sigma
Methyl 3-hydroxydodecanoate	3-OH 12:0	16.32	Biotrend
Methyl 11,12-methyleneoctadecanoate	19:0cyc	17.02	Biotrend
Methyl <i>cis</i> -9, <i>cis</i> -12-octadecadienate	18:2cc	17.19	Sigma
Methyl eicosonate	20:0	17.75	Sigma
Methyl docosanoate	22:0	21.60	Sigma

The degree of saturation was determined according to the method of Heipieper *et al.* (1996). The degree of saturation of the membrane fatty acids of the bacterium *Pseudomonas putida* CP1 was defined as the ratio between the two saturated fatty acids, C16:0 and C18:0 and the unsaturated fatty acids 16:1 *trans*, 16:1 *cis*, 18:1 *trans*, and 18:1 *cis* of the extracts.

The *trans/cis* ratio of unsaturated fatty acids was determined according to the method of Heipieper *et al.* (1995). The *trans/cis* ratio of unsaturated fatty acids was defined as the ratio between the amounts of the *trans* mono-unsaturated fatty acids (16:1*trans*Δ9, 18:1*trans*Δ9 and 18:1*trans*Δ11) and the *cis* isomers of mono-unsaturated fatty acids (16:1*cis*Δ9, 18:1*cis*Δ9, 18:1*cis*Δ11) of the bacterium *P. putida* CP1.

2.2.10 Data analysis

The results presented were the mean of duplicate treatments and all experiments were repeated to confirm the data obtained. Standard errors were determined using a Sigma Plot, Version 1.02, Computer Package (Jandal Scientific Corporation). In all cases, the standard errors between runs were found to be less than 5%.

Calculation of specific growth rate: Specific growth rates were determined by plotting the log of cell number against time during exponential growth. The regression coefficient and slopes were calculated using a Sigma Plot, Version 1.02 Computer Package (Jandal Scientific Corporation). The resulting slope being equal to the specific growth rate, μ .

Calculation of substrate removal rate: Rates of substrate removal were calculated following the lag period. Overall removal rates were expressed as mg l^{-1} substrates removed per unit time. Specific rates of substrate removal were expressed as mg l^{-1} substrate removed per unit time per unit biomass. The lag period was defined as

follows: in a plot of substrate concentration versus time, the straight line was extrapolated to the initial substrate concentration (S_0) and the intercept of the time axis was taken to be the length of the lag period, L (Fig. 11).

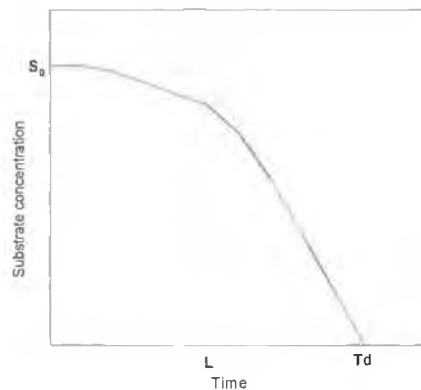


Fig. 11. Definition of lag period, L .

Calculation of growth yield: Yield was calculated using either the dry weight (mg) of cells or the number of cells obtained by using the standard plate count method and the direct epifluorescence filtration technique.

When the change in dry weight (mg) of the organism was used to calculate yield, the result was expressed as either mg of dry cell weight produced per mmol of substrate utilized or mg of dry cell weight produced per mg of substrate utilized.

$$\text{Yield} = \frac{\text{mg of cell dry weight produced}}{\text{mmol of substrate utilized}} \quad \text{or} \quad \text{Yield} = \frac{\text{mg of cell dry weight produced}}{\text{mg of substrate utilized}}$$

When cell number was used to calculate growth yield, the yield of the organism was expressed in terms of number of cells produced either per mmol or mg of substrate utilized.

$$\text{Yield} = \frac{\text{Number of cell produced}}{\text{mmol of substrate utilized}} \quad \text{or} \quad \text{Yield} = \frac{\text{Number of cell produced}}{\text{mg of substrate utilized}}$$

Chapter 3

Results

3. Results

3.1 The growth of *Pseudomonas putida* CP1 on mono-chlorophenols in the presence and absence of additional nutrients

P. putida CP1 was grown on various concentrations of 4-chlorophenol, 3-chlorophenol and 2-chlorophenol when supplied as the sole carbon source and when the medium was supplemented with glucose, fructose and yeast extract in order to investigate the influence of additional nutrients on the degradation of the chlorophenols by the bacterium. Samples were collected at various time intervals and analysed for OD, pH, substrate removal and chloride release.

3.1.1 The removal of various concentrations of mono-chlorophenols by *Pseudomonas putida* CP1 when supplied as the sole carbon source

The removal of 4-chlorophenol (80 to 350 ppm), 3-chlorophenol (100 to 225 ppm) and 2-chlorophenol (100 to 350 ppm) by *P. putida* CP1 is illustrated in Fig. 12. 4-chlorophenol was removed most readily followed by 2-chlorophenol and then 3-chlorophenol. Removal was generally preceded by a lag. The duration of the lag increased with increasing concentrations of substrate (Table 5). In the case of 4-chlorophenol, the organism degraded 80, 200, 250 and 300 ppm completely within 22, 46, 46 and 72 hours, respectively. Higher concentrations of the substrate inhibited the organism. The duration of the lag period extended from 6 hours for 200 ppm to 22 hours for 300 ppm. Following the lag, the rate of substrate removal increased with increasing concentrations up to a maximum value of $10.01 \text{ mg l}^{-1} \text{ h}^{-1}$ at 250 ppm. Above this concentration, the rate of substrate removal decreased and was inhibited above 300 ppm. At 350 ppm of 4-chlorophenol, there was no effective 4-chlorophenol removal and inhibition of growth was observed as the turbidity of the culture medium decreased with time.

A similar pattern of removal was observed with a range of 2-chlorophenol concentrations (100 to 350 ppm). While the duration of the lag period was similar to that observed for 4-chlorophenol, the rate of substrate removal was significantly lower. *P. putida* CP1 degraded 100, 200 and 250 ppm of 2-chlorophenol within 72, 100 and 165 hours, respectively. The rate of substrate removal reached a maximum value, $2.65 \text{ mg l}^{-1} \text{ h}^{-1}$, with 100 ppm. At 300 ppm 2-chlorophenol only 35% of substrate was removed after 215 hours but at 350 ppm of 2-chlorophenol there was no effective 2-chlorophenol removal found and inhibition of growth was indicated by a decrease in culture turbidity.

Of the three mono-chlorophenols, the organism degraded 3-chlorophenol slowest. In all concentrations of substrate investigated, the lag was 22 hours where removal of substrate was observed. 3-chlorophenol was not removed above 200 ppm. *P. putida* CP1 degraded 100, 150, 175 and 200 ppm of 3-chlorophenol within 72, 100, 150 and 215 hours, respectively. The maximum rate of removal, $1.84 \text{ mg l}^{-1} \text{ h}^{-1}$, was obtained with 100 ppm.

Removal of the mono-chlorophenols was accompanied by a stoichiometric amount of chloride release (data not presented). In all cases there was very little change in pH (0.05 to 0.15 unit). *P. putida* CP1 formed clumps of cells in the culture broth when grown on concentrations of 4-chlorophenol above 200 ppm and on all concentrations of 2- and 3-chlorophenol investigated. As 200 ppm of all three isomers of mono-chlorophenols were found to be removed completely by *P. putida* CP1, 200 ppm of mono-chlorophenols was selected for further study.

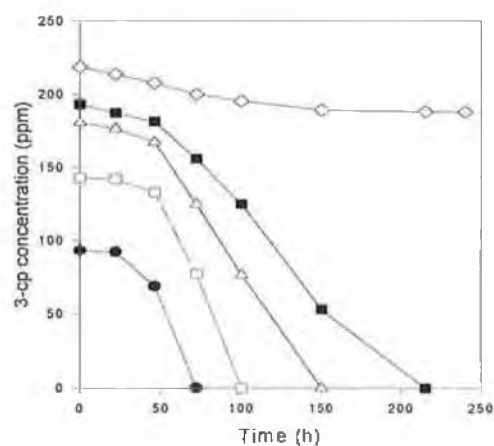
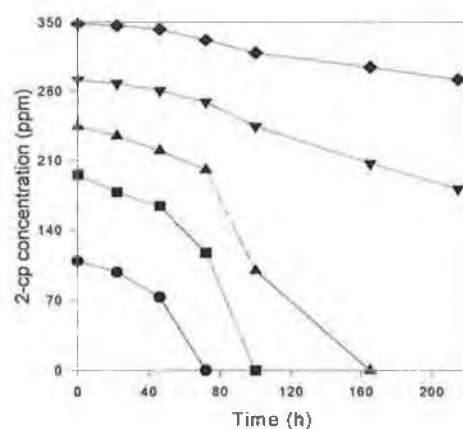
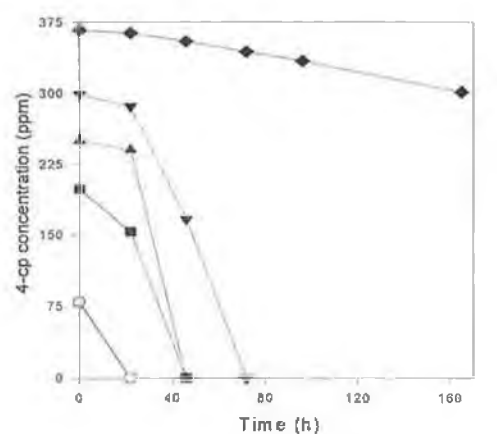


Fig. 12. The removal of mono-chlorophenols by *P. putida* CP1 when grown aerobically at 30°C. Symbols: O, 80 ppm; ●, 100 ppm; □, 150 ppm; Δ, 175 ppm; ■, 200 ppm; ◇, 225 ppm; ▲, 250 ppm; ▼, 300 ppm; ◆, 350 ppm.

Table 5: Summary table for the lag period, rates of removal and pH change in the removal of various concentrations of mono-chlorophenols by *P. putida* CP1 at 30°C.

Mono-chlorophenol	Initial concentration (ppm)	Lag period (h)	Removal rate (mg l⁻¹ h⁻¹)	Δ pH
4-chlorophenol	80	-----	3.61 ± 1.50	-----
	200	6	4.64 ± 0.02	- 0.12
	250	22	10.01 ± 0.16	- 0.15
	300	22	5.72 ± 0.12	- 0.14
	350	Inhibition	Inhibition	- 0.15
2-chlorophenol	100	-----	2.65 ± 0.07	-----
	200	9	2.05 ± 0.01	- 0.13
	250	22	1.99 ± 0.16	- 0.12
	300	22	1.06 ± 0.02	- 0.11
	350	Inhibition	Inhibition	- 0.15
3-chlorophenol	100	22	1.84 ± 0.03	- 0.15
	150	22	1.81 ± 0.01	- 0.08
	175	22	1.28 ± 0.03	- 0.09
	200	22	1.02 ± 0.01	- 0.09
	225	Inhibition	Inhibition	- 0.11

± = Standard error

3.1.2 The growth of *Pseudomonas putida* CP1 on glucose and on mono-chlorophenols in the presence of glucose

It was of interest to investigate the influence of glucose on the degradation of mono-chlorophenols. The growth of the organism on various concentrations of glucose was first investigated and then the degradation of mono-chlorophenols in the presence of glucose was studied.

3.1.2.1 The growth of *Pseudomonas putida* CP1 on various concentrations of glucose

Pseudomonas putida CP1 was incubated in various concentrations (0.05% to 2%, w/v) of glucose containing minimal media. Samples were collected at different time intervals and analysed for OD at 660 nm, pH and glucose concentration. The degradation of various concentrations (0.05% to 2%, w/v) glucose by *Pseudomonas putida* CP1 in minimal media is shown in Fig. 13. No lag in the degradation of glucose was observed. The time of removal of glucose increased with increasing concentrations. The highest concentration of glucose investigated, 2% (w/v), was removed completely in 100 hours, whereas it took 9, 9, 22, 30, 46 hours for complete removal of 0.05%, 0.1%, 0.2%, 5% and 1% (w/v) glucose respectively.

The rate of glucose removal is shown in Table 6. Glucose removal rates increased with increasing concentrations of glucose.

No clumping of bacterial cells was observed when the organism was grown on glucose alone. The organism grew in response to substrate removal (Fig. 13). The level of biomass increased with increasing concentrations of substrate.

A plot of the growth rate (μ) against glucose concentration is illustrated in Fig. 14 and follows the Monod equation.

$$\mu = \frac{\mu_{\max} S}{(S + K_s)}$$

Where, μ_{\max} = Maximum growth rate,

S = Substrate concentration,

K_s = Monod constant.

The maximum rate of growth, μ_{\max} was found to be 0.17 h^{-1} and was obtained when the organism was grown on 0.2% (w/v) glucose. Higher concentrations of glucose resulted in the same rate of growth of the organism (Table 6). The value for K_s , which is equivalent to the substrate concentration at half the maximum growth rate was calculated as 0.27 mg/ml.

The initial pH of the various concentrations of glucose containing media was about 6.85. The pH dropped with glucose utilization (Fig. 13). The drop in pH increased with increasing concentrations of glucose. At the higher concentrations of glucose, the initial fall in pH was followed by a rise in pH when maximum biomass values were achieved.

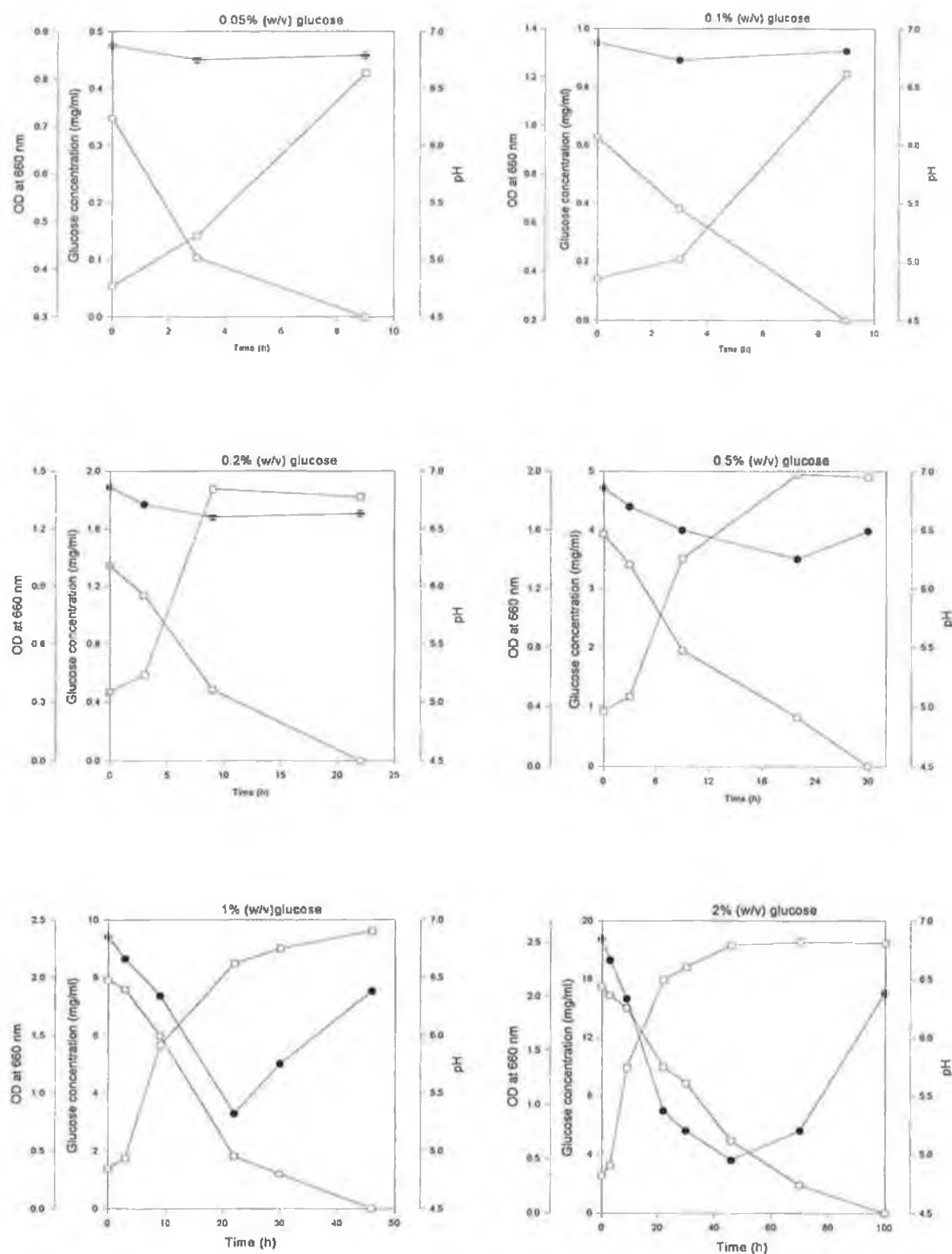


Fig. 13. The removal of various concentrations (0.05% to 2%, w/v) of glucose by *P. putida* CP1 when grown at 30°C. Symbols: ○, mg/ml glucose concentration; □, biomass; ●, pH.

Table 6: Specific growth rates (μ) and glucose removal rates for *P. putida* CP1 grown on a range of glucose concentrations at 30°C.

Glucose Concentration (%)	μ (h^{-1})	Glucose Removal Rate ($\text{mg l}^{-1} \text{h}^{-1}$)	Specific Glucose Removal Rate ($\text{mg l}^{-1} \text{h}^{-1} \text{mg}^{-1}$)
0.05	0.0990 ± 0.005	66.00 ± 2.00	0.133 ± 0.012
0.1	0.1421 ± 0.007	82.00 ± 3.00	0.179 ± 0.006
0.2	0.1703 ± 0.001	95.66 ± 3.50	0.249 ± 0.007
0.5	0.1700 ± 0.002	156.31 ± 3.17	0.665 ± 0.012
1.0	0.1711 ± 0.010	220.40 ± 4.65	1.261 ± 0.023
2.0	0.1645 ± 0.005	220.33 ± 4.50	2.223 ± 0.040

\pm = Standard error

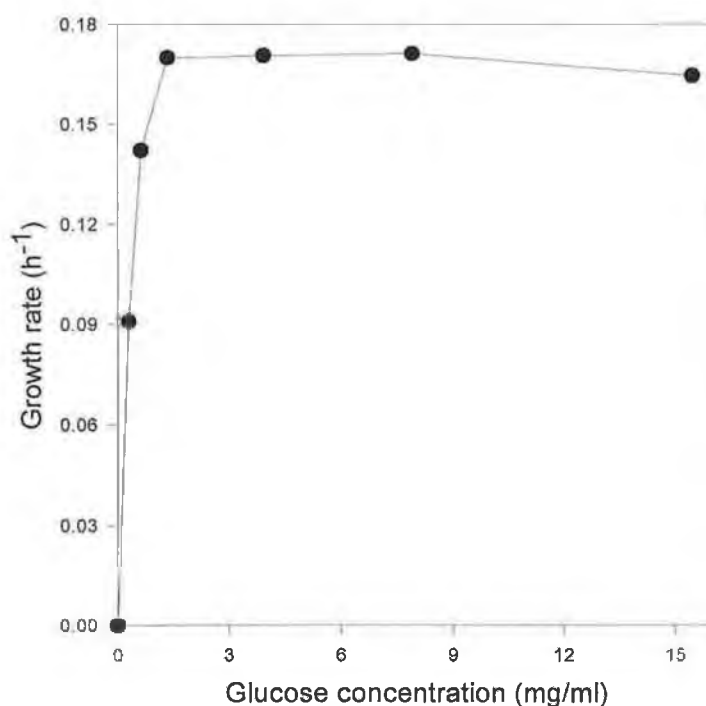


Fig. 14. Specific growth rate (μ) plotted as a function of glucose concentration.

3.1.2.2 The removal of mono-chlorophenols by *Pseudomonas putida* CP1 in the presence of various concentrations of glucose

Degradation of 200 ppm of 4-chlorophenol, 3-chlorophenol and 2-chlorophenol in the presence of various concentrations of glucose (0.05% to 2%, w/v) are shown in Figs. 15, 16 and 17, respectively. Glucose was removed readily by the bacterium with no lag. The rate of removal of the glucose in the presence of chlorophenols was less than that achieved when glucose alone was supplied to the organism (Section 3.1.2.1) and was inhibited at high concentrations of sugar (Table 7). There was a lag in the removal of the chlorophenols, however the duration of the lag was the same in the presence of glucose as in its absence for both 3-chlorophenol and 2-chlorophenol. In the case of 4-chlorophenol the presence of glucose reduced the lag period from 6 hours to 3 hours (Table 7).

The rate of removal of chlorophenols increased at low concentrations of glucose (Table 7). The rate of removal of 4-chlorophenol increased from $4.64 \text{ mg l}^{-1} \text{ h}^{-1}$ in the absence of glucose to $9.58 \text{ mg l}^{-1} \text{ h}^{-1}$ and $9.78 \text{ mg l}^{-1} \text{ h}^{-1}$ in the presence of 0.05% and 0.1% (w/v) glucose, respectively. In the presence of 0.2% and 0.5% (w/v) glucose, the rate of removal of 4-chlorophenol was similar to that in the absence of glucose. However as with glucose, the removal of 4-chlorophenol was inhibited by 1% and 2% (w/v) glucose.

3-chlorophenol was the least readily removed mono-chlorophenol when supplied as the sole carbon source (Section 3.1.1). The rate of removal increased in the presence of glucose concentrations ranging from 0.05% to 0.5% (w/v). Higher concentrations of glucose inhibited substrate removal.

The rate of removal of 2-chlorophenol was half that of 4-chlorophenol and twice that of 3-chlorophenol when supplied as the sole carbon source. The addition of glucose to the medium did not increase the rate of removal significantly. While removal

occurred in the presence of 1% (w/v) glucose, removal was inhibited as with other mono-chlorophenols in the presence of 2% (w/v) glucose.

Where substrate removal was inhibited, a significant fall in pH in the medium was noted (Figs. 15, 16 and 17). In treatments with glucose concentrations up to 0.5% (w/v) there was only a minor drop in pH in the presence of mono-chlorophenols (not more than 0.6 unit). However, a significant pH drop was observed in the case of higher concentrations of glucose. With both 4-chlorophenol and 3-chlorophenol, the pH values fell to nearly 4 in both the 1% and 2% (w/v) glucose containing media. In the case of 2-chlorophenol with 1% (w/v) glucose a pH drop of 0.8 unit was observed. However in the presence of 2% (w/v) glucose the pH fell by 4 units. There was no removal of substrate when the pH dropped substantially.

Monitoring growth by measuring optical density showed that the growth of the organism increased with increasing levels of glucose (Fig. 18). Clumping was observed in all treatments at 24 hours. The size of the clumps decreased with increasing concentrations of glucose.

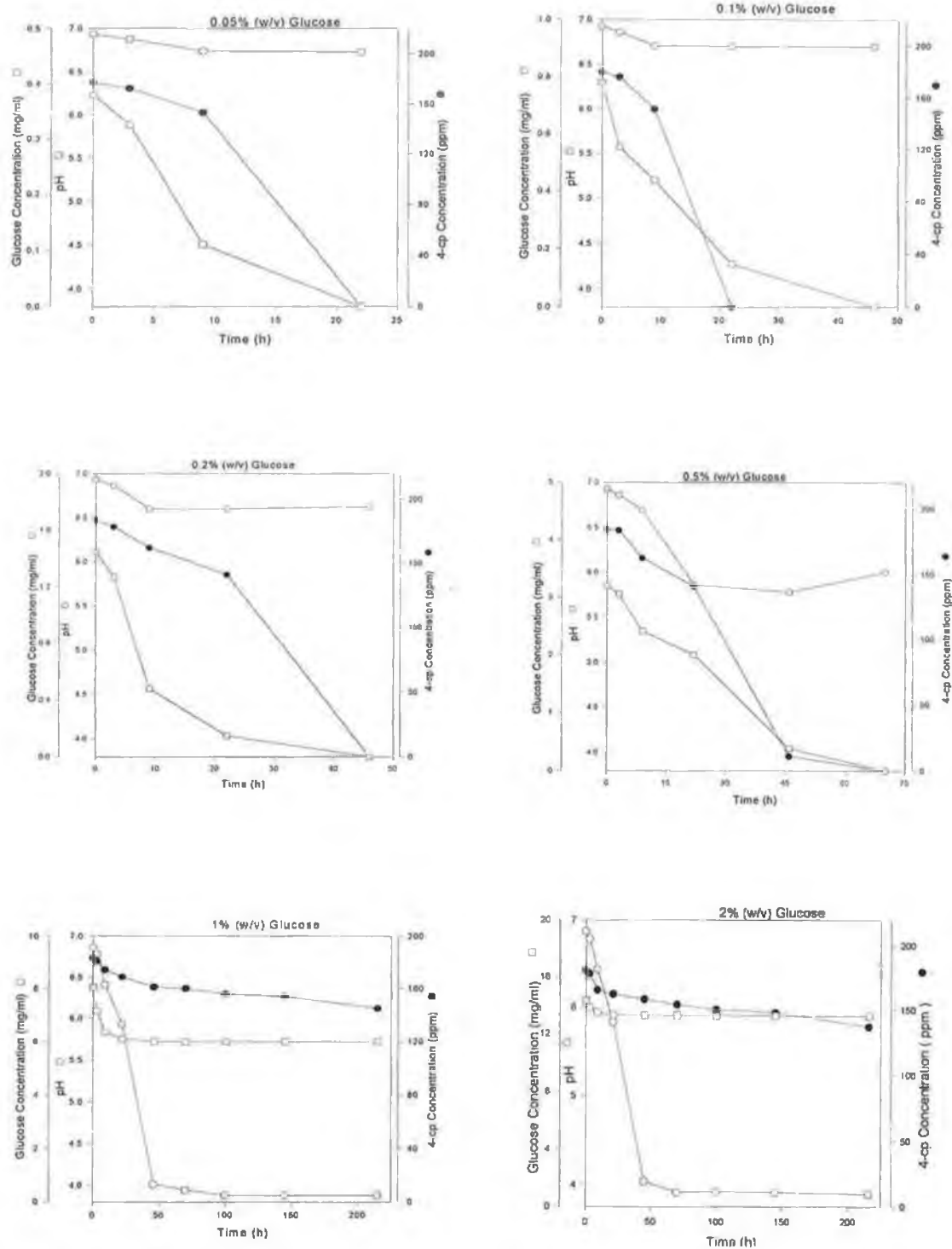


Fig. 15. The removal of 200 ppm 4-chlorophenol by *P. putida* CP1 in the presence of various concentrations (0.05% to 2%, w/v) of glucose in the minimal medium at 30°C. Symbols: □, glucose concentration; ●, 4-chlorophenol concentration; O, pH.

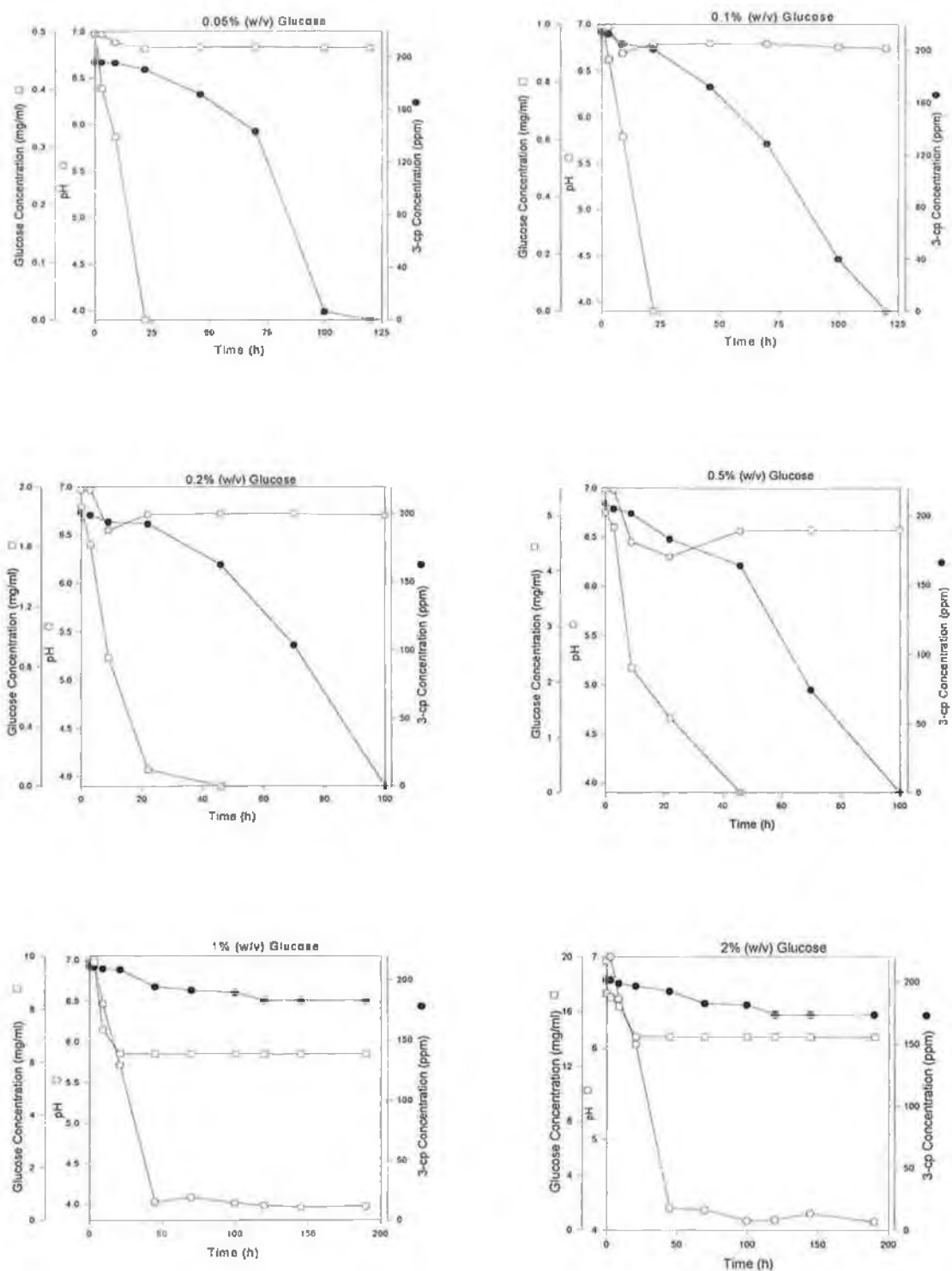


Fig. 16. The removal of 200 ppm 3-chlorophenol by *P. putida* CP1 in the presence of various concentrations (0.05% to 2%, w/v) of glucose in the minimal medium at 30°C. Symbols: □, glucose concentration; ●, 3-chlorophenol concentration; ○, pH.

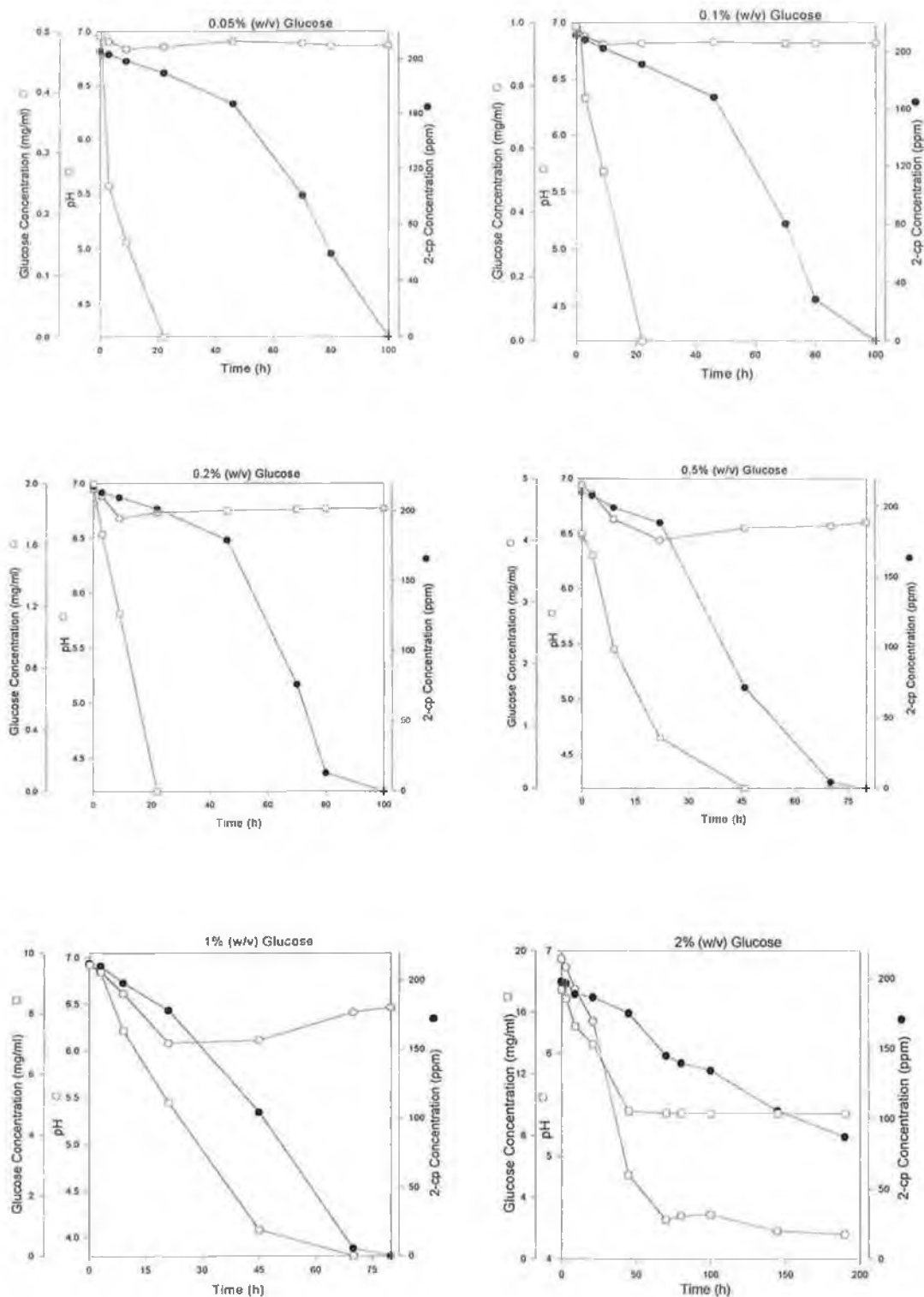


Fig. 17. The removal of 200 ppm 2-chlorophenol by *P. putida* CP1 in the presence of various concentrations (0.05% to 2%, w/v) of glucose in the minimal medium at 30°C. Symbols: □, glucose concentration; ●, 2-chlorophenol concentration; O, pH.

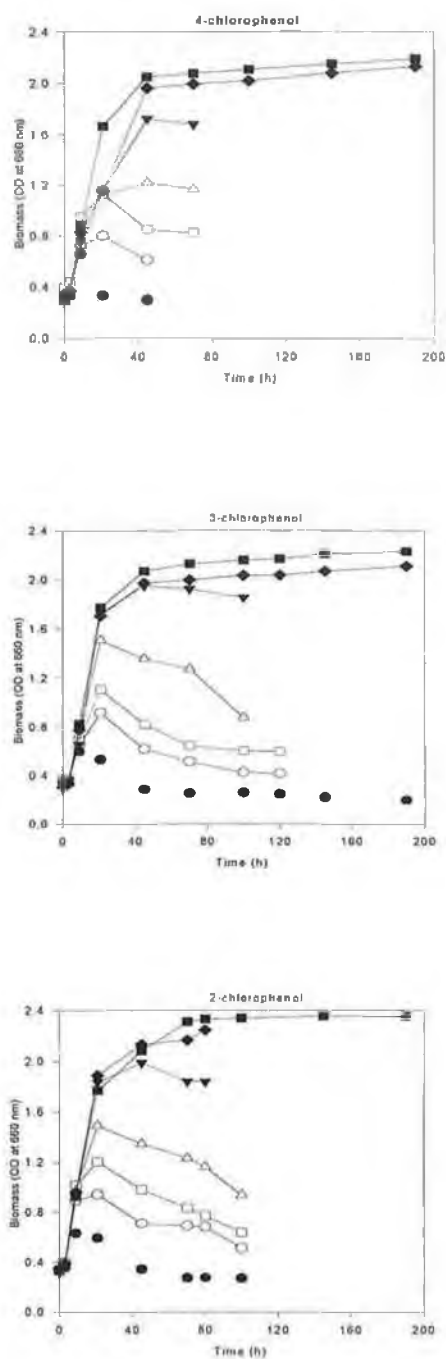


Fig. 18. Changes in optical density at 660 nm during growth of *P. putida* CP1 on 200 ppm mono-chlorophenols in the presence of various concentrations of glucose at 30°C. Symbols: ●, Without glucose; ○, with 0.05% (w/v) glucose; □, with 0.1% (w/v) glucose; △, with 0.2% (w/v) glucose; ▼, with 0.5% (w/v) glucose; ◆, with 1% (w/v) glucose and ■, with 2% (w/v) glucose.

Table 7: Lag values and substrate removal rates when *P. putida* CP1 was grown on 200 ppm mono-chlorophenols (cp) and various concentrations of glucose (G) at 30°C.

Treatment	Lag -cp (h)	cp removal rate (mg l ⁻¹ h ⁻¹) / % Removed	Specific-cp removal rate (mg l ⁻¹ h ⁻¹ mg ⁻¹)	G removal rate (mg l ⁻¹ h ⁻¹) / % Removed	Specific-G removal rate (mg l ⁻¹ h ⁻¹ mg ⁻¹)
4-cp only	6	4.64 ± 0.020	0.0082 ± 0.0001	-----	-----
+ 0.05% (w/v) G	3	9.58 ± 0.275	0.0176 ± 0.0005	30.00 ± 4.61	0.0732 ± 0.009
+ 0.1% (w/v) G	3	9.77 ± 0.070	0.0153 ± 0.0001	38.55 ± 1.18	0.1010 ± 0.002
+ 0.2% (w/v) G	3	4.25 ± 0.169	0.0076 ± 0.0003	107.33 ± 1.56	0.2500 ± 0.004
+ 0.5% (w/v) G	3	4.08 ± 0.040	0.0067 ± 0.0000	66.66 ± 4.19	0.1186 ± 0.006
+ 1% (w/v) G	3	Inhibition (21%)	-----	Inhibition (25%)	-----
+ 2% (w/v) G	3	Inhibition (24%)	-----	Inhibition (14%)	-----
3-cp only	21	1.03 ± 0.010	0.00210 ± 0.0000	-----	-----
+ 0.05% (w/v) G	21	1.92 ± 0.040	0.00303 ± 0.0000	24.57 ± 1.11	0.0425 ± 0.002
+ 0.1% (w/v) G	21	2.03 ± 0.030	0.00290 ± 0.0000	46.66 ± 1.28	0.0916 ± 0.002
+ 0.2% (w/v) G	21	2.44 ± 0.005	0.00164 ± 0.0000	83.32 ± 1.52	0.1726 ± 0.004
+ 0.5% (w/v) G	21	2.30 ± 0.169	0.00153 ± 0.0000	176.12 ± 2.85	0.4440 ± 0.007
+ 1% (w/v) G	21	Inhibition (14%)	-----	Inhibition (38%)	-----
+ 2% (w/v) G	21	Inhibition (14%)	-----	Inhibition (18%)	-----
2-cp only	9	2.11 ± 0.005	0.0035 ± 0.0000	-----	-----
+ 0.05% (w/v) G	9	2.18 ± 0.100	0.0030 ± 0.0000	42.11 ± 3.93	0.0908 ± 0.006
+ 0.1% (w/v) G	9	2.22 ± 0.090	0.0028 ± 0.0001	53.09 ± 3.03	0.1795 ± 0.009
+ 0.2% (w/v) G	9	2.31 ± 0.040	0.0024 ± 0.0000	93.09 ± 4.78	0.2980 ± 0.012
+ 0.5% (w/v) G	9	2.69 ± 0.169	0.0039 ± 0.0002	156.62 ± 4.03	0.3270 ± 0.008
+ 1% (w/v) G	9	2.73 ± 0.042	0.0035 ± 0.0003	194.38 ± 3.95	0.6976 ± 0.014
+ 2% (w/v) G	9	Inhibition (56%)	-----	Inhibition (50%)	-----

± = Standard error

3.1.2.3 The removal of mono-chlorophenols by *Pseudomonas putida* CP1 in the presence of inhibitory concentrations of glucose – an investigation of pH control

In order to investigate the role of pH in the removal of chlorophenols with high concentrations of glucose, the control of pH using buffers was investigated. A study was carried out to investigate a suitable buffering system both in terms of growth and substrate removal by *P. putida* CP1. As 4-chlorophenol was the most readily degraded isomer, it was selected for this study.

3.1.2.3.1 Choice of buffer

The effect of Citric acid- Na_2HPO_4 buffer, Phosphate buffers and Sodium phosphate buffer on the degradation of 4-chlorophenol by *P. putida* CP1 was investigated (Fig. 19). The concentration of buffer in all cases was 0.2M. Substrate removal was monitored together with pH and growth for a 48h period. The pH in all the systems remained at 6.95 ± 0.05 for the duration of the run.

There was no growth or substrate removal in the presence of the Citric acid- Na_2HPO_4 buffer (Fig. 19). Growth and substrate removal in the presence of the phosphate buffer and the sodium phosphate buffer was similar to the treatment with no buffer and complete removal of 4-chlorophenol was achieved within 46 hours. It was decided to use the sodium phosphate buffer in further studies.

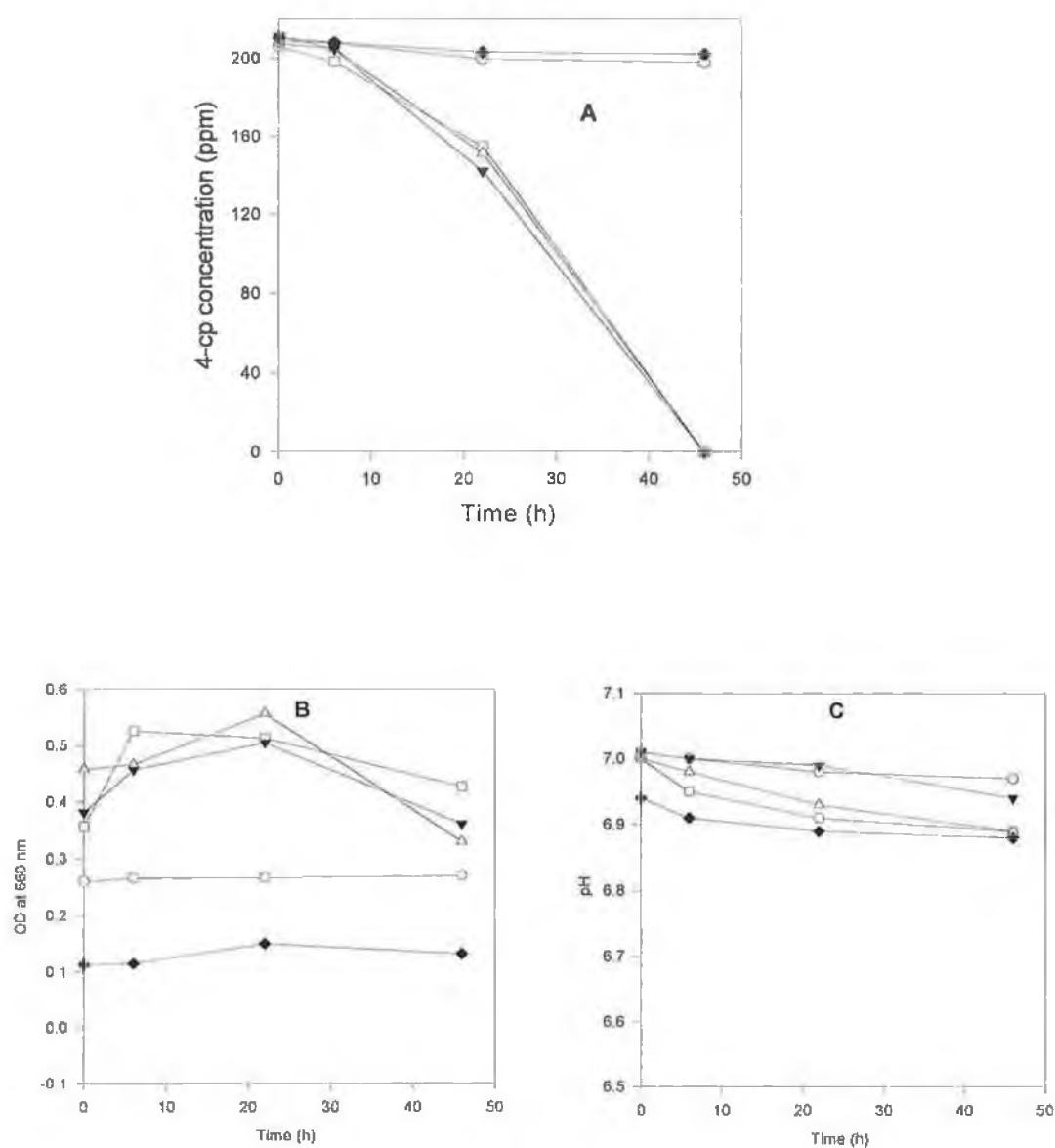


Fig. 19. Effect of different buffers on the degradation of 200 ppm 4-chlorophenol by *P. putida* CP1 (A, chlorophenol removal; B, changes in biomass; C, changes in pH). Symbols: O, without CP1; □, without buffer; Δ, with phosphate buffer; ▼, with sodium phosphate buffer; and ◆, with citric acid-Na₂HPO₄.

3.1.2.3.2 Effect of different concentrations of sodium phosphate buffer on the degradation of 200 ppm 4-chlorophenol

The effect of different concentrations of sodium phosphate buffer (0.1 M, 0.2 M, 0.4 M and 0.6 M) on the removal of 200 ppm 4-chlorophenol was investigated both in the absence (Fig. 20) and presence (Fig. 21) of 1% (w/v) glucose in the minimal medium. Substrate removal together with pH and growth was also measured (Figs. 20 and 21).

In the absence of buffer, 4-chlorophenol was removed when glucose was not present. The presence of glucose caused the pH to fall. A fall in pH inhibited both growth and all substrate removal.

While the 0.6M buffer controlled the pH both in the presence and absence of glucose, there was no growth or substrate removal. The ability of the buffer to control the pH in the presence of glucose decreased with decreasing concentrations of buffer. Substrate removal was optimal in the presence of 0.1M and 0.2 M buffer. The 0.2M concentration was chosen for further studies.

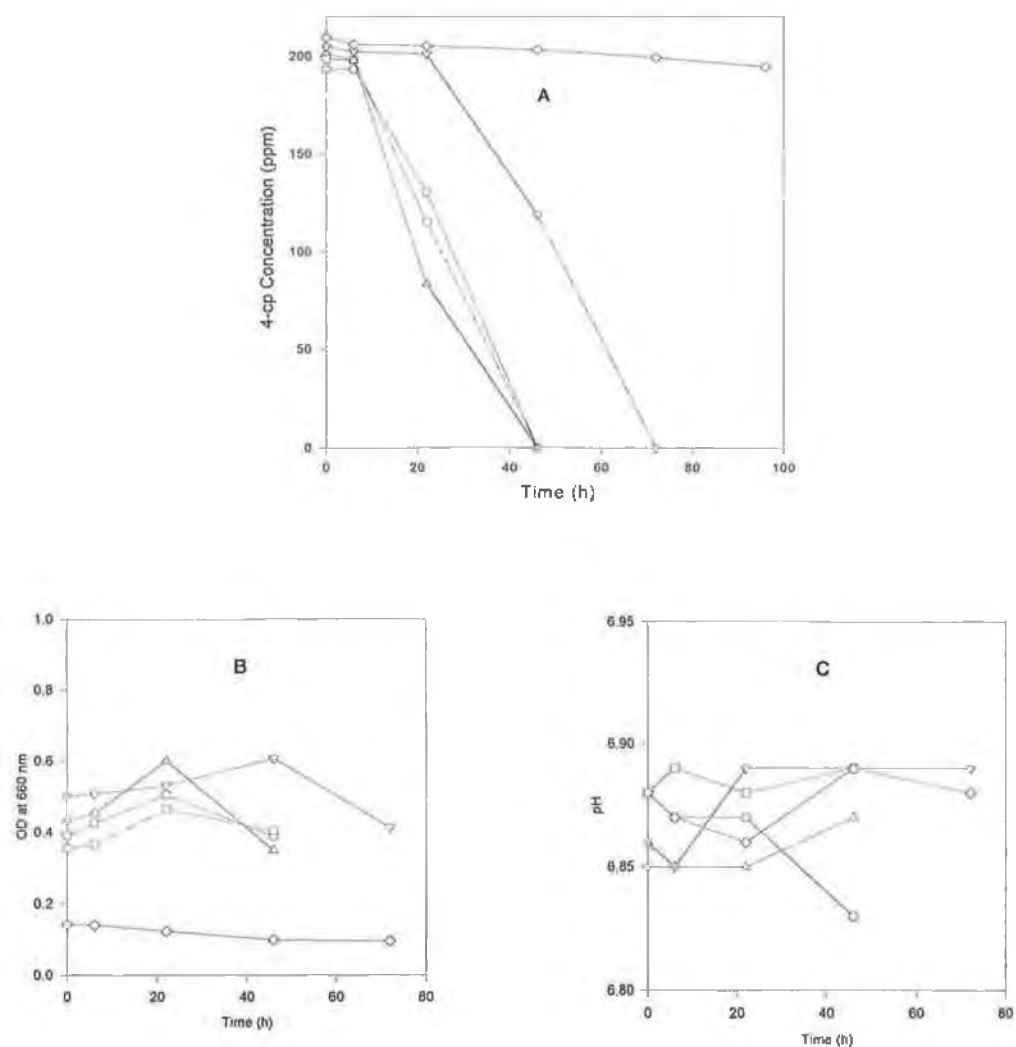


Fig. 20. Effect of different concentrations of sodium phosphate buffer (SPB) on the degradation of 200 ppm 4-chlorophenol by *P. putida* CP1 (A, chlorophenol removal; B, changes in biomass; C, changes in pH). Symbols: O, 4-cp only; □, 4-cp in 0.1 M SPB; Δ, 4-cp in 0.2 M SPB; ▽, 4-cp in 0.4 M SPB; ◇, 4-cp in 0.6 M SPB.

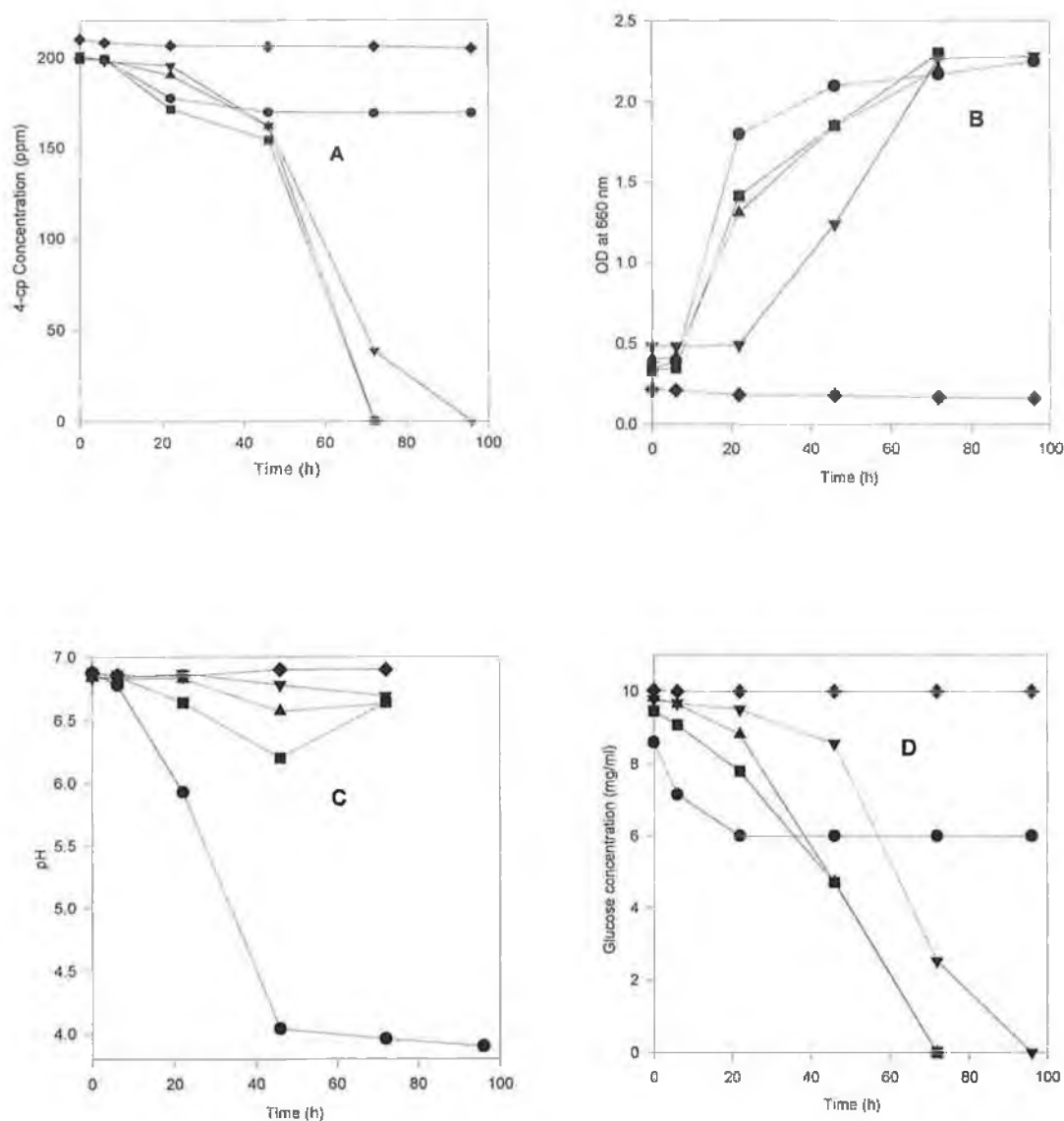


Fig. 21. Effect of different concentrations of sodium phosphate buffer (SPB) on the degradation of 200 ppm 4-chlorophenol by *P. putida* CPI in the presence of 1% (w/v) glucose (A, chlorophenol removal; B, changes in biomass; C, changes in pH; D, glucose removal). Symbols: ●, 4-cp + 1% (w/v) glucose; ■, 4-cp + 1% (w/v) glucose in 0.1 M SPB; ▲ 4-cp + 1% (w/v) glucose in 0.2 M SPB; ▼ 4-cp + 1% (w/v) glucose in 0.4 M SPB; ◆, 4-cp + 1% (w/v) glucose in 0.6 M SPB.

3.1.2.3.3 The removal of 200 ppm mono-chlorophenols by *Pseudomonas putida* CP1 in the presence of 1% and 2% (w/v) glucose with pH control

Degradation of 200 ppm of 4-chlorophenol, 3-chlorophenol and 2-chlorophenol in the presence of 1% and 2% (w/v) glucose was investigated by controlling the pH with 0.2 M sodium phosphate buffer (Fig. 22 and Fig. 23). In all systems both glucose and chlorophenols were removed by the organism. The chlorophenols were completely removed in the order of: 4-chlorophenol >2-chlorophenol >3-chlorophenol but the rate of removal was greater in the presence of 1% (w/v) glucose than in the presence of 2% (w/v) glucose (Table 8).

In the presence of 1% (w/v) glucose the sugar was removed preferentially by the organism. In the case of 2% (w/v) glucose, a residual amount of glucose remained following chlorophenol removal. The buffer worked more effectively in the presence of the lower concentration of sugar. The rate of removal of glucose was similar for any one concentration of glucose regardless of the chlorophenol present (Table 8). There was very slight flocculation of the organism when grown in the presence of chlorophenols and high concentrations of sugar with pH control. Growth as recorded in optical density units was similar in all systems (Fig. 24).

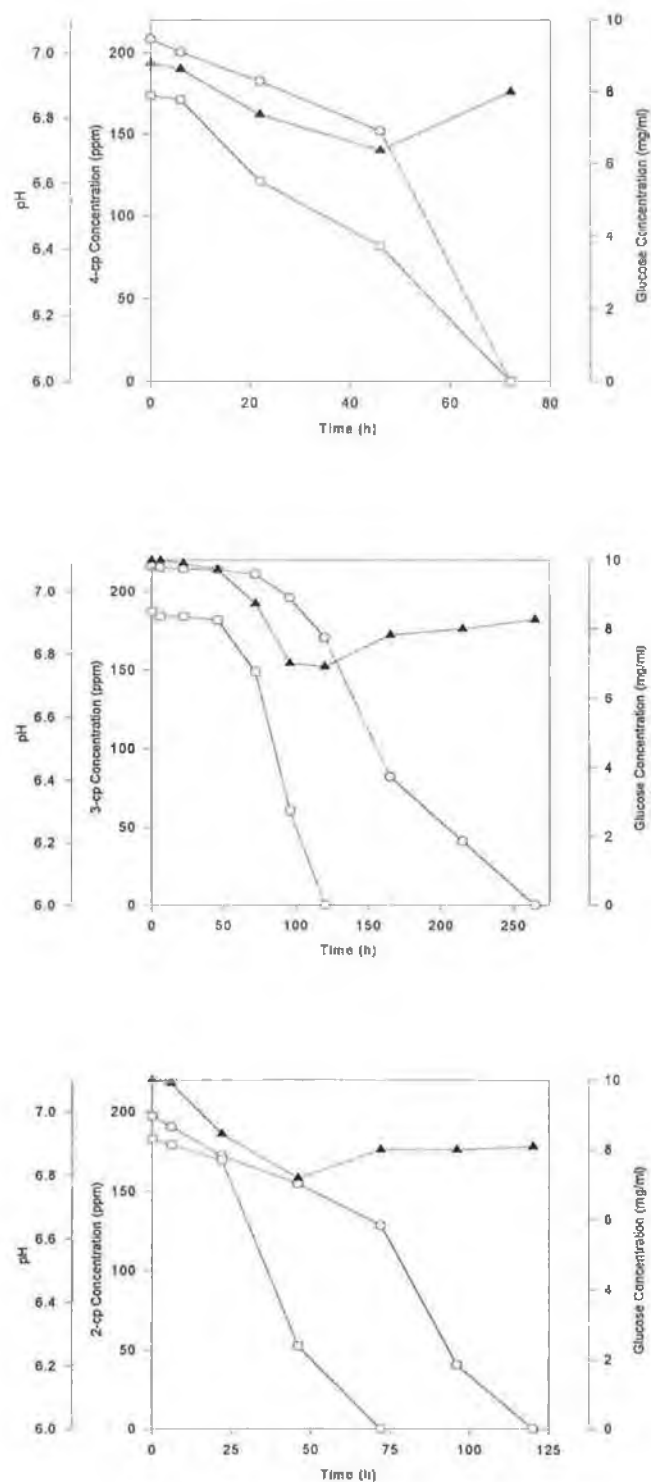


Fig. 22. The removal of 200 ppm mono-chlorophenols in the presence of 1% (w/v) glucose and 0.2 M sodium phosphate buffer at pH 7. Symbols: O, mono-chlorophenol; □, Glucose; ▲, pH.

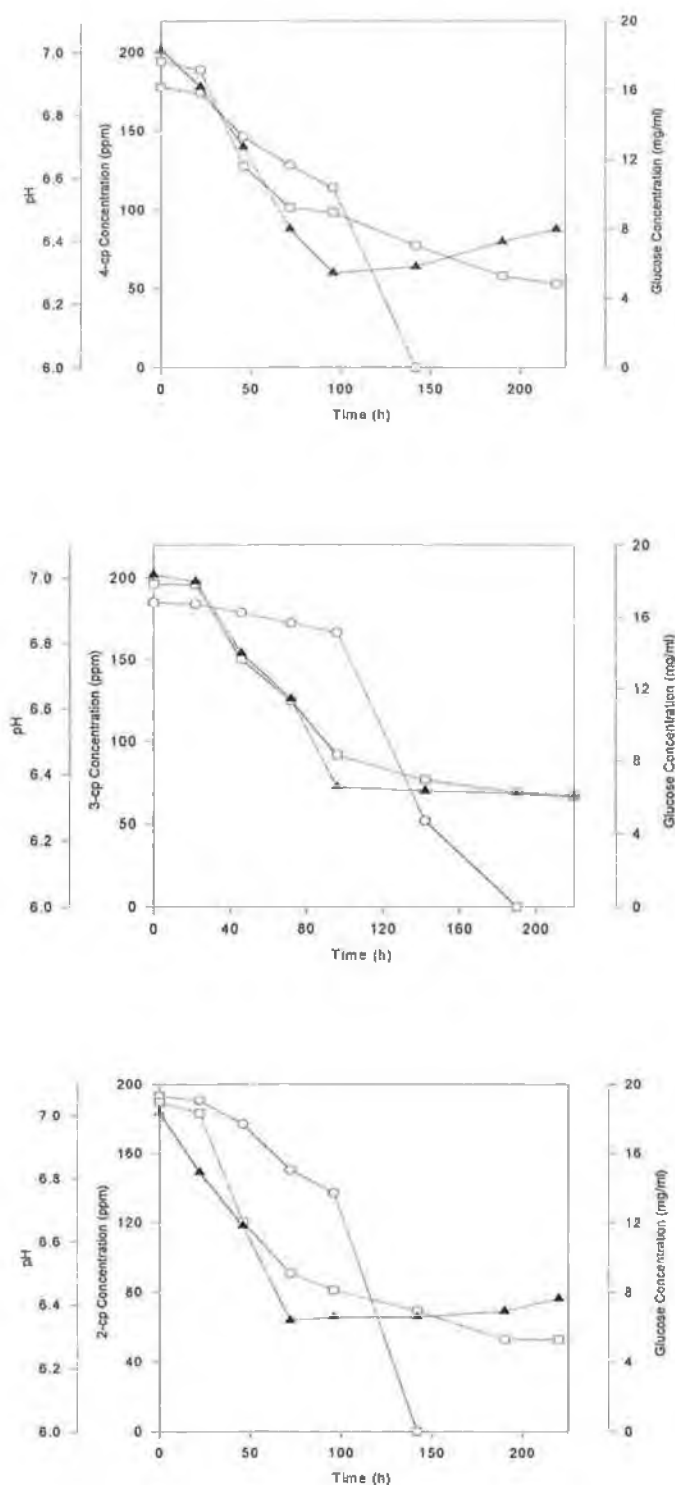


Fig. 23. The removal of 200 ppm mono-chlorophenols in the presence of 2% (w/v) glucose and 0.2 M sodium phosphate buffer at pH 7. Symbols: O, mono-chlorophenol; □, Glucose; ▲, pH.

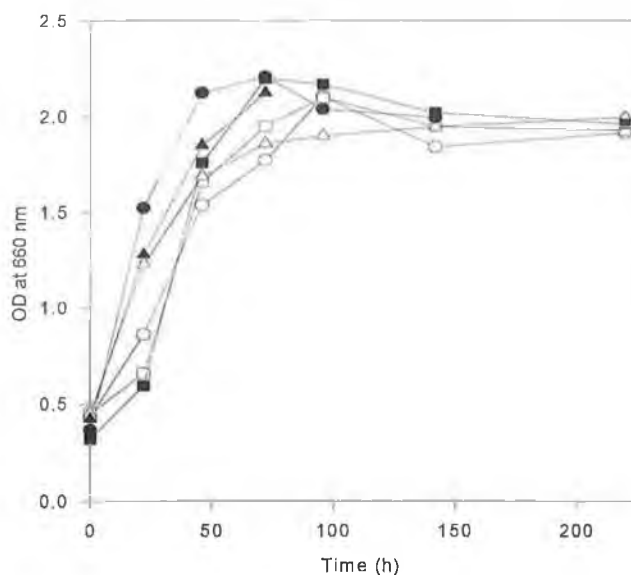


Fig. 24. Changes in optical density at 660 nm during growth of CP1 on 200 ppm mono-chlorophenols and 1% or 2% (w/v) glucose in buffering system. Symbols: ▲, 4-cp + 1% (w/v) glucose; ■, 3-cp + 1% (w/v) glucose; ●, 2-cp + 1% (w/v) glucose; Δ, 4-cp + 2% (w/v) glucose; ○, 3-cp + 2% (w/v) glucose and □, 2-cp + 2% (w/v) glucose.

Table 8: Rates of removal of 200 ppm mono-chlorophenols by *P. putida* CP1 in presence of 1% and 2% (w/v) glucose (G) and 0.2 M sodium phosphate buffer at pH 7.0.

Treatment	Chlorophenol removal rate		Glucose removal rate	
	mg l ⁻¹ h ⁻¹	mg l ⁻¹ h ⁻¹ mg ⁻¹	mg l ⁻¹ h ⁻¹	mg l ⁻¹ h ⁻¹ mg ⁻¹
4-cp + 1% (w/v) G + buffer	3.03 ± 0.02	0.00618 ± 0.0004	117.86 ± 4.24	0.236 ± 0.008
4-cp + 2% (w/v) G + buffer	1.45 ± 0.07	0.00192 ± 0.0000	162.42 ± 3.03	0.260 ± 0.004
3-cp + 1% (w/v) G + buffer	1.31 ± 0.03	0.00093 ± 0.0002	111.54 ± 4.08	0.320 ± 0.011
3-cp + 2% (w/v) G + buffer	1.24 ± 0.05	0.00283 ± 0.0000	121.52 ± 2.04	0.447 ± 0.007
2-cp + 1% (w/v) G + buffer	1.67 ± 0.02	0.00629 ± 0.0000	127.07 ± 1.26	0.452 ± 0.003
2-cp + 2% (w/v) G + buffer	1.34 ± 0.04	0.00219 ± 0.0000	185.02 ± 3.53	0.454 ± 0.009

± = Standard error

3.1.3 The growth of *Pseudomonas putida* CP1 on fructose and the influence of fructose on the removal of mono-chlorophenols

3.1.3.1 The growth of *P. putida* CP1 on various concentrations of fructose

P. putida CP1 was incubated in minimal medium containing various concentrations of fructose (0.05% to 2%, w/v). Samples were collected at different time intervals and analysed for OD at 660 nm, pH and reducing sugar concentration (Fig. 25).

The organism grew in response to fructose removal. Growth increased with increasing concentrations of sugar. The organism was found to clump in the presence of fructose. Clumping was noted after 21 hours incubation. The size of the clumps was found to be of similar size in all concentrations of sugar. There was no significant change in the pH of the medium even at the higher concentrations of fructose. The pH of the lower concentrations of fructose (0.05% to 0.2%, w/v) reduced only slightly (0.05 to 0.25 unit), but with higher concentrations (0.5% to 2%, w/v) the drop in pH was around 0.6 unit. In all cases there was a lag of 9 hours in the removal of fructose. Following the lag the rate of fructose removal increased with increasing concentrations of sugar (Table 9).

Table 9: Lag periods and removal rates of various concentrations (0.05% to 2%, w/v) of fructose by *P. putida* CP1 when grown at 30⁰C. \pm = Standard error

Fructose Concentration (%)	Lag Period (h)	Fructose Removal Rate (mg l ⁻¹ h ⁻¹)	Specific Fructose Removal Rate (mg l ⁻¹ h ⁻¹ mg ⁻¹)
0.05	9	16.33 \pm 0.27	0.049 \pm 0.0008
0.1	9	35.52 \pm 1.26	0.065 \pm 0.0018
0.2	9	44.00 \pm 1.69	0.095 \pm 0.0043
0.5	9	89.71 \pm 3.43	0.194 \pm 0.0065
1.0	9	114.37 \pm 1.50	0.226 \pm 0.0029
2.0	9	110.86 \pm 3.05	0.333 \pm 0.0081

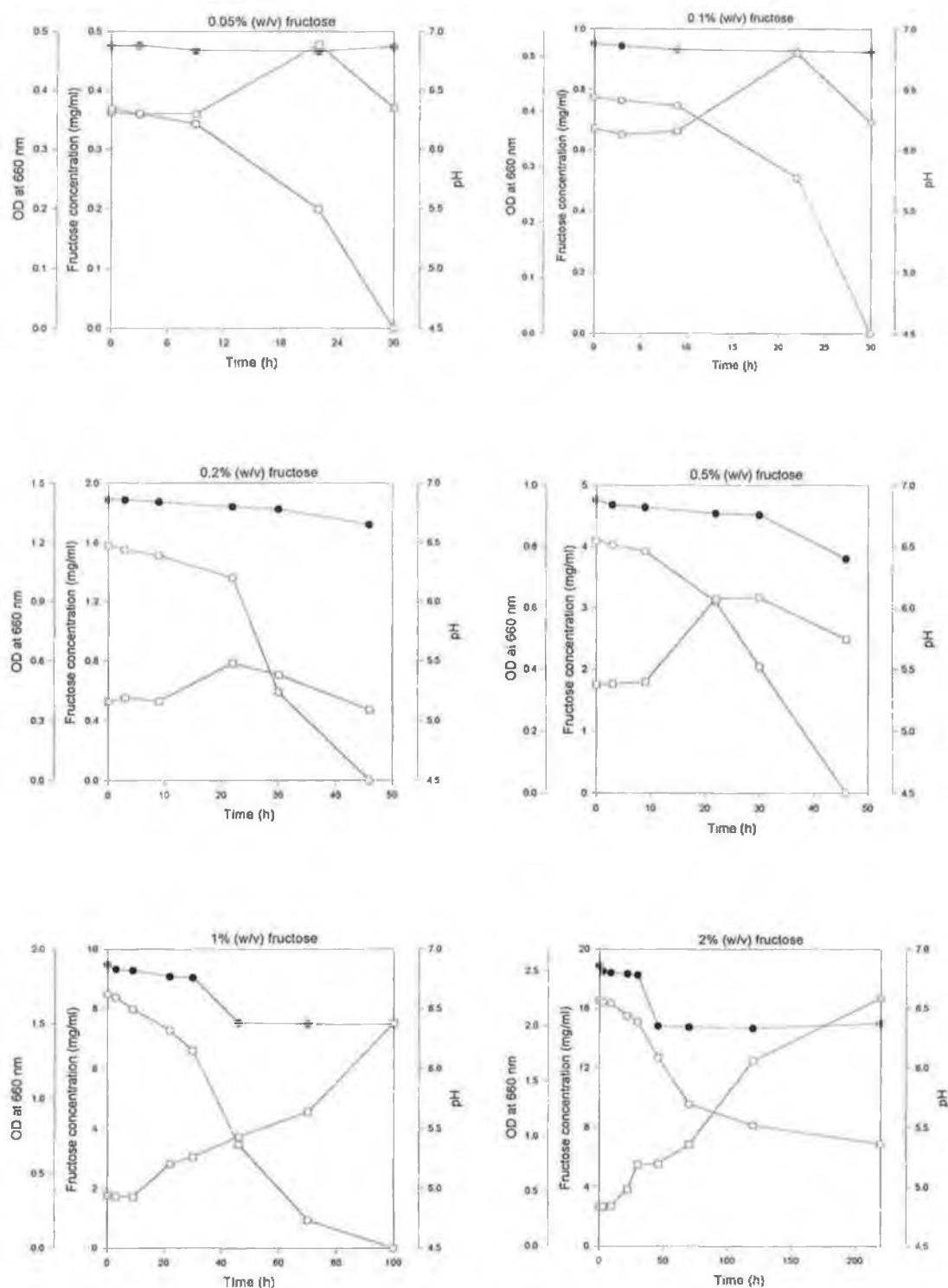


Fig. 25. The removal of various concentrations (0.05% to 2%, w/v) of fructose by *P. putida* CP1 when grown at 30°C. Symbols: ○, fructose concentration; □, OD at 660 nm; ●, pH.

3.1.3.2 Effect of the presence of various concentrations of fructose on 200 ppm 4-chlorophenol, 3-chlorophenol and 2-chlorophenol removal by *Pseudomonas putida* CP1

The effect of the presence of different concentrations (0.05% to 2%, w/v) of fructose on the degradation of 200 ppm of 4-chlorophenol, 3-chlorophenol and 2-chlorophenol is shown in Figs. 26, 27 and 28, respectively.

The organism grew in response to substrate removal. Growth increased with increasing concentrations of substrate and was greater in the presence of chlorophenols and fructose than in the presence of either substrate alone. The growth patterns were similar for all three isomers. Clumping of the cells was minimized when the two substrates were present.

With all isomers of mono-chlorophenol, the pH dropped only slightly in the presence of the various concentrations of fructose.

There was a similar time lag in the removal of both substrates. However the lag for chlorophenol degradation was less than that in the absence of sugar. The rate of removal of chlorophenol increased in the presence of fructose. Unlike glucose, inhibition of substrate removal was not found with fructose (Table 10). 4-chlorophenol was removed faster than the other two isomers. The rate of removal was similar for all concentrations of sugar. While the rates of removal of 2-chlorophenol and 3-chlorophenol were similar, the removal of 2-chlorophenol was greatly enhanced at the higher concentrations of sugar.

The rate of removal of fructose increased with increasing concentrations of sugar. The growth rate of the organism was similar for all treatments (Table 10).

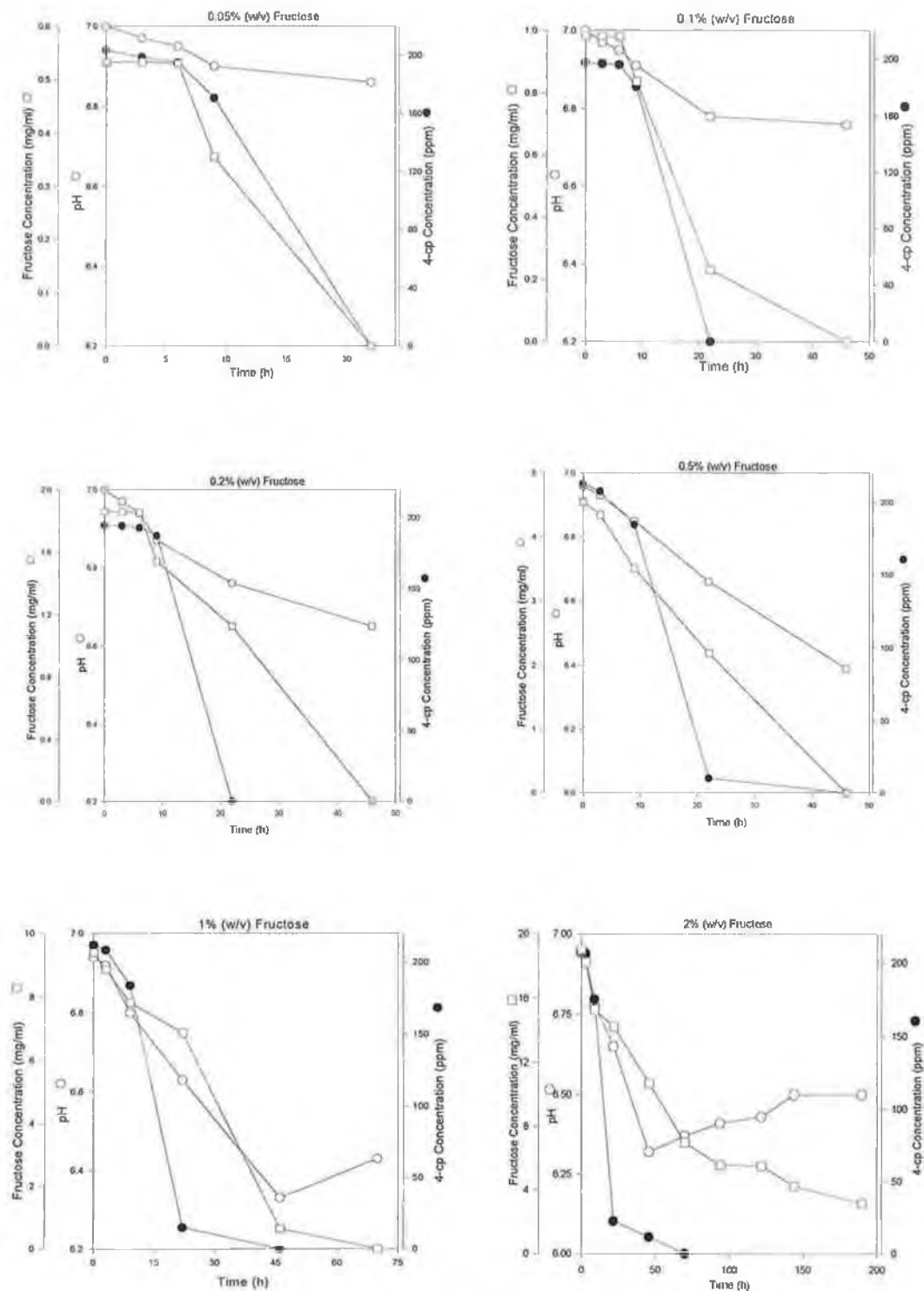


Fig. 26. The removal of 200 ppm 4-chlorophenol by *P. putida* CP1 in the presence of various concentrations (0.05% to 2%, w/v) of fructose at 30°C. Symbols: □ fructose concentration; ●, 4-chlorophenol concentration; ○, pH.

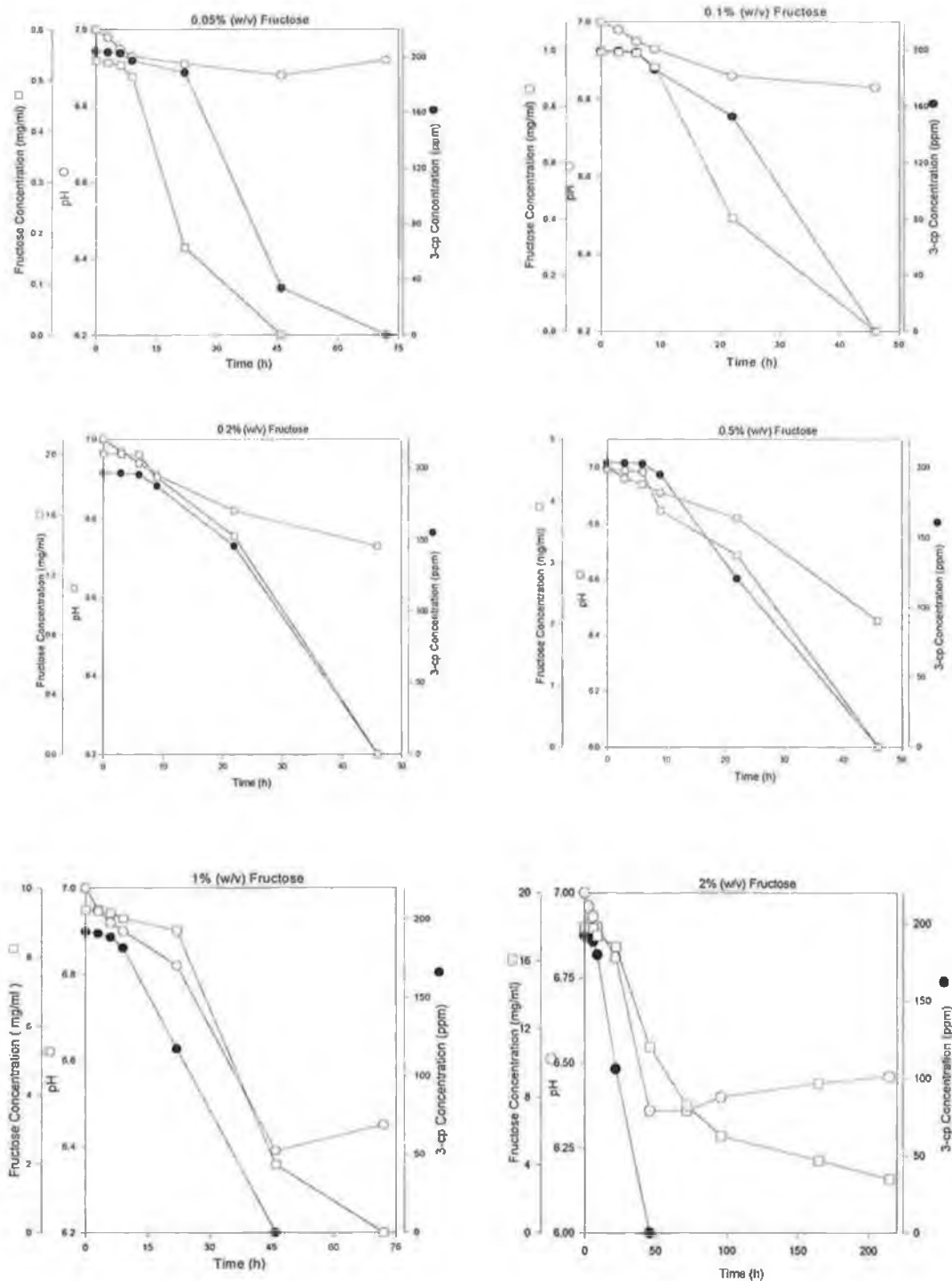


Fig. 27. The removal of 200 ppm 3-chlorophenol by *P. putida* CP1 in the presence of various concentrations (0.05% to 2%, w/v) of fructose at 30°C. Symbols: □, fructose concentration; ●, 3-chlorophenol concentration; ○, pH.

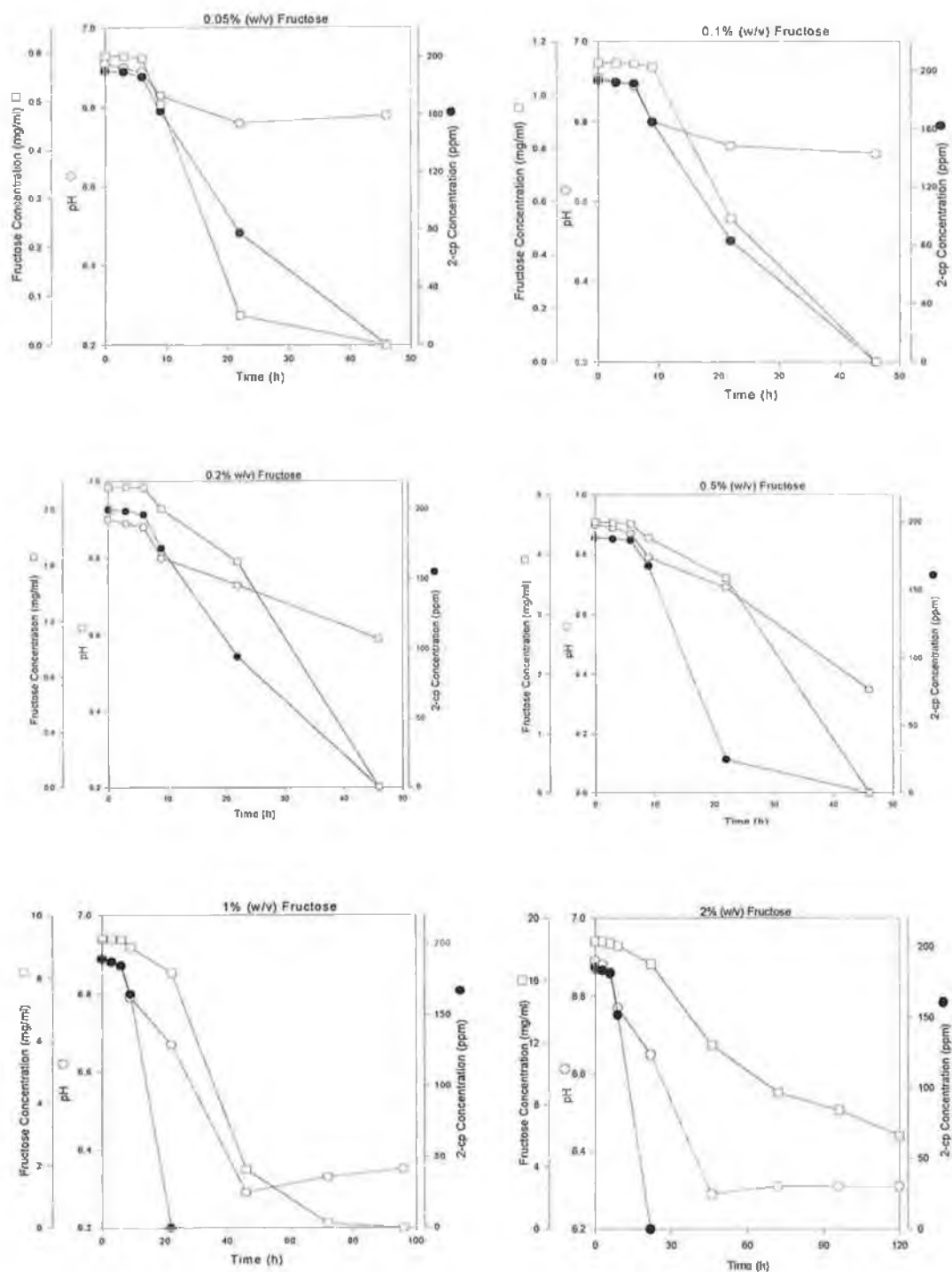


Fig. 28. The removal of 200 ppm 2-chlorophenol by *P. putida* CP1 in the presence of various concentrations (0.05% to 2%, w/v) of fructose at 30°C. Symbols: \square , fructose concentration; \bullet , 2-chlorophenol concentration; \circ , pH.

Table 10: Lag values, growth rates and substrates removal rates when *P. putida* CP1 was grown on 200 ppm mono-chlorophenols (cp) and various concentrations of fructose (F) at 30°C.

Treatment	Lag -cp h	Lag -F h	Growth rate (μ) h ⁻¹	Chlorophenol removal rate		Fructose removal rate	
				mg l ⁻¹ h ⁻¹	mg l ⁻¹ h ⁻¹ mg ⁻¹	mg l ⁻¹ h ⁻¹	mg l ⁻¹ h ⁻¹ mg ⁻¹
4-cp only	6	---	0.383 ± 0.020	4.64 ± 0.020	0.0082 ± 0.0004	-----	-----
+ 0.05% (w/v) F	6	6	0.343 ± 0.001	12.19 ± 0.03	0.0206 ± 0.0002	33.31 ± 1.31	0.052 ± 0.002
+ 0.1% (w/v) F	6	6	0.373 ± 0.005	12.24 ± 0.00	0.0217 ± 0.0000	46.87 ± 0.13	0.097 ± 0.000
+ 0.2% (w/v) F	6	6	0.406 ± 0.008	12.08 ± 0.10	0.0225 ± 0.0002	58.87 ± 0.59	0.125 ± 0.001
+ 0.5% (w/v) F	6	6	0.370 ± 0.001	12.10 ± 0.06	0.0211 ± 0.0000	92.60 ± 1.00	0.269 ± 0.002
+ 1% (w/v) F	6	6	0.331 ± 0.002	12.12 ± 0.03	0.0219 ± 0.0000	154.97 ± 4.50	0.529 ± 0.018
+ 2% (w/v) F	6	6	0.300 ± 0.000	12.04 ± 0.03	0.0229 ± 0.0001	127.58 ± 1.05	0.625 ± 0.004
3-cp only	22	---	0.306 ± 0.025	1.02 ± 0.01	0.0021 ± 0.0000	-----	-----
+ 0.05% (w/v) F	9	9	0.370 ± 0.004	3.78 ± 0.02	0.0070 ± 0.0000	25.84 ± 1.51	0.019 ± 0.001
+ 0.1% (w/v) F	9	9	0.383 ± 0.020	5.03 ± 0.12	0.0079 ± 0.0002	41.38 ± 3.00	0.038 ± 0.002
+ 0.2% (w/v) F	9	9	0.400 ± 0.003	5.07 ± 0.00	0.0074 ± 0.0000	49.97 ± 0.59	0.073 ± 0.000
+ 0.5% (w/v) F	9	9	0.436 ± 0.007	5.07 ± 0.15	0.0076 ± 0.0002	104.05 ± 2.94	0.144 ± 0.004
+ 1% (w/v) F	9	9	0.310 ± 0.002	4.91 ± 0.03	0.0076 ± 0.0000	190.27 ± 1.35	0.360 ± 0.002
+ 2% (w/v) F	9	9	0.293 ± 0.015	4.86 ± 0.04	0.0077 ± 0.0000	158.10 ± 1.01	0.615 ± 0.003
2-cp only	9	---	0.385 ± 0.015	2.11 ± 0.01	0.0040 ± 0.0000	-----	-----
+ 0.05% (w/v) F	6	6	0.401 ± 0.007	4.66 ± 0.02	0.0120 ± 0.0000	32.87 ± 0.25	0.060 ± 0.000
+ 0.1% (w/v) F	6	6	0.415 ± 0.000	4.78 ± 0.03	0.0130 ± 0.0000	37.29 ± 2.07	0.076 ± 0.004
+ 0.2% (w/v) F	6	6	0.425 ± 0.008	4.89 ± 0.15	0.0130 ± 0.0004	54.05 ± 0.01	0.103 ± 0.000
+ 0.5% (w/v) F	6	6	0.461 ± 0.003	5.05 ± 0.01	0.0170 ± 0.0003	115.92 ± 0.86	0.188 ± 0.001
+ 1% (w/v) F	6	6	0.411 ± 0.001	11.56 ± 0.19	0.0190 ± 0.0003	190.51 ± 0.80	0.333 ± 0.002
+ 2% (w/v) F	6	6	0.401 ± 0.002	11.33 ± 0.15	0.0190 ± 0.0002	173.43 ± 0.78	0.491 ± 0.003

± = Standard error

3.1.3.3 Degradation of higher concentrations of mono-chlorophenols by *Pseudomonas putida* CP1 in the presence of 1% (w/v) fructose

As the presence of fructose was found to enhance the degradation of mono-chlorophenols by *P. putida* CP1, the effect of 1% (w/v) fructose on the degradation of higher concentrations, >200 ppm, of 4-chlorophenol, 3-chlorophenol and 2-chlorophenol by *P. putida* CP1 was investigated.

The removal of various concentrations of 4-chlorophenol, 3-chlorophenol and 2-chlorophenol by *P. putida* CP1 in the presence of 1% (w/v) fructose is shown in Figs. 29, 30 and 31, respectively. Substrate removal together with the pH of the growth medium is noted. There was a lag in the degradation of the chlorophenols and fructose – the lag time increased with the increasing concentration of mono-chlorophenols (Table 11).

In general the rates of chlorophenol and fructose removal decreased above 200 ppm. Chlorophenol removal was inhibited at 400 ppm for 4-chlorophenol, 300 ppm for 3-chlorophenol and 500 ppm for 2-chlorophenol. In these systems, growth and fructose removal was also inhibited. Where substrate removal was inhibited there was a decrease in optical density suggesting cell lysis.

The growth rate of the organism decreased above 200 ppm (Table 11). The pH of the medium fell slightly, about 0.6 unit, with substrate metabolism. There was no pH change in the absence of substrate removal.

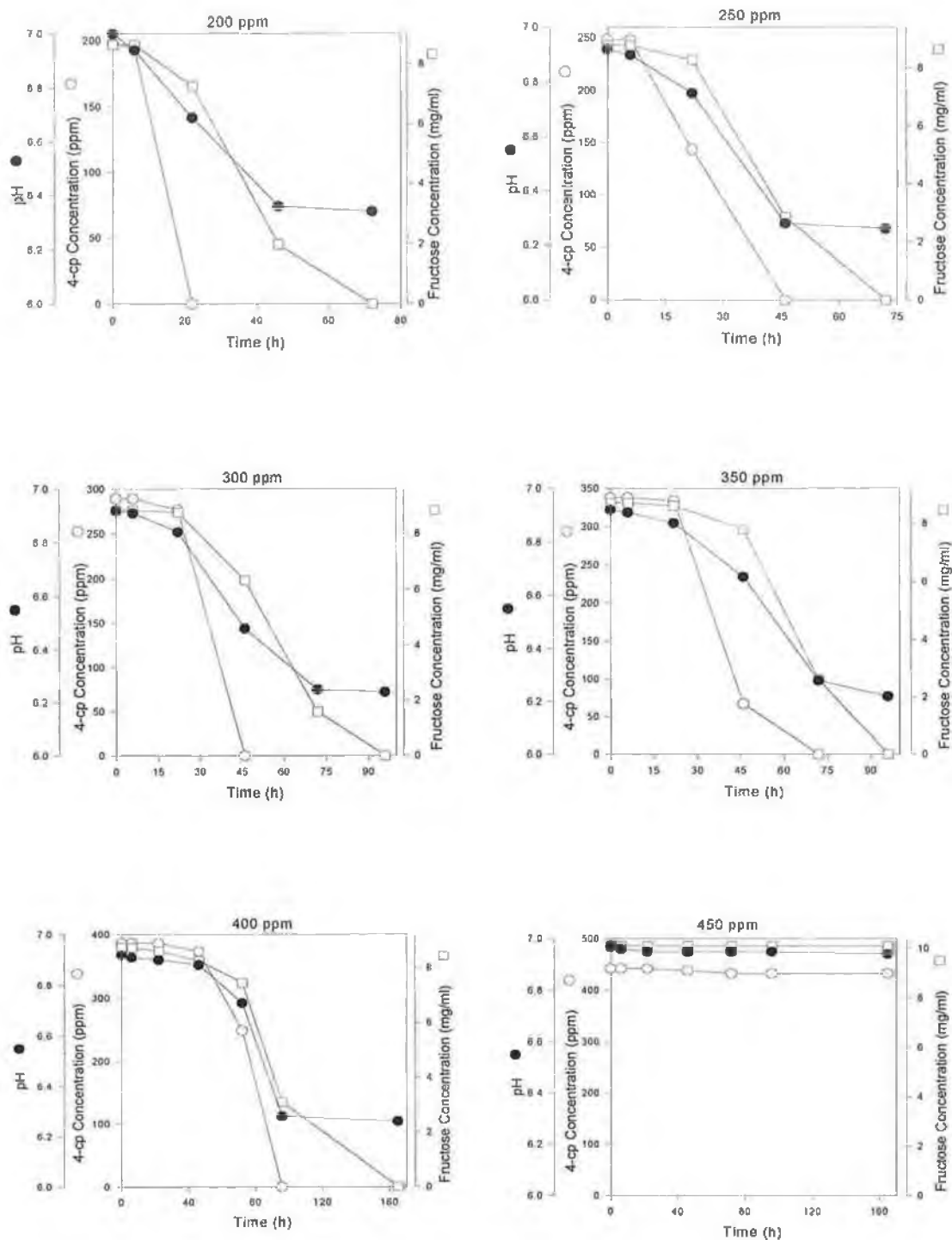


Fig. 29. The removal of high concentrations of 4-chlorophenol by *P. putida* CP1 in the presence of 1% (w/v) fructose. Symbols: O, chlorophenol concentration; □, fructose concentration; ●, pH

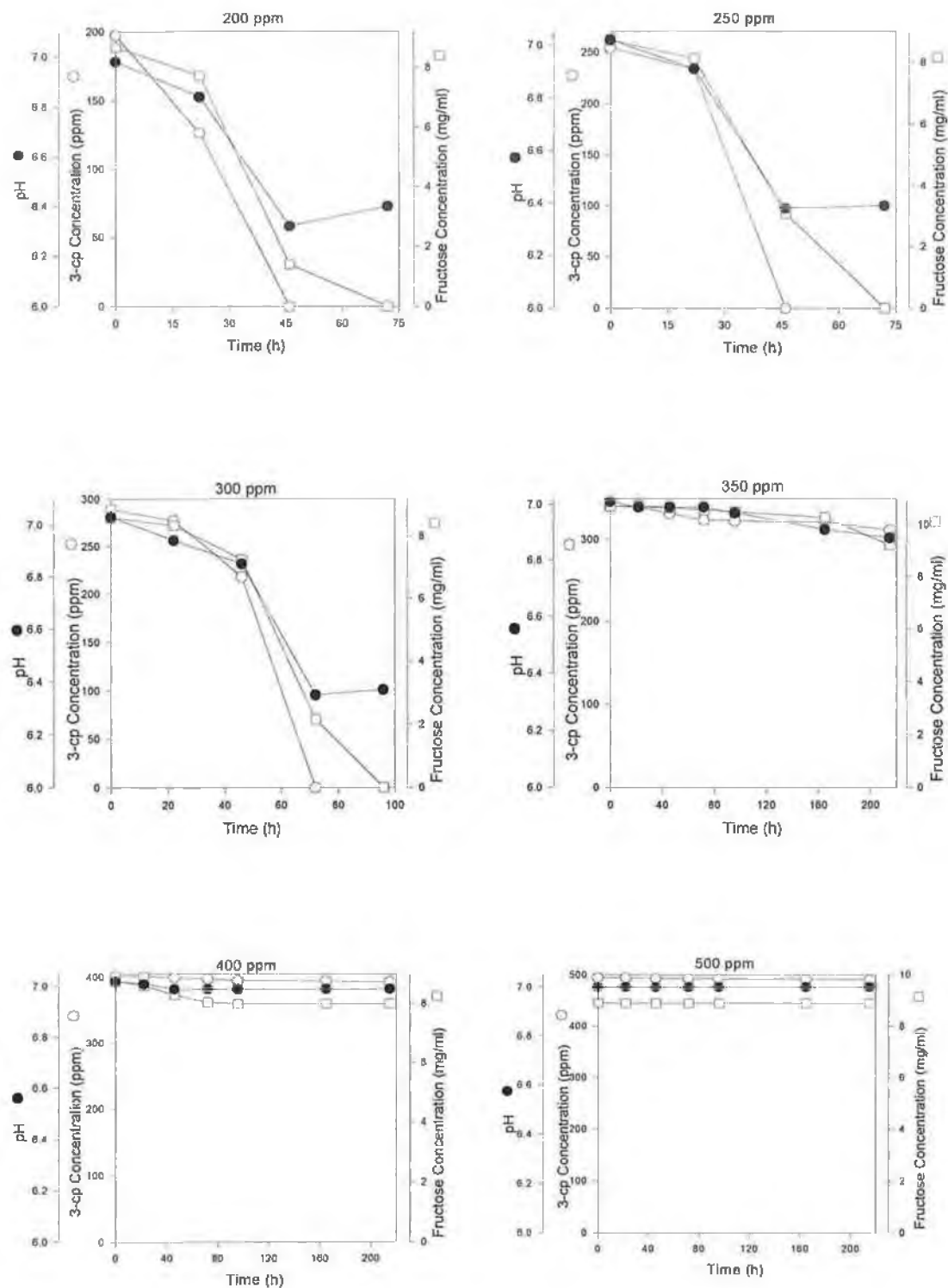


Fig. 30. The removal of high concentrations of 3-chlorophenol by *P. putida* CP1 in the presence of 1% (w/v) fructose. Symbols: O, chlorophenol concentration; □ fructose concentration; ●, pH

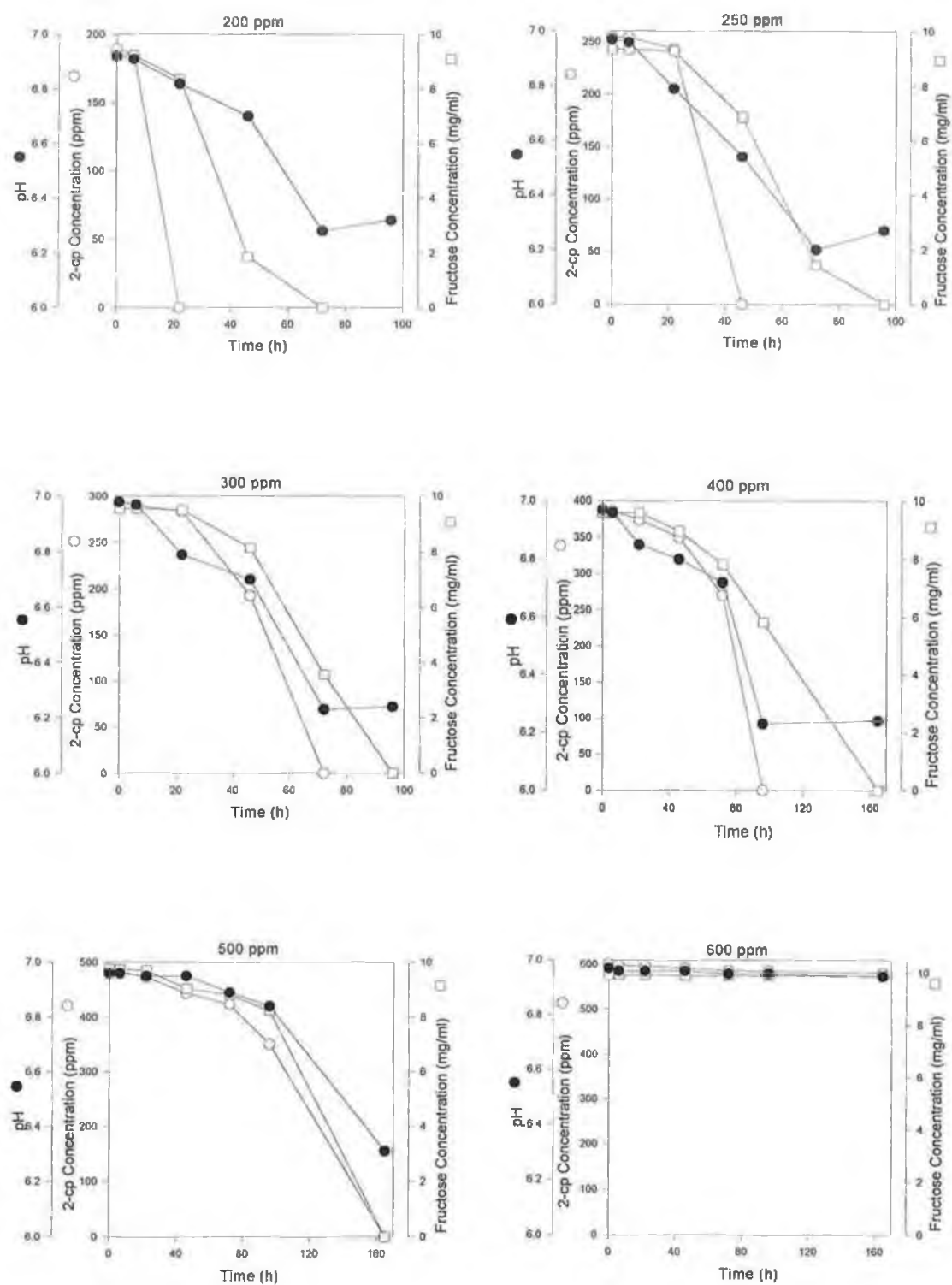


Fig. 31. The removal of high concentrations of 2-chlorophenol by *P. putida* CP1 in the presence of 1% (w/v) fructose. Symbols: O, chlorophenol concentration; □ fructose concentration; ●, pH

Table 11: Lag values, growth rates and substrates removal rates when *P. putida* CP1 was grown on high concentrations of chlorophenols (cp) and 1% (w/v) fructose (F) at 30°C.

cp conc.	Lag -cp	Lag -F	Growth rate (μ)	Chlorophenol removal rate		Fructose removal rate	
ppm	h	h	h^{-1}	$\text{mg l}^{-1} \text{h}^{-1}$	$\text{mg l}^{-1} \text{h}^{-1} \text{mg}^{-1}$	$\text{mg l}^{-1} \text{h}^{-1}$	$\text{mg l}^{-1} \text{h}^{-1} \text{mg}^{-1}$
4-cp							
200	6	6	0.105 ± 0.009	12.12 ± 0.03	0.021 ± 0.000	154.97 ± 4.50	0.729 ± 0.011
250	6	22	0.092 ± 0.002	7.29 ± 0.05	0.011 ± 0.000	148.08 ± 2.50	0.378 ± 0.006
300	9	22	0.086 ± 0.002	7.20 ± 0.11	0.014 ± 0.000	143.40 ± 3.80	0.238 ± 0.006
350	22	22	0.053 ± 0.001	7.68 ± 0.49	0.016 ± 0.000	119.60 ± 0.15	0.236 ± 0.000
400	46	46	0.047 ± 0.001	7.46 ± 0.03	0.011 ± 0.000	103.00 ± 0.05	0.174 ± 0.000
450	-----	-----	0.000 ± 0.000	Inhibition	Inhibition	Inhibition	Inhibition
3-cp							
200	9	9	0.102 ± 0.006	4.91 ± 0.03	0.007 ± 0.000	190.27 ± 1.35	0.360 ± 0.003
250	9	9	0.093 ± 0.003	5.54 ± 0.05	0.010 ± 0.000	162.80 ± 0.65	0.331 ± 0.002
300	22	22	0.079 ± 0.001	5.49 ± 0.07	0.007 ± 0.000	123.80 ± 4.50	0.200 ± 0.007
350	----	----	0.000 ± 0.000	Inhibition	Inhibition	Inhibition	Inhibition
400	----	----	0.000 ± 0.000	Inhibition	Inhibition	Inhibition	Inhibition
500	-----	----	0.000 ± 0.000	Inhibition	Inhibition	Inhibition	Inhibition
2-cp							
200	6	6	0.099 ± 0.002	11.56 ± 0.195	0.019 ± 0.000	190.56 ± 0.80	0.333 ± 0.001
250	6	22	0.088 ± 0.002	10.70 ± 0.135	0.013 ± 0.000	156.80 ± 1.60	0.275 ± 0.003
300	6	22	0.086 ± 0.001	5.67 ± 0.02	0.011 ± 0.000	119.05 ± 3.50	0.215 ± 0.006
400	22	22	0.036 ± 0.000	5.05 ± 0.175	0.009 ± 0.000	84.08 ± 0.375	0.176 ± 0.000
500	22	22	0.020 ± 0.000	4.54 ± 0.03	0.008 ± 0.000	67.35 ± 3.43	0.173 ± 0.007
600	-----	----	0.000 ± 0.000	Inhibition	Inhibition	Inhibition	Inhibition
1% (w/v) F	-----	9	-----	-----	-----	114.37 ± 1.50	0.226 ± 0.003

\pm = Standard error

3.1.4 Removal of various concentrations (0.01% to 1%, w/v) of yeast extract by *Pseudomonas putida* CP1

The utilization of various concentrations (0.01% to 1%, w/v) of yeast extract by *P. putida* CP1 was investigated (Fig. 32). COD removal, OD at 660 nm, and changes in pH were also determined.

Substrate removal was measured in terms of COD. There was no lag in substrate removal however, except for 0.01% (w/v) yeast extract, there were increasing levels of residual COD with increasing concentrations of yeast extract. The specific rate of COD removal increased proportionally (linearly) with increasing concentrations of yeast extract (Fig. 33).

A decrease in COD was accompanied by an increase in biomass. There was no clumping of the cells. Growth increased with increasing concentrations of yeast extract. A plot of growth rate versus yeast extract concentration (Fig. 34) illustrates a maximum rate of growth, μ_{\max} of 0.3926 h^{-1} with 0.2% (w/v) yeast extract. K_s , which is equivalent to the substrate concentration at half-maximum growth rate ($\frac{1}{2}\mu_{\max} = 0.1963 \text{ h}^{-1}$) was calculated at 0.035% (w/v).

In all cases the pH of the growth medium increased. The increase was greatest, up to 8.15, at the highest concentration of yeast extract.

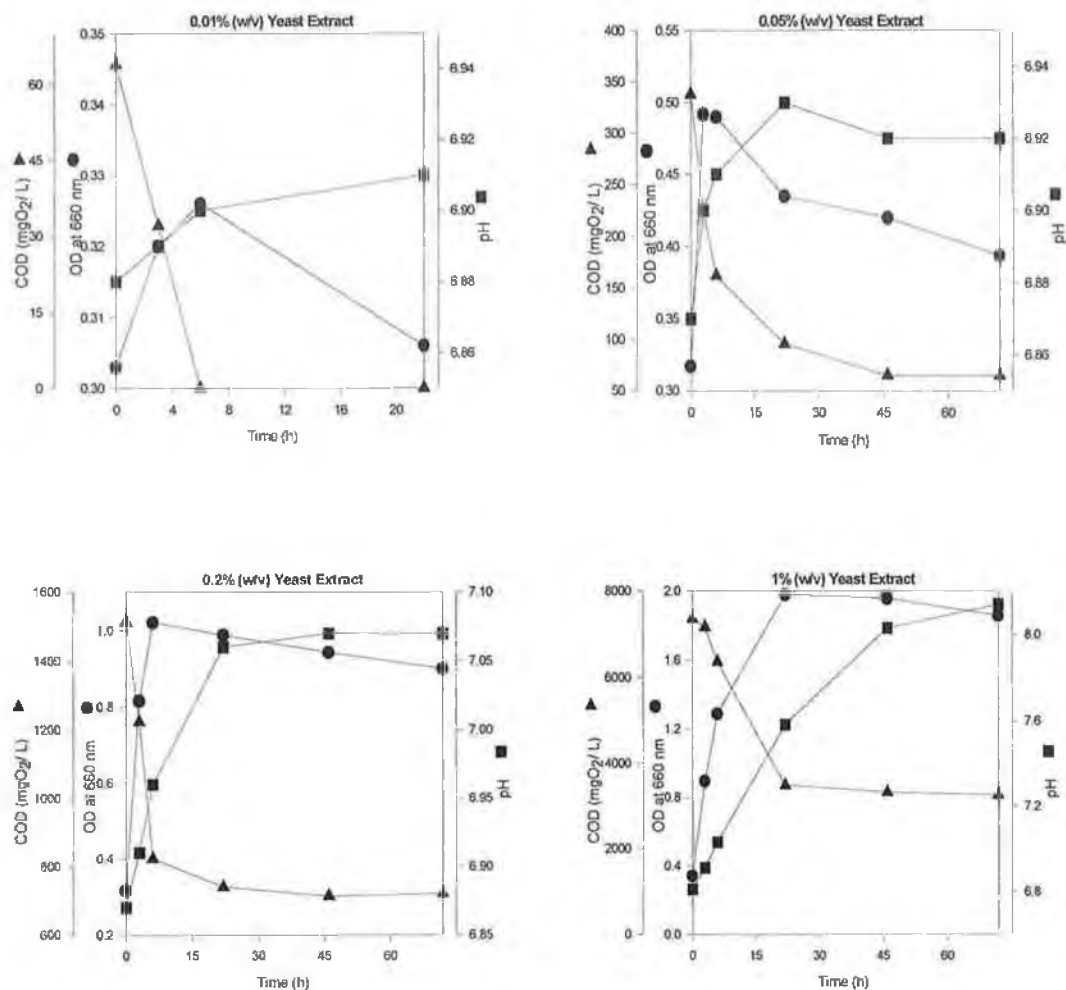


Fig. 32. Removal of COD, changes in biomass and pH on various concentrations (0.01% to 1%, w/v) of yeast extract containing minimal media by *P. putida* CP1. Symbols: ●, biomass; ▲, COD; ■, pH.

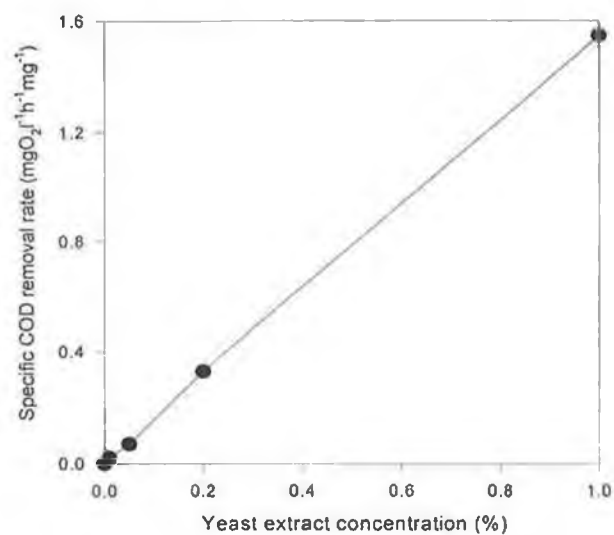


Fig. 33. Specific COD removal rates by *P. putida* CPI grown on various concentrations (0.01% to 1%, w/v) of yeast extract.

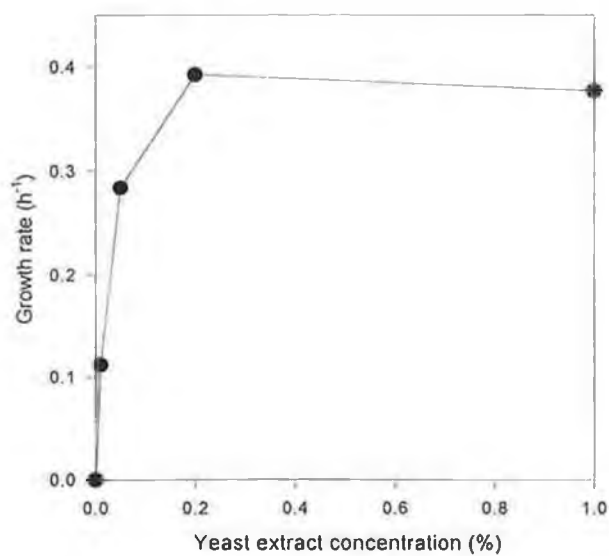


Fig. 34. Specific growth rates (μ) plotted as a function of yeast extract concentration.

3.1.4.1 Effect of the presence of various concentrations of yeast extract on removal of 200 ppm mono-chlorophenols by *Pseudomonas putida* CP1

The removal of 200 ppm mono-chlorophenols in the presence of various concentrations (0.01 to 1%, w/v) of yeast extract was investigated. COD removal, chlorophenol removal, OD at 660nm, changes in pH and chloride release were also determined (Figs. 35, 36, 37, 38 and Table 12).

The organism grew in response to substrate removal. Growth increased with increasing concentrations of yeast extract. The organism flocculated when grown in the presence of chlorophenol as the sole source of carbon. Clumping was most marked in the presence of 2- and 3-chlorophenol and less in the presence of 4-chlorophenol. When yeast extract was introduced to the medium, the degree of flocculation decreased with increasing concentrations of yeast extract. However, unlike the situation with chlorophenol alone, the clumping in the presence of both substrates was most marked in the presence of 4-chlorophenol and less in the presence of 2- and 3-chlorophenol.

The change in pH was very slight in the controls and this was also the case in the treatments containing less than 0.2% (w/v) yeast extract. Above that concentration of yeast extract, the pH increased slightly with increasing concentrations of yeast extract.

The removal of substrate was monitored by measuring both chlorophenol levels and COD. The removal of chlorophenols was measured directly. However, the removal of yeast extract was measured by monitoring the removal of COD which was also a measure of chlorophenol removal.

COD removal mirrored chlorophenol removal in the control flasks and the substrate was completely removed. When yeast extract was present, the COD increased with

increasing concentrations of yeast extract. However, while the chlorophenols were completely removed, there was residual COD, which increased with increasing concentrations of yeast extract.

There was a lag in the removal of chlorophenol in all cases. However, the lag in the removal of chlorophenol decreased with increasing concentrations of yeast extract.

The rate of removal of chlorophenol increased with increasing concentrations of yeast extract up to a value of 0.5% (w/v). At 1% (w/v) yeast extract there was a decrease in the rate of chlorophenol removal. The rate of removal of COD however continued to increase with an increase in COD concentration. In the absence of yeast extract, there was a stoichiometric release of chloride. Chloride was also released from yeast extract, however, in the presence of yeast extract, the release was not always stoichiometric (Table 12).

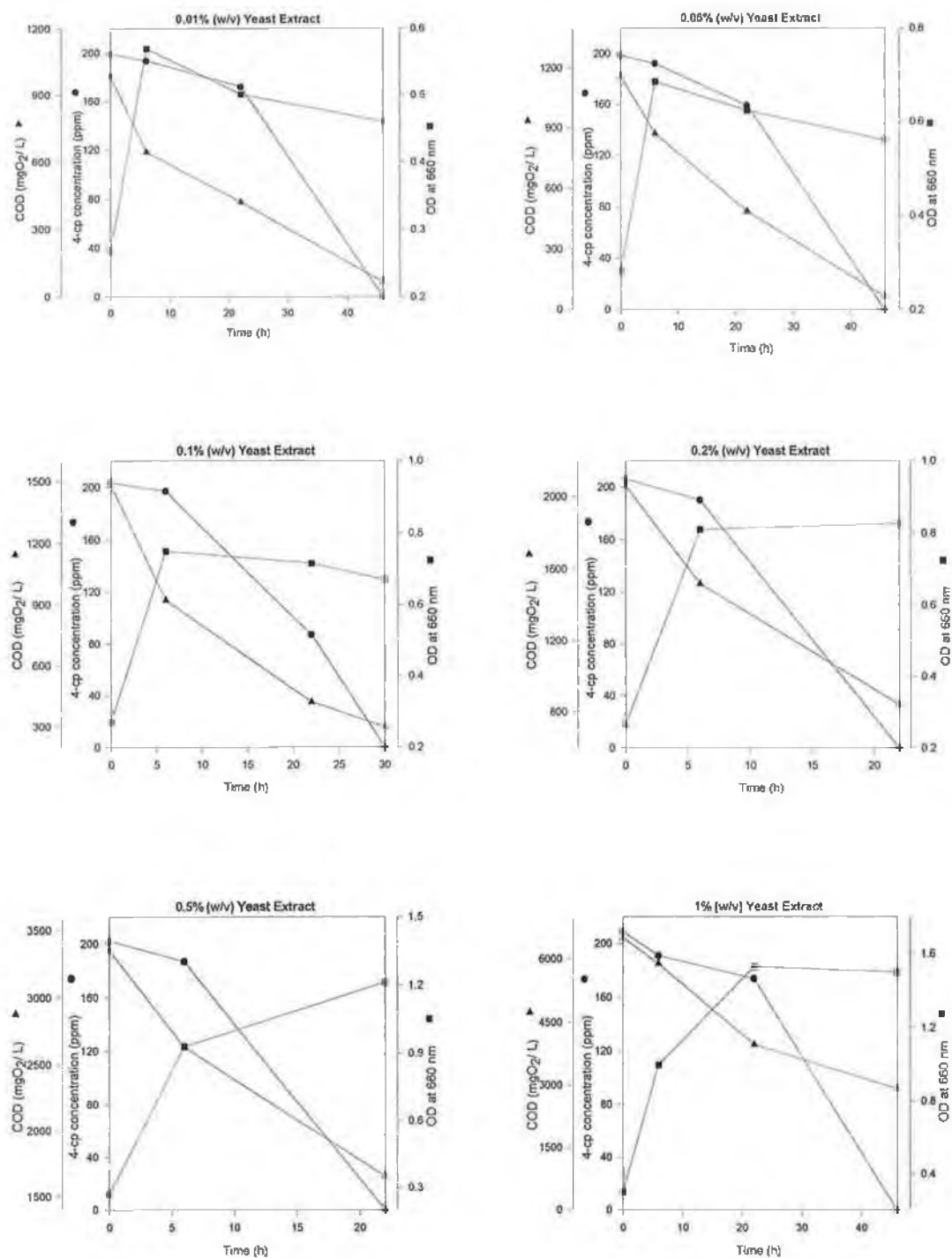


Fig. 35. The removal of 200 ppm 4-chlorophenol by *P. putida* CP1 in the presence of various concentrations (0.01% - 1%, w/v) of yeast extract. Symbols: ●, 4-chlorophenol concentration; ▲, COD; ■, OD at 660 nm.

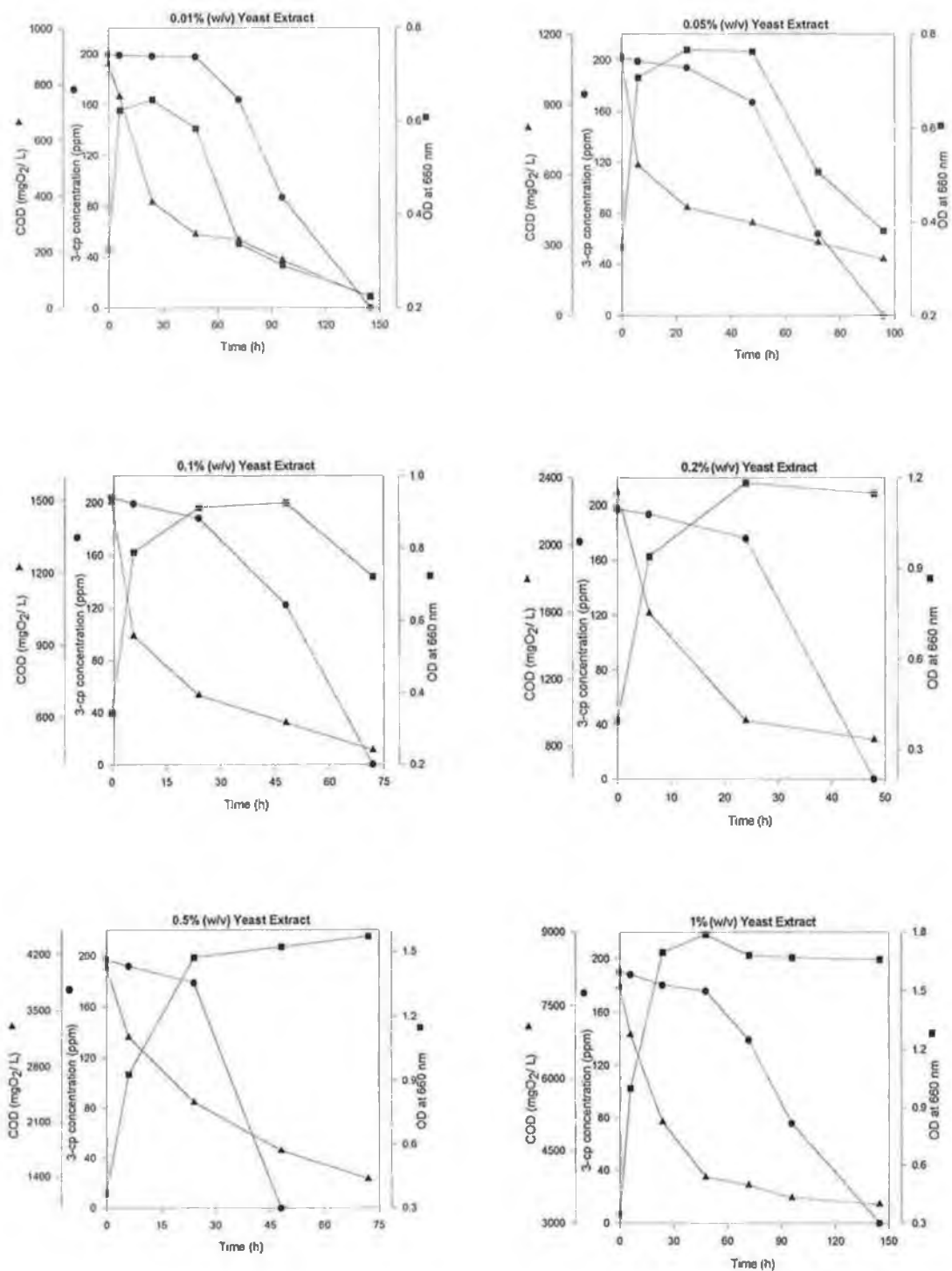


Fig. 36. The removal of 200 ppm 3-chlorophenol by *P. putida* CP1 in the presence of various concentrations (0.01% - 1%, w/v) of yeast extract. Symbols: ●, 3-chlorophenol concentration; ▲, COD; ■, OD at 660 nm.

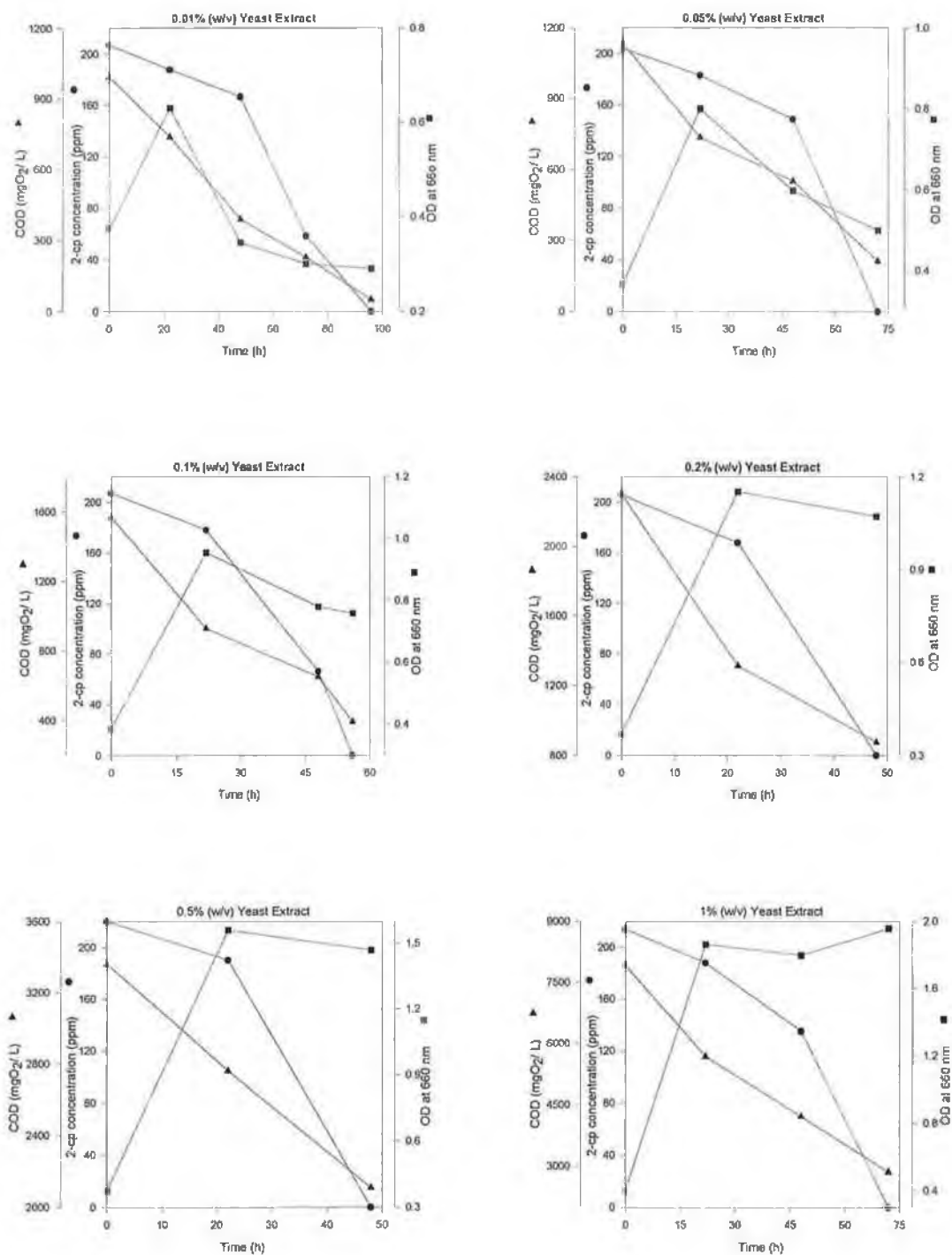


Fig. 37. The removal of 200 ppm 2-chlorophenol by *P. putida* CP1 in the presence of various concentrations (0.01% - 1%, w/v) of yeast extract. Symbols: ●, 2-chlorophenol concentration; ▲, COD; ■, OD at 660 nm.

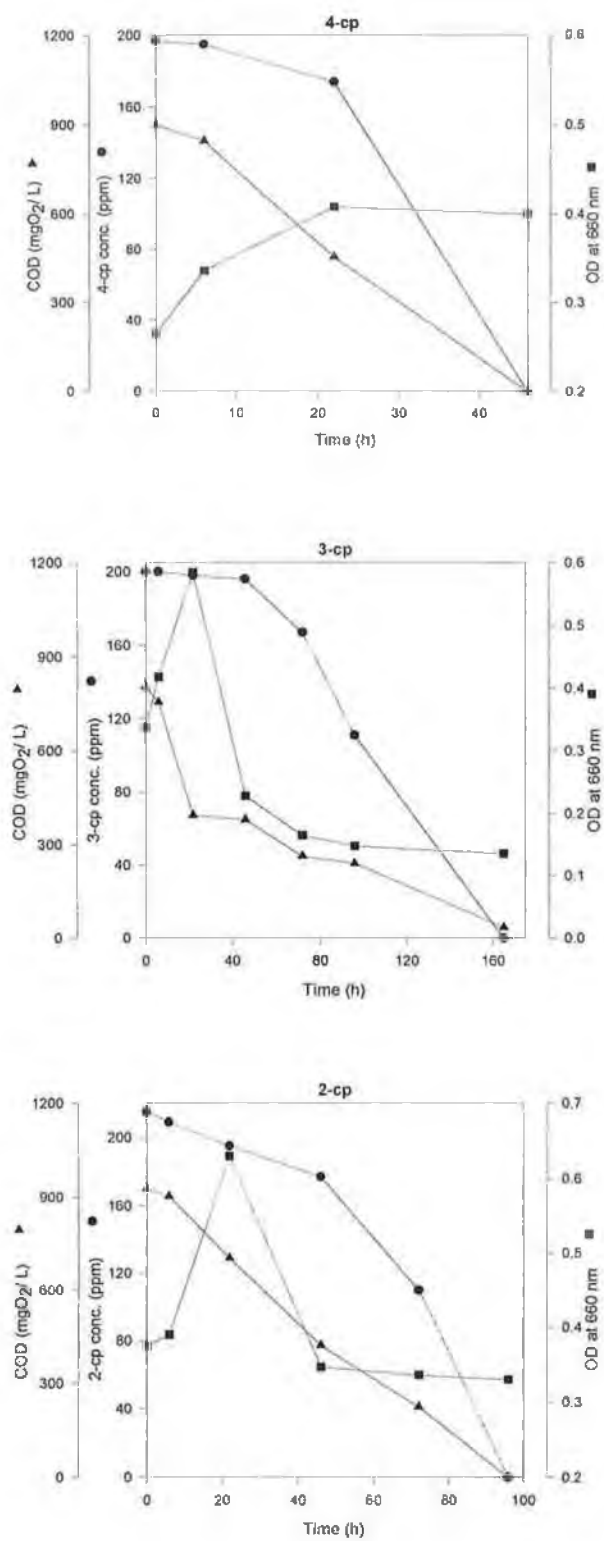


Fig. 38. The removal of 200 ppm mono-chlorophenols by *P. putida* CP1. Symbols: ●, mono-chlorophenol concentration; ▲, COD; ■, OD at 660 nm.

Table 12: Lag periods, C:N, changes in chloride and pH, and rates of chlorophenol and COD removal by *P. putida* CP1 when grown on 200 ppm mono-chlorophenols in the presence of various concentration of yeast extract (YE).

Treatment	Lag -cp (h)	C:N	ΔCl^-	ΔpH	Chlorophenol removal rate		COD removal rate	
					$\text{mg l}^{-1} \text{h}^{-1}$	$\text{mg l}^{-1} \text{h}^{-1} \text{mg}^{-1}$	$\text{mg O}_2 \text{ l}^{-1} \text{h}^{-1}$	$\text{mg O}_2 \text{ l}^{-1} \text{h}^{-1} \text{mg}^{-1}$
4-cp only	6	0.427	62.05 ± 3.50	- 0.10	4.64 ± 0.02	0.0082 ± 0.0000	20.35 ± 1.48	0.052 ± 0.004
+ 0.01% (w/v) YE	6	0.479	75.30 ± 4.00	- 0.10	4.82 ± 0.02	0.0101 ± 0.0000	23.36 ± 1.46	0.067 ± 0.004
+ 0.05% (w/v) YE	6	0.648	90.00 ± 4.00	- 0.08	4.80 ± 0.11	0.0105 ± 0.0002	30.61 ± 0.01	0.082 ± 0.000
+ 0.1% (w/v) YE	6	0.804	82.05 ± 0.15	- 0.03	7.71 ± 0.10	0.0088 ± 0.0002	47.27 ± 0.09	0.122 ± 0.000
+ 0.2% (w/v) YE	3	1.013	76.10 ± 4.10	+ 0.05	10.70 ± 0.26	0.0125 ± 0.0003	55.39 ± 1.46	0.162 ± 0.004
+ 0.5% (w/v) YE	3	1.291	80.00 ± 3.00	+ 0.29	10.48 ± 0.04	0.0104 ± 0.0004	76.50 ± 4.50	0.279 ± 0.015
+ 1% (w/v) YE	3	1.459	43.50 ± 0.50	+ 0.98	4.76 ± 0.02	0.0061 ± 0.0000	129.58 ± 5.5	0.483 ± 0.016
3-cp only	22	0.427	59.50 ± 0.50	- 0.10	1.03 ± 0.01	0.0021 ± 0.0000	10.01 ± 0.50	0.024 ± 0.001
+ 0.01% (w/v) YE	22	0.479	72.50 ± 0.50	- 0.09	1.59 ± 0.15	0.0025 ± 0.0002	12.69 ± 0.68	0.043 ± 0.002
+ 0.05% (w/v) YE	22	0.648	83.15 ± 3.75	- 0.08	2.68 ± 0.03	0.0028 ± 0.0000	14.86 ± 0.00	0.053 ± 0.000
+ 0.1% (w/v) YE	6	0.804	86.40 ± 5.00	- 0.03	3.01 ± 0.05	0.0031 ± 0.0000	19.13 ± 0.70	0.076 ± 0.003
+ 0.2% (w/v) YE	6	1.013	117.5 ± 0.35	+ 0.08	4.60 ± 0.00	0.0044 ± 0.0000	30.78 ± 0.37	0.114 ± 0.001
+ 0.5% (w/v) YE	6	1.291	108.5 ± 0.50	+ 0.41	4.57 ± 0.01	0.0043 ± 0.0000	47.96 ± 0.09	0.220 ± 0.000
+ 1% (w/v) YE	22	1.459	127.5 ± 5.50	+ 1.33	1.76 ± 0.02	0.0017 ± 0.0000	82.13 ± 1.67	0.424 ± 0.007
2-cp only	9	0.427	59.3 ± 0.00	- 0.10	2.11 ± 0.010	0.0035 ± 0.0000	10.25 ± 0.33	0.021 ± 0.000
+ 0.01% (w/v) YE	9	0.479	72.05 ± 0.35	- 0.09	2.22 ± 0.015	0.0036 ± 0.0000	12.59 ± 0.68	0.026 ± 0.001
+ 0.05% (w/v) YE	6	0.648	80.05 ± 4.24	- 0.06	2.98 ± 0.025	0.0038 ± 0.0000	18.18 ± 2.18	0.058 ± 0.006
+ 0.1% (w/v) YE	6	0.804	81.3 ± 1.70	- 0.03	3.94 ± 0.07	0.0037 ± 0.0001	29.09 ± 2.91	0.082 ± 0.008
+ 0.2% (w/v) YE	6	1.013	102.5 ± 3.50	+ 0.06	4.44 ± 0.100	0.0041 ± 0.0000	45.08 ± 1.45	0.134 ± 0.000
+ 0.5% (w/v) YE	6	1.291	111.5 ± 5.50	+ 0.32	4.83 ± 0.03	0.0041 ± 0.0000	56.18 ± 1.14	0.214 ± 0.003
+ 1% (w/v) YE	6	1.459	46.00 ± 0.50	+ 1.03	2.99 ± 0.15	0.0021 ± 0.0001	101.5 ± 7.25	0.448 ± 0.025

\pm = Standard error

3.1.4.2 Effect of the presence of 0.1% (w/v) yeast extract plus 0.2% (w/v) fructose on the removal of 200 ppm 4-chlorophenol, 3-chlorophenol and 2-chlorophenol by *Pseudomonas putida* CP1

There was always some residual COD when chlorophenol removal was investigated in the presence of yeast extract. It was thought that this might have been due to a less than optimal carbon: nitrogen ratio. An investigation was carried out to study chlorophenol removal in the presence of a more favourable C:N ratio. A combination of yeast extract (0.1%, w/v) and fructose (0.2%, w/v) with a C:N ratio 3:1 was chosen.

The effect of the presence of a combination of 0.1% (w/v) yeast extract and 0.2% (w/v) fructose on the degradation of 200 ppm of mono-chlorophenol was investigated (Figs. 39, 40 and 41). Substrate removal was measured in terms of COD, reducing sugars and chlorophenol removal. The pH and OD of the culture were also measured.

The carbon to nitrogen ratio (C:N) for each system is listed in Table 13. There was little change in pH in any of the systems.

The removal of chlorophenol, COD and fructose when present was concurrent. The rate of chlorophenol removal was fastest for 4-chlorophenol, then 2-chlorophenol and finally 3-chlorophenol. The rate of removal increased in the presence of an additional carbon source. The rate of removal was greater in the presence of fructose than yeast extract but was greatest in the presence of both substrates. The order in terms of rate of removal continued to be 4-chlorophenol > 2-chlorophenol > 3-chlorophenol even in the presence of additional carbon sources.

The rate of removal of COD increased with increasing concentrations of COD. The rate of removal of fructose increased in the presence of yeast extract.

The organism clumped when grown in the presence of chlorophenols alone. As before the degree of clumping was greater in the presence of 2-chlorophenol and 3-chlorophenol than 4-chlorophenol. The degree of clumping decreased with the addition of carbon. Clumping was reduced in the presence of both fructose and yeast extract when added individually to the medium. However the degree of clumping in this instance was greater for 4-chlorophenol than with 2- and 3-chlorophenol. When both substrates were added to the growth medium there was no clumping of the organism in the presence of 2- and 3-chlorophenol and only slight clumping in the presence of 4-chlorophenol.

The degree of growth and the rate of growth increased with increasing COD concentrations in the case of 3-chlorophenol. Whereas for both 4-chlorophenol and 2-chlorophenol the maximum growth was found in the presence of both yeast extract and fructose, followed by yeast extract, which was followed by fructose (Fig. 42 and Table 13).

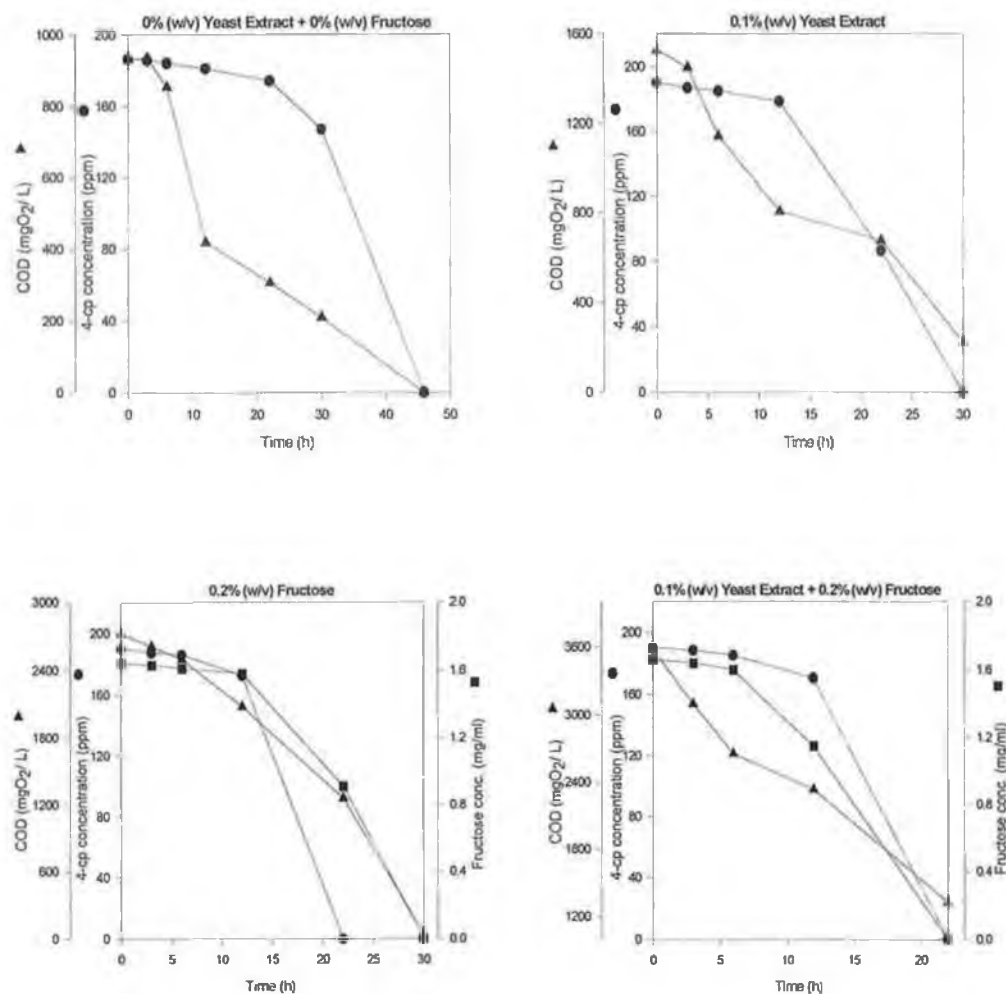


Fig. 39. The removal of 200 ppm 4-chlorophenol by *P. putida* CP1 in the presence of 0.1% (w/v) yeast extract plus 0.2% (w/v) fructose. Symbols: ●, 4-chlorophenol concentration; ▲, COD; ■, fructose concentration.

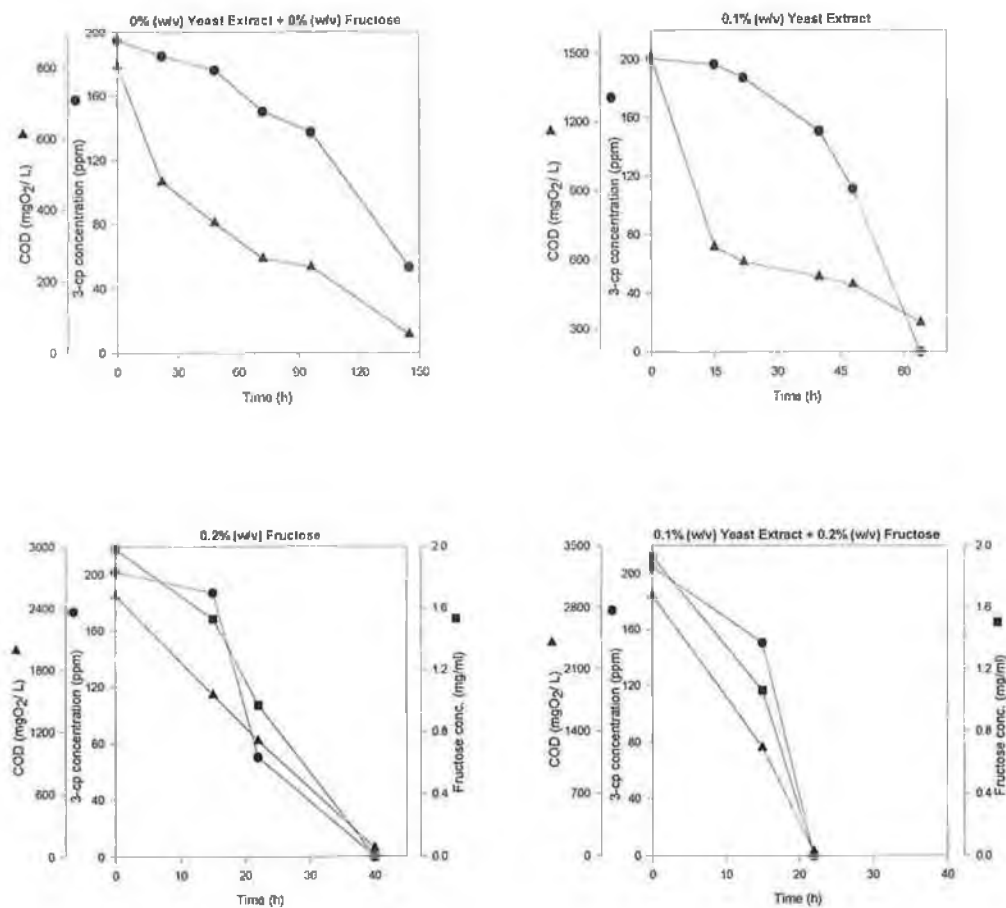


Fig. 40. The Removal of 200 ppm 3-chlorophenol by *P. putida* CP1 in the presence of 0.1% (w/v) yeast extract plus 0.2% (w/v) fructose. Symbols: ●, 3-chlorophenol concentration; ▲, COD; ■, fructose concentration.

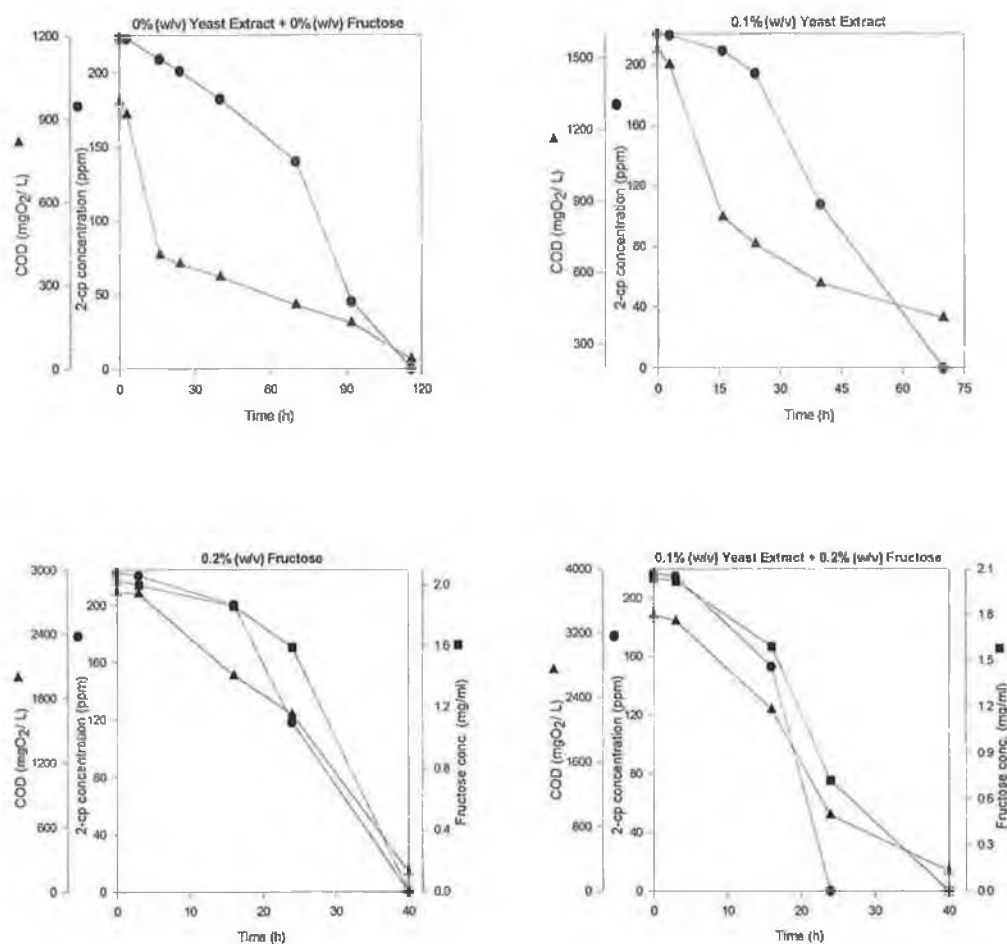


Fig. 41. The removal of 200 ppm 2-chlorophenol by *P. putida* CP1 in the presence of 0.1% (w/v) yeast extract plus 0.2% (w/v) fructose. Symbols: ●, 2-chlorophenol concentration; ▲, COD; ■, fructose concentration.

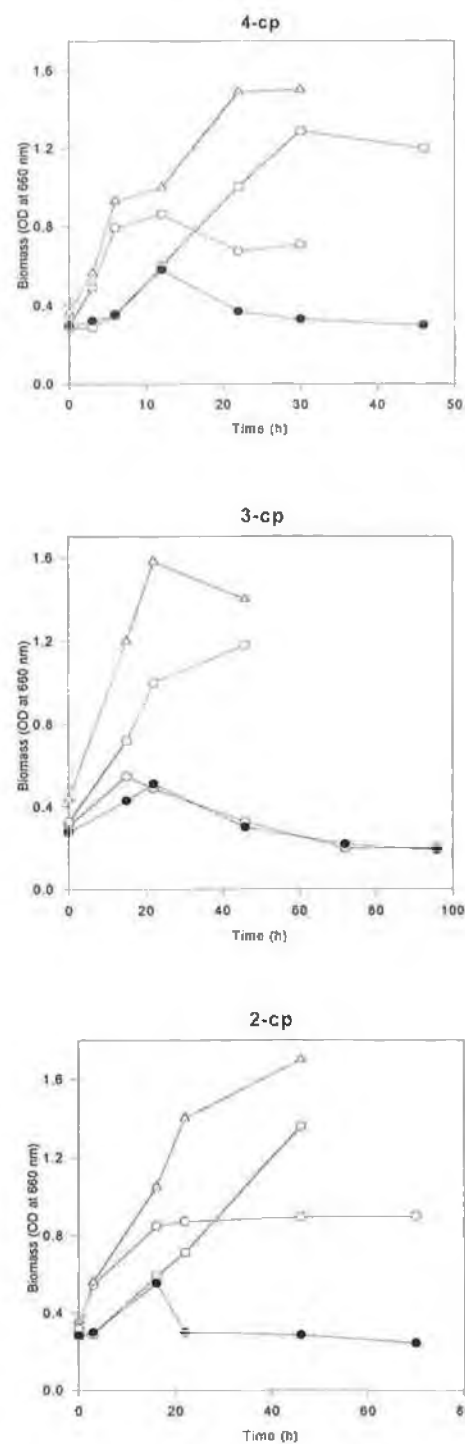


Fig. 42. Changes in biomass during growth of CP1 on 200 ppm mono-chlorophenols with the combination of 0.1% (w/v) yeast extract and 0.2% (w/v) fructose. Symbols: ●, mono-chlorophenols alone; ○, with 0.1% (w/v) yeast extract; □, with 0.2% (w/v) fructose; Δ, with 0.1% (w/v) yeast extract and 0.2% (w/v) fructose.

Table 13: Growth rate, C:N, changes in pH and rates of removal of chlorophenols, COD and fructose during growth of *P. putida* CP1 on 200 ppm mono-chlorophenols in the presence and absence of 0.1% (w/v) yeast extract (YE) and 0.2% (w/v) fructose (F).

Treatment	Total COD mgO ₂ l ⁻¹	μ h ⁻¹	C:N	Δ pH	cp removal rate		COD removal rate		F removal rate	
					mg l ⁻¹ h ⁻¹	mg l ⁻¹ h ⁻¹ mg ⁻¹	mgO ₂ l ⁻¹ h ⁻¹	mgO ₂ l ⁻¹ h ⁻¹ mg ⁻¹	mg l ⁻¹ h ⁻¹	mg l ⁻¹ h ⁻¹ mg ⁻¹
4-cp only	934.23	0.011 ± 0.0001	0.427	- 0.11	4.64 ± 0.02	0.0082 ± 0.0000	20.35 ± 1.480	0.053 ± 0.0037	-----	-----
+ 0.1% (w/v) YE	1530.03	0.071 ± 0.0060	0.804	- 0.03	7.71 ± 0.10	0.0088 ± 0.0001	47.27 ± 0.095	0.123 ± 0.0002	-----	-----
+ 0.2% (w/v) F	2722.15	0.025 ± 0.0001	3.480	- 0.28	11.62 ± 0.10	0.0230 ± 0.0002	66.62 ± 2.020	0.170 ± 0.0051	67.00 ± 0.59	0.125 ± 0.0014
+ 0.1% (w/v) YE + 0.2% (w/v) F	3583.89	0.082 ± 0.0015	2.960	- 0.20	11.68 ± 0.06	0.0114 ± 0.0000	102.05 ± 4.09	0.223 ± 0.0087	100.37 ± 0.65	0.144 ± 0.0012
3-cp only	863.30	0.012 ± 0.0000	0.427	- 0.09	1.03 ± 0.01	0.0021 ± 0.0000	10.01 ± 0.05	0.024 ± 0.0001	-----	-----
+ 0.1% (w/v) YE	1484.89	0.016 ± 0.0001	0.804	- 0.01	3.01 ± 0.05	0.0031 ± 0.0000	19.13 ± 0.70	0.076 ± 0.0028	-----	-----
+ 0.2% (w/v) F	2658.90	0.022 ± 0.0004	3.480	- 0.25	5.07 ± 0.01	0.0074 ± 0.0000	63.54 ± 1.27	0.202 ± 0.0039	49.97 ± 0.59	0.073 ± 0.0003
+ 0.1% (w/v) YE + 0.2% (w/v) F	3021.58	0.026 ± 0.0002	2.960	- 0.14	9.31 ± 0.30	0.0147 ± 0.0004	70.18 ± 2.96	0.220 ± 0.0094	87.68 ± 0.65	0.153 ± 0.0010
2-cp only	963.27	0.014 ± 0.0001	0.427	- 0.10	2.11 ± 0.01	0.0035 ± 0.0000	10.25 ± 0.33	0.021 ± 0.0006	-----	-----
+ 0.1% (w/v) YE	1534.69	0.023 ± 0.0002	0.804	- 0.02	3.94 ± 0.07	0.0037 ± 0.0000	29.09 ± 2.91	0.082 ± 0.0084	-----	-----
+ 0.2% (w/v) F	2791.84	0.020 ± 0.0001	3.480	- 0.34	4.89 ± 0.15	0.0131 ± 0.0003	65.32 ± 0.10	0.162 ± 0.0002	77.62 ± 0.15	0.007 ± 0.0001
+ 0.1% (w/v) YE + 0.2% (w/v) F	3510.20	0.025 ± 0.0006	2.960	- 0.29	11.34 ± 0.10	0.0138 ± 0.0001	79.58 ± 0.56	0.224 ± 0.0015	108.75 ± 0.65	0.117 ± 0.0007

± = Standard error

3.2 The monitoring of growth and cell morphology of *Pseudomonas putida* CP1 when grown on various substrates

It had been noted that *P. putida* CP1 flocculated when grown under certain environmental conditions. This caused difficulties for monitoring growth using optical density measurements. Other methods of monitoring growth were investigated.

P. putida CP1 was grown on glucose (0.5%, w/v), 4-, 3- and 2-chlorophenol (200 ppm), phenol (500 ppm) and a combination of phenol (500 ppm) and glucose (0.5%, w/v). The organism was grown in shake flask culture at 30⁰C with no pH control. The time of incubation varied with the substrate. Substrate removal was monitored together with pH, growth and cell shape. Growth was measured using dry weight measurements, the standard plate count (SPC) method and the direct epifluorescence filtration technique (DEFT). Both viable and non-viable numbers of cells were recorded using the DEFT method. Cell shape was observed when using the DEFT method.

3.2.1 Growth of *Pseudomonas putida* CP1 on 0.5% (w/v) glucose

No clumping of the organism was observed when the organism was grown on 0.5% (w/v) glucose. Changes in glucose concentration, pH and dry weight measurements during growth are shown in Fig. 43. The organism utilised the glucose readily and there was a linear removal of substrate. The glucose was completely removed in 22 hours.

The organism grew in response to substrate removal reaching a maximum value at 22 hour. After that point the biomass decreased. This pattern was observed regardless of the method used to monitor growth (Fig. 44). Fewer viable cells were counted using

the direct epifluorescence filtration technique than using the standard plate count method. Non-viable cells were detected at 46 hours but not at 22 hours.

Fig. 45 shows the cell shape of the organism at 0, 22 and 46 hours when grown on 0.5% (w/v) glucose. *Pseudomonas putida* CP1 retained its rod shape as long as glucose was in the medium. A change in cell shape from rod to coccus and a reduction in cell size were evident at 46 hours when no glucose remained in the medium. The pH of the medium fell by 0.7 units at 22 hours and then increased by 0.1 units at 46 hours.

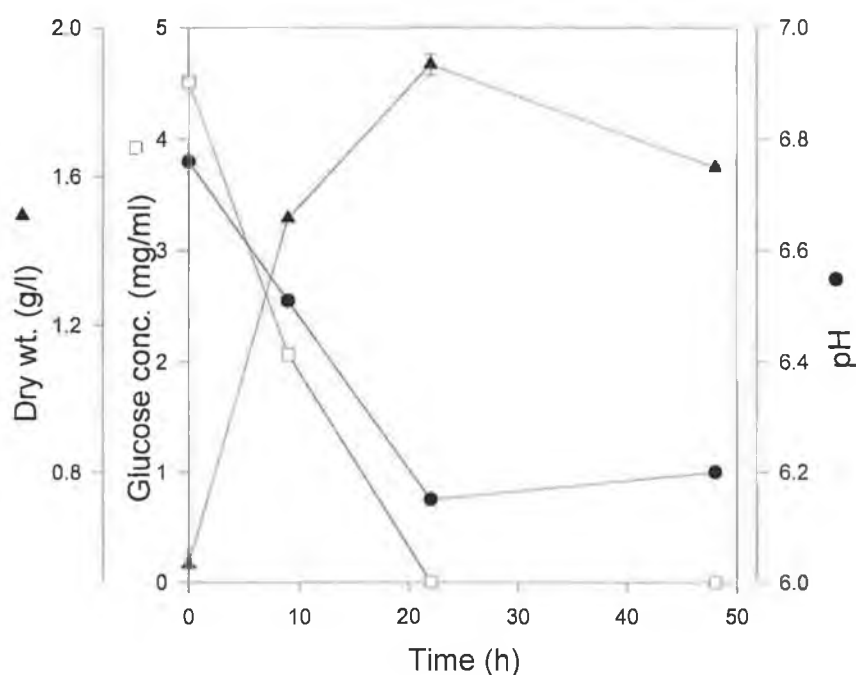


Fig. 43. Changes in glucose concentration, dry weight and pH during growth of *P. putida* CP1 on 0.5% (w/v) glucose containing minimal media. Symbols: \square , glucose concentration; \bullet , pH and \blacktriangle , dry weight.

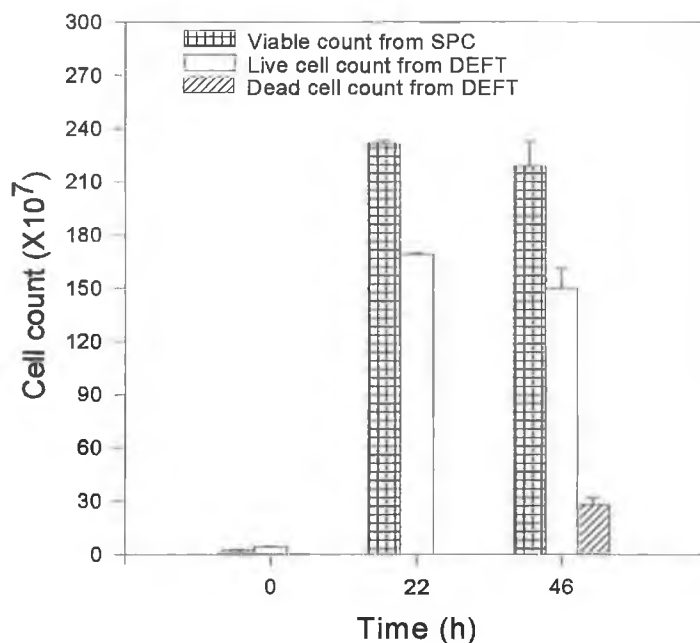


Fig. 44. Measurement of growth of *P. putida* CP1 on 0.5% (w/v) glucose by the standard plate count (SPC) method and the direct epifluorescence filtration technique (DEFT).

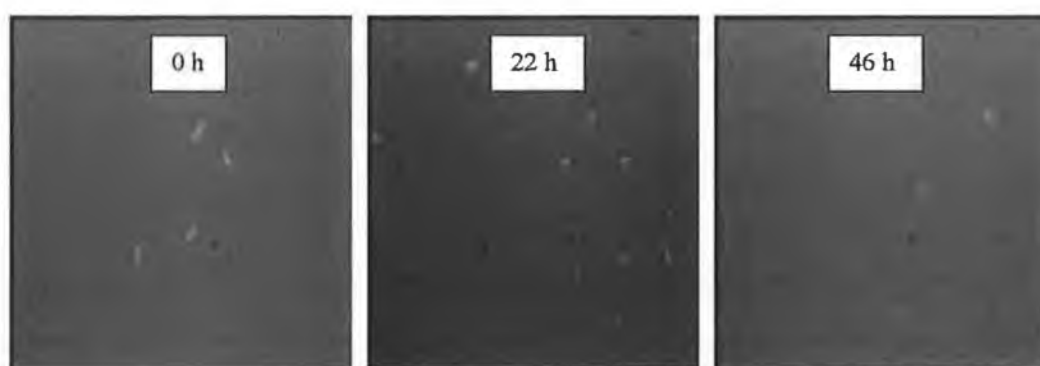


Fig. 45. Epifluorescence micrographs of *P. putida* CP1 grown on 0.5% (w/v) glucose- containing minimal media at different time intervals (magnification 1000 x).

3.2.2 Growth of *Pseudomonas putida* CP1 on 200 ppm 4-chlorophenol, 3-chlorophenol and 2-chlorophenol

Fig. 46 shows the utilization of mono-chlorophenols by CP1 and changes in dry weight and pH during growth of *Pseudomonas putida* CP1 on 200 ppm mono-chlorophenols. Substrate removal was as outlined in previous experiments. The organism flocculated when grown on mono-chlorophenols as described in earlier experiments. There was negligible change in pH. When growth of the organism was monitored using dry weight measurements, the biomass was seen to decrease in value with time as substrate was removed from the medium. The dry weight initially increased but dropped steadily after 22 hours. There was a drop of 0.12, 0.21 and 0.16 g/l of biomass with the growth and complete removal of 4-, 3- and 2-chlorophenol respectively. Thus, the greatest drop in dry weight was found with 3-chlorophenol followed by 2-chlorophenol, followed by 4-chlorophenol.

A change in bacterial cell number during growth on 200 ppm mono-chlorophenols containing minimal medium is shown in Fig 47. In the case of the standard plate count method the numbers of cells initially decreased. The numbers of cells recovered and increased with time as the mono-chlorophenol was removed from the medium. The numbers of viable cells observed using the direct epifluorescence filtration technique was always greater than the numbers counted using the standard plate count method. No non-viable cells were noted until the end of a run when the particular mono-chlorophenol was removed from the medium.

The change in cell shape of the organism at 0, 22 and 46 hours on all three mono-chlorophenols is outlined in Fig. 48. The organism was rod shaped at the beginning of the experiment. Following 22 hours growth, the cell shape was coccus in all cases. At 46 hours the shape remained coccus but smaller than at 22 hours. The increase in cell number and a drop in dry weight correlated with changes in cell shape and a reduction in cell size.

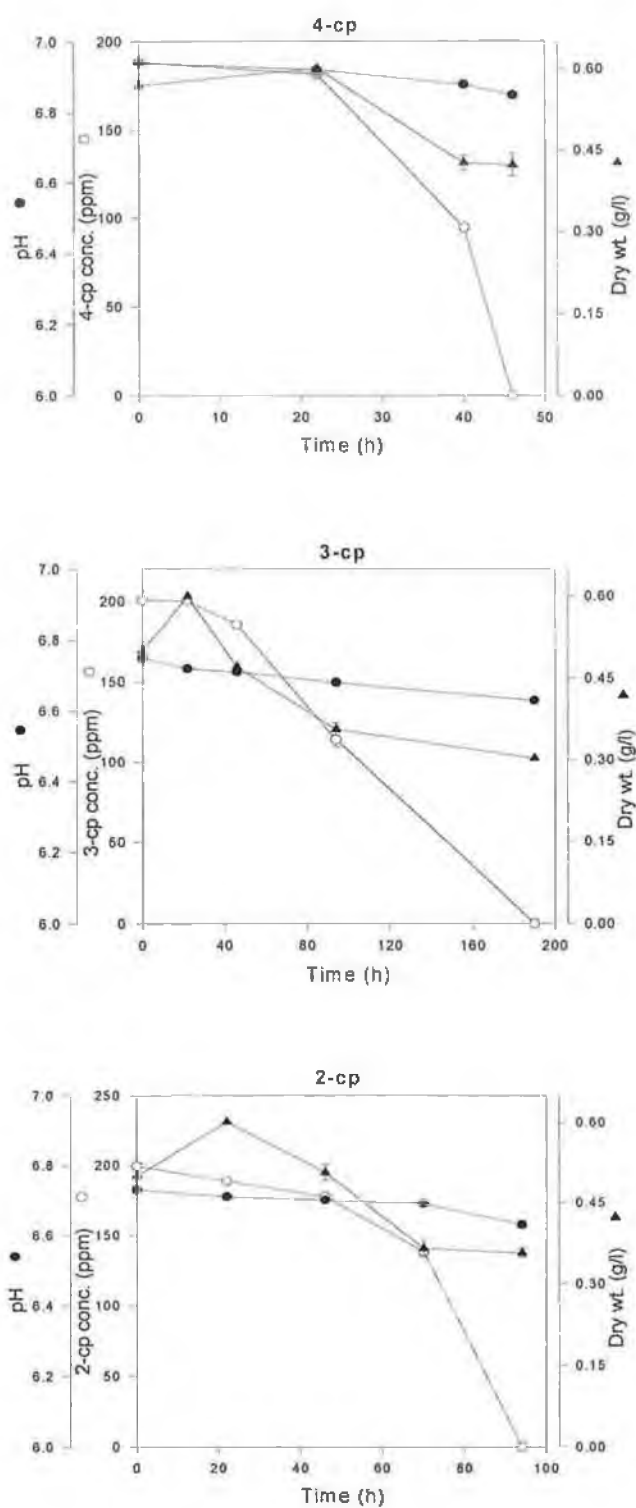


Fig. 46. Changes in mono-chlorophenol concentration, dry weight and pH during growth of *P. putida* CP1 on 200 ppm mono-chlorophenols. Symbols: O, mono-chlorophenol concentration; ●, pH and ▲, dry weight.

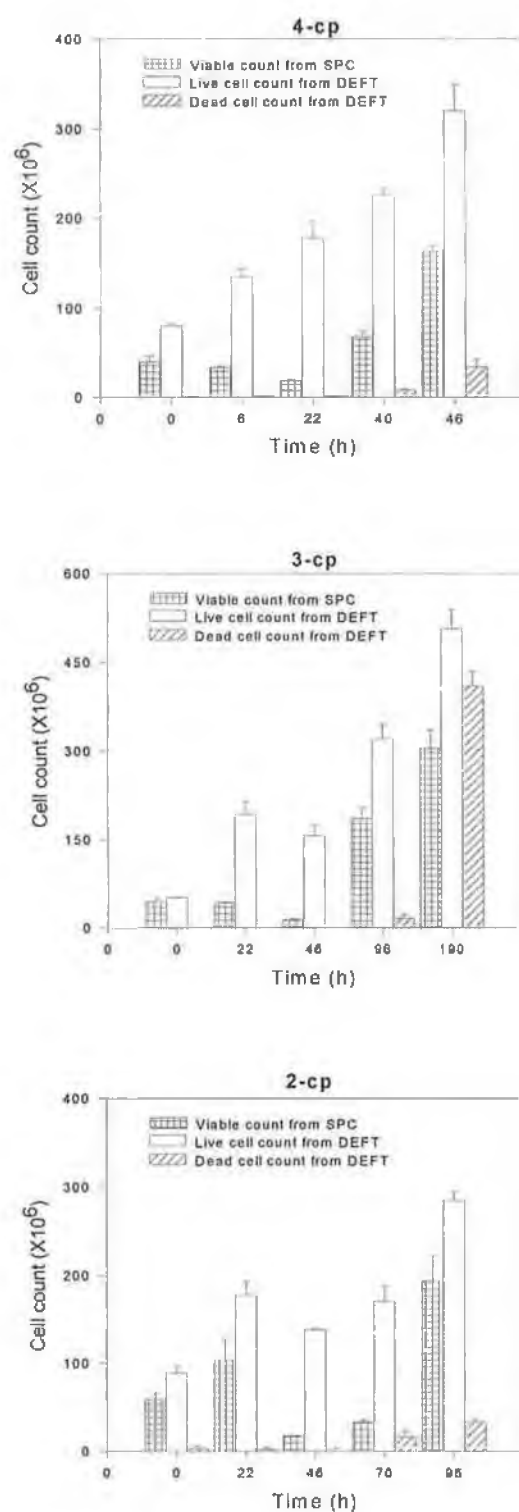


Fig. 47. Measurement of growth of CP1 on 200 ppm mono-chlorophenols by the standard plate count (SPC) method and the direct epifluorescence filtration technique (DEFT).

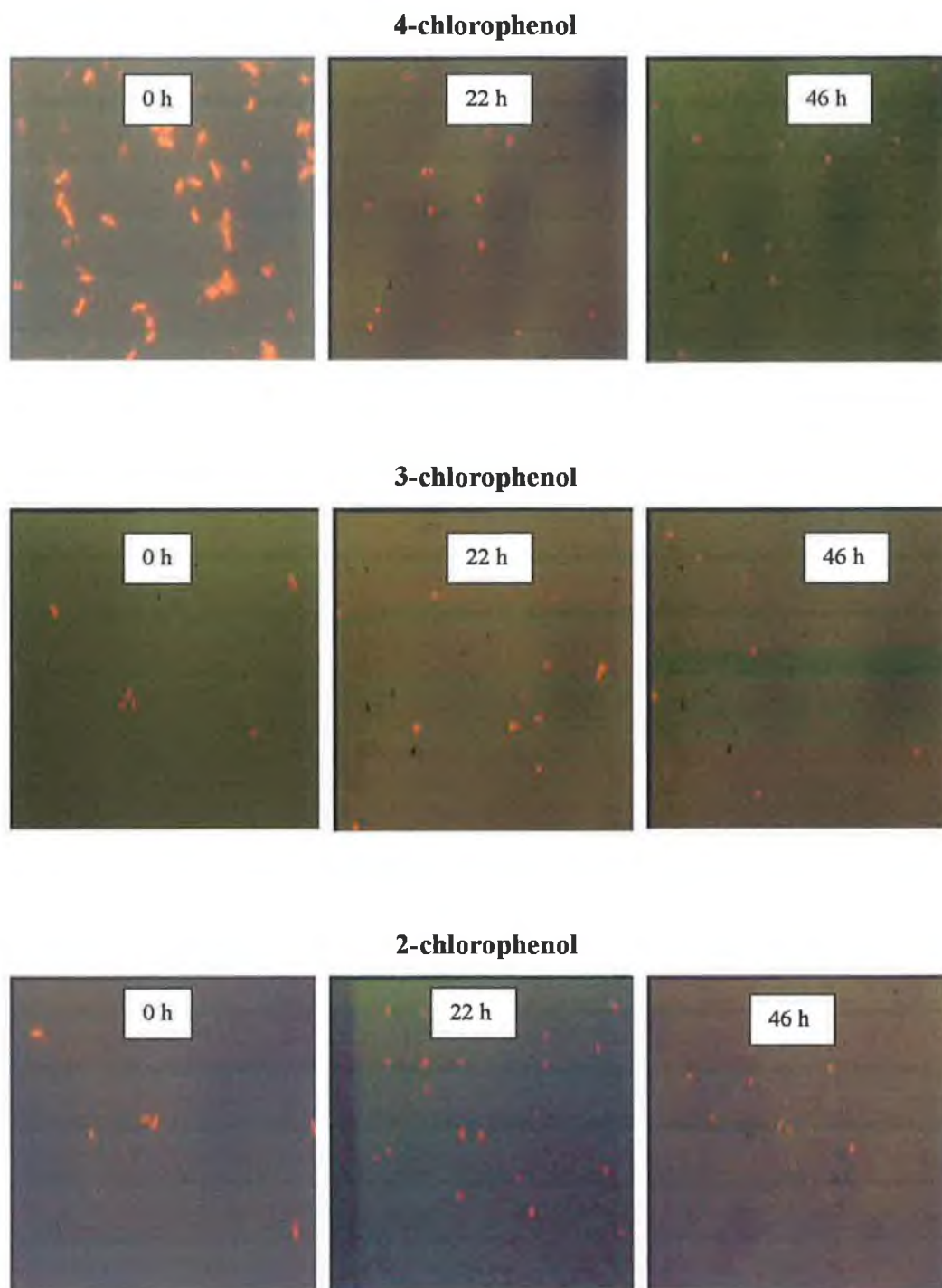


Fig 48. Epifluorescence micrographs of *P. putida* CP1 grown on 200 ppm mono-chlorophenols-containing minimal media at different time intervals (magnification 1000 x).

3.2.3 Growth of *Pseudomonas putida* CP1 on 500 ppm phenol

When 500 ppm phenol was used as the sole carbon source for *Pseudomonas putida* CP1, no flocculation of the cells was observed. Fig. 49 shows the changes in phenol concentration, pH and dry weight during growth of *Pseudomonas putida* CP1 on 500 ppm phenol. There was negligible change in the pH of the medium.

The organism grew in response to substrate removal. Using all three methods of measuring growth, the levels of biomass increased at 22 hour and then decreased slightly as the levels of substrate decreased below 20%. While the total numbers of cells detected using the direct epifluorescence filtration technique were greater than the number detected using the standard plate count method, the numbers of viable cells detected were lower. A significant number of dead cells were detected at 22 hours and the numbers of dead cells doubled at 46 hours. Bacterial numbers increased around 2 log in phenol in both the standard plate count method and the direct epifluorescence filtration technique (Fig. 50).

Fig. 51 shows the change in cell shape of the organism while growing on phenol. As with the mono-chlorophenols, the organism changed from a rod shape to a coccus shape at 22 hours. At 46 hours the cell shape was still round but smaller than at 22 hours.

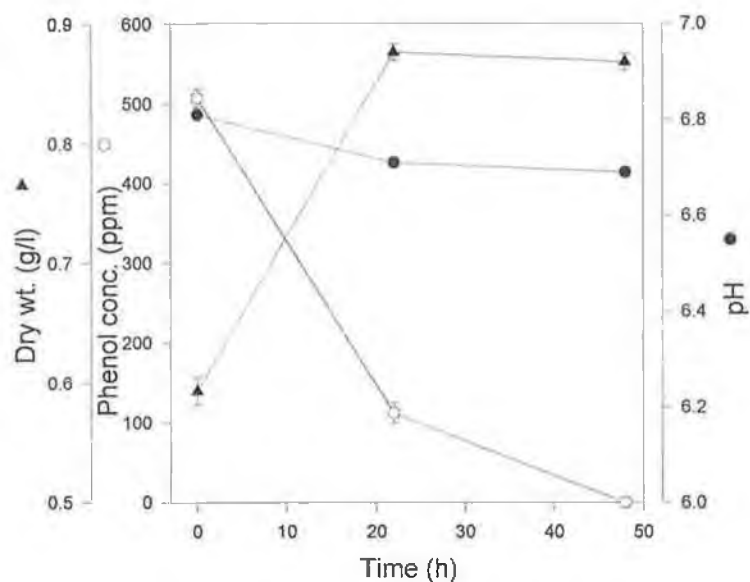


Fig. 49. Changes in phenol concentration, pH and dry weight during growth of *P. putida* CP1 on 500 ppm phenol. Symbols: O, phenol concentration; ●, pH and ▲, dry weight.

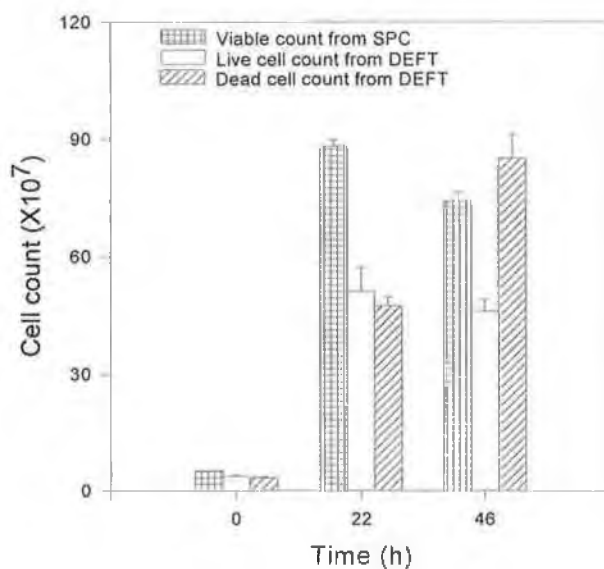


Fig. 50. Measurement of growth of *P. putida* CP1 on 500 ppm phenol by the standard plate count (SPC) method and the direct epifluorescence filtration technique (DEFT).

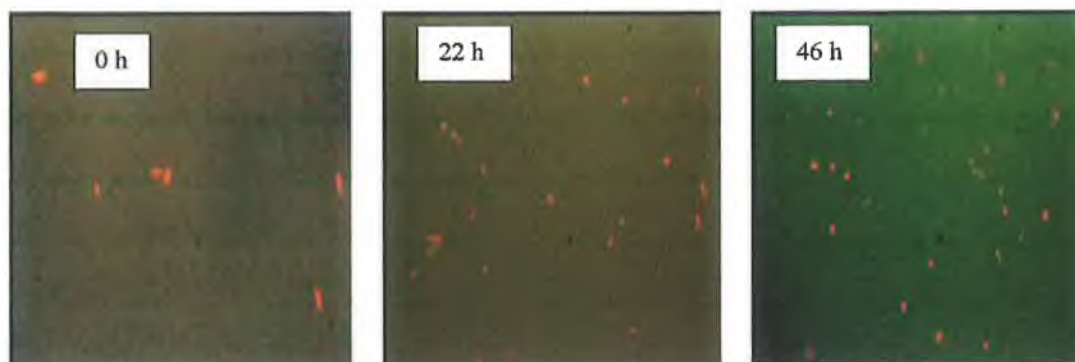


Fig. 51. Epifluorescence micrographs of *P. putida* CP1 grown on 500 ppm phenol-containing minimal media at different time intervals (magnification 1000 x).

3.2.4 Growth of *Pseudomonas putida* CP1 on 500 ppm phenol plus 0.5% (w/v) glucose

Changes in phenol concentration, glucose concentration, pH and dry weight during growth of the organism are shown in Fig. 52. The two substrates were removed concurrently from the medium. The organism grew in response to substrate removal. This was recorded as an increase in both dry weight and in cell numbers. There was 0.50 unit change in pH was observed in the presence of both substrates.

While there was slight flocculation of the cells at 22 hours there was no flocculation of the bacterium at 46 hours. The numbers of cells detected using both the standard plate count method and the direct epifluorescence filtration technique were similar. Few non-viable cells were detected (Fig. 53).

Fig. 54 shows the change in shape of the organism with time. The rod shape of the bacterium is observed initially and at 22 hours. At 46 hours, however, the shape of the cell is coccus shaped and there is a reduction in cell size.

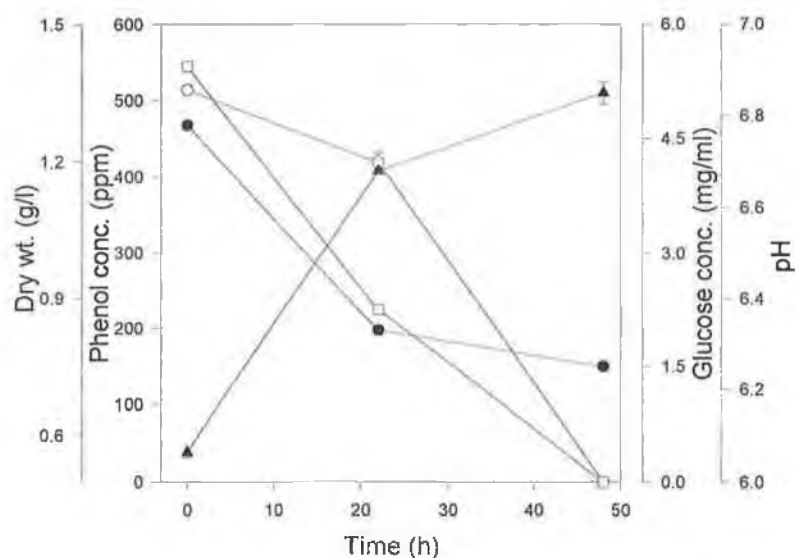


Fig. 52. Changes in phenol concentration, glucose concentration, dry weight and pH during growth of *P. putida* CP1 on 500 ppm phenol plus 0.5% (w/v) glucose. Symbols: O, phenol concentration; □, glucose concentration; ●, pH and ▲, dry weight.

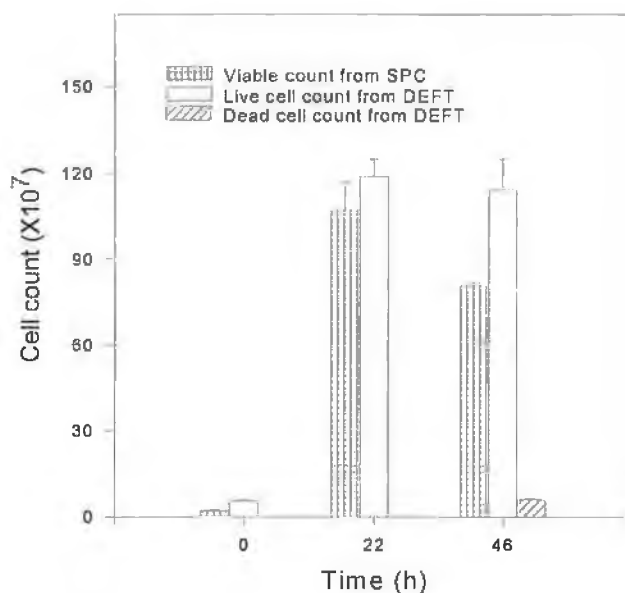


Fig. 53. Measurement of growth of CP1 on 500 ppm phenol plus 0.5% (w/v) glucose by standard plate count (SPC) method and direct epifluorescence filtration technique (DEFT).

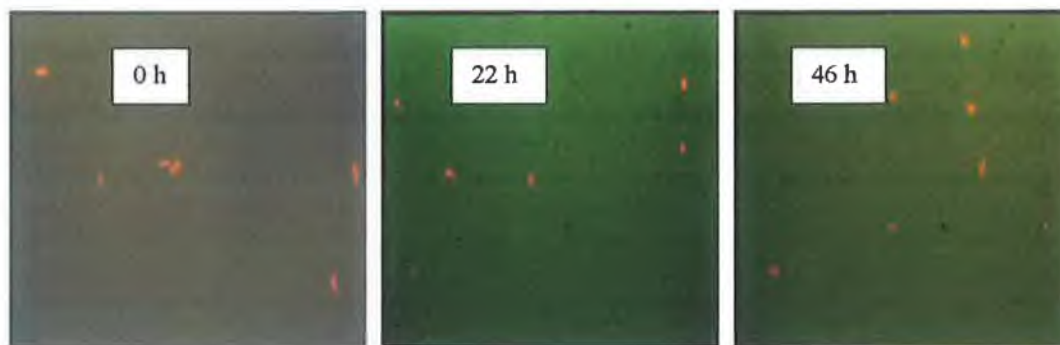


Fig. 54. Epifluorescence micrographs of *P. putida* CP1 grown on 500 ppm phenol plus 0.5% (w/v) glucose-containing minimal media at different time intervals (magnification 1000 x).

3.2.5 The determination of growth parameters of *Pseudomonas putida* CP1 using various approaches

P. putida CP1 was grown on various substrates and the growth response was measured in terms of dry weight and cell number and used to calculate the growth rate (μ) and the growth yield (Y) (Table 14).

In the case of dry weight, while there was an increase in dry weight when the organism was grown on glucose (0.5%, w/v), 500 ppm phenol and glucose plus phenol, while growth on the mono-chlorophenols (200 ppm) resulted in a decrease in dry weight of the organism. The greatest increase in dry weight was obtained when the organism was grown on glucose, followed by phenol and glucose combined and then phenol alone.

This trend was also obtained when growth was measured using cell number. The greatest increase in cell number was obtained when the organism was grown on glucose. The difference in the number of cells when the organism was grown on phenol and phenol plus glucose was more accurately determined when the direct cell

count method was used. Unlike with dry weight, a positive response was obtained for cell number when the organism was grown on the monochlorophenols.

The growth rate (μ) of the organism was calculated in three ways using the number of cells as determined by the standard plate count method and the direct epifluorescence filtration technique (Table 14). The highest value was obtained when the organism was grown on glucose, then glucose with phenol, followed by phenol and the chlorophenols. In the case of chlorophenols, the highest growth rate was obtained with 4-chlorophenol. Similar values were obtained with 3-chlorophenol and 2-chlorophenol. The values obtained for all substrates were similar regardless of the method used for the measurement of cell numbers.

The method of measuring cell growth influenced the calculation of yield. The growth yield of the organism was expressed in terms of number of cells produced either per mmol or per mg of substrate utilized. When the change in dry weight of the organism was used, the yield was greatest when the organism was grown on phenol followed by glucose and then phenol with glucose. Negative values were obtained for the organism when grown on the monochlorophenols (Table 14).

When cell number was used to calculate growth yield the value varied depending on the method used for the measurement of cell numbers. The greatest growth yield in terms of cell number was found with 3-chlorophenol, when the direct epifluorescence filtration technique was used to measure cell number. But the greatest growth yield in terms of cell number was found with phenol, when the standard plate count was used to measure cell number (Fig. 55).

Table 14: Changes in dry cell mass, cell shape-size, cell number, growth yield and growth rate of *P. putida* CP1 when grown on 0.5% (w/v) glucose, 200 ppm mono-chlorophenols, 500 ppm phenol and 0.5% (w/v) glucose plus 500 ppm phenol.

Treatment	Time (h)	Δ cell shape -size	Δ cell dry weight (g/l)	Δ cell no. SPC $\times 10^8$ (cfu/ml)	Δ cell no. DEFT		Growth Yield		Growth rate μ (h ⁻¹)		
					Viable	Total			SPC	DEFT Viable	DEFT Total
					$\times 10^8$	$\times 10^8$	$\frac{\text{mg}}{\text{mmol}}$	$\frac{\text{mg}}{\text{mg}}$			
0.5% (w/v) glucose	22	-	+1.36 \pm 0.029	22.9 \pm 0.15	16.5 \pm 0.10	16.5 \pm 0.10	+ 54.33 \pm 2.200	+ 0.391 \pm 0.004	0.09 \pm 0.001	0.072 \pm 0.000	0.072 \pm 0.000
200 ppm 4-cholorophenol	46	+	-0.136 \pm 0.011	1.25 \pm 0.06	2.39 \pm 0.12	2.59 \pm 0.165	- 92.82 \pm 1.500	- 0.721 \pm 0.003	0.013 \pm 0.000	0.013 \pm 0.000	0.014 \pm 0.000
3-chlorophenol	190	+	-0.206 \pm 0.001	2.59 \pm 0.02	4.54 \pm 0.02	8.66 \pm 0.245	- 133.25 \pm 3.350	- 1.036 \pm 0.002	0.0057 \pm 0.000	0.0055 \pm 0.000	0.0065 \pm 0.000
2-chlorophenol	96	+	-0.145 \pm 0.009	1.35 \pm 0.20	1.96 \pm 0.10	2.29 \pm 0.125	- 93.480 \pm 2.250	- 0.726 \pm 0.005	0.006 \pm 0.000	0.0052 \pm 0.000	0.0061 \pm 0.000
500 ppm phenol	22	+	+0.269 \pm 0.014	7.80 \pm 0.17	4.73 \pm 0.30	9.24 \pm 0.02	+ 63.98 \pm 1.650	+ 0.681 \pm 0.001	0.0560 \pm 0.000	0.052 \pm 0.000	0.061 \pm 0.000
0.5% (w/v) glucose + 500 ppm phenol	46	+	+0.788 \pm 0.023	7.84 \pm 0.11	10.8 \pm 0.20	11.4 \pm 0.025	+29.65 \pm 0.350	+0.176 \pm 0.000	0.070 \pm 0.000	0.060 \pm 0.000	0.061 \pm 0.000

\pm = Standard error

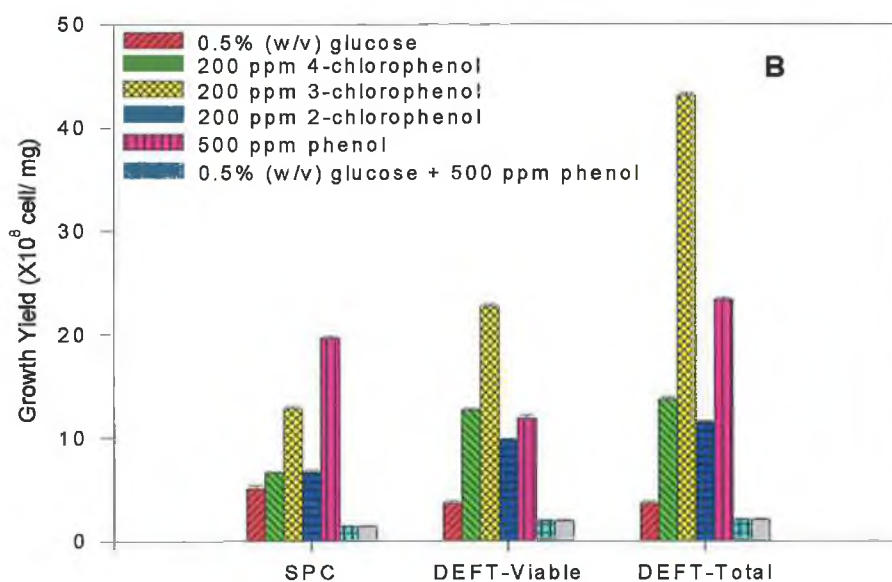
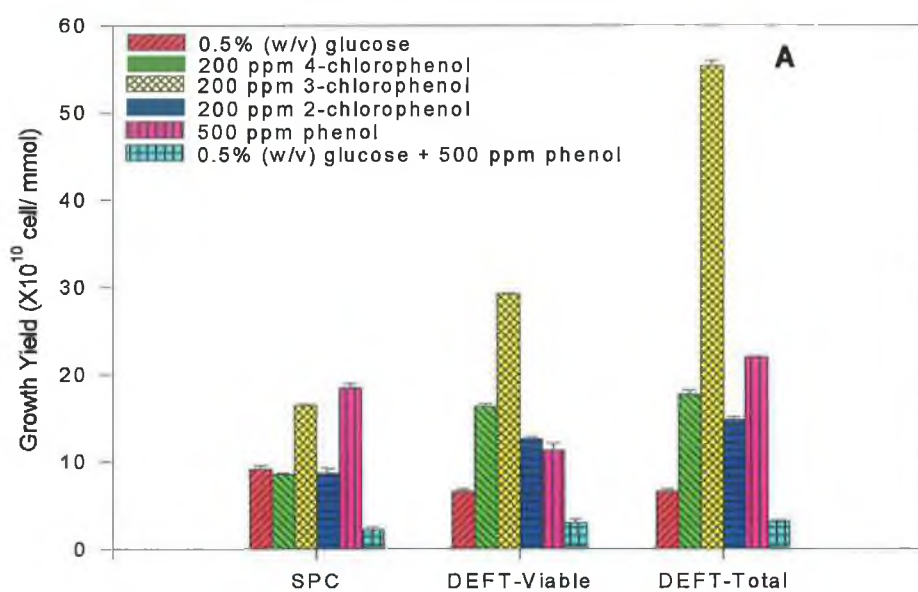


Fig. 55. Growth yield of *P. putida* CP1 when grown on various substrates in terms of number of cells produced per mmol (A) and per mg (B) of substrate utilized.

3.3 Analysis of fatty acid profiles of *Pseudomonas putida* CP1 when grown on various substrates

The fatty acid profile of *P. putida* CP1 was determined at 18, 40 and 70 hours when grown on various substrates. The phospholipid fraction was also investigated by growing the organism in 2-chlorophenol (Fig. 56 to Fig. 62). The main fatty acids detected were the even-numbered saturated fatty acids (SFA) and the monounsaturated fatty acids (MUFA) of the range C₁₀-C₁₈.

The levels of the individual fatty acids detected when the organism was grown on various substrates are described in the following sections. The substrates used are abbreviated in the figures as follows:

<u>Name of Substrate</u>	<u>Abbreviation</u>
1. nutrient broth	NB
2. 0.5% (w/v) glucose	0.5 G
3. 0.2% (w/v) glucose	0.2 G
4. 0.2% (w/v) fructose	0.2 F
5. 500 ppm phenol	P
6. 500 ppm phenol with 0.5% (w/v) glucose	P+G
7. 50 ppm 4-chlorophenol	4cp 1
8. 100 ppm 4-chlorophenol	4cp 2
9. 200 ppm 4-chlorophenol	4cp 3
10. 200 ppm 4-chlorophenol with 0.5% (w/v) glucose	4cp 3+G
11. 200 ppm 2-chlorophenol	2cp
12. phospholipid fraction of 200 ppm 2-chlorophenol	2cppl

C12 fatty acid:

Dodecanoic acid (lauric acid, C12:0) was detected in all samples except the 2-chlorophenol phospholipid fraction. The highest levels, 6% - 12% were detected in the presence of nutrient broth and the sugars glucose and fructose. Lower levels <4% were detected in the presence of phenol and chlorophenols. The levels increased when glucose was added to the phenolic substrate (Fig. 56).

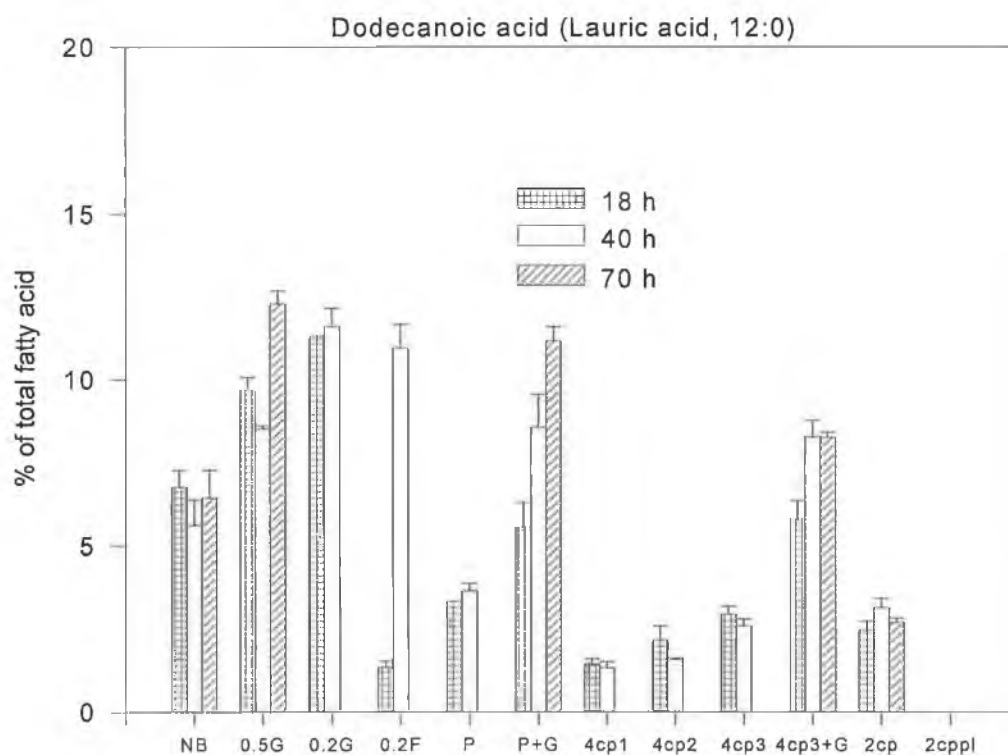


Fig. 56. Changes in dodecanoic (lauric) acid composition during growth of *P. putida* CP1 on various substrates.

C14 fatty Acids:

In the case of the C14 fatty acids, low levels of tetradecanoic (myristic) acid (14:0) and *cis*-9-tetradecanoic acid (14:1c) were detected. However, the total level of these fatty acids never exceeded 3%. The fatty acids were also detected in the phospholipid fraction when the organism was grown on 2-chlorophenol (Fig. 57).

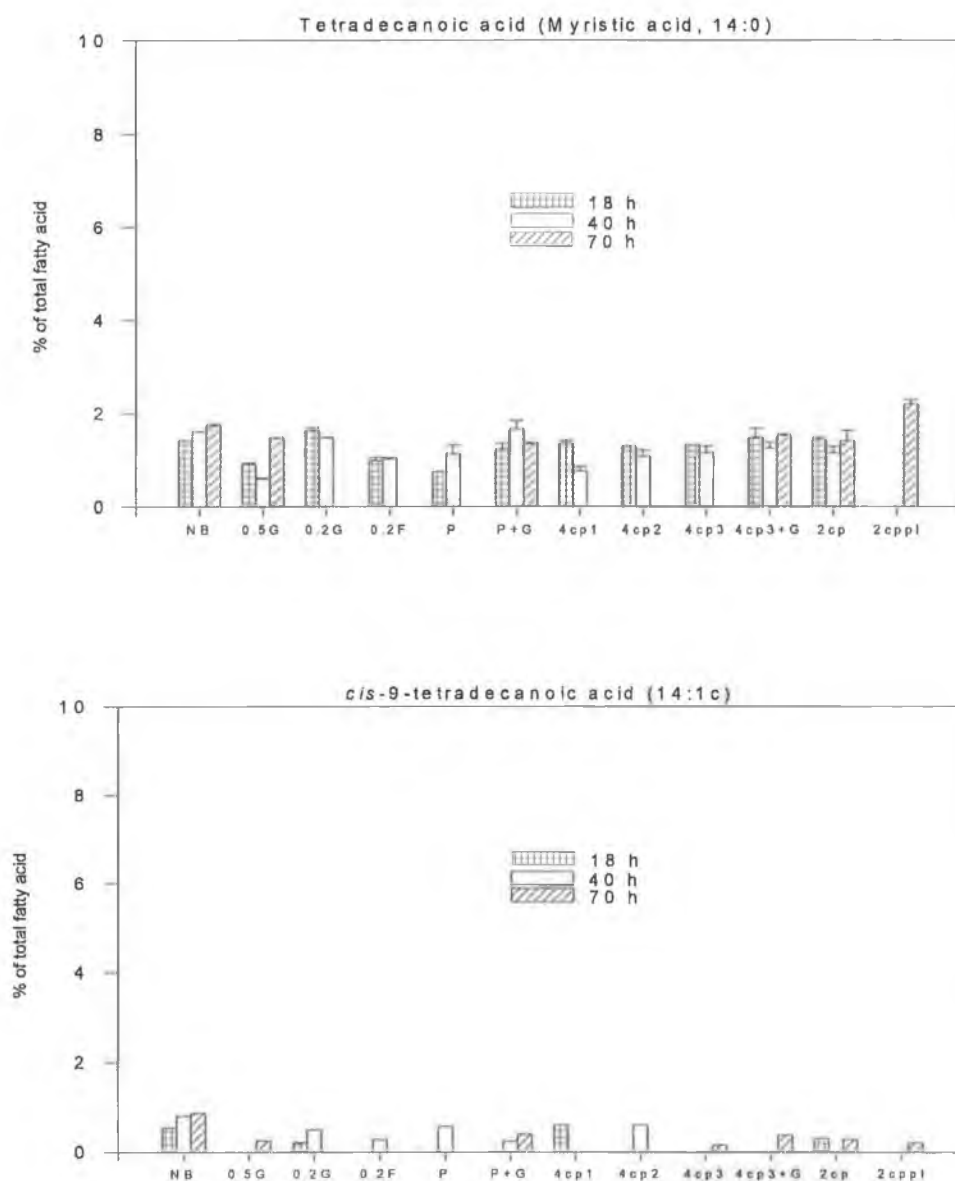


Fig. 57. Changes in tetradecanoic (myristic) acid and *cis*-9-tetradecanoic acid composition during growth of *P. putida* CP1 on various substrates.

C16 fatty acids:

Three fatty acids were identified in the C16 fraction. They were the saturated fatty acid hexadecanoic acid (16:0) and the unsaturated *cis*-9-hexadecanoic acid (16:1c) and *trans*-9-hexadecanoic acid (16:1t). The C16 fraction made up 40% to 70% of the total fatty acids depending on the growth substrate. Levels of palmitic acid were consistently high and similar for all substrates. A high level >35% of this fatty acid was also detected on the phospholipid fraction. The levels of *cis*-9-hexadecanoic acid were high initially and then decreased with time of incubation. The levels on chlorophenols were lower than on other substrates. The pattern was reversed for the *trans* isomer. The levels of the fatty acid were low, less than 2%, when the organism was grown on nutrient broth and increased in the presence of the phenolics. The highest levels, in the order of 20%, were detected when the organism was grown on 2-chlorophenol (Fig. 58).

C18 fatty acids:

The C18 fraction comprised five fatty acids. One saturated fatty acid, octadecanoic acid or stearic acid (18:0) and four unsaturated fatty acids – *cis*-vaccinic acid (18:1c) and *trans*-vaccinic acid (18:1t), oleic acid (18:1co) and linoleic acid (18:2cc). Less than 2% stearic acid was detected when the organism was grown on nutrient broth, sugars or phenol. Higher levels, about 5%, were detected when the organism was grown on the mono-chlorophenols. The highest level, 15%, was detected in the phospholipid fraction. A similar pattern, but with lower values, was observed for the unsaturated fatty acids except for *cis*-vaccinic acid. In the case of the latter, similar levels of the fatty acid ranging from 8% to 10% were detected regardless of the substrate. The level of this fatty acid decreased with time of incubation (Figs. 59 and 60).

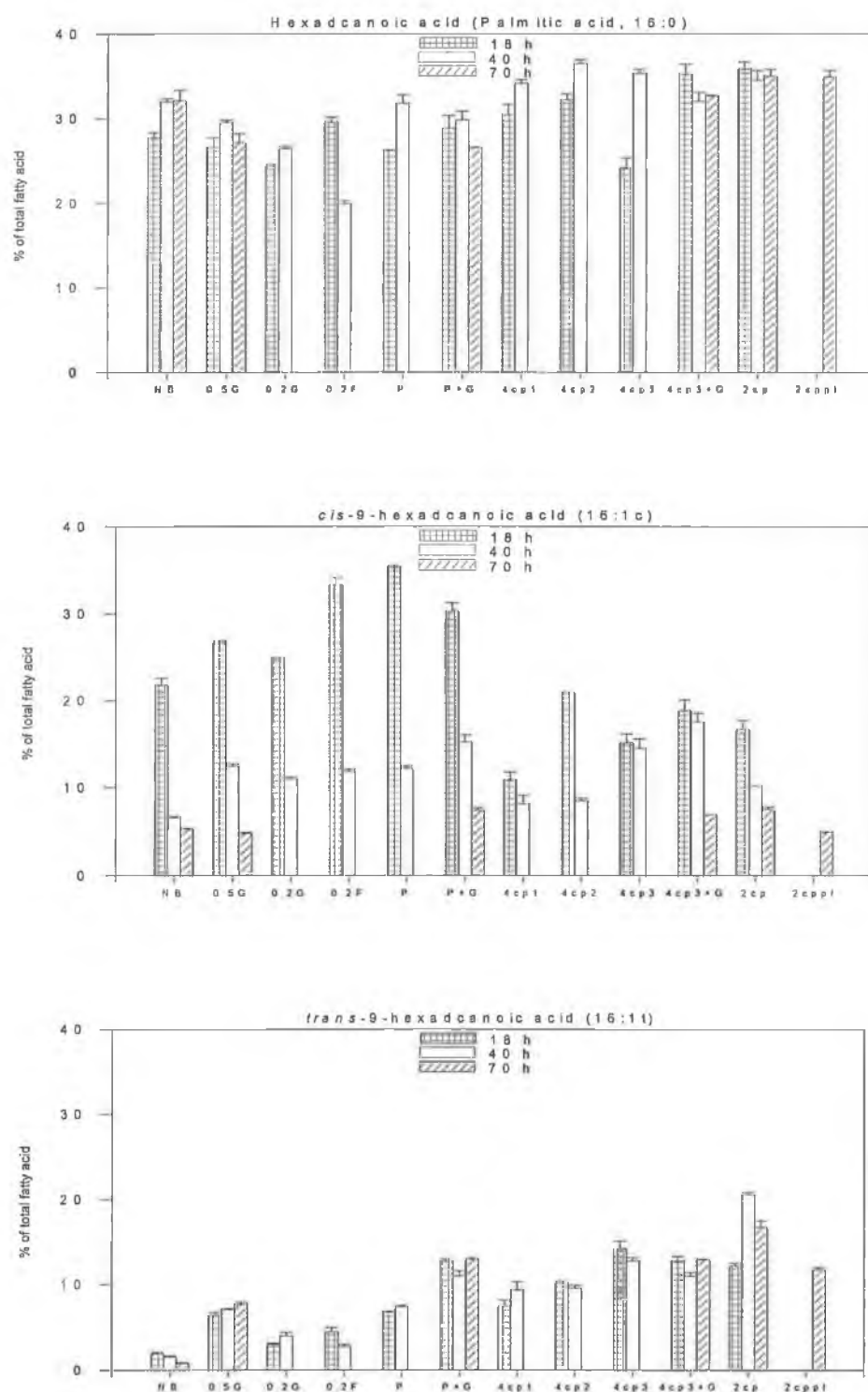


Fig. 58. Changes in hexadecanoic (palmitic) acid, *cis*-9-hexadecanoic acid and *trans*-9-hexadecanoic acid composition during growth of *P. putida* CP1 on various substrates.

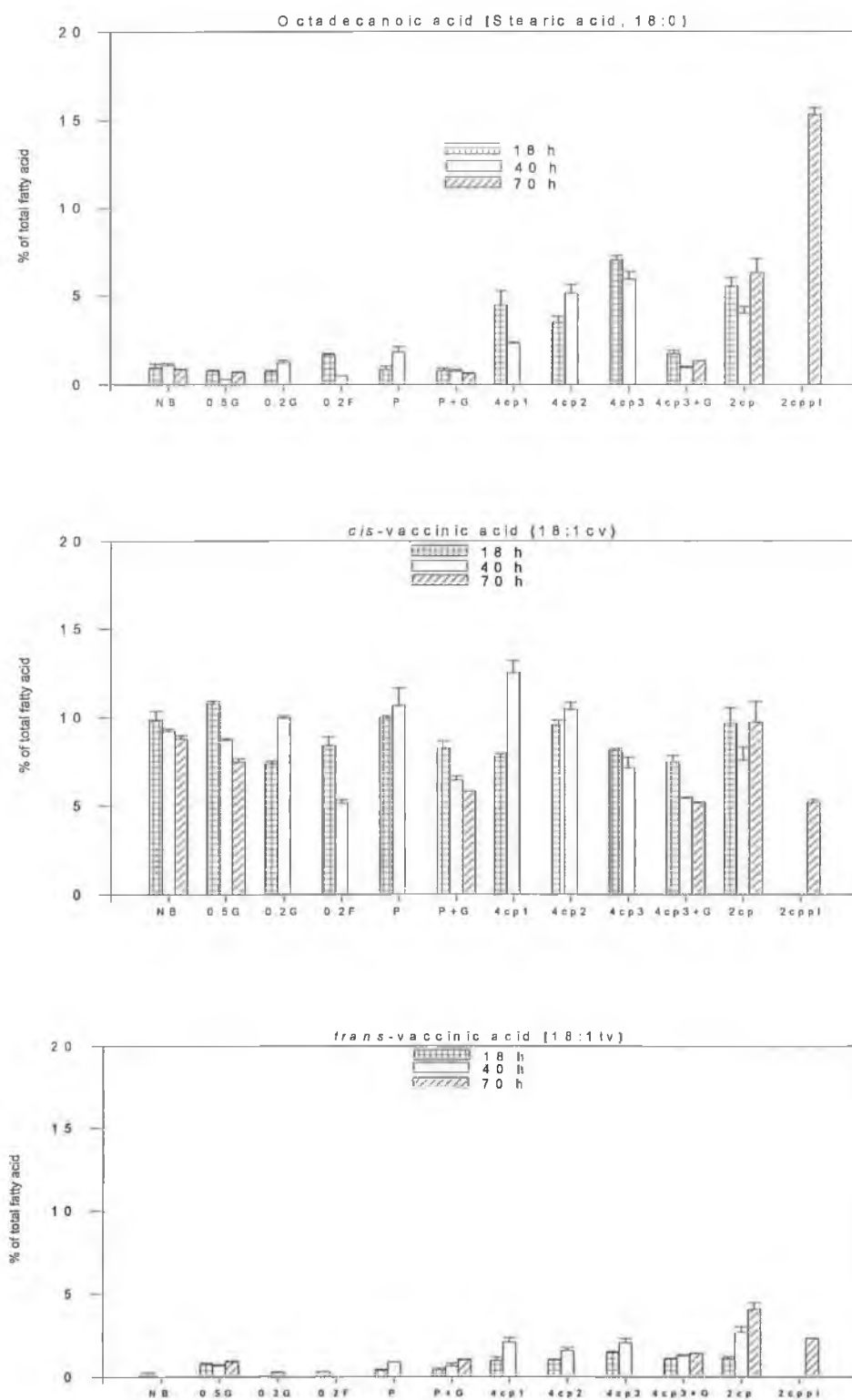


Fig. 59. Changes in octadecanoic (stearic) acid, *cis*-9-octadecanoic acid and *trans*-9-octadecanoic acid composition during growth of *P. putida* CP1 on various substrates.

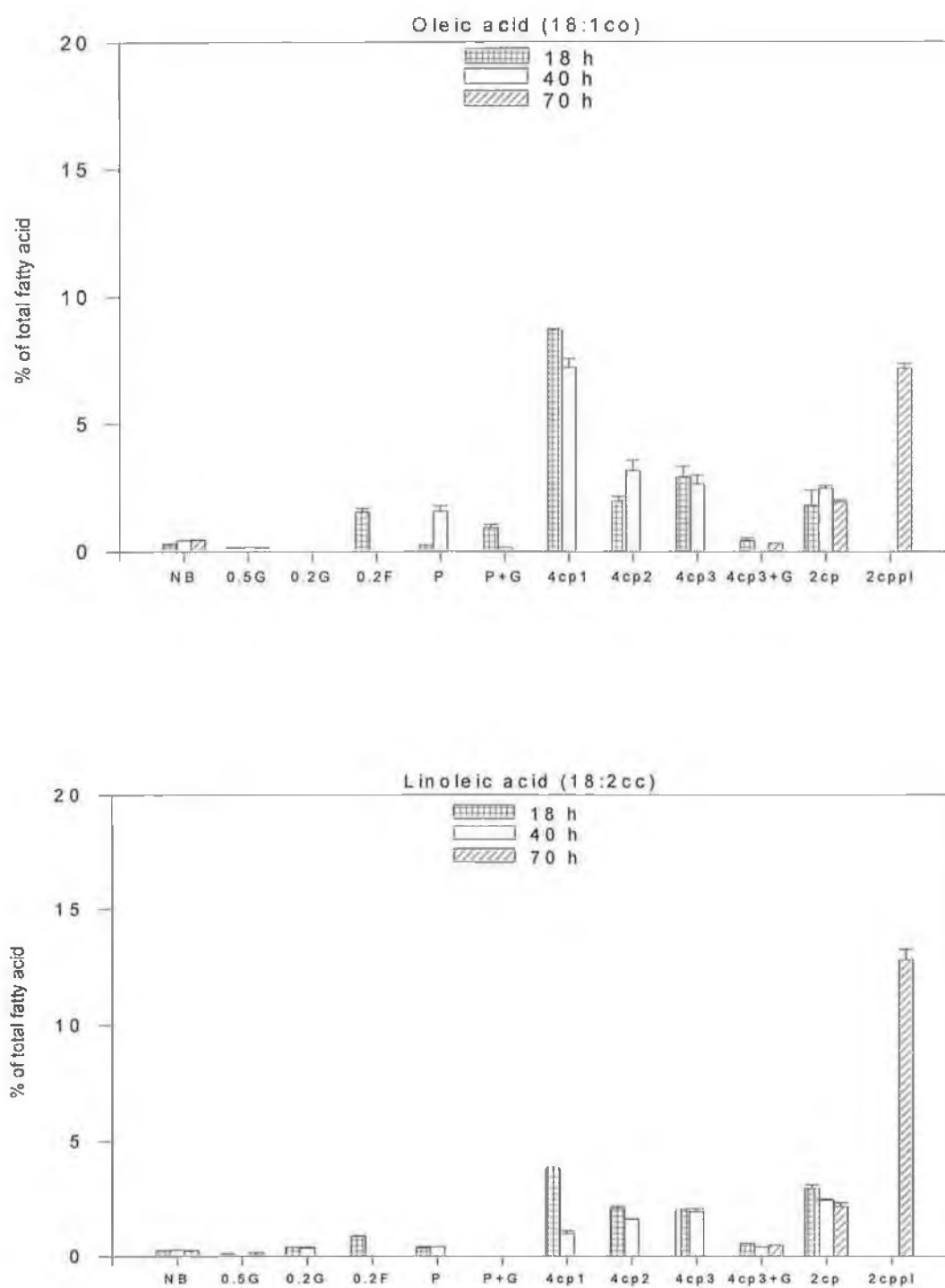


Fig. 60. Changes in oleic acid and linoleic acid composition during growth of *P. putida* CP1 on various substrates.

Cyclopropane fatty acids:

In the cyclopropane fatty acid fraction, there were just traces of the *cis*-11,12-methyleneoctadecanoic acid (C19:0 cyclo). However higher levels of *cis*-9,10-methylenehexadecanoic acid (C17:0 cyclo) were detected. The levels of this fatty acid increased with time of incubation. Lower levels of the fatty acid were detected when the organism was grown on phenolic substrates. Less than 2% cyclo fatty acids were detected in the phospholipid fraction (Fig. 61).

Hydroxy fatty acids:

Two fatty acids were identified in the hydroxy fatty acid fraction. Low levels, less than 3%, of 3-hydroxydodecanoic acid (3-OH, C12:0) were detected. The levels did not vary significantly for the different substrates. Higher levels, 4% to 10%, of 3-hydroxydecanoic acid (3-OH, C10:0) were detected. The levels of this fatty acid decreased when the organism was grown on phenolics and in particular chlorophenols. No hydroxy fatty acids were detected in the phospholipid fraction (Fig. 62).

Total levels of various fatty acids, saturated: unsaturated ratio and *trans*: *cis* ratio:

The total levels of the fatty acids fractions, C12-C18, are described in Tables 15 and 16. 16 carbon fatty acids were found to be the most abundant, followed by cyclopropane fatty acids, which was followed by 18 carbon fatty acids.

Tables 15 and 16 also shows the ratio of the saturated to unsaturated fatty acids and the ratio of *trans* to *cis* fatty acids when *P. putida* CP1 was grown on various substrates. The initial ratio of saturated to unsaturated fatty acids did not vary significantly for the different substrates, however, the ratio increased with increasing

incubation time. With the exception of nutrient broth, the ratio of *trans* to *cis* fatty acids increased with increasing incubation time. The increase was more marked in the presence of phenolic substrates and the greatest increase was with 2-chlorophenol.

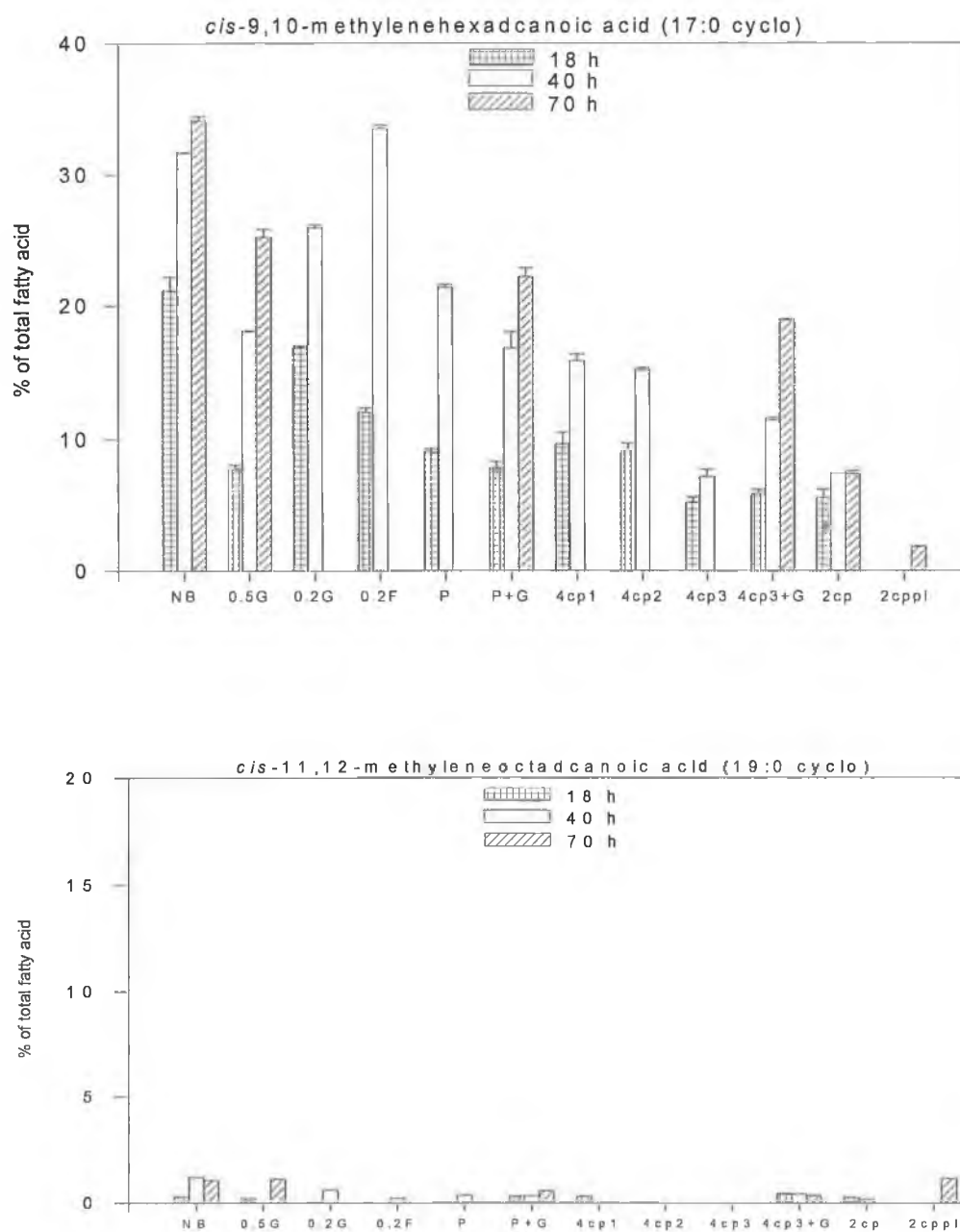


Fig. 61. Changes in cyclopropane fatty acids composition during growth of *P. putida* CP1 on various substrates.

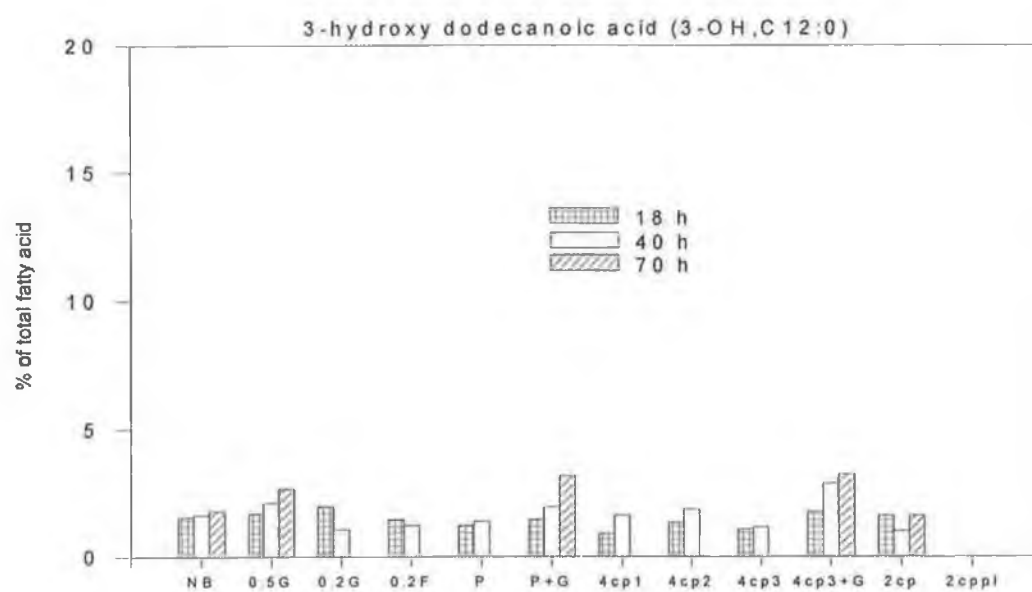
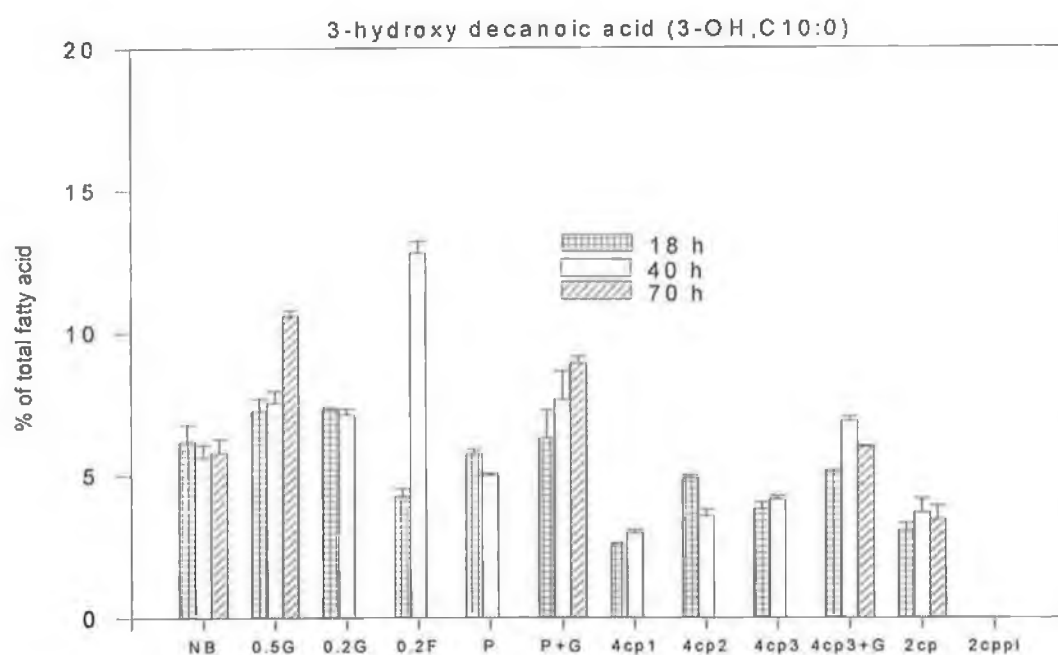


Fig. 62. Changes in hydroxy fatty acids composition during growth of *P. putida* CP1 on various substrates.

Table 15: Fatty acid composition of CP1 when it was grown on nutrient broth, 0.5% (w/v) glucose, 0.2% (w/v) glucose, 0.2% (w/v) fructose, 500 ppm phenol and 500 ppm phenol plus 0.5% (w/v) glucose.

Fatty Acid	% of Peak Area														
	Nutrient Broth			0.5% glucose			0.2% glucose		0.2% fructose		500 ppm phenol		500 ppm phenol plus 0.5% glucose		
	18h	40h	70h	18h	40h	70h	18h	40h	18h	40h	18h	40h	18h	40h	70h
Total C12	6.77	6.53	6.44	9.67	8.52	12.29	11.23	11.6	1.34	10.95	3.33	3.64	5.55	8.55	11.16
Total C14	1.95	2.40	2.60	0.90	1.59	1.70	1.84	1.97	0.98	1.30	0.74	1.69	1.27	1.89	1.74
Total C16	51.44	40.09	38.22	59.81	49.50	39.66	52.16	41.43	67.26	34.52	68.28	51.31	67.52	55.03	45.1
Total C18	11.29	10.72	10.29	12.45	7.81	9.7	8.55	9.79	12.75	6.52	11.44	13.11	10.45	8.15	7.61
Total Cyclo	20.52	32.88	35.18	7.89	14.07	26.32	16.94	27.02	12.00	33.72	8.99	21.72	7.96	17.11	22.73
Total Hydroxy	7.73	7.25	7.56	9.24	9.35	13.24	9.30	8.18	5.71	13.02	6.95	6.36	7.74	9.60	12.09
Sat/Unsat	0.84 ± 0.030	1.86 ± 0.010	2.16 ± 0.025	0.60 ± 0.025	0.81 ± 0.005	1.33 ± 0.010	0.71 ± 0.005	1.17 ± 0.030	0.64 ± 0.005	1.03 ± 0.020	0.52 ± 0.005	1.03 ± 0.005	0.60 ± 0.035	0.94 ± 0.050	1.06 ± 0.005
Trans/cis	0.07 ± 0.005	0.08 ± 0.010	0.06 ± 0.005	0.19 ± 0.015	0.37 ± 0.000	0.69 ± 0.015	0.10 ± 0.005	0.25 ± 0.025	0.11 ± 0.020	0.16 ± 0.010	0.16 ± 0.000	0.34 ± 0.000	0.22 ± 0.010	0.53 ± 0.055	0.93 ± 0.010

± = Standard error

Table 16: Fatty acid composition of *P. putida* CP1 when it was grown on 50, 100, 200 ppm 4-chlorophenol, 200 ppm 4-chlorophenol plus 0.5% (w/v) glucose and 200 ppm 2-chlorophenol at different time intervals.

Fatty Acid	% of Peak Area												
	50 ppm 4-cp		100 ppm 4-cp		200 ppm 4-cp		200 ppm 4-cp plus 0.5% glucose			200 ppm 2-cp			200 ppm 2-cp (pl)
	18h	40h	18h	40h	18h	40h	18h	40h	70h	18h	40h	70h	70h
Total C12	1.44	1.32	2.16	1.59	2.94	2.59	5.79	8.27	8.25	2.45	3.14	2.69	0.0
Total C14	1.73	0.76	1.27	1.67	1.31	1.15	1.48	1.25	1.90	1.63	1.15	1.69	2.39
Total C16	48.69	51.56	63.13	54.52	53.56	62.56	66.74	60.44	52.54	64.67	65.28	59.27	51.63
Total C18	4.46	2.30	3.48	5.12	7.02	5.94	12.36	7.97	9.2	20.62	19.2	23.18	42.78
Total Cyclo	9.90	15.85	9.08	15.16	5.23	7.16	6.23	11.83	19.27	5.82	7.61	7.37	2.98
Total Hydroxy	3.70	4.58	6.24	5.44	4.89	5.31	6.88	9.76	9.23	4.68	4.62	5.09	0.0
Sat/Unsat	0.80 ±	0.93 ±	0.82 ±	1.25 ±	0.98 ±	1.05 ±	0.87 ±	0.85 ±	1.27 ±	1.00 ±	0.89 ±	1.04 ±	1.60 ±
	0.020	0.015	0.010	0.025	0.010	0.035	0.030	0.050	0.000	0.040	0.045	0.040	0.070
Trans/cis	0.27 ±	0.41 ±	0.35 ±	0.50 ±	0.60 ±	0.61 ±	0.47 ±	0.53 ±	1.15 ±	0.47 ±	1.15 ±	1.09 ±	0.83 ±
	0.030	0.000	0.015	0.020	0.040	0.005	0.050	0.045	0.005	0.005	0.030	0.020	0.010

± = Standard error

Chapter 4

Discussion

4. Discussion

There is concern over mono-chlorophenols due to their persistence in the environment. Few microorganisms have been isolated capable of degrading all three isomers of mono-chlorophenols (Menke and Rehm, 1992; Fava *et al.*, 1995a; Zaitsev *et al.*, 1995; Lu *et al.*, 1996; Pérez *et al.*, 1997). *Pseudomonas putida* CP1 was able to grow and completely degrade up to 300, 250 and 200 ppm of 4-chlorophenol, 2-chlorophenol and 3-chlorophenol, respectively, when supplied as the sole source of carbon and energy. Beyond these levels, chlorophenol degradation did not occur to any measurable degree. During the degradation of mono-chlorophenols there was a very slight reduction in pH, which was due to the release of chloride ions in the medium and the subsequent production of acidic metabolites. It was found that 4-chlorophenol was the most readily removed, followed by 2-chlorophenol, 3-chlorophenol being the most toxic for *P. putida* CP1.

P. putida CP1 contained a broad-spectrum phenol hydroxylase which allowed it to grow on all three mono-chlorophenol isomers (Farrell, 2000). The differences in the degradation between the mono-chlorophenols themselves seemed to be due to the position of the chloro-substituent. During hydroxylation of the mono-chlorophenols by phenol mono-oxygenase, corresponding chlorocatechols were produced with the introduction of a second hydroxyl group, at a position determined by that of the first two substituents. Menke and Rehm (1992) described the theoretical order of degradability of chlorophenols by considering the reaction-decelerating effect of the –Cl ion and the reaction-accelerating effect of the –OH group position. They reported the order of mineralization as: phenol > 4-chlorophenol > 2-chlorophenol > 3-chlorophenol as described in this study and that of others in the laboratory (Farrell and Quilty, 2002). Lu and Tsai (1993) also reported the same order of microbial degradation of phenolic compounds by an unacclimated culture. However, the sequence of mono-chlorophenol degradation was different for *Pseudomonas pickettii* LD1 (Fava *et al.*, 1995a). Fava *et al.* found that 2-chlorophenol was more readily degraded than 4-chlorophenol which was more readily degraded than 3-chlorophenol.

As the enzyme activity is complex and affected not only by substrate structure but also by the induction of catabolic enzymes, the order of degradability may vary among the different species of bacteria depending on the regulatory enzymes.

P. putida CP1 degraded all three isomers of mono-chlorophenol at a concentration comparable to and even higher than those reported for other strains (Menke and Rehm, 1992; Fava *et al.*, 1995a; Zaitsev *et al.*, 1995; Lu *et al.*, 1996). Among the few bacterial species which have been described capable of degrading all three isomers of mono-chlorophenols, *Pseudomonas testosteroni* was able to remove 10 ppm of all three isomers (Lu *et al.*, 1996). In 1995 Fava *et al.* described the total removal of 195.74 mg l⁻¹ 2-chlorophenol, 73.88 mg l⁻¹ 3-chlorophenol and 97.22 mg l⁻¹ 4-chlorophenol by *Pseudomonas picketti* strain LD1 (later the organism was reclassified as *Ralstonia picketti*) under batch culture conditions. Moreover, *Pseudomonas picketti* strain LD1 was also able to metabolize other organic compounds, like benzoic acid, hydroxybenzoic acid and hydroquinone. *Rhodococcus opacus* strain GM-14 was isolated by selective enrichment from contaminated soil with chlorobenzene as the sole source of carbon and energy (Zaitsev *et al.*, 1995). It utilized an exceptionally wide spectrum of haloaromatic substrates. *Rhodococcus opacus* GM-14 grew on 48 of 117 different aromatic and haloaromatic compounds. It utilized phenol at concentrations up to 1200 mg l⁻¹, 3- and 4-methylphenols up to 500 mg l⁻¹, 2- and 4-chlorophenols up to 250 mg l⁻¹, and 3-chlorophenol up to 100 mg l⁻¹. Menke and Rehm (1992) described the complete removal of 58.33 mg l⁻¹ 4-chlorophenol and 38.88 mg l⁻¹ 2-chlorophenol by the constructed strain *Alcaligenes* sp A7-2. However, that constructed strain only degraded 3-chlorophenol cometabolically either in combination with phenol or 2-chlorophenol at a concentration of 12.96 mg l⁻¹. The fungus *Phanerochaete chrysosporium* degraded a maximum concentration of 150 mg l⁻¹ 4-chlorophenol and 100 mg l⁻¹ of 2- and 3-chlorophenol in static cultures (Pérez *et al.*, 1997).

Mono-chlorophenol removal by *P. putida* CP1 was always preceded by a lag and the duration of the lag period varied with the isomer and concentration of the mono-

chlorophenol. For any given concentration of chlorophenol, the lag was always greater for 3- and 2- chlorophenol than for 4-chlorophenol. This result was also observed by Zaitsev *et al.* (1995) who reported lag periods for phenol and mono-chlorophenol grown cells of *Rhodococcus opacus* GM-14. Following the lag, the rate of substrate removal increased with increasing concentrations of chlorophenol up to a maximum value of 10.01 mg l⁻¹ h⁻¹ at 250 ppm, 2.65 mg l⁻¹ h⁻¹ at 100 ppm and 1.84 mg l⁻¹ h⁻¹ at 100 ppm for 4-chlorophenol, 2-chlorophenol and 3-chlorophenol respectively. Above these concentrations, the rate of substrate removal decreased. The rate of removal of 200 ppm 2-chlorophenol was half that of 200 ppm 4-chlorophenol and twice that of 200 ppm 3-chlorophenol when supplied as the sole carbon source. The rate of removal of all isomers of mono-chlorophenol by *P. putida* CP1 was higher than the rate observed for various strains of immobilized bacteria (Lu *et al.*, 1996). The rate of removal of 4-chlorophenol by *P. putida* CP1 was greater than that observed in batch culture for *Pseudomonas* sp. TCP114 (Bae *et al.*, 1997b) and similar to that observed in the continuous fermentation of 4-chlorophenol by *Alcaligenes* sp. A 7-2 (Westmeier and Rehm, 1985). In 1988 Goulding *et al.* reported removal rates of 4.8, 5.5 and 5.1 mg l⁻¹ h⁻¹ for 200 ppm 4-chlorophenol, 3-chlorophenol and 2-chlorophenol respectively by a mixed culture of bacteria.

The degradation of toxic chemicals in the presence of an easily degradable alternative carbon source is of great practical importance in the biological treatment of waste water. This study found that the addition of glucose at certain concentrations could enhance the degradation of mono-chlorophenols. Removal of substrate was expressed both in terms of unit volume and in terms of biomass. According to Wang *et al.* (1996) removal rates in terms of unit biomass (specific removal rate) are always more accurate than overall removal rates for drawing conclusions about mixed substrate degradation. The specific rate of removal of 4-chlorophenol doubled when 0.05% and 0.1% (w/v) glucose was added to the medium. Above 0.1% (w/v) glucose addition there was no further increase in the removal rate and the removal of 4-chlorophenol was inhibited by 1% and 2% (w/v) glucose. The specific rate of removal of 3-chlorophenol increased 1.5 times in the presence of glucose concentrations ranging

from 0.05% to 0.1% (w/v), further addition of glucose to the medium did not increase the specific rate of removal significantly. The specific rate of removal of 2-chlorophenol did not increase significantly by the addition of glucose and the removal rates were very similar in the presence of various concentrations of glucose.

There are several reports of the effect of glucose on the biodegradation of chlorophenols by different microorganisms. Janke *et al.* (1988), Topp and Hanson (1990), Yu and Ward (1994), and Loh and Wang (1998) all reported the enhancement of mono-chlorophenol degradation in the presence of glucose. Janke *et al.* (1988) reported that the addition of glucose (0.5 –1.0 g/l) resulted in an increased turnover rate for 2- and 3-chloroaniline as well as 3- and 4-chlorophenol by *Rhodoccus* sp. An117 and An213. Topp and Hanson (1990) and Yu and Ward (1994) reported that the rate of penta-chlorophenol degradation could be increased by the addition of glucose. In 1998 Loh and Wang reported the enhancement of the biodegradation of 4-chlorophenol by *Pseudomonas putida* ATCC 49451 following the addition of glucose and sodium glutamate. Reber and Kaiser (1981) reported that the presence of glucose in the medium accelerated the ability of *Pseudomonas putida* strains to oxidize aromatic substrates. Hess *et al.* (1993) reported enhanced 2,4-dinitrophenol degradation by the addition of glucose at a concentration of 50, 100, 500 mg/l by *Rhodococcus* and *Janthinobacterium* species.

Topp *et al.* (1988) observed the influence of readily available carbon sources like glutamate, aspartate, succinate, acetate, glucose and cellobiose on pentachlorophenol metabolism by a *Flavobacterium* sp. They suggested that the addition of more readily available carbon sources could facilitate substrate removal by enhancing growth and thereby attenuating the toxicity of pentachlorophenol. The enhanced removal of mono-chlorophenols in this study was also accompanied by an increase in biomass.

Suppression of the degradation of phenolic compounds in the presence of more easily degradable carbon sources was also well documented by a number of investigators (Goulding *et al.*, 1988; Swindoll *et al.*, 1988; Chitra *et al.*, 1995; O'Sullivan, 1998).

Goulding *et al.* (1988), reported the repression of 3-chlorobenzoic acid degradation by mixed cultures by more easily degradable carbon sources. Swindoll *et al.* (1988) also reported similar repressive effects on the mineralization of *p*-nitrophenol, phenol and toluene by mixed cultures. O'Sullivan (1998), reported an inhibitory effect on the removal of phenol and mono-chlorophenols by a mixed microbial population in the presence of glucose. This study reported a reduction in the rate of removal as well as the percentage removal in the presence of glucose. The presence of glucose exerted repressive effects on phenol removal by *Pseudomonas pictorum* (Chitra *et al.*, 1995). Reduction of phenol removal rates in the presence of various concentrations of glucose was also reported for heterogeneous populations (Rozich and Colvin, 1986). Hess *et al.* (1993) reported that 10 mg/l glucose had no effect and 1000 mg/l glucose inhibited the degradation of 2,4-dinitrophenol by *Rhodococcus* and *Janthinobacterium* sp.

There was also a lag in the removal of chlorophenols by *P. putida* CP1 in the presence of glucose. The duration of the lag was the same in the presence of glucose as in its absence for both 3-chlorophenol and 2-chlorophenol. However, in the case of 4-chlorophenol the presence of glucose reduced the lag period from 6 hours to 3 hours suggesting that any enhancement of substrate removal by an additional carbon source is influenced by the relative toxicity of the primary substrate.

Glucose supplementation of the medium above 1% (w/v) caused a significant drop in pH. The drop in pH may be due to the formation of acidic products like citric acid, pyruvic acid, lactic acid and acetic acid during glucose metabolism (Solé *et al.*, 2000). Substrate degradation was inhibited when the pH of the medium dropped. By regulating the pH with buffer the pH effect was alleviated and substrate removal was restored. The complete degradation of 200 ppm mono-chlorophenols in the presence of glucose by regulating the pH of the medium indicated the role of pH in the biodegradation of mono-chlorophenols. The specific rate of removal of glucose was similar for any one concentration of glucose regardless of the chlorophenol present.

There are several other reports of pH regulation in glucose and organic pollutant containing media (Loh and Wang, 1998; Wang and Loh, 1999). They also found the enhancement of degradation rates of phenol or 4-chlorophenol in the presence of glucose and concomitant complete degradation of phenol and 4-chlorophenol can be achieved by regulating the pH of the medium. Wang and Loh (1999) reported that when 0.1% w/v glucose was used as an added growth substrate for *Pseudomonas putida* ATCC 49451, only 78% and 43% of the initial 4-chlorophenol concentrations of 100 and 200 mg l⁻¹, respectively, were transformed before the pH dropped to below 4.5 and stopped all reactions. But by maintaining the medium pH, complete removal of 4-chlorophenol was achieved even at the high initial concentration of 200 mg l⁻¹.

Among the three different buffers tested for suitability, sodium phosphate buffer and phosphate buffer were found suitable in terms of pH control and growth of *P. putida* CP1 and there was no growth or substrate removal in the presence of the Citric acid- Na₂HPO₄ buffer. Sodium phosphate buffer was selected for further study. Wang and Loh (1999) also used a phosphate buffer for maintaining the pH in a 4-chlorophenol and glucose containing medium. The duration of the lag period in the buffer containing media increased. This was attributed to the time taken by *P. putida* CP1 to adapt to higher concentrations of salts. When different concentrations of sodium phosphate buffer were tested for suitability it was found that 0.2 M buffer was the most suitable.

The degradation of fructose by *P. putida* CP1 unlike glucose was preceded by a lag period. When mono-chlorophenol removal was studied in the presence of fructose, there was a lag in the removal of each substrate. However, fructose addition reduced the lag in the removal of the chlorophenols. Following the lag, fructose removal rates increased with increasing concentrations of fructose. Fructose exerted a stimulatory effect on the degradation of mono-chlorophenols by *P. putida* CP1 as the removal of all three mono-chlorophenol isomers was enhanced by the presence of various concentrations of fructose and this stimulatory effect was greater than that exerted by glucose. Although the removal rates of mono-chlorophenols increased significantly

following the addition of fructose, the growth rate of the organism was similar on all substrates.

There is little literature on the effect of fructose on the transformation of chlorophenols. O'Sullivan (1998) noticed the effect of various concentrations of fructose on the removal of phenol and mono-chlorophenols by a commercial mixed culture, Biolyte HAB, and reported that increasing concentrations of fructose facilitated transformation of the phenolics by HAB. While there was preferential utilization of glucose by *P. putida* CP1 in the presence of mono-chlorophenols, there was simultaneous utilization of fructose and mono-chlorophenols. A similar finding was also observed by O'Sullivan (1998) for a mixed microbial population.

Unlike glucose, there was no significant drop in pH with fructose metabolism either when supplied on it's own or in combination with the mono-chlorophenols. The differences observed between glucose and fructose utilization by *P. putida* CP1 can be explained on the basis of sugar metabolism. Fructose metabolism by *Pseudomonas* spp. has been distinguished from that of other carbohydrates. While carbohydrate uptake normally occurs via active transport, a PEP-dependent phosphotransferase system has been demonstrated in *Pseudomonas* grown on fructose, pointing to the transport of this sugar into the cell by vectoral phosphorylation. The sugar accumulates in the cell as fructose-1-phosphate, which is converted to fructose-1,6-diphosphate and then fructose-6-phosphate before subsequent metabolism via the Entner-Doudoroff pathway (Lessie and Phibbs, 1984; Temple *et al*, 1998).

As fructose was found to enhance the degradation of chlorophenols by *P. putida* CP1, an attempt was made to degrade higher concentrations of mono-chlorophenols in the presence of 1% (w/v) fructose. Whereas in the absence of an alternative carbon source *P. putida* CP1 was only able to degrade 300, 200 and 250 ppm of 4-chlorophenol, 3-chlorophenol and 2-chlorophenol respectively, it was found that in the presence of 1% w/v fructose, *P. putida* CP1 was able to completely degrade 400, 300 and 500 ppm of 4-chlorophenol, 3-chlorophenol and 2-chlorophenol respectively.

Beyond these limits, mono-chlorophenol concentrations had a toxic effect on growth and therefore inhibited the degradation of both fructose and mono-chlorophenols. Although the organism could degrade higher concentrations of mono-chlorophenols in the presence of 1% (w/v) fructose, growth rates and removal rates decreased with increasing concentrations of mono-chlorophenols and lag periods also increased. Thus the results show that *P. putida* CP1 is able to tolerate higher levels of mono-chlorophenols when supplemented with 1% (w/v) fructose as an additional carbon sources.

With the oxidation of sugar, there is the formation of large quantities of NADH and NADPH (Gottschalk, 1986) and also increased growth of the organism. NADH or NADPH is the required cofactor for the key enzyme hydroxylase in chlorophenol metabolism (Spain and Gibson, 1988; Sáez and Rittmann, 1991 & 1993). Thus an increase in mono-chlorophenol removal in the presence of sugar can be explained by the increased production of cofactors such as NADH as well as the increase in biomass. In the absence of sugar, the NADH required for the transformation of mono-chlorophenols is produced through endogenous respiration by the oxidation of biomass (Sáez and Rittmann, 1991). Sáez and Rittmann also reported that the transformation of 4-chlorophenol was controlled by the 4-chlorophenol/biomass ratio. When the ratio was low the system was uninhibited but when the ratio was high the system was inhibited. In this study, it was found that easily degradable alternative substrates decreased the ratio of chlorophenol/biomass by increasing the growth and therefore, mono-chlorophenols degradation was increased.

In the presence of all three isomers of mono-chlorophenols, there was repression of glucose removal. However no repression of fructose removal was observed in the presence of mono-chlorophenols. Instead there was enhancement of fructose degradation. Bali and Şengül (2002) also reported the inhibition of glucose oxidation by 4-chlorophenol by using a mixed culture. The difference between glucose and fructose utilization may be related to the transport mechanism of fructose into the *P. putida* CP1 cells, which is different to that of glucose transport (Lessie and Phibbs,

1984; Temple *et al.*, 1998). The drop in pH may be another cause of repression of glucose removal.

A number of reports were published on the important role of yeast extract on the degradation of xenobiotic compounds by bacteria (Madsen and Aamand, 1992; van den Wijngaard *et al.*, 1993; Yu and Ward, 1994; Fava *et al.*, 1995b). Therefore the effect of yeast extract on the degradation of mono-chlorophenols by *P. putida* CP1 was investigated.

When yeast extract was used as the sole carbon and energy source in the minimal medium, there was no lag in the utilization of substrate. When chlorophenol removal was studied in the presence of yeast extract there was a lag in the removal of chlorophenol but not yeast extract. However, the lag in the removal of chlorophenol decreased with increasing concentrations of yeast extract. The rate of removal of chlorophenol increased with increasing concentrations of yeast extract up to a value of 0.5% (w/v). At 1% (w/v) yeast extract there was a decrease in the rate of chlorophenol removal. An increase in the pH of the growth medium was observed with 1% w/v yeast extract. This was attributed to the utilization of amino acids, peptides and proteins and the subsequent production of nitrogenous metabolites. The rate of removal of chlorophenols decreased with the increase in pH.

In the absence of yeast extract, a stoichiometric release of chloride accompanied the degradation of mono-chlorophenols. Chloride was also released from yeast extract, however chloride release from this complex substrate was not found to be stoichiometric. As yeast extract contains complex sources of nutrients the chloride produced following degradation may have been bound by some of the constituents of the yeast extract.

Other investigators showed that yeast extract and possible constituents of yeast extract may enhance the survival of xenobiotic degrading organisms (van den Wijngaard *et al.*, 1993; Armenante *et al.*, 1995; Fava *et al.*, 1995b). The results of

their work also indicated that the complex mixture, yeast extract enhances the growth and dechlorination activity of the organisms. van den Wijngaard *et al.* (1993) observed the effect of yeast extract and vitamins on the growth of *Xanthobacter autotrophicus* GJ10 on degradation of 1,2-dichloroethane in batch culture. They found that addition of 10 mg l⁻¹ yeast extract in the medium enhanced the growth and they also reported that the vitamin biotin was required for the optimum growth of the organism. The effect of 50 mg l⁻¹ yeast extract on the growth of *Pseudomonas* sp. CPE2 and *Alcaligenes* sp. CPE3 on chlorobenzoic acid was observed by a group of investigators (Armenante *et al.*, 1995; Fava *et al.*, 1995b). They reported inhibitory growth kinetics in the absence of yeast extract and noninhibitory kinetics in the presence of yeast extract and also found a significant increase in the affinity of the strains for chlorobenzoic acid in the presence of yeast extract and an overall increase in the rate of chlorobenzoic acid utilization and dechlorination.

Loh and Tan (2000) reported an improvement of phenol degradation rates when yeast extract was supplemented at concentrations from 0.2 g l⁻¹ to about 2 g l⁻¹, but a deterioration with further increases to 4 g l⁻¹. Yuan *et al.* (2000), reported the enhancement of phenanthrene degradation by the individual addition of yeast extract, acetate, glucose or pyruvate and among them yeast extract was the most stimulatory. Cometabolism of phenol and benzoate by *Azospirillum* strains in the presence of yeast extract was also reported (Barkovskii *et al.*, 1995). Usually under normal conditions in the environment a significant proportion of xenobiotic biodegradation occurs by cometabolism and many microorganisms was reported to utilize different isomers of mono-chlorophenol as a cometabolite (Menke and Rehm, 1992; Hill *et al.*, 1996; Kim and Hao, 1999).

Addition of easily degradable carbon sources may facilitate a reduction in the toxicity of chlorophenols and alleviate substrate inhibition of growth thereby increasing the degradation of chlorophenols. Conventional carbon sources may also have some other effects, as they may provide the reducing power for the degradation of recalcitrant

organic compounds (Perkins *et al.*, 1994) or in some cases act as inducing agents for biodegradative enzymes (Chaudhuri and Wiesmann, 1995).

There was always some residual COD remaining in the yeast extract and mono-chlorophenol containing media possibly due to the imbalance of the carbon and nitrogen ratio. Moreover the addition of fructose was found to be stimulatory for the degradation of mono-chlorophenols. Therefore an attempt was made to observe the effect of a combination of yeast extract and fructose on the degradation of mono-chlorophenols by *P. putida* CP1 using an optimal carbon nitrogen ratio (Stanbury and Whitaker, 1984).

The combination of yeast extract and fructose was found to enhance the degradation of chlorophenols by *P. putida* CP1 and improved COD removal was also observed. The removal of chlorophenol, COD and fructose when present was concurrent. The rate of chlorophenol removal was fastest for 4-chlorophenol, then 2-chlorophenol and finally 3-chlorophenol. The rate of removal increased in the presence of an additional carbon source. The rate of removal was greater in the presence of fructose than yeast extract but was greatest in the presence of both substrates. The order in terms of rate of removal continued to be 4-chlorophenol > 2-chlorophenol > 3-chlorophenol even in the presence of additional carbon sources.

There is no other known report of the use of a combination of fructose and yeast extract as a supplement for the degradation of chlorophenols. Yu and Ward (1994) reported the use of various combinations of carbon and nitrogen sources including glucose and yeast extract supplementation for the degradation of penta-chlorophenol by mixed bacterial cultures and noticed significant levels of enhancement. They reported maximum penta-chlorophenol degradation with the medium supplemented with glucose and peptone. Other investigators also reported the extensive dechlorination of trichlorophenol in media containing complex carbon sources like yeast extract, peptone and casamino acids (Madsen and Aamand, 1992).

The growth of *P. putida* CP1 on both glucose and yeast extract followed Monod kinetics. The maximum growth rate μ_{\max} with glucose was 0.17 h^{-1} and with yeast extract was 0.3926 h^{-1} . The increase in growth rate on yeast extract could be attributed to the presence of readily oxidizable carbon and nitrogen, vitamins and trace elements. The Monod relationship between specific growth rate and glucose concentration was also found for *Pseudomonas aeruginosa* PU21 (Chang and Hong, 1995) and *Pseudomonas putida* ATCC 17514 (Wang *et al.*, 1996). In their studies values for μ_{\max} ranged from 0.7 to 1.0 h^{-1} . Jahn *et al.* (1999) reported the maximum growth rate, $\mu_{\max} 0.28 \text{ h}^{-1}$ for batch cultures of glucose grown cells of a *Pseudomonas putida* species. However the maximum growth rate may vary from strain to strain for the same substrate depending on the environmental conditions of growth.

As *P. putida* CP1 formed flocs during growth on mono-chlorophenol it was difficult to measure growth by using conventional growth measurement procedures, such as optical density measurements. Flocculation caused problems especially in sampling. Therefore, an attempt was made to deflocculate the flocs by using a sonicator and a deflocculating agent Ethylenediaminetetraacetic acid (EDTA) (results not presented). As there are some reports of the use of sodium tripolyphosphate to enhance dispersion of flocs of phenol degrading bacteria (Fujita *et al.*, 1994; Soda *et al.*, 1998), an attempt was also made to observe the effect of the surfactant sodium tripolyphosphate on deflocculation as well. Although EDTA treatment caused partial dispersion of *P. putida* CP1 flocs, it also caused lysis of the cell at a concentration greater than 10 mM . Therefore, the EDTA treatment was not useful for the dispersion of *P. putida* CP1 flocs. EDTA breaks up flocs by tying up multi-valent inorganic ions, e.g., calcium, which is responsible for floc aggregation. Silverstein *et al.* (1994) reported that a 1% (w/v) EDTA solution was effective in breaking up sludge flocs, but their treatment also resulted in cell lysis.

Pike *et al.* (1972) found that the addition of 5 mg l^{-1} sodium tripolyphosphate before homogenization was suitable for the disruption of flocs and the enumeration of aerobic bacteria in activated sludge. However, preliminary studies in this work

showed that the surfactant sodium tripolyphosphate had no effect on the dispersion of the *P. putida* CP1 flocs.

Sonication was found to be the best method for the dispersion of *P. putida* CP1 flocs (results not presented). Release of bacteria from flocs depends on both the intensity and duration of sonication. High power (100 W) sonication caused dispersion of cells and cell lysis, but sonication at a low power (50 W) for 30 seconds was found to be effective for the dispersions of cells and caused no cell lysis. Therefore sonication at low power (50 W) for 30 seconds was selected. Several other investigators used sonication as a method for the dispersion of flocs of activated sludge by using comparable intensities and time (King and Forster, 1990; Jorand *et al.*, 1994; Jorand *et al.*, 1995; Snidaro *et al.*, 1997). Jorand *et al.* (1994) reported that the sonication of activated sludge at 37 W for 60 seconds was the best method for the dispersion of flocs with minimal cell lysis.

The physical method of sonication was selected because sample contamination could be avoided and so the subsequent study of various parameters would not be hampered. Sonication may cause cell lysis. Activity of the intracellular enzyme glucose-6-phosphate-dehydrogenase (G6PDH) was used as a measure of cell lysis, because this enzyme is normally present within the cell. If G6PDH is detected in the sonicated samples, cell lysis is assumed to have occurred. The activity of glucose-6-phosphate dehydrogenase was not detected in low power output sonicated samples. Biggs and Lant (2000) and Frølund *et al.* (1996) also used a similar technique for the identification of cell lysis in activated sludge.

Biomass estimation is important in microbial and other bioprocesses. Its determination leads to an understanding of the efficiency of a biological system. Although biomass concentration is a simple measure, it is a key variable in measuring rates of growth and product synthesis, yield coefficients, and also for the calculation of specific rates and mass balance in any bioprocess. Thus an accurate method for real-time biomass estimation during a bioprocess is an important goal to be achieved.

Despite the many promising classical methods available, evaluation of microbial growth in bioprocesses may sometimes become laborious, impracticable and give erroneous values (Singh *et al.*, 1994).

Classical methods for biomass determination may be based on cell number or cell mass. Methods dependent on cell number are observational, based on physical and microbial activity. These methods include total and viable counting of cells. Total count usually does not differentiate between active and dead cell populations whereas a few methods may provide the counts of viable or active cells. However, viable counts do not distinguish between cells and clumps of cells. Viable counts usually underestimate the microbial community when compared to direct count methods. Indirect methods usually estimate some component of the cell. They do not require visual or cultural examination of the organisms and depend upon a specific chemical component that is only present in the cells (White, 1988; Herbert, 1990).

The application of epifluorescence microscopy is one of the best direct methods for counting microorganisms (Herbert, 1990). The direct epifluorescence filtration technique is a rapid and sensitive enumeration technique, which allows microscopic counting of bacteria retained on a filter. The method involves staining of microbes with a fluorochrome, collecting cells on a membrane filter and finally counting using epifluorescence microscopy (Hobbie *et al.*, 1977; Bittton *et al.*, 1993; Standard Methods for the Examination of Water and Wastewater, 1998). With this procedure, fluorochrome (acridine orange or diamino-2-phenylindole) is added to the sample for a contact time of a few minutes and then filtered through a polycarbonate membrane. The membrane is rinsed with equal volumes of sterilized water equal to the total sample volume to remove excess stain.

Acridine orange is one of the most commonly used metachromatic fluorochromes. Acridine orange binds to DNA and RNA and fluoresces when excited with light at a wavelength of 436 or 490 nm. Generally the DNA-AO complex fluoresces greenish yellow while the RNA-AO complexes fluoresces red. Investigators suggested that

live cells with predominance of RNA fluoresce orange-red, whereas dead cells with predominance of DNA fluoresce green (Hobbie *et al.*, 1977; Bitton *et al.*, 1993).

When *P. putida* CP1 was grown on a variety of substrates, variations in cell numbers were found between the standard plate count (SPC) method and the direct epifluorescence filtration technique (DEFT). This may be due to the presence of viable but non-culturable cells of *P. putida* CP1. Direct cell counts of bacteria in water and wastewater usually exceed counts obtained from heterotrophic plate counts and the most probable number method because unlike those procedures, direct counts preclude errors caused by viability-related phenomena such as selectivity of growth media, cell clumping, and slow growth rates (Standard Methods for the Examination of Water and Wastewater, 1998)

Conventional culturable techniques have been shown to have many shortcomings. This study highlights the value of using the direct epifluorescent filtration technique for real time measurement of biomass in environmental samples. Monitoring growth is frequently done by measuring dry weight. In this study it was noted that an increase in cell numbers for the organism grown on mono-chlorophenols was accompanied by a decrease in the dry weight. This was a most unusual and unexpected result. Image analysis showed that the shape of the bacterial cell changed from rod shape to coccus shape when the organism was grown under certain environmental conditions. The change in shape was associated with environmental stress. In certain instances, not only did the cell change shape but the volume of the cell decreased. This explained the decrease in dry weight while there was an increase in cell number and again highlighted the need for real time measurement of cell numbers.

Givskov *et al.* (1994) also reported changes in cell shape and size leading to an increase in cell number but not an increase in cell mass with *Pseudomonas putida* KT2442. A decrease in cell size accompanied by an increase in population during starvation of marine bacteria was reported by Amy and Morita (1983) and Novitsky and Morita (1976). Size reduction was also reported for starved cells of *Pseudomonas*

aeruginosa and *Pseudomonas fluorescens* by Mueller (1996) and Makarov *et al.* (1998).

Shrinkage in size due to starvation was also reported for *Pseudomonas* strain A by Sanin *et al.* (2003). Electron micro-photographic studies of their work showed that *Pseudomonas* strain A tended to increase its surface area to volume ratio as a starvation response and increased the ability to transport nutrients into the cell with minimum energy consumption. Several other investigators reported the changes in cell shape of *Escherichia coli* and *Vibrio* from cylindrical to small spheric during the transition phase between growth and non growth phases to adjust their metabolic rate to a lower level (Kjelleberg *et al.*, 1987; Nyström *et al.*, 1991; Siegele and Kolter, 1992).

The effect of low nutrient or nutrient starvation on *Salmonella typhimurium* was investigated by Galdiereo *et al.* (1994). They reported the degenerated cellular forms, a coccoidal cell shape and a decrease in cellular volume. They explained the changes in cell shape and size as the rapid decline in bacterial carbohydrates and lipids and the gradual decline of protein and DNA in starvation. They also reported that during starvation the cell envelope structure becomes less rigid, which leads to the changes in cell shape and size that might account for the pleomorphism. Mårdén *et al.* (1985) reported the formation and release of small vesicles on the outer cell layer of starved bacterial isolates. This formation and release of vesicles was related to the continuous size reduction during starvation. Formation of vesicles is a mechanism for the release of excess outer layer material, which leads to the continuous reduction in size. Several other investigators also reported the rapid cell fragmentation and subsequent formation of smaller cells during nutrient depletion (Novitsky and Morita, 1978; Dawson *et al.*, 1981; Kjellberg *et al.*, 1983). Kjelleberg and Hermansson (1984) described how starvation confers a considerable stress upon the cell envelope, which then triggers the fragmentation and continuous size reduction.

Thus it is suggested that the findings from the present study show that the round morphology can be assumed by the rod-shaped bacterium *P. putida* CP1 as a consequence of alterations in the shape determining mechanism due to the nutritional stress.

In this study growth yield was expressed in a variety of ways. In the first instance, yield was expressed as mg of dry cell mass produced either per mmol or mg of substrate utilized. Both approaches have been reported in the literature. Pirt (1975) and Isken *et al.* (1999) reported yield values for bacteria grown on glucose in terms of g/g comparable to those values obtained in this study. The growth yield data for the growth of CP1 on phenol 0.68mg/mg was also comparable to the data from previous investigators for other *P. putida* strains which ranged from 0.52 to 0.80 g/g. (Hill and Robinson, 1975; Yang and Humphrey, 1975; Kotturi *et al.*, 1991; Dikshitulu *et al.*, 1993; Şeker *et al.*, 1997; Reardon *et al.*, 2000).

Growth yield of the organism was also expressed in terms of numbers of cells produced either per mg or per mmol of substrate utilized. This approach has also been reported in the literature (Shreve and Vogel, 1993 and Frías *et al.*, 1994). Shreve and Vogel (1993), reported the yield coefficient of 1.44×10^{10} cells/mmol for toluene grown cells of *Pseudomonas* strain K3-2. A value comparable to that was obtained when *P. putida* CP1 was grown on phenol and mono-chlorophenols. In this study, it was determined that growth on toxic substrates was more accurately measured using a direct cell count method such as the direct epifluorescence filtration technique (DEFT). This needs to be considered when evaluating yield and other growth parameters when cultivating bacteria on toxic substrates.

Growth media and the stage of growth influenced the fatty acid composition of *P. putida* CP1. When the fatty acid profile of *P. putida* CP1 was examined, the main fatty acids detected were the even-numbered saturated fatty acids (SFA) and the monounsaturated fatty acids (MUFA) of the range C₁₀-C₁₈. These fatty acids are characteristic of many Gram-negative bacteria (Wilkinson, 1988) and the fatty acid

profile of *P. putida* CP1 is very similar to other reference Pseudomonad strains: *Pseudomonas putida* mt-2, *Pseudomonas putida* F1, *Burkholderia cepacia* G4, *Burkholderia pickettii* PKO1, and *Pseudomonas mendocina* KR1 (Fang *et al.*, 2000b).

Dodecanoic (lauric) acid was detected when the organism was grown on various substrates. Levels of this fatty acid were higher with nutrient broth and easily degradable substrates than on the phenolics. However dodecanoic acid was absent from the phospholipid fraction of 2-chlorophenol grown cells. Smirnova (2001) also reported the absence of dodecanoic acid in the phospholipid fraction of *Pseudomonas syringae*. Dodecanoic acid was also detected in the lipopolysaccharide component of *Pseudomonas putida* IH-2000 (Kobayashi *et al.*, 1999).

Low levels of tetradecanoic (myristic) acid (14:0) and *cis*-9-tetradecanoic acid (14:1c) were detected in whole cells and in the phospholipid fraction when *P. Putida* CP1 was grown on various substrates including phenolic compounds. Similar low levels of 14 carbon fatty acids were also detected in five reference Pseudomonad strains by Fang *et al.* (2000b).

The most abundant fatty acids were the 16 carbon fatty acids in *P. putida* CP1. They were the saturated fatty acid hexadecanoic (palmitic) acid (16:0) and the unsaturated *cis*-9-hexadecanoic acid (16:1c) and *trans*-9-hexadecanoic acid (16:1t). The 16 carbon fatty acids comprised 40% to 70% of the total fatty acids depending on the growth substrate. Levels of hexadecanoic acid were consistently high and similar for all substrates. The levels of *cis*-9-hexadecanoic acid were high initially and then decreased with the time of incubation. The levels of these fatty acids detected when the organism was grown on chlorophenols were lower than on other substrates. Higher levels of *trans*-9-hexadecanoic acid was detected when the organism was grown on phenolics, but the levels of this fatty acid were low, when the organism was grown on nutrient broth and sugars. A similar proportion of 16 carbon fatty acids was detected in cells of *Pseudomonas putida* IH-2000 grown on organic solvents

(Kobayashi *et al.*, 1999). Comparable levels of hexadecanoic acid (16:0) and the unsaturated *cis*-9-hexadecanoic acid (16:1c) and *trans*-9-hexadecanoic acid (16:1t) were also detected in glucose (0.27% w/v) grown cells of the solvent tolerant strains *P. putida* S12, *P. putida* PpG1 and *P. putida* Idaho (Weber *et al.*, 1994). When glucose grown cells of these *Pseudomonas* strains were compared to acetate-grown (0.342% w/v) cells, an increase in the amount of *trans*-9-hexadecanoic acid and a decrease in the amount of *cis*-9-hexadecanoic acid was seen and a further increase in *trans/cis* ratio was observed when the organisms were grown on acetate in the presence of toluene. The findings of this study were very similar to their observations as higher levels of *trans*-9-hexadecanoic acid were detected when the organism was grown on phenolics and the levels of this fatty acid fell when the organism was grown on nutrient broth and sugars.

Five 18 carbon fatty acids were the third major fatty acid detected in *P. putida* CP1 cells. These fatty acids comprised one saturated fatty acid, octadecanoic acid or stearic acid (18:0) and four unsaturated fatty acids— *cis*-vaccinic acid (18:1c) and *trans*-vaccinic acid (18:1t), oleic acid (18:1co) and linoleic acid (18:2cc). Lower levels (<2%) of stearic acid were detected when the organism was grown on nutrient broth, sugars or phenol, but higher levels (about 5%) were detected when the organism was grown on the mono-chlorophenols. The highest level, 15%, was detected in the phospholipid fraction. A similar pattern, but with lower values, was observed for the unsaturated fatty acids except for *cis*-vaccinic acid. In the case of the latter, similar levels of the fatty acid ranging from 8% to 10% were detected regardless of the substrate. The level of this fatty acid decreased with time of incubation. Comparable levels of octadecanoic acid (18:0) and *cis*-vaccinic acid (18:1c) and *trans*-vaccinic acid (18:1t) were also detected on glucose (0.27% w/v) grown cells of the solvent tolerant strains *P. putida* S12, *P. putida* PpG1 and *P. putida* Idaho by Weber *et al.* (1994). However, oleic acid and linoleic acid were not detected in these *Pseudomonas* strains.

Oleic acid (C18:1 9-*cis*), which is a typical product of aerobic fatty acid synthesis was found in *P. putida* CP1. This fatty acid is usually found in those bacterial species having a fatty acid desaturase system. The positions of double bonds of microbial 18 carbon fatty acids depends on the biosynthetic route by which they are produced (Morita *et al.*, 1992; Keweloh and Heipieper, 1996). During anaerobic metabolism a C2-elongation of palmitoleic acid (C16:1 9-*cis*) leads to *cis*-vaccenic acid (C18:1 *cis*-11), whereas in the case of aerobic pathways the product is oleic acid (C18:1 9-*cis*). Therefore, the occurrence of both *cis*-vaccenic acid as well as oleic acid indicates the presence of both pathways for *P. putida* CP1.

Production of the polyunsaturated fatty acid linoleic acid (C18:2, 9-*cis*, 12-*cis*) was found in chlorophenol containing media. Polyunsaturated fatty acids are synthesized by very few bacteria (Henderson *et al.*, 1993; Hamamoto *et al.*, 1994; Ramos *et al.*, 1997; Fang *et al.*, 2000a). The role of polyunsaturated fatty acids in the growth of cells at low temperatures and in salt stress has been described (Sakamoto *et al.*, 1998; Allakhverdiev *et al.*, 1999). Sakamoto *et al.* (1998) described that tolerance of low temperature was enhanced by the introduction of a second double bond into the fatty acids in *Synechococcus* sp. PCC 7942. Allakhverdiev *et al.* (1999) showed that the presence of polyunsaturated fatty acids in membrane lipids is important for the tolerance of the oxygen-evolving machinery to salt stress. The importance of polyunsaturated fatty acids in the tolerance of other kinds of stress has yet to be investigated.

Cyclopropane fatty acids were the second major fatty acid detected in *P. putida* CP1 cells. There were just traces of *cis*-11,12-methyleneoctadecanoic acid (C19:0 cyclo), but higher levels of *cis*-9,10-methylenehexadecanoic acid (C17:0 cyclo) were detected. Lower levels of the fatty acid were detected when the organism was grown on phenolic substrates. Less than 2% cyclo fatty acids were detected in the phospholipid fraction. The proportion of cyclopropane fatty acids increased with the age of the organisms. Some other investigators also reported the production of

increased proportions of cyclopropane fatty acid at the stationary phase of growth (Guckert *et al.*, 1986; Magnuson *et al.*, 1993).

Two hydroxy fatty acids were identified in *P. putida* CP1 cells, however these fatty acids were absent in the phospholipid fraction. Only trace amounts of 3-hydroxydodecanoic acid (3-OH, C12:0) were detected when the organism was grown on various substrates. But higher levels of 3-hydroxydecanoic acid (3-OH, C10:0) were detected on nutrient broth and sugar grown cells and the levels of this fatty acid decreased when the organism was grown on phenolics and in particular chlorophenols. The level of hydroxy fatty acids also increased when glucose was added to media containing phenolics. Hydroxy fatty acids have been reported in several other *Pseudomonas* spp. (Wilkinson, 1988; Smirnova, 2001). The highest reported levels of hydroxy fatty acids in any bacterium have been reported in *Pseudomonas ovalis* IAM 1177. This bacterium contained 3-hydroxy-decanoic acid, 3-hydroxy-dodecanoic acid and 2-hydroxy-dodecanoic acid in the lipophilic part of the lipopoly-saccharide fraction, which comprised 80% of the total fatty acids (Kawahara *et al.*, 1979). 3-hydroxydecanoic acid, 2-hydroxydecanoic acid and 3-hydroxydodecanoic acid were also detected in the lipopolysaccharide fraction of *Pseudomonas putida* IH-2000 (Kobayashi *et al.*, 1999).

The initial ratio of saturated to unsaturated fatty acids did not vary significantly for the different substrates, however the ratio increased with increasing incubation time. An increase in the relative percentage of saturated fatty acids was observed in general, but particularly octadecanoic acid which was observed in the mono-chlorophenol grown cells of *P. putida* CP1. An increase in the degree of saturation of membrane lipids with a subsequent decrease in membrane fluidity has been reported (Keweloh *et al.*, 1991; Weber and de Bont, 1996). Thus it can be expected that the increase in saturated fatty acids in *P. putida* CP1 increased the rigidity of the membrane.

With the exception of nutrient broth, the ratio of *trans* to *cis* fatty acids increased with increasing incubation time. The increase was more marked in the presence of phenolic substrates and the greatest increase was detected with 2-chlorophenol. The proportion of *trans* unsaturated fatty acids in the membrane indicates the level of toxicity of the solvent towards the organisms. This observation was similar to that of Weber *et al.* (1994). Conversion of *cis* to *trans* is the mechanism for membrane adaptation of *P. putida* CP1 during stressful growth on toxic chemicals. The behaviour of *trans* unsaturated fatty acids in the cell membrane resembles that of saturated fatty acids in that they possess an extended configuration. Therefore the effect of converting *cis*-unsaturated fatty acids to *trans*-unsaturated fatty acids is similar to that seen for the substitution of unsaturated with saturated fatty acids resulting in a decrease of the membrane lipid fluidity. Several other investigators reported the response of various *Pseudomonas putida* strains to solvents by shifting their *cis* to *trans* ratio when exposed to toxic chemicals including Heipieper *et al.* (1992), Heipieper and de Bont (1994), Weber *et al.* (1994), Ramos *et al.* (1995), Pinkart *et al.* (1996), Pinkart and White (1997), Ramos *et al.* (1997) and Loffhagen *et al.* (2001).

The *cis* to *trans* isomerization has been reported by many investigators as a general index of starvation or stress (Guckert *et al.*, 1986, 1987; Heipieper *et al.*, 1996; Pinkart *et al.*, 1996). In this study *cis* to *trans* isomerisation was also noted with pH change. The relative amounts of *trans* fatty acids strongly depend on the physiological conditions of the cells, such as growth rate, medium composition and environmental factors (Heipieper *et al.*, 1996). *Trans/cis* ratio of greater than 0.1 in environmental samples is an index for starvation or stress (Guckert *et al.*, 1986). Loffhagen *et al.* (2001) suggested the suitability of using the ratio of *trans/cis* of unsaturated fatty acids as an indicator of the toxicity of membrane active hazardous chemicals.

Trace amounts of *trans* unsaturated fatty acids were found in *P. putida* CP1 in this study even when the organism was grown on a complex medium like nutrient broth

where the organism was not under stress. The occurrence of *trans* fatty acids in bacteria is rare and has only been reported for a few bacteria including pseudomonads, vibrios and methylotrophs (Shanklin and Cahoon, 1998) and *Rhodococcus* (Tsitko *et al.*, 1999). These bacteria contained a mixture of *trans* and *cis* isomers of unsaturated fatty acids and under various environmental stress conditions, there was an increased level of *trans* isomers at the expense of *cis* isomers. Isomerisation of membrane unsaturated fatty acids from *cis* to *trans* increased in this study in the presence of environmental stress. This could account for the corresponding change in the shape from rod to coccus.

The occurrence of *trans* fatty acids was also reported for a few bacteria which used both aerobic and anaerobic routes of fatty acid biosynthesis (Keweloh and Heipieper, 1996). Thus the occurrence of both oleic acid and *trans* unsaturated fatty acids in the membrane of *P. putida* CP1 suggested the presence of both an aerobic and an anaerobic route of fatty acid biosynthesis.

P. putida CP1 flocculated and formed clumps when grown in the presence of 2-chlorophenol, 3-chlorophenol and higher concentrations (≥ 200 ppm) of 4-chlorophenol. The clumping size increased with increasing concentrations of chlorophenol up to a limit, which varied for the different isomers. Dawson *et al.* (1981) reported that aggregation of microbial cells was a tactic for survival during starvation. Few dead cells were observed when the organism was grown on chlorophenols. However, dead cells were found when the organism was grown on phenol. While the organism flocculated on chlorophenols, no flocculation was observed when the bacterium was grown on phenol. This could explain the differences observed in cell viability in that flocculation was affording a protective mechanism to the cell (Farrell and Quilty, 2002). A further explanation could be that toxicity led to the complete lysis of cells which would not be detected using the direct epifluorescence filtration technique.

In the case of carbon stress, excess nitrogen is channeled into protein formation. These excess proteins contribute to the extracellular polymer matrix and increase the hydrophobicity of the cell wall. As the hydrophobicity increased the aggregation also increased (Dignac *et al.*, 1998; Farrell and Quilty, 2002 and Sanin *et al.*, 2003).

P. putida CP1 flocculated with fructose but did not flocculate with glucose. This suggested that the organism was under stress which could be attributed to the more complex mode of transport of this particular sugar into the cell (Lessie and Phibbs, 1984; Temple *et al.*, 1998).

When a readily available carbon source was combined in the growth medium with chlorophenol, clumping was reduced. The reduction in clump size increased with increasing additions of additional carbon. In the presence of a readily degradable carbon source, excess carbon is channeled to the production of extracellular carbohydrates, causing an increase in hydrophilic components. When these hydrophilic components cover the hydrophobic sites of the bacterial surface hydrophobicity is lowered (Durmaz and Sanin, 2001; Sanin *et al.*, 2003).

As the organism formed flocs with chlorophenols but not with phenol or with easily degradable substrates, it was thought that there may be some variation in the cell membrane structure especially in terms of the membrane fatty acid composition when the organism was grown on various substrates. This, however, was not found to be the case. Thus there was no obvious link between flocculation and changes in cell membrane fatty acid composition.

Many investigators have reported the production of extracellular polysaccharide during growth of certain *Pseudomonas* sp. under stressful conditions and have noted the role of extracellular polysaccharides in aggregation (Wrangstadh *et al.*, 1986). Sanin *et al.* (2003), showed with electron microphotographs that surface characteristics and extracellular polymer production changed during carbon and nitrogen starvation. When the *Pseudomonas* strain A, was starved for carbon an overall slight change in

hydrophobicity was observed and cells tended to be more clumped together rather than being more dispersed as prior to starvation. Jahn *et al.* (1999) also pointed out the involvement of extracellular polysaccharides, outer membrane protein and DNA in aggregation of a *Pseudomonas putida* species.

In a separate study in our laboratory it was found that the production of extracellular polysaccharides, DNA and changes in membrane proteins are responsible for the aggregation of *P. putida* CP1 on mono-chlorophenols. DNA was found to play a particularly important role. Other investigators also reported DNA as a flocculation factor for *Pseudomonas* sp. (Sakka and Takahashi, 1981). They worked with the deoxyribonuclease (DNase)- susceptible floc forming bacterium *Pseudomonas* sp. strain C-120 and concluded that the component involved in flocculation of that organism was highly polymerized double stranded DNA.

Sadasivan and Neyra (1985) observed the phenomena of flocculation and floc formation by *Azospirillum brasilense* Sp7 (ATCC 29145) and *Azospirillum lipoferum* Sp59b (ATCC 29707) in aerobic liquid cultures. They noted that a combination of fructose and nitrate was the most effective in terms of floc yields and concluded that the overproduction of exocellular polymers induced flocculent growth. They also noted that the microscopic appearance of the vegetative cells was found to change upon flocculation. Cells grown on nutrient broth appeared to be rods and did not form any kind of aggregation. But when transferred from nutrient broth to minimal medium containing fructose (8 mM) and KNO₃ (0.5 mM), the cells assumed a more oval to spherical shape within a period of 2 to 4 hours and then aggregation appeared with respect to culture age of the organisms.

In other studies, a number of bacterial species were found to have enhanced adhesion capabilities following starvation or when present in low nutrient conditions. It was reported that the alteration in morphology from rod to coccus related to an increase in attachment activity (Dawson *et al.*, 1981; Kjelleberg *et al.*, 1983; Kjelleberg and Hermansson, 1984; Kjelleberg *et al.*, 1985; James *et al.*, 1995). The change in cell

shape from rod to coccus was observed for *P. putida* CP1 when grown under a variety of environmental conditions. In some cases flocculation of the cells occurred e.g. growth on chlorophenols and in others flocculation did not occur e.g. when the culture was grown on phenol. The change in cell shape was always observed when the cells flocculated. Thus the transition of cell shape from rod to coccus may have increased the attachment activity and triggered the cells to flocculate.

Chapter 5

Conclusions

5. Conclusions

1. *P. putida* CP1 grew on all three mono-chlorophenol isomers when supplied as the sole source of carbon and energy. The biodegradability of the mono-chlorophenols followed the order: 4-chlorophenol > 2-chlorophenol > 3-chlorophenol.
2. *P. putida* CP1 was able to degrade 300 ppm 4-chlorophenol, 250 ppm 2-chlorophenol and 200 ppm of 3-chlorophenol. In the presence of 1% (w/v) fructose the organism could degrade 400 ppm 4-chlorophenol, 500 ppm 2-chlorophenol and 300 ppm 3-chlorophenol.
3. The degradation of chlorophenols was enhanced in the presence of low concentrations of glucose (below 0.5%, w/v). Higher concentrations of glucose caused a significant drop in pH and substrate removal was inhibited. When the pH was controlled complete substrate removal did occur but at a slower rate than that observed with lower concentrations of sugar.
4. Optimal chlorophenol removal was observed in the presence of fructose. The rate of removal was greater than that observed with glucose or yeast extract.
5. A change in cell shape from rod to coccus was noted when the organism was exposed to environmental stress.
6. Isomerization of membrane unsaturated fatty acids from *cis* to *trans* increased in the presence of environmental stress and could be linked to changes in cell shape.
7. The ratio of saturated to unsaturated fatty acids in the cell were similar regardless of substrate but increased with increase in incubation time.

8. The cells of *P. putida* CP1 aggregated and formed clumps in response to environmental stress.
9. The change in cell shape was accompanied by a reduction in cell size. This result together with cell aggregation affected the measurement of growth parameters in the system.
10. Monitoring growth of the bacterium under conditions of environmental stress was best achieved using real time measurement. The direct epifluorescence filtration technique was found to be suitable.

Chapter 6

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6. Bibliography

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Chapter 7

Appendix A

7. Appendix A

List of the chemicals, sources and grades.

<u>Chemical</u>	<u>Source</u>	<u>Grade/Purity</u>
2-chlorophenol	Sigma Chemical Co.	99%
3,5-dinitrosalicylic acid	Sigma Chemical Co.	>98%
3-chlorophenol	Aldrich Chemical Co.	99%
4-aminioantipyrine	Sigma Chemical Co.	>99%
4-chlorophenol	Sigma Chemical Co.	99%
Acridine orange	Sigma Chemical Co.	98%
Ammonium chloride	Riedel-de Haën	99.5%
Ammonium hydroxide	Sigma Chemical Co.	28.1%
Ammonium sulfate	Sigma Chemical Co.	99%
CaCl ₂	Sigma Chemical Co.	96%
CaCl ₂ .2H ₂ O	Riedel-de Haën	99%
Chloroform	Lab-Scan	HPLC/ 99.8%
Citric acid	BDH Chemical Ltd.	99.5%
CoCl ₂ .6H ₂ O	BDH Chemical Ltd.	97%
D-Fructose	BDH Chemical Ltd.	99%
D-Glucose	Riedel-de Haën	>99%
Diethylether	Lab-Scan	HPLC/ 99.5%
Ethanol	Aldrich Chemical Co.	HPLC/ 99.9%
Ethylenediaminetetraacetic acid	Sigma Chemical Co.	99%
Fe(NH ₄) ₂ (SO ₄) ₂ .6H ₂ O	Riedel-de Haën	99.5%
FeSO ₄ .7H ₂ O	Riedel-de Haën	99.5%
HgSO ₄	Riedel-de Haën	99%
Hydrochloric acid	Aldrich Chemical Co.	37%
K ₂ Cr ₂ O ₇	Riedel-de Haën	99.5%
K ₂ HPO ₄	Sigma Chemical Co.	99.7%
KH ₂ PO ₄	Sigma Chemical Co.	99.2%

<u>Chemical</u>	<u>Source</u>	<u>Grade/Purity</u>
Methanol	Aldrich Chemical Co.	HPLC/ 99.9%
Methyl 2-hydroxydecanoate	Sigma Chemical Co.	>98%
Methyl 2-hydroxydodecanoate	Biotrend Chemikalien GmbH	>98%
Methyl 3-hydroxydecanoate	Biotrend Chemikalien GmbH	>98%
Methyl 3-hydroxydodecanoate	Biotrend Chemikalien GmbH	>98%
Methyl <i>cis</i> -11,12-methyleneoctadecanoate	Biotrend Chemikalien GmbH	>98%
Methyl <i>cis</i> -11-octadecenoate	Sigma Chemical Co.	99%
Methyl <i>cis</i> -9, <i>cis</i> -12-octadecadienate	Sigma Chemical Co.	99%
Methyl <i>cis</i> -9,10-methylenehexadecanoate	Biotrend Chemikalien GmbH	>98%
Methyl <i>cis</i> -9-hexadecanoate	Sigma Chemical Co.	99%
Methyl <i>cis</i> -9-octadecenoate	Sigma Chemical Co.	99%
Methyl <i>cis</i> -9-tetradecanoate	Sigma Chemical Co.	99%
Methyl docosanoate	Sigma Chemical Co.	99%
Methyl dodecanoate	Sigma Chemical Co.	99%
Methyl eicosonate	Sigma Chemical Co.	99%
Methyl hexadecanoate	Sigma Chemical Co.	99%
Methyl octadecanoate	Sigma Chemical Co.	99%
Methyl tetradecanoate	Sigma Chemical Co.	99%
Methyl <i>trans</i> -11-octadecenoate	Sigma Chemical Co.	99%
Methyl <i>trans</i> -9-hexadecanoate	Sigma Chemical Co.	99%
Methyl <i>trans</i> -9-octadecanoate	Sigma Chemical Co.	99%
Methyl <i>trans</i> -9-tetradecanoate	Sigma Chemical Co.	99%
MgSO ₄ .6H ₂ O	Riedel-de Haën	99%
MnCl ₂ .4H ₂ O	Sigma Chemical Co.	99.4%
Na ₂ HPO ₄	Sigma Chemical Co.	100.3%
Na ₂ MoO ₄ .2H ₂ O	Sigma Chemical Co.	99.9%
NAD	Sigma Chemical Co.	98%

<u>Chemical</u>	<u>Source</u>	<u>Grade/Purity</u>
NADP	Sigma Chemical Co.	95%
NaH ₂ PO ₄	Sigma Chemical Co.	99%
NH ₃	Riedel-de Haën	32%
n-Hexane	Lab Scan	HPLC/ 95%
Phenol	Riedel-de Haën	99.5%
Potassium ferricyanide	Sigma Chemical Co.	99%
Potassium hydrogen phthalate	Riedel-de Haën	99.5%
Potassium sodium tartarate	Riedel-de Haën	99.5%
Silver sulfate	Sigma Chemical Co.	100.1%
Sodium chloride	Sigma Chemical Co.	99.5%
Sodium hydroxide	Sigma Chemical Co.	98%
Sodium nitrate	Sigma Chemical Co.	99.5%
Sodium sulphate	Sigma Chemical Co.	99%
Sulphuric acid	Aldrich Chemical Co.	95-98%