Bioremediation and Detoxification of Chlorinated Phenols in Tannery Effluent

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CERTIFICATE

This is to certify that the thesis entitled "**Bioremediation and Detoxification of Chlorinated Phenols in Tannery Effluent,**" submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy (PhD) at the School of Environmental Sciences, Jawaharlal Nehru University, New Delhi, India is a bonafide research carried out by **Ashwani Sharma**. The work is original and has not been submitted in part or full, for any other degree and diploma in any university.

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To my parents and teachers

O young scholar!

When you leave your vidyalaya and enter into the larger society, do not deviate from the path of truth and righteousness. Continue to seek your own well-being as well as of others. Do not procrastinate either in the study of scriptures or in the propagation of their knowledge.

TAITTIRIYA UPANISHAD

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Ashwani Sharma

New Delhi

We don't see words in nature but always only the initial letters of words, and when we set out to spell, we find that the so-called new words are in their turn merely the initial letters of others.

LICHTENBERG

NOTATION INDEX

ASP	activated sludge process
ASR	aerobic sequential reactors
ATCC	American type culture collection
BOD	biochemical oxygen demand
CFU	colony forming unit
COD	chemical oxygen demand
CETP	common effluent treatment plant
DGGE	denaturing gradient gel electrophoresis
DiCH	dichlorohydroquinone
DNA	deoxyribo nucleic acid
DO	dissolved oxygen
ECD	electron capture detection
EPA	Environmental Protection Agency
EU	European Union
FAD	flavin adenine dinucleotide
GC	gas chromatography
HPLC	high performance liquid chromatography
MLD	million liters per day
MS	mass spectrometer
MSM	mineral salts medium
NADPH	nicotinamide adenine dinucleotide phosphate
NIST	National Institute of Standards and Technology
NMR	nuclear magnetic resonance
PCP	pentachlorophenol

PCR	polymerase chain reaction
ppb	parts per billion
ppm	parts per million
rDNA	ribosomal deoxyribo nucleic acid
RNA	ribo nucleic acid
SCGE	single cell gel electrophoresis
TeCH	tetrachloro-*p*-hydroquinone
TDS	total dissolved solids
TS	total solids
TSS	total suspended solids
UASB	up-flow anaerobic sludge blanket
UV	ultra violet
VSS	volatile suspended solids

CONTENTS

INTRODUCTION

CHAPTER 1

INTRODUCTION

Of all the planet's renewable resources, water has a unique place. It is essential for sustaining all forms of life, food production, economic development, and for general well being. It is impossible to substitute for most of its uses, difficult to de-pollute, expensive to transport, and it is truly a unique gift to mankind from nature. Water is also one of the most manageable of the natural resources as it is capable of diversion, transport, storage, and recycling. All these properties impart to water its great utility for human beings (Kumar *et al.*, 2005). Today water resources have become one of the most exploited and vulnerable natural systems. Pollution of the water bodies is ever-increasing; due to rapid population growth, industrial proliferations, urbanization, increasing living standards and wide spheres of human activities. Time is perhaps not too far when pure and clean water, particularly in densely populated, industrialized water scarce areas may be inadequate for maintaining the normal living standards.

Glimpses of ugly figured pollution have been visible since long due to fast industrial development. The rapid industrialization and growth in the population substantially alters the nature of interactions between humans and their environment. Life is based on the circulation of elements, which means the exchange of substances between living creature and nature. The environment changes so rapidly because of humans activity that the equilibrium established in nature during past several years does not have time to restore. Water the elixir of life, is being contaminated more and more with time by various xenobiotics, in the natural water. The relatively sudden introduction of recalcitrant xenobiotic have overwhelmed the self-cleansing capacity of recipient ecosystem and thus resulted in

accumulation of pollutant to problematic or even harmful level (Aggarwal, 1996; Rao, 1998).

Water pollution world-over and particularly in India has now reached a crisis point. Almost every river system in India is now polluted to a considerable extent. As assessed by the National Environmental Engineering Research Institute (NEERI), Nagpur nearly 70% of water in India is polluted. There are about 3,200 major industries in India, in addition to a large number of small industries. The Union Ministry of Environment and Forests, Government of India (MoEF, GoI) has identified seventeen most polluting industries and has directed them to install pollution control equipments or face punitive action, including their forcible closures (Martin, 1998).

Leather industry, consisting of around 2500 registered tanneries in India, which are mainly located in clusters at Jajmau–Kanpur (Uttar Pradesh), Kolkata (West Bengal), Chennai (Tamil Nadu), Maharashtra and Karnataka is a highly polluting industry (More *et al.*, 2001). Annually, about 80 million pieces of skins are processed by the tanning industries for manufacturing semi finished and finished leather (Wiegant *et al.*, 1999; Sreeram and Ramasami, 2003; Stoop, 2003). Approximately around 7 lakh tons of hides and skins are processed each year in India that releases around 75,000 m^3 per day of toxic liquid effluents into near by water bodies (Sahasranaman and Buljan, 2000; Tare *et al.*, 2003). The leather processing requires a large quantity of chemicals and water (Chhonkar, 2000). Different chemicals are used at different leather processing stage since two types of tanning modes are followed in India. Consequently, two types of effluents are discharged from tanneries viz., effluents from vegetable tanning and chrome tanning (Babu *et al.*, 1994; Srivastava and Pathak, 1997).

Almost 80% to 90% tanneries use trivalent chromium (basic chromium sulfate) as a tanning agent. Indian leather industry makes

use of nearly 40,000 tons of basic chromium sulfate every year. The conventional chrome tanning practices lead to an uptake of only 50% to 70% by the skin/hides, leaving behind 12,000–20,000 tons of chromium slats in the effluents (Basu and Chakraborty, 1989; Rao *et al.*, 1998).

A large variety of chemicals are being synthesized and produced every year. Compounds are discharged into the environment during their manufacturing and use of these chemicals. Over last decade, chlorophenolic compounds have been used extensively as wide spectrum biocides in industries and agriculture. These are among the most persistent environmental pollutants because of their physicochemical characteristics (Annachhatre and Gheewala, 1996). The toxicity of these compounds tends to increase with relative degree of chlorination (Reineke and Knackmuss, 1988; Fetzner and Lingens, 1994). It is generally believed that these compounds are of precisely the right size of fit as the key in the lock in some vital molecules of the living cells and by finally attaching to their active site, they interfere in normal functioning of the cell (Alexander, 1981).

The chlorophenolic compounds are major environmental contaminants giving global concern mainly due to use of these chemicals as wide spectrum biocides in industries and agriculture (Vallecillo *et al.*, 1999). The most common sources of chlorinated phenols in the environment includes; production of chlorine from bleaching of pulp, combustion of organic matter, partial transformation of phenoxy pesticides, treatment of timber against moulds, fungi and insects and preservation of raw hides in leather tanning industries.

Among chlorinated phenols, pentachlorophenol (PCP) and its sodium salt have been widely used as wood and leather preservative owing to their toxic effect on bacteria, mould, algae and fungi (Kaoa *et al.*, 2004). PCP is used as a biocide in leather processing (tanneries), textile cardboard, wood industries and it is also unintentionally

formed in pulp and paper mill industry effluent. PCP is toxic to all life forms, as it is an inhibitor of oxidative phosphorylation (Yang *et al.*, 2006). Despite a widespread ban on PCP, it is still been used by tanneries across India as a biocidal leather/hides preserving agent. The main reasons for using PCP as leather preserving agent are: its availability in the grey market; its ease of using; cheap price; availability of its sodium salt, which is equally toxic and is still not banned in countries like India. The PCP and its sodium salt are used in the early stages of tanning process, it is at this stage that the raw hides are most vulnerable to fungal and moulds attack. Once the hides are infected with fungus, its further processing produces an inferior quality of finished leather. The finished leather, after tanning process is relatively resistant to fungal and moulds attack. Since the PCP is mixed in water for it application on hides, the same is discharged along with other liquid effluents. Also, chlorinated phenols including PCP are not removed by wastewater treatment units, thus persisting in the environment and find its way into the food chain.

Distribution of PCP along with other chlorinated phenol in biosphere, their toxicity and persistence in environment has caused public concern over the harmful effect on the quality of life (Alexender, 1973). The worldwide production of Na-PCP was 150,000 tons per year in early eighties (Edgehill and Finn, 1983). It is estimated that 300–400 tons of PCP has been used annually by industries in U.K. over the recent years. Out of the total PCP produced, 80% was used in wood preserving industries (Arsenault, 1976). Since few microorganisms can decompose them, the more highly chlorinated phenols like PCP tend to accumulate in the environment (Alexander, 1985). An organic chemical introduced into terrestrial or aquatic environment may be subject to non-enzymatic or enzymatic reaction brought about by the inhabitant of the indigenous microbial population.

As a result of the extensive use of chlorophenols including PCP over the past decades, PCP and other phenolic metabolites are widely

distributed in various environmental compartments. The various transportation and transformation processes that are involved in the distribution of a pollutant in the environment are schematized in Figure 1.1.

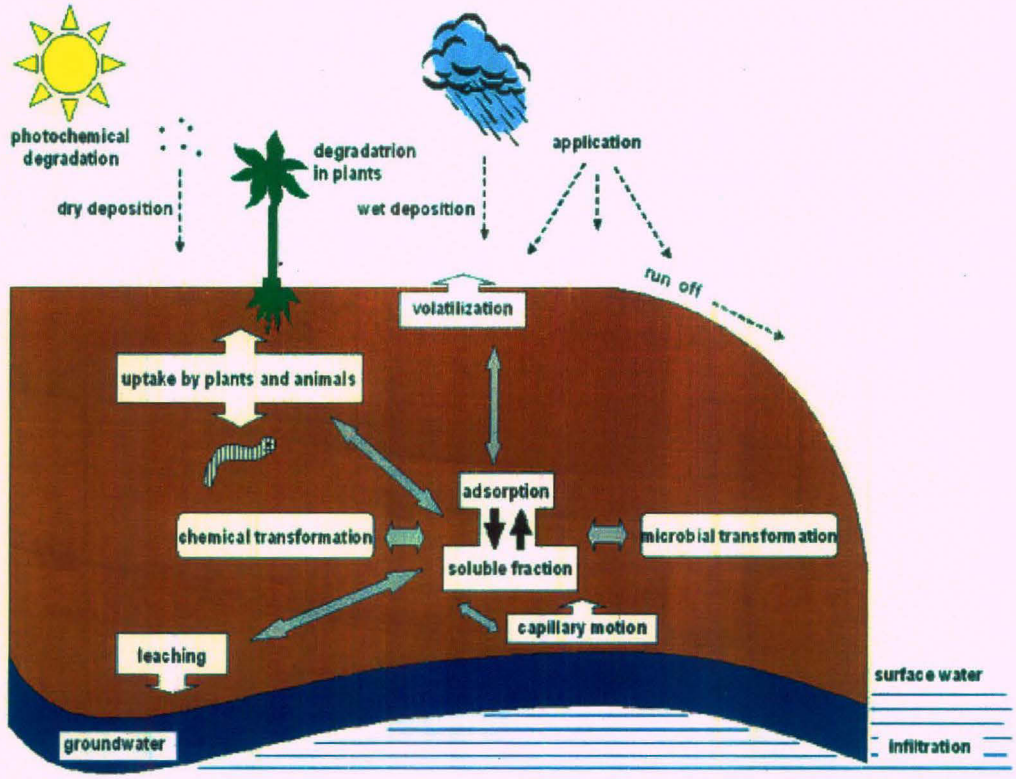

Figure 1.1 Once in the environment, pollutants is subject to various transportation and transformation processes (HCN, 1996)

Usage presents a potential and an actual problem of environmental contamination. The toxic effect of PCP is inactivation of respiratory enzymes uncoupling of oxidative phosphorylation, inhibition of glycolytic phosphorylation and damage to mitochondrial structure (Weinbach, 1957). The symptoms of illness occur at concentration of 4 to 8 mg/100 ml blood and doze ranging from 20–40 ppm may cause even death. The toxic effects are apparently caused by

entry of PCP in body by inhalation, absorption and feeding on contaminated food (Gilbert *et al.*, 1990; Shen *et al.*, 2005; Yang *et al.*, 2006). Since 1971, use of PCP had been restricted due to its proven toxicity to biota, its environmental persistence and at each trophic levels and production dibenzo-*p*-dioxins as by-product of its synthesis (Weiss *et al.*, 1982; Bhattacharya, 1996; McCarthy *et al.*, 1997). PCP is also listed as a priority contaminant by the United States Environmental Protection agency (USEPA). It is also listed as the 31st most hazardous substance in the US federal register (USEPA, 1991).

Microorganisms, which evolved more than three billion years ago, have developed strategies from obtaining energy from virtually every compound. They play a crucial role in the sustainability of the biosphere. The abundance of microbes, together with their ability for horizontal gene transfer and high growth rate, allows them to evolve quickly and to adapt with changing environmental conditions (De Lorenzo, 2001; Lovley, 2003). Proper identification of potential microbes and their hidden genetic resources should be deciphered and utilized. In general, bacteria are classified on the basis of its morphology (size, shape etc), staining (Gram), growth, nutritional requirements and physiological characteristics (temperature, pH, salt tolerance, antibiotic sensitivity). Bacterial communities have been compared on the basis of 16S rRNA genes (rDNA sequence) to investigate the evolutionary divergence. In situ and reverse transcription polymerase chain reaction (PCR), fluorescence in situ hybridization (FISH) techniques are used in single cell level detection of bacteria. Quantitative determinations of microbial community similarities have been made based on denaturing gradient gel electrophoresis (DGGE) banding profiles of PCR-amplified rDNA fragments without culturing them (LaPara *et al.*, 2000).

Microbes represent almost half of planets' biomass, we only know as little as 1% to 5% of the microbial diversity of the biosphere and majority of them could not be cultured in laboratory, which

reveals the largely unexplored pool of the microbial world (Amann *et al.*, 1995; Torsvik *et al.*, 2002; Curtis *et al.*, 2002). Culture independent molecular studies could provide a complete understanding of microbial community, without interference in their metabolic interactions at community level.

Human perturbations of the environment, particularly introduction of xenobiotic chemicals have created new opportunities for microorganisms to evolve metabolic pathways in order to either exploit them as new carbon source or to detoxify these toxic compounds (Sheeley, 2000). Bioremediation, a process that exploit the catalytic abilities of living organisms is an important tool in our attempt to mitigate environmental pollution (Gadd and Christpher, 1993; Glazer and Nikaido, 1995). Successful bioremediation depends on the availability in bio-treatment will be fascinating only when it use maximum input along with the indigenous microorganisms *in situ* (Karmanev and Sampson, 1998). Bacteria of different genera existing in close proximity are thought to aid each other in growth and survival via gene transfer and metabolic cross feeding. The latter case has been relatively well studied with bacteria that provide amino acids or vitamins to other strains with biosynthetic deficiencies. More recently, there has been interest in elucidating microbial metabolic cooperativity that is functional in the catabolism of organic compound (De Suiza *et al.*, 1998).

Various bioreactors have been designed for the application of microbial consortium for the treatment of chlorinated phenols including PCP. Bench scale continuous flow activated sludge reactors were used to study the removal of PCP. Various bioreactors have been designed and applied by the workers for the treatment of toxicant by the microorganism (Shukla *et al.*, 2001; Tare *et al.*, 2003; Farabegoli *et al.*, 2004; Lefebvre *et al.*, 2005; Taseli and Gokcay, 2005; Zilouei *et al.*, 2006; Barrera and Urbina, 2008).

The PCP degradation pathways offer a fascinating example of nature's ability to patch together a novel metabolic pathway by recruiting catalytic promiscuous progenitor enzymes to perform new functions (McCarty *et al.*, 1997). The PCP is converted to tetrachlorohydroquinone (TeCH) by PCP 4-monooxygenase enzyme. The second enzyme involved in the pathway of PCP degradation is TeCH dehalogenase, which catalyzes sequential dehalogenation of TeCH to 2,3,6-trichloro-*p*-hydroquinone and then to 2,6-dichloro-*p*-hydroquinone (2,6-DiCH). The enzyme responsible for further degradation of PCP is DiCH chlorohydroxylase, which is encoded by *pcpA* gene, it catalyzes the conversion of 2,6-DiCH to 2-chloro-maleylacetate (Xun and Orser, 1991).

There are very few reports of genetic analysis of the PCP degradation because of the lack of a gene transfer system for PCP degrading microorganisms (Xun and Orser, 1991). The genetic analysis has indicated the involvement of genomic DNA for PCP degradation, but genes are not transferable from one strain to another. Nevertheless, degrading genes present in plasmid confer considerable significance in mobility and can be transferred to indigenous population during bioremediation of contaminants at the industrial site. The plasmid may posses the necessary characteristics for growth and survival of microorganisms in the contaminated environment and thus establishes a stable array of host during bioremediation (Thakur *et al.*, 2001).

There are numerous physical and chemical parameters that influence the kinetics of the PCP metabolisms in soil and natural water by microorganisms. The parameter that might be of significance is the presence of readily metabolizable carbon source or co-metabolite. Through a variety of mechanisms, the metabolisms of one compound may often by enhanced or inhibited by that of the second compound (Edward *et al.*, 1998). The examination of the interaction of

PCP and alternative carbon and nitrogen source on the survival and activities of microorganisms is therefore the need of hour.

In light of the mobility of conventional treatment to completely detoxify the tannery effluent, a wide range of bioremediation strategies are being developed to treat contaminated soil and wastewater. Selecting most appropriate strategies to treat specific site can be guided by considering three basic principles, the amenability of the pollutant for biological transformation to less toxic product (biochemistry), the accessibility of the contaminants to microorganisms (bioavailability) and, the opportunity for optimization for the biological activity (bioactivity) (Blackburn and Hafker, 1993).

To eliminate above discussed problems bioreactors have been designed to support immobilization of microbial cell on solid supports. Bioreactor is an important biotechnological approach introduced recently in bioremediation studies. Since then this novel technique has been employed in various biotechnological applications. The microbial growth in the bioreactor ascertains the utilization of compounds in the effluent. Alternatively, a bioreactor may be designed in a way that maximizes enzymes production but minimizes inhibitory interaction between the inducers and pollutant (Van *et al.*, 1993). Bioreactor using immobilized cells has been proved to be advantageous over conventional effluent treatment technologies. Among various types of bioreactor, sequential bioreactors are considered best because of the enhancement of retention time of effluent, which enables microbial consortium to sustain the shock load of high PCP concentration (Shukla *et al.*, 2001). In addition such types of bioreactor will be true representatives of scale up processes of industrial effluent treatment.

Microorganisms in the effluent are responsible for biochemical transformation of many recalcitrant compounds. In recently years pollution induced changes in the structure of microbial communities

has been widely recognized to remove such type of recalcitrant compound and the compounds formed after degradation. But it is often difficult to define or quantitatively describe the development of potential microorganisms and to elucidate structure and function of microbial community (Alexander, 1981).

In the light of facts mentioned above, it is important to elucidate such biological interaction with tannery effluent in a continuously operating system in order to develop a potential microbial consortium for the removal of chlorinated phenols (PCP) and other toxicants. The study could help in developing strategies for enhanced degradation on a large scale removal of PCP from the environment. Therefore, specific objectives of the present investigation are:

- Characterization of physico-chemical parameters of tannery effluent.
- Enrichment and characterization of chlorinated phenols degrading bacterial community in the chemostat with pentachlorophenol as sole source of carbon and energy.
- Optimization and elucidation of the pathways for biodegradation of pentachlorophenol
- Purification and characterization of the monooxygenase enzyme involved in the biodegradation of pentachlorophenol
- Biological treatment of tannery effluent in aerobic sequential reactors
- Detoxification analysis of chlorinated phenols and its metabolites in the tannery effluent

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE

2.1 Industrial development and water quality

The rapid industrialization and growth in the population substantially alter the nature of interaction of man and his environment. Life is based on the circulation of elements, which means the exchange of substance between man and nature. The environment changes so rapidly because of man's activity that the equilibrium established in nature during centuries does not have time to restore. Water the elixir of life is being contaminated more and more with time by various xenobiotic in the natural water. The relatively sudden introduction of recalcitrant xenobiotics have overwhelmed the self-cleaning capacity of recipient ecosystem and thus resulted in accumulation of pollutants to problematic or even harmful level. The great challenges dominate the scene as one contemplates the global environment in the first few decades of 21st century. First and foremost is a huge legacy of industrial pollution, which is not being responded. The systematic pollution of our environment through industrial effluent is one of the biggest hazards that humanity face towards. People are becoming increasingly aware of the threat posed by pollution; India is no exception (Aggarwal, 1996).

Modern technological developments have multiplied the hazards to which human beings are exposed. More than 5 million chemicals have been synthesized in the world in last 50 years and 50,000 to 70,000 chemicals are used extensively in millions of different commercial products without the availability of proper toxicological information on the majority of chemicals. Much of this growth can be

attributed partly the needs of growing population and partly to development.

Science and technology has made it possible to bring to all people of the earth, the prosperity, well being and opportunities undreamed of by earlier generation has also produced a series of depending environment imbalance which are underming the basic foundation for a successful future.

India today is one of the first ten industrialized countries of the world. Today we have good industrial infrastructure in core industries like chemicals, fertilizers, petroleum, leather industries etc. Though three fourth volume of the wastewater is generated from municipal sources, industrial wastewater contribute over half of the pollution load and major portion of this originates from large and medium scale industries (Martin, 1998).

2.2 Status of tanneries in India

Tanning is an ancient craft in India and had been practiced for many centuries at the village level. The industry flourishes in conditions of natural advantage offered by large cattle population, which supply raw materials i.e. hides and skins (Nandy *et al.*, 1999). Tanneries are significant in terms of its contribution to total Indian exports and employment opportunities for people of economically weaker populations. However, sustenance of tanneries, particularly of the small units, is becoming increasingly difficult because of alarming levels of environmental pollution caused by various tanning operations and practices (Tare *et al.*, 2003).

Tanning industry is scattered unevenly in the country and it exists in the large, medium and small cottage section. Annually, about 80 million pieces of hides and 130 million pieces of skin are processed by tanning industry of the country for manufacturing semi finished and finished leather. There are about 680 leather-finishing units in

our country producing about 100 million square meter of finished leather per annum. During tannery processing 30 to 40 liter effluent per kg of skin or hide is discharged, however in the case of finishing the quantity is reached up to 50 liters/kg (Sreeram and Ramasami, 2003; Stoop, 2003). The pollutants from large number of tanneries in the country have caused damage of water courses, affecting drinking water supply and irrigation. Tannery consume huge amount of water and consequently generate large volume of wastewater, which contains various organic and inorganic material as well as toxic trace material (Kaul, 1993; Chhonkar, 2000).

2.3 Process of leather production and tannery waste

Leather production involves tanning, which is a chemical process of converting the semi soluble protein 'collagen' present in the corium of animal skins and hides into tough, flexible and highly durable leather (Nandy *et al.*, 1999). Tanning is a very complex process, which involves various steps. These steps are summarized in Figure 2.1.

2.3.1 Types of tanning processes

Two types of tanning processes are mainly followed in India viz., chrome tanning and vegetable tanning (Chhonkar *et al.*, 2000). More than 250 chemicals are used in tanning at different leather processing stage. A schematic overview of leather production from raw hides is given in Table 2.1.

2.3.1.1 Chrome tanning process

Salts of chromium are used as agents in this type of tanning process. The effluent from chrome tanning generally contains much higher concentrations of total dissolved salts, suspended solids, chloride etc, as compared to those vegetable tanning (Vajpayee *et al.*, 1995). A comparative study of the values of different parameters in effluent is presented in Table 2.2 and Table 2.3.

Table 2.1 Chemicals used during various stages in leather processing

S. No.	Process	Chemicals used
1	Curling and preservation	Sodium chloride, pentachlorophenol (PCP)
2	Unhairing	Sodium sulfate
3	Deliming	Ammonium chloride and ammonium sulfate
4	Degreasing	Alkyl-phenyl ethoxylate
5	Pre-tanning	Basic chromium sulfate
6	Tanning	Mineral tannages
7	Fat liquoring	Synthetic liquors, surfactants from petrochemicals
8	Finishing	Cadmium and lead chrome pigment, nitrocellulose, liquor emulsions, water proofing agents, formaldehyde, acrylic and poly urethane benzidine

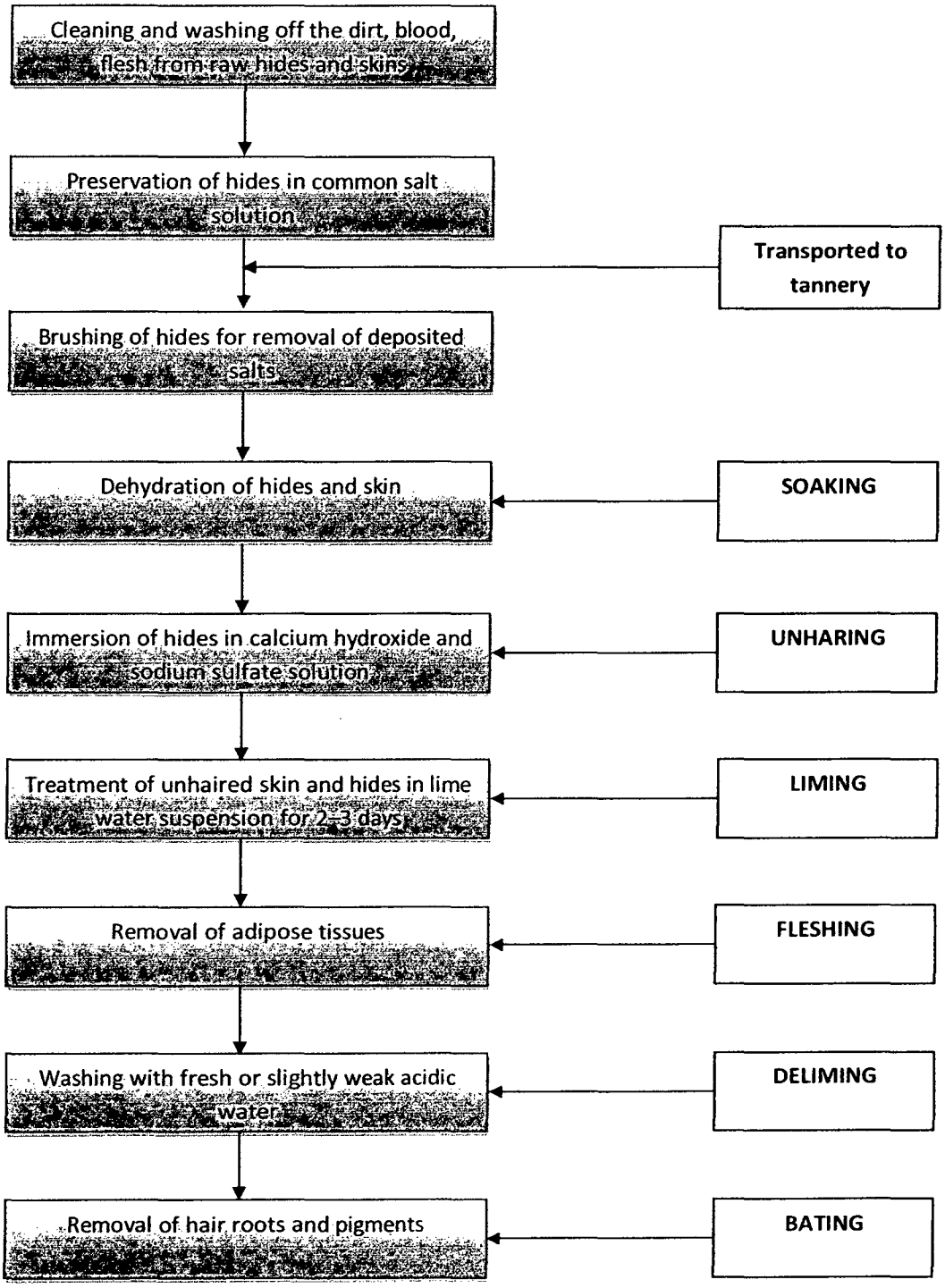

Figure 2.1 Schematic representation of a leather production process

Table 2.2 Effluent characteristics of chrome tanning process

Parameters	Values				
	Balashauri and Devi, 1994	**Murugesan *et al.*, 1996**	**Sujatha and Gupta, 1996**	**Srivastava and Pathak, 1997**	**Chonnkar *et al.*, 2000**
pH	10.0	8.4	7.5–10.0	2.6–4.0	8.67
DO	–	–	–	–	10
BOD	3215	1650	2000–3000	800–3500	980
COD	9650	3905	5100–7200	1916	2080
Chloride	1624	3560	1600–26200	–	2148
Total Nitrogen	–	726	216	–	56
Phosphate	2	–	–	–	15
Sulfate	36	1220	–	–	–
Sulfide	–	40	–	–	4.7
Chromium	150	6.5	–	500–5500	0.705
Nitrate	1.5	–	–	–	–
Carbonate	32.5	–	–	–	–
Bicarbonate	60	–	–	–	–
TS	–	20772	–	–	–
TDS	–	19852	9000–20000	–	8.35
TSS	15786	920	1250–6000	1500–2400	–
VSS	–	285	–	–	–
Tannins	2160	–	–	–	–
Alkalinity	743	2060	–	–	–
Oil and grease	56	–	–	–	–

All values except pH are expressed in mg/l; (–), not detected

Table 2.3 Characteristics of chrome tanning effluent at different leather processing stages

Parameters	Soaking	Liming	Deliming	Pickling	Chrome tanning	Neutralization	Dyeing and fat liquoring	Composite
pH	8.04	12.75	8	3.5	3.0	6.2	3.9	8.2
Acidity	–	–	–	6700	7600	2968	6071	–
Alkalinity	1550	2300	1500	–	–	–	–	17500
Total solids	20600	27600	34800	86600	89892	6200	19400	4400
Dissolved solids	19200	14800	20100	76800	13624	5200	17200	4000
Suspended solids	1400	12800	14700	9800	76268	1000	2200	400
Chloride	17060	1732.5	–	13190	29069	–	–	–
COD	5333	10800	2560	1562	3250	1642	1785	3950
BOD	3354	6793	1610	750	1265	753	840	1965

All values except pH are expressed in mg/l; (–), not detected

2.3.1.2 Vegetable tanning process

The vegetable tanning industry is one of the important industries of our country. The complex wastewater from this industry was ranked among most polluting of all industrial wastewater. The complexity is evident on account for presence of excess concentration of suspended and dissolved form of organic solids, inorganic impurities and numerous chemical additives used in converting raw hides to leather (Routh, 1999). There is a significant difference in respect to pollution load between effluent generated from vegetable tanning and chrome tanning process (Srinivas *et al.*, 1984). The effluent of vegetable tanning is highly complex. Various works have reported the values of different parameters of effluent emanating from vegetable tanning. A comparative study of two different effluents is presented in Table 2.4.

Table 2.4 Characteristics of effluent from vegetable tanning process

Parameters	**Values**	
	Srivastava and Pathak, 1997	**Chhonkar *et al.*, 2000**
Color	Brownish	Brownish
pH	7.3–8.4	6.0
DO	–	–
BOD	320-700	7687
COD	1555–3310	9589
Nitrogen	1275	–
Phosphorus	1.26	–
Sulfide	1170	–
Chloride	3010–6213	–
Tannin	480	127
TSS	33610	57
Potassium	–	638
Magnesium	–	1543
Manganese	–	0.67
Zinc	–	2.56
Lead	–	0.23
Calcium	–	4.0
Sodium	–	5380

All values except pH are expressed in mg/l; (–), not detected

Farooque, (1994) analyzed the tannery effluents collected from fill and draw tank inside the tannery premises, Lucknow, and reported that these effluents contain considerable quantities of chlorides (2148 mg/l), nitrogen (56 mg/l), phosphate (15 mg/l) and sulfide (4.7 mg/l) along with excess of water soluble salts, BOD, COD and sodium. However, Gupta and Sujatha, (1996) reported much higher concentrations of total nitrogen (216 mg/l) in effluent collected from small tannery industries of Tamil Nadu.

2.4 Impact of tannery effluent

According to Srinivas et *al.*, (1984), the effluent arising from North Arcot district of Tamil Nadu are generally discharged into neighboring field to irrigation tanks in untreated form, this effluent finally goes to river and infiltrate down to contaminate ground-water. Similarly chemical pollution problems arising from the discharge of untreated effluents of about 9000 cubic meter per day from Jajmau tanneries near Kanpur (U.P.) directly into the river Ganga, which is a serious concern. In Jajmau, around 375 tanneries operate with an average daily rawhide processing capacity of 320 tons. Approximately 315 tanneries convey 7.75 million liters per day (MLD) of effluent to the effluent treatment plant (Shukla *et al.*, 2001; Tare *et al.*, 2003).

The continue letting out of tannery effluent with so much organic load in large volume in water sources affected the quality of irrigation water and productivity of soil. Different types of chemicals are used in the processing of skin and hides and through many compound and ions are discharged (Singram, 1996). The organic matters present in effluent undergo oxidation and cause depletions in dissolved oxygen. Depletions in oxygen cause inability of various aquatic biota to survive.

2.4.1 Impact of effluent on soil health

The addition of tannery effluents is reported to have undesirable effect on soil properties. The total porosity and hydraulic conductivity of soil

decrease as a result of addition of effluent, while bulk density of soil gets increased (Singram, 1996). This may be attributing to the twin effect of direct accumulation of large quantities of organic and inorganic materials as well as the interaction of Na with exchange complex, which causes the deflocculation.

Karunyal *et al.*, (1994) have also studied the effect of vegetable tannin on the elemental status of garden soil and reported that soil treated with tannery effluent was rich in magnesium, manganese, iron, sodium and potassium. However the information pertaining to the effect of tannery effluent on soil health on long-term basis is not available on literature (Chhonkar *et al.*, 2000).

2.4.2 Impact of effluent on crops

Thangapandian *et al.*, (1995) studied the cytological effect of tannery effluents on *Allium ceppa* and have reported that the mitotic process was severely affected (48%) by the effluent. Anaphase was much affected (54%) followed by the segregation stage, the telophase, and least effect was on prophase (13%). The induction of mitotic aberration was around 8.8%.

Among the growth processes, seed germination and early seedling growth have been considered critical for raising a successful agricultural crop. Saxena *et al.*, (1984) have studied the impact of untreated tannery effluent on germinations and seedling growth of four, important crop of India, extensively cultivated for domestic consumption and marketing. The maximum retardation of seed germination was observed in pea (33.6%) followed by grave (23.2%), black gram (15.4%) and green gram (8.2%) and subsequent growth of seedlings were also adversely affected by tannery effluent. In the case of paddy, *Leucaena* sp. and *Acacia* sp., the germination of seed was reported to be completely prevented when 100 per cent tannery effluent were used. However at lower concentration of tannery effluent

the inhibitory effect of the effluents on seed germination followed the order: *Leucaena* sp.>*Acacia* sp.>Paddy.

Attempts have also been made to compare the effect of tannery effluent on the growth of pulses and cereal crops. The retardation of germination and overall seedling growth of crop can be attributed to high salinity and other ingredients of effluent like sodium chloride, PCP or chromium etc (Chhonkar *et al.*, 2000).

2.4.3 Impact of effluents on water bodies

Serious deterioration in the quality of ground water occurs as result of the effluents discharged from the tanneries. Consequently the agricultural production, in the Palar basin was reported to reduce to one fourth within a span of 12 to 15 years, with as large as 40,000 acres of fertile land becoming unproductive due to sodium hazard (Srinivas, 1984).

As mentioned earlier in the text, the problem of high concentration of chlorinated phenols, chromium and high COD are major problems associated with tannery effluents. Various attempts have been made by workers to remove major contaminants of the tannery effluent using biological methods, chemical methods or combination of both the methods (Table 2.5).

2.5 Chlorinated compounds and their significance

A large variety of chemicals are being synthesized and produced every year. Compounds are discharged into the environment during their manufacturing and use of these chemicals. Over last decade, chlorophenolic compounds have been used extensively as wide spectrum biocides in industries and agriculture. These are among the most persistent environmental pollutants because of their physico-chemical characteristics (Annachhatre and Gheewala, 1996). The toxicity of these compounds tends to increase with relative degree of chlorination (Reineke and Knackmuss, 1988). It is generally believed

that these compounds are of precisely the right size of fit as the key in the lock in some vital molecules of the living cells and by finally attaching to their active site, they interfere in normal functioning of the cell (Alexander, 1981).

Table 2.5 Major studies carried out for the treatment of tannery effluent

Treatment	**References**
Treatment of tannery effluent by *E. crassipes*	Vivekanandan, 1984
Coupled aerobic and anaerobic treatment of toxic wastewater	Guiot *et al.*, 1993
Performance evaluation of full scale wastewater treatment facility for finished leather industry	Kaul *et al.*, 1993
Aerobic bio-oxidation of post anaerobic tannery effluents	Nandy *et al.*, 1993
Removal of chromium from tannery effluent by using water weeds	Singram, 1994
Bioremediation of tannery effluent by aquatic macrophytes	Vajpayee *et al.*, 1995
High rate of algal oxidation pond for the treatment of tannery effluents	Rose *et al.*, 1996
Bioremediation of chromium from water and soil by vascular aquatic plants	Chandra *et al.*, 1997
Enrichment and characterization of pentachlorophenol degrading microbial community for the treatment of tannery effluent	Shukla *et al.*, 2001
Case studies on biological treatment of tannery effluents in India	Tare *et al.*, 2003
Biological treatment of tannery wastewater in the presence of chromium	Farabegoli *et al.*, 2004
Halophilic biological treatment of tannery soak liquor in a sequencing batch reactor	Lefebvre *et al.*, 2005
Hexavalent chromium removal by a *Trichoderma inhamatum* fungal strain isolated from tannery effluent	Barrera and Urbina, 2008

2.6 Pentachlorophenol and its significance

Pentachlorophenol (CAS No. 87–86–5) is a halogenated man-made organic chemical with a molecular weight of 266.35 and a chemical formula of C_6HCl_5O. In its pure form it is a colorless or white powder (needle-like crystalline structure), but the crude product may be dark gray to brown in color. PCP has a boiling point of 309–310°C, a melting point of 190–191°C, a density of 1.978 g/ml and a vapor pressure of 0.00011 mm Hg. PCP is almost insoluble in water, freely soluble in alcohol and ether, and moderately soluble in benzene (Budavari *et al.*, 1989).

The primary route of exposure for PCP involves occupational settings although environmental exposure may be relevant in the vicinity of spills, waste sites, or around sites where the chemical had been applied as a biocide (ATSDR, 1989).

Among chlorinated phenols; PCP and its sodium salts have been widely used as wood and leather preservative, owing to their toxic effect on bacteria, mould, fungi and algae (Kaoa *et al.*, 2004). PCP is toxic to all life forms, as it is an inhibitor of oxidative phosphorylation (Yang *et al.*, 2006). Its excessive exposure could cause cancer. Shukla *et al.*, (2000) has reported significant amount of PCP in the tannery effluent after extraction and analysis by HPLC.

The world-wide production of PCP and Na-PCP is 150,000 tons/year (Edgehill and Finn, 1983). The United States Environmental Protection agency has registered 578 products containing PCP (Saber and Crawford, 1985). It is estimated that 300–400 tons of PCP is used annually by U.K. industries over recent years. Over 100 tons are released directly via spillages, volatilization or associated with wastewater. The USEPA has regarded PCP as priority toxic chemical (Wild *et al.*, 1992).

PCP has been extensively used in the construction and lumber industries and in homes to control mold and termite infestation, and

for the control of powder post beetles and wood boring insects (Monsanto, 1963). Chloranil (tetrachloro-*p*-hydroquinone) an oxidation product of PCP has been used as a fungicide under the trade name of "spergon" to protect seeds and bulbs. Sodium PCP has been used as a molluscaside for the destruction of the snails, intermediate host of human schistosomes (Bevenue and Beckman, 1967). A chemical with so wide and so varied usage present a potential problem of environment contamination.

2.6.1 Properties of PCP

PCP was previously used extensively as a biocide and is still in use as a wood preservative but this use is restricted to certified applicators (ATSDR, 1989). Commercial PCP preparations contain 85% to 99% pentachlorophenol and various impurities including tetrachlorophenol (4-12%), trichlorophenol (<0.1–10%), chlorinated phenoxyphenols (1%–5%), and polychlorinated dibenzo-*p*-dioxins and dibenzofurans (<1%) (Jorens and Schepens, 1993). The presence of these contaminants makes the assessment of toxicity difficult for technical grade PCP (Williams, 1982).

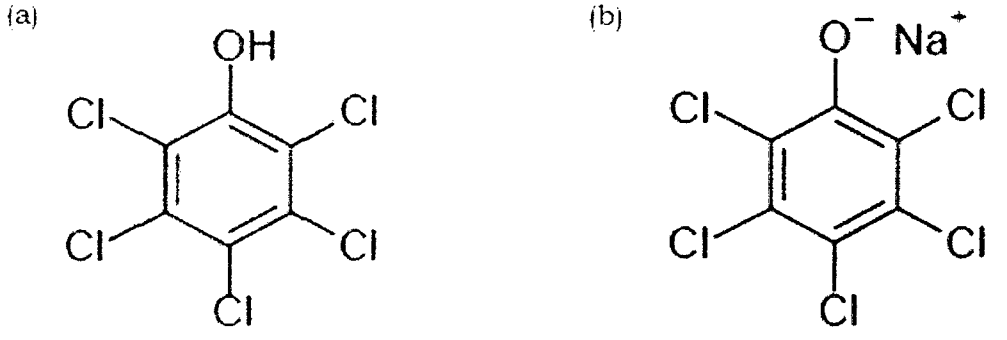

Figure 2.1 Chemical structure of PCP; (a); sodium-PCP, (b)

2.6.2 Physical properties of PCP

PCP forms colorless solid needle like monoclinic crystals with a phenolic odor and insoluble in water. Therefore, the rapidly water-soluble salt (Na-PCP) is Substituted in many industrial application. PCP is rapidly and extensively degraded by U.V. radiation and by sunlight (when it is in soluble form) to several identifiable products (Hamadmad, 1966). The degradation of PCP by U.V. radiation or by sunlight is first order reaction (Hiatt *et al.*, 1960). Kuwahara (1965) exposed the aqueous solution of Na-PCP to sun light for 10 days and found that decomposition products like chloranilic acid and chlorinated benzoquinons are formed.

2.6.3 Chemical properties of PCP

Pure PCP is relatively inert and is not subjected to coupling or substitution reactions, common to most phenols (Monsanto, 1958). It is a weak acid ($k_a = 10^{-5}$) and reacts with strong bases to give corresponding water-soluble salt.

Strong oxidizing agents decompose PCP. For example nitric acid treatment converts PCP to a mixture of tetrachloro-*ortho* and *p*-quinones, which can be measured color-metrically (Monsanto, 1963). Among its salt sodium and potassium salts are highly water soluble, ammonium salt 1% soluble in water and other metallic salt such as silver (golden yellow crystals) copper (purple crystals) and mercury (lemon yellow crystals) the solubility is of the same order of magnitude as PCP. PCP can be readily converted to the either derivative, a property that is utilized for its analysis by gas chromatography. Physical and chemical properties of pentachlorophenol and sodium pentachlorophenate are given in Table 2.6.

Table 2.6 Physical and chemical properties of pentachlorophenol and sodium pentachlorophenate

Property	**Pentachlorophenol (PCP)**	**Sodium pentachlorophenate (Na-PCP)**
Molecular weight	266.35	288.34
Color	Colorless or white (pure); dark grey to brown (crude)	White or tan
Physical state	Crystalline solid (pure); pellets or powder (crude)	Flakes or powder
Melting point	190°C	No data
Boiling point	309–310°C	No data
Density	1.978 g/ml at 22°C	No data
Odor	Phenolic; pungent	No data
Odor threshold:		
Water	0.857 mg/l at 30°C 12 mg/l at 60°C	No data
Solubility:		
Water	14 mg/l at 20°C	330,000 mg/l at 25°C
Organic solvent(s)	Very soluble in alcohol and ether; soluble in benzene; slightly soluble in petroleum ether	Soluble in acetone and ethanol
Partial coefficients:		
Log K_{OW}	5.01	No data
Log K_{OC}	4.5	No data
Vapor pressure (25°C)	0.00011 mmHg	No data
Photolysis	$T_{1/2}$ = 48 h	$T_{1/2}$ = 0.70 h
Auto-ignition temperature	No data	No data
Flash point	No data	No data
Flammability limits	No data	No data
Incompatibilities	No data	No data
Conversion factor	No data	No data
Explosive limits	No data	No data

2.6.4 PCP in the environment

PCP is found in all environmental media (air, soil, and water) as a result of its past widespread use. In addition, a number of other chemicals, including hexachlorobenzene, pentachlorobenzene, and benzene hexachloride isomers, are known to be metabolized to PCP.

PCP is stable to hydrolysis and oxidation, but the compound is rapidly photolyzed by sunlight and can be metabolized by microorganisms, animals, and plants. Adsorption to soils and sediments is more likely to occur under acidic conditions than under neutral or basic conditions. The compound has been found to bioaccumulate to modest levels (e.g., bioconcentration factors of <1,000), but food chain biomagnification has not been observed. In recent decades, PCP has been widely detected in human urine, blood, and adipose tissue among members of the general population. Human exposure to PCP is believed to occur via inhalation of indoor and workplace air, ingestion of contaminated water and food, and direct dermal contact with PCP-treated wood products.

PCP is ubiquitously distributed in the environment. It has been detected in surface waters and sediments, rainwater, drinking water, aquatic organisms, soils, and food, as well as in human milk, adipose tissue, and urine. The compound has been identified in at least 313 of the 1,585 hazardous waste sites on the NPL (HazDat, 2001).

2.6.4.1 PCP in atmosphere

PCP is released directly into the atmosphere via volatilization from treated wood products. Evaporation of PCP-treated industrial process waters from cooling towers was an additional source of historical atmospheric releases of the compound. Historical atmospheric releases included those from cooling towers, where PCP and its sodium salt were used as slimicides in cooling tower waters. However, PCP and its salt are no longer commonly used for this purpose since the early 1980s, when its use was restricted (EPA, 1984b).

Emissions during production are considered to be relatively insignificant in volume, and are geographically restricted to the Vulcan Materials facility in Wichita, Kansas (SRI, 1998). Physical removal mechanisms, such as wet deposition, are important processes that decrease PCP concentrations in the atmosphere. PCP may be formed during the incineration of chlorine-containing waste material. Heeb *et al.*, (1995) found that PCP constituted 8% of polychlorinated phenols formed in the flue gas and 10% of the stack gas during the incineration of chlorine-containing waste material. It may also be released in stack emissions as a result of pyrolysis of polyvinyl chlorides (Blankenship *et al.*, 1994).

2.6.4.2 PCP in water

PCP releases to surface water occur through direct discharge and direct entry from numerous nonpoint sources, including treated wood. In addition, it is transported to surface waters from the atmosphere by wet deposition and from soil by runoff and leaching. Approximately 90% of wood-treatment plants evaporate their wastewater and, consequently, have no direct discharge to surface waters. The remainder of the plants discharges to municipal wastewater treatment facilities.

Chlorination of phenolic compounds during water treatment has been reported to produce detectable levels of PCP (Detrick, 1977; Smith *et al.*, 1976). In addition, common pesticides such as lindane, hexachlorobenzene, pentachlorobenzene, and pentachloronitrobenzene are known to be metabolized to PCP by plants, animals, and/or microorganisms, but the contribution of the metabolism of these pesticides to environmental levels of PCP is unknown (Dougherty, 1978).

2.6.4.3 PCP in soil

PCP is released to soils as a result of its past use as a herbicide, leaching from treated wood products, atmospheric deposition in precipitation (such as rain and snow), spills at industrial facilities using PCP, and at hazardous waste sites. Most of the PCP removed

from effluent streams by wastewater treatment processes is adsorbed to sludge solids.

In soils and sediments, PCP is metabolized by acclimated microbes, under both aerobic and anaerobic conditions, or is adsorbed. PCP may also be methylated to form pentachloroanisole, a more lipid soluble compound. Adsorption of PCP in soils is pH dependent. The compound has a pK_a value of 4.7 and consequently exists in the ionic forms at environmentally relevant pH values. For example, at pH 4.7, PCP is 50% ionized, whereas at pH 6.7, the compound is about 99% ionized (Crosby, 1981).

2.6.5 Toxicology of PCP

PCP is highly toxic and recalcitrant compound. The recalcitrant nature of this compound is due to carbon halogen bond and low electron density of aromatic ring (Allison *et al.*, 1983). Several illness and some deaths have resulted from the careless handling of this hazardous chemical. The contamination of water with toxic chemicals from run off waste, sewage and industrial wastes and its effect on the environment has created considerable interest.

PCP and its biotransformation products manifest adverse or toxic effect. The PCP gets access in the body system through inhalation and skin. It has been observed that lipoproteins have high affinity with PCP and the bonding between blood carrier is reversible (Gomez *et al.*, 1991).

PCP may cause irritation of the skin, conjunctiva and upper respiratory tract and also cause demonstrable systemic absorption. Exfoliation of the epidermal layer of human hands occurred at 1–2 days after adjusting a weir dispensing 20% Na-PCP. Nine men died after dipping timber by hand and without any protection in a 1.5–2% solution for periods varying 3 to 30 days with average 13 days. Both hemolytic anemia and aplastic anemia have been reported to be associated with exposure to PCP (Hassan *et al.*, 1985). The occupationally exposed cases had a significantly higher mean level of

urinary PCP than the controls in 88 wood treaters in Hawaii (Gilbert *et al.*, 1990).

2.6.6 Metabolism of PCP

Results from animal and human studies indicate that PCP is not extensively metabolized, as evidenced by a large portion of the administered dose being excreted in urine unchanged in all species studied. However, available human and animal data indicate that metabolism of PCP does occur in the liver, and the major pathways are conjugation to form glucuronide and oxidative dechlorination to form TeCH. A summary of possible metabolic pathways for pentachlorophenol is presented in Figure 2.2.

UDP-glucuronosyl transferase and sulfotransferases are involved in phase II metabolism of PCP. Both of these enzymes are thought to be developmentally regulated (Leeder and Kearns, 1997). Although the ontogeny of UDP-glucuronosyl transferase is isoform-specific, the adult level of activity seems to be achieved in humans by 6–18 months of age. Ontogeny for the sulfotransferases seems to be more rapid than that for UDP-glucuronosyl transferase, and the activity for some isoforms of sulfotransferase may exceed adult levels during infancy and early childhood (Leeder and Kearns, 1997). Mehmood *et al.*, (1996) has provided evidence that human cytochrome P450 3A4 may metabolize PCP to TeCH/TCHQ in phase I metabolism of PCP; however, the initial purity of the PCP used in this study was not indicated.

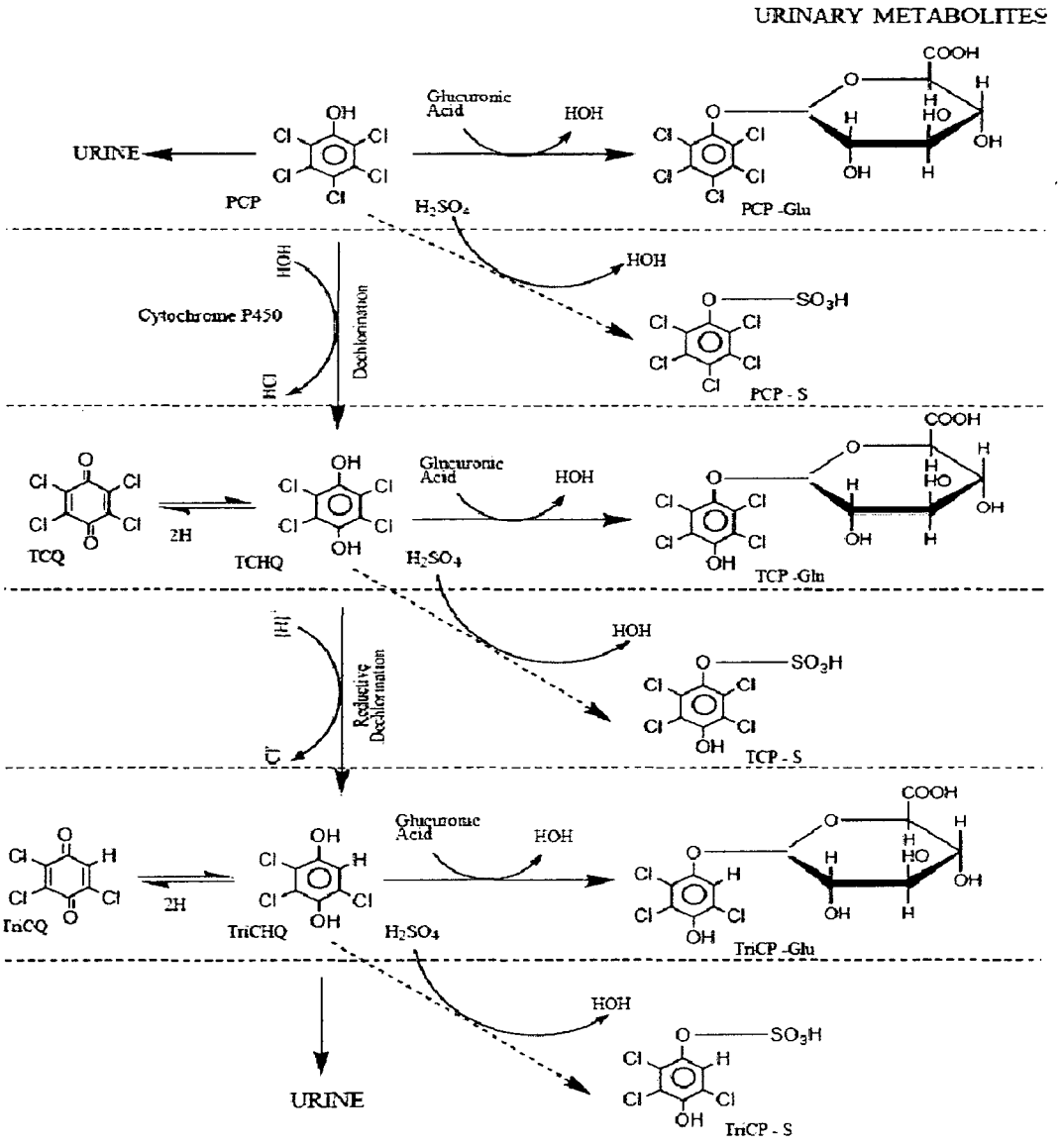

Figure 2.2 Proposed metabolic scheme for pentachlorophenol in body (ATSDR, 2001)

PCP = pentachlorophenol; PCP-Glu = pentachlorophenol-β-glucuronide; PCP-S = pentachlorophenylsulfate; TCHQ = tetrachloro-p-hydroquinone; TCP-Glu = tetrachlorophenol-β-glucuronide; TCP-S = tetrachlorophenylsulfate; TCQ = tetrachloroquinone; Tri CHQ = trichloro-p-hydroquinone; Tri CP-Glu = trichlorophenyl-β-glucuronide; Tri CP-S = trichlorophenylsulfate; Tri CQ = trichloro-p-quinone.

2.7 Methods for analysis of pentachlorophenol

A number of methods for qualitative and quantitative determination of PCP have been used.

2.7.1 Isolation and analysis of PCP

The primary problem of PCP residue analysis is the isolation of compound from extraneous material prior to use of any chromatographic or colorimetric procedure. The residue must be in solution in either an aqueous or organic solvent vehicle. A liquid-liquid portioning procedure may be sufficient for the isolation of PCP from extraneous constituents, which are in solution.

2.7.1.1 Amino antipyrine method

Uede *et al.*, (1962) examined drinking water for PCP examination. They extracted the PCP with dichloromethane by acidifying the sample with hydrochloric acid. The PCP in the sample was measured at 580 nm by 4- amino antipyrine method using 8 % potassium ferricyanide as oxidizing agent and 0.13% sodium carbonate as pH controlling agent. Goto *et al.*, (1963) devised another modified procedure for PCP analysis in water sample. The phenol was extracted in xylene containing small amount of hydrochloric acid. PCP was measured by taking OD at 574 nm using 0.15 m disodium hydrogen phosphate as pH controlling agent. Boric acid for pH adjustment and 10 per cent potassium ferricyanide for oxidation of PCP was used in xylene by Akisada (1964). Concentration of PCP was measured using xylene as blank at 470 mm.

2.7.1.2 Nitric acid oxidation method

Uede *et al.*, (1962) extracted PCP from water by acidifying the sample using benzene. The PCP extracted in benzene was further oxidized by 20 per cent nitric acid and measuring OD at 470 nm. Nitric acid oxidized PCP to a mixture of tetrachloro *ortho* and *para* quinones, which produce an intense yellowish red color and can be measured

using suitable colorimeter or spectrophotometer at 450 nm (Monsanto, 1963).

2.7.1.3 Ion exchange chromatography

Skelly, (1961) used a gradient elution procedure for quantitative separation of PCP. Aluminum oxide plates with gypsum binder were used for separation. The plate was initially developed perpendicularly using a mixture of sodium hydroxide and acetone. The dried plate was sprayed with either silver nitrate reagent and exposed to UV light or with 4-amino antipyrine.

2.7.1.4 Gas chromatography

An electron capture detector and column of five percent Dow–11 silicon and PEGA at 170°C and 180°C respectively were used by Kanazawa and Tawahara, (1966) for the measurement of PCP after extraction. The linearity range for the PCP ether was 0.02 to 0.6 nanograms (ng) on the DOW–11 calcium and 0.02 to 10 mg on the PEGA column.

Kilgore and Cheng, (1966) analyzed fruits for PCP residues by gas chromatography using a glass column containing five percent DOW–11 silicon grease and electron capture detector. The PCP residue was converted to its ether derivatives with diazomethane and analyzed at 180°C. The lower limit of detection in the fruits was 0.01 ppm with an average recovery of PCP added to the fruits was 84%.

2.7.1.5 HPLC method

Ugland *et al.*, (1981) separated mono, di, tri, tetrachloro and pentachlorophenol by the high performance liquid chromatography using three different systems, adsorption chromatography on silica and reversed phase chromatography on a polar bonded phase (Amino alkyl) and a non-polar bonded phase (octadecyl). For quantitative analysis of PCP after extraction in dichloromethane, samples containing PCP were separated by reverse phase HPLC with STR ODS

column size 150 × 3.0 mm. The mobile phase was methanol and was detected at 224 nm (Thakur *et al.*, 2002).

2.7.1.6 Other methods

Aggarwal and Gupta, (1998) used a new, simple and sensitive spectrophotometeric method for the determination of PCP. The method is based on the reaction of PCP with concentrated nitric acid to form chloranil, which liberates iodine from potassium iodide. The liberated iodine then selectively oxidizes leucocrystal violet to form crystal violet, which has an absorption maximum at 592 nm.

The extraction of PCP from contaminated soils was evaluated using water ethanol mixtures as solvents. A mixed solvent containing equal proportion of water and ethanol, a water miscible mixture proved effective in extracting PCP from both spiked and field contaminated soil (Khodadoust *et al.*, 1999).

In a model experimental system of Morimoto *et al.*, (2000), 90% of the PCP reacted but almost no transformed PCP was detected by GC/MS analysis. Most of the transformed PCP was detected in the polymeric products for the determination of organic absorbable organic halide (AOX) in gel permeation chromatography (GPC).

2.7.2 Detection and monitoring PCP

Many of the analytical methods used for environmental samples are the methods approved by federal agencies and organizations such as EPA and the National Institute for Occupational Safety and Health (NIOSH). Other methods presented in this chapter are those that are approved by groups such as the Association of Official Analytical Chemists (AOAC) and the American Public Health Association (APHA). Additionally, analytical methods are included that modify previously used methods to obtain lower detection limits, and/or to improve accuracy and precision.

2.7.2.1 Detection of PCP from biological materials

Exposure to PCP is most commonly evaluated by analysis of urine, blood, feces, or adipose or other tissues, using gas chromatography (GC) combined with electron capture detection (ECD) or high-performance liquid chromatography (HPLC) combined with ultraviolet (UV) detection. Recovery is generally high and sensitivity using GC/ECD and HPLC/UV is in the parts per billion (ppb) range. Some efforts are currently in development to detect PCP metabolites in urine as a biological marker.

Many purification schemes take advantage of the fact that PCP is a weak organic acid. These methods involve extracting the compound into the organic phase under acidic conditions, and/or extracting into alkaline solution as phenolate salts (Chou and Bailey, 1986; EPA 1986). Thus, the standard methods involve multiple extractions, with potential for sample loss; some of these methods derivatize PCP prior to analysis (EPA, 1980; NIOSH, 1984). Derivatization often involves diazomethane or diazoethane, which are toxic substances (Bevenue *et al.*, 1968; Shafik, 1973; Morgade et al., 1980; Holler *et al.*, 1989; Wagner *et al.*, 1991). Hexane extraction, cleanup on thin layer chromatography (TLC) plates, and HPLC/UV detection were used to isolate and characterize PCP in human fat, demonstrating that PCP is present in human adipose tissue as an ester of palmitic acid (Ansari *et al.*, 1985). Fatty acid conjugates of PCP and other chlorinated phenols could be separated by reverse-phase HPLC (Kaphalia, 1991). TLC followed by GC/ECD was used to analyze PCP in adipose tissue (Ohe, 1979).

2.7.2.2 Detection of PCP from environmental samples

Concerns about contamination of environmental media, plants, and animals with PCP have led to the need for more rapid, sensitive, and selective methods of analysis. As with biological samples, the most common methods of analysis are GC/ECD, high resolution gas

chromatography (HRGC)/ECD, and HPLC/UV detection. Methods are available that detect in water or sediment at the 1–10 ppb range.

PCP could be detected in marine water at concentrations ranging from 0.2 to 200 ppb in volumes as small as 5 ml using a simplified monitoring procedure with HPLC/UV detection (Giam *et al.*, 1980). This method reduces costs and analysis time, and can also be used in other aquatic toxicity studies. Differential pulse polarography was used for direct determination of trace amounts of PCP (Wade *et al.*, 1979). It was demonstrated that PCP is electrochemically reduced and direct determinations are possible at levels as low as 0.27 ppm. HPLC/UV was used to distinguish among 10 different phenolic compounds at mg/l levels in water (Realini, 1981). HPLC/UV was also used to measure chlorinated phenols in surface-treated lumber and to distinguish between tetra and pentachlorophenol (Daniels and Swan, 1979). Automated HPLC is 10 times faster than wet chemical techniques. Once the method for analysis has been established and tested thoroughly, the HPLC method requires neither extensive pretreatment nor highly trained laboratory personnel (Ervin and McGinnis, 1980).

PCP could be separated from acidic pesticides and other organic acids possibly present in a mill effluent by extraction with an acetylating agent (Rudling, 1970). A similar single step extraction and acetylation procedure was used to determine several chlorinated phenolic compounds in paper mill effluent without interference (Lee *et al.*, 1989). GC/MS has been used to measure PCP in honey (Muiño and Lozano, 1991). This method is simple, accurate, and rapid. Its sensitivity is in the low ppb range.

2.8 Physico-chemical method for removing pentachlorophenol

Several physicochemical processes have been developed for removal of toxic xenobiotics from industrial effluent

2.8.1 Physical removal of PCP

Traditional clean-up methods for removal of PCP from effluent include use of activated charcoal. Traditional clean up methods for removal of

PCP from the effluent included use of activated charcoal. Edgehill, (1998) studied the potential of using carbonized pine bark as a substitute for activated carbon. The absorption capacity for phenol and PCP at pH 2 and pH 8 were evaluated. The adsorption capacity of carbonized bark was much lower than for phenol. The decomposition of PCP by sonication was investigated at two frequencies (20 and 500 MHz) and two concentrations (20 and 60 fm). Observed by products of sonication include tetrachlorobenzoquinone, tetrachlorocatechol, oxalate and chloride (Weavers *et al.*, 2000).

2.8.2 Chemical removal of PCP

Various chemical techniques are used to remove PCP from effluent such as coagulation, precipitation, reverse osmosis etc. A chemical method for oxidative degradation of PCP in soil under unsaturated condition and neutral pH was developed. Reagents used were heme (Fe^{2+}) as a catalyst and hydrogen peroxide as an oxidant. Chen *et al.*, (1999) reported that home and peroxide (Fenton reagent) could degrade PCP efficiently in short period of time either in liquid or unsaturated soil systems. However, the problems as underlying the industrial non-acceptability of the physicochemical treatment technology are those associated with coast and reliability.

2.8.3 Biological removal of PCP

Biological removal of PCP includes degradation with bacterial or fungal isolates, either in suspended or immobilized forms. Pure culture studies provided a wealth of knowledge of basic principles, degradation pathways and involved enzymes and their regulation. They have led to successful application of microbial strains in industrial processes, for example in reactors or in "end of pipe" applications. The studies highlighted the relationship between one degrading organism and the chemical of interest. But in nature, most environments support the growth of a wide range of microorganisms having many different metabolic capabilities. Relationships and interactions occur between organisms, which grow in close proximity

to each other. It has been shown that these relationships between the populations result in beneficial effects which make the associations more successful than any of the individual populations alone.

2.8.3.1 Fungal degradation of PCP

Various fungi have been isolated and applied and their potentialities for the bioremediation of PCP have been tested. Different fungi exhibited varying rates and extent of PCP degradation.

Mileski *et al.*, (1988) have demonstrated the disappearance and mineralization of (^{14}C) PCP in nitrogen-limited culture by the while rot fungus *Phanerochaete chrysosporium.* Toxicity studies showed that PCP concentrations of more than 4 mg/l prevented growth when fungal cultures were initiated by inoculation with spores. The lethal effect of PCP could, however, be prevented by allowing fungus to establish a mycelia mat before adding PCP, resulting mineralization of PCP at concentration as high as 500 mg/l.

The ability of two white rot fungi to deplete PCP from soil, which was contaminated with a commercial wood preservative, was examined in a field study by Lamar and Dietrich, (1990, 1992). Inoculation of soil containing 250–400 mg of PCP per gram with either *P. chrysosporium* or *P. sordida* resulted in an overall decrease of PCP by 88–91% soil within 6–5 weeks.

Liang *et al.*, (1994) evaluated the effects of bioaugmentation of PCP contaminated soil with *P. chrysosporium* using a chemical mass balance approach. This mass balance analysis indicated that the soil aeration might enhance removal of PCP and its chemical intermediates through a process of facilitated transport and/or volatilization.

The ability of 12 species of white rot fungi to absorb and remove PCP was studied as a formation of species and culture condition. *P. chrysosporium, Trametes versicolor,* and four *Ganoderma* sp. removed up to 50% of PCP within 24 h. though *Inonotus rickii* achieved a 96%

overall reduction (Logan *et al.*, 1994). After long pre incubation periods (8–20 days), all species reduced PCP by up to 50% with in 12 days of PCP addition.

Laugero, *et al.*, (1997) compared the degradation of PCP and its metabolite pentachloroanisole by *P. chrysosporium* in static and agitated immobilized cultures. Results clearly established the advantage of an immobilized culture for mineralization of PCP, 23% of CO_2 was released for fungus immobilized on stainless steel mesh ring as compared with 11% for static culture.

2.8.3.2 Bacterial degradation of PCP

Traditionally, the activities of microorganisms relevant to biodegradation have been studied in pure monocultures. Enrichment cultures were followed by selection with the aim to isolate a pure culture with the capacity to use the required compound as growth substrate. Biodegradation mechanisms were determined by elucidating the catabolic sequence, purifying and identifying intermediate metabolites, assaying and characterizing the enzymes involved in the pathway and determining the factors controlling the regulation of pathway expression. These approaches have provided a deeper insight into the individual metabolic pathways and underlying mechanisms. These pure culture studies revealed that (among others) two main criteria influencing the degradability of xenobiotics are the structure of the pollutant and the adaptation and genetical composition of the microorganisms.

Polychlorinated phenols such as the trichlorophenols, the tetrachlorophenols, and pentachlorophenol have been used extensively since the 1920s as preservatives to prevent fungal attack on wood (Colosio *et al.*, 1993). During this time, they have become serious environmental contaminants. PCP-degrading bacteria are present in soils worldwide (Saber and Crawford, 1985; Tiirola *et al.*, 2002a; Kao *et al.*, 2005; Yang *et al.*, 2006; Mahmood *et al.*, 2005).

Although monochlorophenols and dichlorophenols are produced naturally by some fungi and insects (Gribble, 1996), natural sources of PCP are not known; therefore, the degradation pathway(s) employed by bacteria to degrade PCP likely evolved during the approximately 60 years since the human introduction of PCP into the environment (Copley, 2000). PCP-degrading pure cultures may be useful for bioaugmentation in the remediation of PCP-contaminated soils and waters (Saber and Crawford, 1985; Bielefeldt and Cort, 2005).

A variety of bacteria have been isolated and applied for the degradation of PCP. The degradation of PCP by a *Flavobacterium* sp. was investigated using PCP a sole carbon source, the degradation actively increased with PCP concentration. However a lag phase was observed which was more pronounced at higher concentration of PCP (Gonzalez and Hu, 1991). Edgehill, (1996) studied degradation of PCP by *Arthrobacter* strain with naturally immobilized cells on glass bead in a column. PCP was removed from mineral salt medium 4–5 days after inoculation of the column with PCP acclimatized cells. Four strains of bacteria, *Arthrobacter, Flavobacterium, Pseudomonas* and *Sphingmonas* were isolated by Ederer *et al.*, (1997) from different geographical region of U.S.A.

P. aeruginosa completely degraded PCP up to 800 mg/l in 6 days. The PCP was degraded to CO_2 with release of chloride (Premalatha and Rajkumar, 1994). Mineralization of PCP by *Pseudomonas* sp. was demonstrated by Rescnick and Chapman, (1994) by loss of detectable PCP from growth medium, stoichiometric of chloride release and formation of biomass consistent with the concentration of PCP mineralized.

Saboo *et al.*, (1998) isolated bacterium from a PCP contaminated site which can grew in the presence of 50 mg PCP per ml but were not able to degrade it in either liquid medium or in the presence of 1% sterile potting solid support.

A PCP degrading bacterium was isolated from possible PCP contaminated soil form Pusan, Korea and identified as a member of the genus *Pseudomonas*. This strain was able to degrade a much higher concentration of PCP (4000 mg/l) than any previously reported PCP-degrading bacteria and fungi. It was suggested that this strain could be used for the bioremediation of highly PCP-contaminated soils, water or wood products (Lee *et al.*, 1998).

Crawford and Ederer, (1999) isolated four PCP degrading bacteria from geographically diverse area which have been examined in detail as regards to their physiology and phylogeny. According to traditional biochemical methods, these strains had been classified as members of genera *Arthobacteria, Flavobacterium, Pseudomonas* and *Sphingomonas*.

2.8.3.3 Degradation by consortia

In the field of biodegradation, the cooperation between different microorganisms is of specific interest because synergistic (both populations benefit from the relationship) interactions occur. Although undefined communities, which are treated as integral biocatalytic "back boxes," have allowed us to handle, in many instances, contaminated streams or sites, the lack of knowledge of the interrelationships among community members as well as of community function regulation and control often resulted in unexplainable failures or malfunctioning of the treatment systems. In most cases to date, bioremediation has depended on the ability of naturally existing microbial communities to degrade hazardous waste chemicals under environmental conditions that have been managed to enhance their activity. This approach has worked successfully for certain types of contaminants under a variety of site conditions. Treatable wastes include petroleum products, creosote and non-chlorinated solvents.

One solution to enhance bioremediation of xenobiotics in the environment is to make use of microbial consortia (a collection of organisms that have some functional association with each other). In many situations, a network of microorganisms is required for complete degradation of refractory molecules or to stabilize the degradation process. Often a community or consortium performed better than pure cultures, both in attaining maximal cell densities and in removing the pollutant. For example, substrate mixtures present in industrial waste streams and contaminated waste sites often require activity by mixtures of microorganisms to be completely mineralized. More examples are listed in Table 2.7.

Table 2.7 Examples of consortia capable of completely degrading pollutants

Pollutant	**Consortium/community composition**	**References**
4-aminobenzene-sulfonic acid	*Hydrogenophaga palleronni* *Agrobacterium radiobacter*	Feigel and Knackmuss, 1993
3,6-dichloro-2-methoxybenzoic acid	*Pseudomons paucimobilis* *Achromobacter* sp. *Flavobacterium* sp.	Fogarty and Tuovinen, 1995
Nitrate ester	*Arthrobacter ilicis*; *Agrobacterium radiobacter*	Ramos *et al.*, 1996
Pentachlorophenol	*Flavobacterium* sp. *Agrobacterium radiobacter* *Pseudomonas* sp.	Yu and Shephered, 1997
Monochloro-dibenzofuran	*Sphingomonas* sp. RW16 *Psedomonas* sp. RW10	Wittich *et al.*, 1999
3-chlorobiphenyl	*Burkholdria* sp. LB400; *Pseudomonas* sp. B13 (FR1)	Nielsen *et al.*, 2000
Fluorobenzene	*Sphingobacterium/Flavobacterium*; *Alcaligenes* spp.	Carvalho *et al.*, 2002

Mixed culture or consortia might overcome some of the problems degradative monocultures face in the environmental such as nutrient limitation and non-utilizable or toxic substrates. Consortia may be based on the exchange of specific nutrients, removal of growth inhibitory products and co metabolism (Slater and Lovatt, 1984). The association of microorganism to completely mineralize the toxic compound by sequential metabolism is referred as microbial communities, consortia, syntrophic association or synergistic association.

Le and Carberry, (1992) conducted biodegradation experiments in completely mixed batch reactor using selective microbial consortium and a stock culture of activated sludge. Comparable biodegradation has also been carried out, following chemical oxidation and pretreatment method.

The potential dechlorination of PCP in a soil by a DCP adopted consortium was investigated. Results show that PCP dechlorination was enhanced under sulfate reduction and methanogenic conditions but inhibited under denitrifying conditions within a 20 days incubation period (Chang *et al.*, 1996).

A PCP degrading mixed culture contained three predominant strains, identified as *Flavobacterium gleum*, *Agrobacterium radiobacter* and *Pseudomonas* sp. The relative abilities of the three strains to degrade PCP were tested individually and in combination. Rates of PCP degradation by individual isolates were lower than the three isolate combined (Yu and Shepherd, 1997).

Reactors operated under aerobic/ methanogenic and anaerobic/denitrifying conditions were inoculated with bacterial consortia isolated from PCP contaminated site or from anaerobic granular sludge. This consortium was shown to degrade PCP (Strompl and Thiele, 1997).

2.9 Alternative treatment technology–Bioremediation

Bioremediation is the process by which living organisms degrade hazardous organic contaminants. Microbial metabolism is accepted as a safer and efficient tool for the removal of many such organic pollutants. The relatively inexpensive technology of bioremediation for reclaiming chemically contaminated land has therefore been steadily gaining acceptance since the 1980's. Different bioremediation approaches have been successfully applied for the removal of soils contaminated with a variety of xenobiotic compounds (Newcombe and Crowley, 1999; Top *et al.*, 1999; Cunningham and Philip, 2000; Juhasz *et al.*, 2000; Runes *et al.*, 2001; Manzano *et al.*, 2003). Bioremediation may be applied after excavation of polluted site material and transport to a controlled environment (*ex situ*) or, under relatively natural conditions in the field (*in situ*). Different bioremediation approaches for soil clean-up exist, including natural attenuation and enhanced natural attenuation (biostimulation or bioaugmentation). In other worlds, bioremediation is the process by which microorganism are stimulated and used for degradation of persistent and hazardous organic contaminants to simpler organic compounds, along with the goal of achieving environmental safe levels for their discharge in soil, water. Stimulation is achieved by the addition of nutrients and terminal electron acceptor usually oxygen because most biological reaction occur faster in aerobic than anaerobic conditions.

2.9.1 Bioreactors for the degradation of PCP

The immobilization of microbial cells on solid supports is an important biotechnological approach introduced only recently in bioremediation studies, this novel technique has been employed in various biotechnological applications. Treatment of industrial cells has also been attempted successfully. Bioreactors using immobilized cells have been several advantages over conventional effluent treatment technologies.

Various bioreactors have been designed for the application of microbial consortium for the treatment of PCP. Bench scale continuous flow activated sludge reactors were used to study the removal of PCP. Various bioreactors have been designed and applied by the workers for the treatment of toxicant by the microorganism (Table 2.8).

O'rielly and Crawford, (1989) immobilized *Flavobacterium* cells on polyurethane and studied the degradation activity of cells in semi continuous batch reactor.

The ability of *Arthrobacter* cells to degrade PCP in mineral salt medium was evaluated for immobilized, non-immobilized and co immobilized cells. The immobilized cells were encapsulated in alginate (Lin and Wang, 1991).

A microbial consortium able to degrade PCP in contaminated soil was used in a fed batch bioreactor by Otte *et al.*, (1994). During the 35 days of bioreactor operation residual PCP in solution remained near zero up to a leading rate of 700 mg/l per day.

Fathepure and Tiedje, (1994) investigated the feasibility of treating chloroaliphatic in ground water with a microbial consortium under fixed film conditions. The dechlorination rate of PCP was increased with increasing flow rates up to 50 ml/hr.

Table 2.8 Bioreactors used for the treatment of chlorinated phenols

Type of bioreactor and treatment	References
Removal of organic micropollutants in ppb levels in laboratory activated sludge reactors under various operating condition	Nyholm *et al.*, 1992
Use of a biofilms membrane reactor for the production of lignin peroxidase and treatment of PCP by *Phanerochaete chrysosporium*	Venkatadri *et al.*,1992
Anaerobic dechlorination of PCP in fixed-film and up-flow anaerobic sludge blanket (UASB) reactors	Hendrickson and Ahring, 1992
Reduction of chlorophenol toxicity in lab scale aerobic fluidized bed reactor	Makinen *et al.*, 1993
Removal of organic micro pollutants in activated sludge reactor	Jacobsen *et al.*, 1993
Removal of creosote and PCP in a two stage aerobic bioreactor	Middaugh *et al.*, 1994
Adsorption and biodegradation of PCP by polyurethane immobilized *Flavobacterium*	Hu *et al.*, 1994
Degradation of PCP in bench scale reactors using *Phanerochaete chrysosporium*	Kang and Stevens, 1994
Degradation of PCP by white rot fungi in rotating tube bioreactors	Alleman *et al.*, 1995
Biodegradation of chlorophenols by mixed and pure cultures of bacteria in fluidized bed reactor	Puhakka *et al.*, 1995
Kinetics of multiple compounds degradation with a mixed culture in a continuous flow reactor	Bae *et al.*, 1995
PCP biodegradation kinetics of an oligotrophic fluidized bed enrichment culture	Melin *et al.*, 1997
Biodegradation of PCP by *Flavobacterium* species in batch and immobilized continuous reactors	Lo *et al.*, 1998
Biodegradation of PCP by biofilm developed in the immobilized soil bioreactor	Karmanev and Samson, 1998
Degradation of chlorinated compounds by *Penicillium camemberti* in batch and up-flow column reactors	Taseli and Gokcay, 2005
Biological degradation of chlorophenols in packed-bed bioreactors using mixed bacterial consortia	Zilouei *et al.*, 2006

2.9.1.1 Above-ground bioreactor

AGBS have been used widely to treat industrial effluents. Bioreactor can also be designed to treat contaminated ground water and soil in slurry form and require much the same technology as used in wastewater treatment. The preferred design of these reactors is based on the use of suspended microbial growth or growth on solid support media (Balba and Reas, 1987).

2.9.1.2 Fixed film bioreactor

The solid support media can be granulated charcoal, plastic spheres, glass bead or diatomaceous earth, which provide a large surface area for microbial growth. The microbial inoculum may come from an indigenous population on the contaminated site, from activated sludge or from pure or mixed culture of appropriate organism (Glazer and Nikaido, 1995).

A novel type of bioreactor, the immobilized soil biofilms reactor, was used to biodegrade PCP in aqueous solution (Karmanev and Samson, 1998). An extremely high volumetric PCP degradation rate was obtained of the order 5950 mg/l/day. Biofilm based aerobic biodegradation of PCP was less affected by variations in physico-chemical factors than in free suspended cultures.

Recent developments in molecular biology, ecology and environmental engineering are making bioremediation more attractive and an attainable treatment option. The microorganism in the biofilms employs natural biological processes to efficiently degrade complex chemical process and can remediate high volume of waste more cheaply than other available clean up procedures. Biological treatment in the form of bioreactor with PCP degrading microorganism is worthy of further investigation as a mean of clean up. Its installation and maintenance is not complex and is one of the cost effective clean-up technology at small scale

2.10 Factors affecting pentachlorophenol degradation

2.10.1 Environmental factors

The effect of temperature and surfactant concentration on the degradation of PCP by a pure culture of *Sphingomonas chlorophenolicum* was studied by Cort and Bielefeldt, (2000). An ionic, a cationic, and two nonionic surfactants were tested at concentrations above and below their critical mycelia concentrations. The surfactants caused more inhibition at lower temperatures. The exception was a slight enhancement of PCP degradation at 10°C for low concentration of the nonionic surfactants.

2.10.2 Effect of co-substrate

Gonzalez and Hu, (1991) reported the presence of glutamate as co substrate reduced the lag. Such an elimination of the lag phase appears to be due to maintaining cell viability with the presence of glutamate.

Lu and Chen, (1992) studied the effect of the presence of secondary carbon source on the biodegradation of chlorinated phenols. They found that addition of phenol, an easily biodegradable compound, generally decreased the biodegradation of chlorinated phenols.

Augmentation of PCP in mineral salts medium with glucose and peptone dramatically increased the rate and extent of PCP degradation. Yu and Ward, (1994) reported over a 6–day incubation period, 80% of the initial PCP was degraded. This acclimation protocol reduced the lag phase for PCP degradation and eliminated up to 85.5% of the PCP after 3 days.

Chang *et al.*, (1996) have investigated the addition of lactate; pyruvate and acetate enhanced the PCP utilization by bacteria.

2.11 Pathways for pentachlorophenol biodegradation

Despite the above mentioned problems numerous microorganisms capable to mineralize chloroaromatics have been isolated in the recent decades. The degradation pathways are here shortly introduced, as the members of the consortium inherit the enzymes for these pathways. Depending on the bacterial culture and community, degradation of chlorinated aromatics can proceed along different pathways (van der Meer *et al.*, 1992). The aerobic degradation of natural aromatic compounds usually proceed via their activation by oxygenases and a few central dihydroxylated intermediates such as catechol, chlorocatechol and gentisate, which after dioxyphenolytic ring cleavage are finally channelled into the TCA cycle. As an example phenol, is usually subject to monooxygenation to catechol followed by extradiol cleavage. Benzoate is also usually activated to catechol, degradation however follows an intradiol cleavage route, the 3-oxoadipate pathway. Whereas numerous enzymes capable of activating aromatic compounds such as benzoate dioxygenase are of broad substrate specificity and capable to transform halogenated substrate analogues, neither the catechol *meta*-cleavage pathway nor the 3-oxoadipate pathway are suited for channelling chlorocatechols in the tricarboxylic acids cycle (TCA) cycle. Bacteria capable to degrade chloroaromatics via chlorocatechols usually contain a chlorocatechol *ortho*-cleavage pathway. This pathway comprises a chlorocatechol 1,2-dioxygenase, which differs from catechol 1,2-dioxygenases of the 3-oxoadipate pathway by its broad substrate specificity and capability to cleave 3- and 4-chlorocatechol (4-CC) and various dichlorocatechols with high activity (Dorn & Knackmuss, 1978).

Numerous PCP degraders have been characterized well enough that information about these strains can be used to infer evolutionary relationships of enzymes and genes of the PCP catabolic pathway (Table 2.9). However, a number of interesting but lesser examined

PCP-degrading bacteria have been isolated from various aquatic and soil environments. For example, numerous PCP degraders have been isolated from aquifers contaminated by polychlorophenols (Nohynek *et al.*, 1995; Mannisto *et al.*, 1999; Tiirola *et al.*, 2002a). Such isolates typically degrade 2,4,6-trichlorophenol and 2,3,4,6-tetrachlorophenol, but only some degrade PCP. One isolate identified as *Novosphingobium* sp. strain MT1 (Tiirola *et al.*, 2002a) was found to degrade 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol, and PCP at 8°C and carried a homolog of *pcpB*. Two gram positive isolates obtained from these sites degraded PCP, but 2,3,4,6-tetrachlorophenol was required as an inducer. In another study a PCP-degrading bacterium was isolated from aquifer sediments and tentatively identified as *Pseudomonas mendocina* (strain NSYSU) via biochemical tests and 16S rRNA gene sequencing (Kao *et al.*, 2004). Another strain tentatively identified by biochemical tests as *Pseudomonas fluorescens* was reported to degrade PCP and to harbor a PCP-4-monooxygenase with a molecular weight of 24,000 Da (Shah and Thakur, 2003).

Table 2.9 Well-characterized bacteria that grow on pentachlorophenol

Strains	Other compounds degraded	References
Sphingobium chlorophenolica ATCC 39723 (formerly *Flavobacterium* and *Sphingomonas*)	3,5-dibromo-4-hydroxybenzonitrile), triiodophenol, tribromophenol, trichlorophenol, 2,4,6-trichlorophenol, and 2,3,5,6-tetrachlorophenol	Saber and Crawford, (1985); Steiert and Crawford, (1986); Steiert *et al.*, (1987); Topp *et al.*, (1992); Xun and Orser, (1991a)
Rhodococcus (*Mycobacterium*) *chlorophenolicus*	3,4,5-, 2,3,4,6-, and 2,3,5,6-tetrachlorophenol, and 2,3,5- and 2,3,6-trichlorophenol; chloroguaiacols	Apajalahti *et al.*, (1986); Apajalahti and Salkinoja-Salonen (1987); Häggblom *et al.*, (1994); Briglia *et al.*, (1994)
Sphingomonas spp. UG25 and UG30	*p*-Nitrophenol (PNP), 2,4-dinitrophenol (2,4-NP), *p*-nitrocatechol and 4,6-dinitro-*o*-cresol (DNOC)	Leung *et al.*, (1997)
Arthrobacter ATCC 33790 (renamed *Sphingomonas chlorophenolica*)	Trichlorophenol	Stanlake and Finn, (1982); Ederer *et al.*, (1997)
Pseudomonas SR3 (renamed *Sphingomonas chlorophenolica*)	2,3,5,6-; 2,3,6-; 2,4,6-; 2,4-; and 2,6-chloro-substituted phenols	Resnick and Chapman, (1994); Ederer *et al.*, (1997)
Pseudomonas RA2 (renamed *Sphingomonas chlorophenolica*)	Non-ionic surfactant Tween 20	Radehaus and Schmidt, (1992); Colores *et al.*, (1995); Bielefeldt and Cort, (2005); Ederer *et al.*, (1997)

Nam *et al.*, (2003) reported isolation of a PCP-degrading *Pseudomonas veronii* strain PH-05 from a timber storage yard. This strain was identified by fatty acid analysis and 16S rRNA gene sequencing and was reported to produce tetrachlorocatechol from PCP. This transformation differs from those observed in other PCP-degrading bacteria, which universally metabolize PCP through a chlorinated hydroquinone intermediate. Further investigation of *P. veronii* strain PH-05 is needed to determine if its PCP catabolic pathway is truly different from other gram negative bacteria examined thus far, or if the chlorocatechol intermediate is formed by a yet to be determined side reaction. The authors observed that the production of tetrachlorocatechol accounted for less than half of the consumed PCP and stated that alternative pathways might also be present.

Thakur *et al.*, (2002) isolated a strain identified as *Pseudomonas* sp. strain IST 103 (PCP103) that was capable of growth on PCP and produced a PCP-4-monooxygenase with a molecular size of 30 kDa. Evidence was obtained indicating that a gene encoding the monooxygenase was plasmid-encoded in this strain. Yang *et al.*, (2006) reported isolation of a PCP-degrading bacterium identified as *Sphingomonas chlorophenolica* by 16S rRNA gene analysis. It appears that this strain is similar to other *sphingomonads* studied previously. Martins *et al.*, (1997) isolated three gram negative bacterial strains capable of using PCP as a sole carbon and energy source. These strains were identified as members of the genera *Pseudomonas* (one strain) and *Acinetobacter* (two strains), though it is not clear from their report how these identifications were verified and little work was done to fully characterize their polychlorophenol degrading abilities.

Despite the isolation of many PCP degrading bacteria, a pathway for degradation of PCP is known in detail only for *Sphingobium* (formerly *Sphingomonas*) *chlorophenolicum* ATCC 39723 (Xun and Orser, 1991a, b; Xun *et al.*, 1992a, b, c; Orser *et al.*, 1993; Orser and Lange, 1994; Lange *et al.*, 1996; Dai *et al.*, 2003). This strain was originally isolated by Saber and Crawford before the era of

16S rDNA phylogeny and was identified at the time as a strain of *Flavobacterium* (Saber and Crawford, 1985). In the years since the original isolations of ATCC 39723 and other closely related PCP-degrading bacterial strains and adoption of 16S rDNA phylogeny for bacterial classification, the genus *Sphingomonas* has been divided into multiple genera (Takeuchi *et al.*, 2001). *Sphingomonas chlorophenolica* (Karlson *et al.*, 1996; Ederer *et al.*, 1997) is now classified as a member of the genus *Sphingobium*. The rate-limiting step for PCP degradation in *Sphingobium chlorophenolicum* ATCC 39723 appears to be the *para*-hydroxylation of PCP to tetrachlorohydroquinone (McCarthy *et al.*, 1997).

This step is catalyzed by the enzyme PCP-4-monooxygenase, encoded by the *pcpB* gene (Orser *et al.*, 1993) and works in concert with a tetrachlorobenzoquinone reductase encoded by *pcpD* (Dai *et al.*, 2003). In addition to PCP, the PCP-4-monooxygenase can use trichlorophenols, tetrachlorophenols, and several other halogenated phenols as substrates (Xun *et al.*, 1992a, b, c). The PCP catabolic pathway of *Sphingobium chlorophenolicum* ATCC 39723 is shown in Figure 2.3. In addition to PcpB, several of the other enzymes in the PCP pathway have now been characterized. Tetrachlorohydroquinone reductive dehalogenase (PcpC; Figure 2.3) has been isolated and characterized from *Sphingobium chlorophenolicum* ATCC 39723 (Xun *et al.*, 1992c; Anandarajah *et al.*, 2000) and *Sphingomonas* sp. UG30 (Habash *et al.*, 2002).

PcpC is a member of the glutathione transferases that are widely found in both prokaryotes and eukaryotes. Though the UG30 PcpC shares 94% primary sequence identity with the PcpC from *S. chlorophenolicum* ATCC 39723, there are significant differences between the two enzymes in some of their functional and kinetic properties (Habash *et al.*, 2002). 2,6-Dichloro-*p*-hydroquinone 1,2-dioxygenase (PcpA) has been purified from *Sphingobium* (formerly *Sphingomonas*) *chlorophenolicum* ATCC 39723 (Xun and Orser, 1991b; Ohtsubo *et al.*, 1999; Xun *et al.*, 1999). PcpA shows novel Fe^{2+} and

O_2-dependent ring-cleavage dioxygenase activity against hydroquinone derivatives and within the PCP pathway converts 2,6-DCHQ to 2-chloromaleylacetate (Figure 2.3).

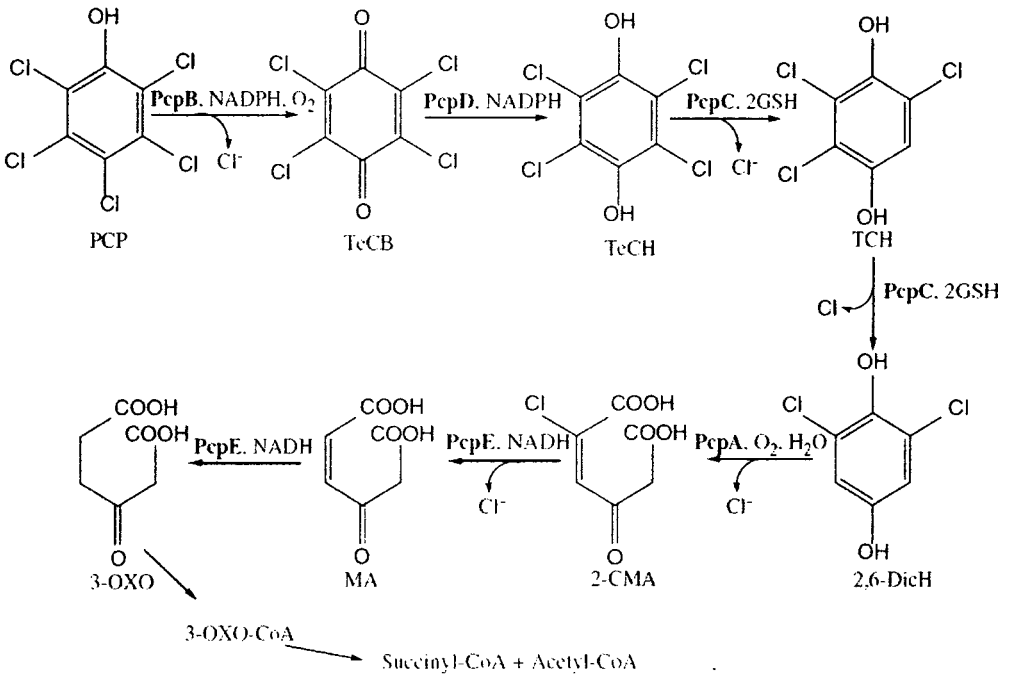

Figure 2.3 Pentachlorophenol degradation pathway of *Sphingobium chlorophenolicum* ATCC 39723. PcpB, PCP hydroxylase; PcpD, TCBQ reductase; PcpC, TCHQ dehalogenase; GSH, glutathione; PcpA, 2,6-DCHQ dioxygenase; PcpE, MA reductase; PCP, pentachlorophenol; TeCB, tetrachloro-*p*-benzoquinone; TeCH, tetrachloro-*p*-hydroquinone; TCH, 2,3,6-trichloro-*p*-hydroquinone; 2,6- DiCH, 2,6-dichloro-*p*-hydroquinone; 2-ClMA, 2-chloromaleylacetic acid; MA, maleylacetic acid; 3-OXO, 3-oxoadipic acid (Cai and Xun, 2002; Dai and Copley, 2004)

2.12 Enzymatic dehalogenation of pentachlorophenol

The enzyme responsible for dehalogenation of PCP was found pentachlorophenol dehalogenase and the product of the enzymatic conversion was tetrachlorohydroquinone (Schenik *et al.*, 1989). NADPH and oxygen were essential for this reaction. PCP from *Arthrobacter* sp. converts PCP to tetrachlorohydroquinone was confirmed by Schenik *et al.*, (1990) by labeling experiments with $H_2{}^{18}O$ or $^{18}O_2$.

Uotila *et al.*, (1991) demonstrated membrane bound dechlorination (para–hydroxylation) of pentachlorophenol and tetrachlorohydroquinone (TeCH) and *o*-methylaion of TeCH by PCP *para*-hydroxylating activity in cell extracts of *R. chlorophenolicus*. PCP hydroxylase purified from *Flavobacterium* sp. converted PCP or 2, 3,5,6-tetrachlorophenol to tetrachlorohydroquinone (TeCH) with the consumption of O_2 and NADPH. The results clearly demonstrate that PCP is oxidatively converted to TeCH by a monooxygenase type enzyme (Xun *et al.*, 1992c).

Lee and Xun, (1997) purified and characterized an enzyme 2,6,dichloro-*p*-hydroquinone chlorohydrolase from *Flavobacterium*. This enzyme converts 2,6-dichloro-*p*-hydroquinone to 6-chlorohydroxyquinol.

The group of Xun *et al.*, (1999) have characterized the enzyme 2,6-dichloro-p-hydroquinone-1,2 dioxygenase. This enzyme is responsible for the conversion of 2,6-dichloro-*p*-hydroquinone to chloromaleylacetate.

2.13 Molecular mechanism of pentachlorophenol degradation

Genetic analysis of the gene involved in PCP degradation has been studied very less because of the lack of gene transfer system for PCP degrading microorganism.

Xun and Orser, (1991b) identified and purified a PCP induced periplasmic protein, pcpA and the cloning and sequencing of the corresponding gene *pcpA* from the microorganism. This pcpA transcript was detected only after PCP induction and the *pcpA* is not part of a poly-cistronic message.

The *pcpB* gene of *Flavobacterium* sp was cloned by Orser *et al.*, (1993). The sequence encoded an open reading frame of 1,614 nucleotides, yielding a predicted translational product of 538 amino acids. Protein database comparisons of the predicted translational products revealed regions of homology with other microbial monooxygenase: phenol 2-monooxygenase, tryptophan 2-monooxygenase.

Karlson *et al.*, (1996) analyzed four PCP degrading bacterial strain, *Arthrobacter, Pseudomonas, Flavobacterium* and *Pseudomonas* by 16S rRNA gene sequence comparisons, REP/ERIC PCR finger printing of genomic DNA and serological typing by reactions to antisera prepared against each strain. A gene probe, prepared by PCR amplification of the PCP 4-monooxygenase gene (*pcpB*) of *Flavobacterium* sp. was used to group PCP degrading strains on the basis of hybridization to the gene probe.

The 16S ribosomal RNA gene sequence of the PCP degrader *Sphingomonas* strain was used to generate specific polymerase chain reaction primers for the detection of the strain (Elsas *et al.*, 1998).

Involvement of low molecular size plasmid in degradation of PCP has been studied by Thakur *et al.*, (2001) by shotgun cloning and characterization of genes by southern blot and sequence analysis indicated similarity with *'thdf'* gene of monooxygenase for degradation of thiophene and furan.

There have been numerous studies related to the DNA sequences of *pcpB* alleles from various PCP-degrading bacteria.

Identical *pcpB* gene sequences have been found in three of four known PCP-degrading strains of *S. chlorophenolicum* (ATCC 39723, SR3, and RA2) (Karlson *et al.*, 1996; Ederer *et al.*, 1997). A *pcpB* gene characterized in *S. chlorophenolicum* ATCC 33790 differs by 10% from the other three sequences (Ederer *et al.*, 1997; Crawford and Ederer, 1999). A variant of *pcpB* was found in the sphingomonad strain UG30 (Cassidy *et al.*, 1999; Leung *et al.*, 1999). The sequence similarity between the UG30 gene and that of ATCC 39723 was 90%, while the similarity between the UG30 and ATCC 33790 genes was 89%. A *pcpB* gene homolog 98% similar to the gene of *S. chlorophenolicum* ATCC 39723 was observed in two proteobacterial strains isolated from soil samples from a PCP-contaminated wood treatment site, though the strains were not PCP degraders (Saboo *et al.*, 1998).

Tiirola *et al.*, (2002a) isolated from polychlorophenol-contaminated groundwater in Finland a strain identified as *Novosphingobium* sp. MT1 that degrades 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol, and PCP at 8°C. The strain carried a homolog of the *pcpB* gene originally observed in *Sphingobium chlorophenolicum.* The full-length *pcpB* allele had approximately 70% identity with the three *pcpB* genes previously sequenced from sphingomonads ATCC 39723, SR3, and RA2 (Karlson *et al.*, 1996; Ederer *et al.*, 1997) and was also closely related to the environmental clones obtained by Beaulieu *et al.*, (2000) from chlorophenol-enriched soil samples. However, spontaneous deletion of *pcpB* from the MT1 genome resulted in the loss of chlorophenol degradation abilities and the exact mechanism of this deletion was not reported.

The Finnish researchers also examined the distribution of *pcpB* in a phylogenetically diverse group of polychlorophenol-degrading sphingomonads isolated from their contaminated groundwater site (Tiirola *et al.*, 2002b). All the isolates shared *pcpB* gene homologs with 98.9 to 100% sequence identity. An analysis of the 16S rRNA gene and *pcpB* phylogenetic trees of these strains suggested that a recent

horizontal transfer of the *pcpB* gene was involved in the evolution of the catabolic pathways for degradation of polychlorinated phenols in these strains. Yan *et al.*, (2006) demonstrated that *Sphingobium chlorophenolicum* ATCC 39723 is quite capable of attaining the ability to degrade a xenobiotic molecule it could not previously degrade by gene acquisition. The gene cassette (*camA camB camC*) encoding a cytochrome P–450 *cam* variant was integrated into a nonessential gene of this PCP degrader by homologous recombination. The recombinant strain could degrade hexachlorobenzene by conversion to PCP.

However, bioremediation is recognized as an economically viable method to treat the PCP and the use of microbial inoculants has been proposed to enhance remediation process. The gain to be achieved by development of bioremediation technologies is immense particularly considering the current scenario of environment pollution. Focused and target oriented interdisciplinary research are required for degradation of polychlorinated compounds in the environment.

ENRICHMENT AND CHARACTERIZATION OF PENTACHLOROPHENOL DEGRADING BACTERIAL CONSORTIUM FROM THE CHEMOSTAT

CHAPTER 3

ENRICHMENT AND CHARACTERIZATION OF PENTACHLOROPHENOL DEGRADING BACTERIAL CONSORTIUM FROM THE CHEMOSTAT

3.1 Introduction

Over the recent decades, significant quantities of industrial, agricultural and domestic chemicals have been released into the environment. Halogenated aromatic compounds constitute one of the largest groups of chemicals used in various industrial applications. The chlorophenolic compounds are major environmental contaminants giving global concern mainly due to use of these compounds as wide spectrum biocides in industry and agriculture (Vallecillo *et al.*, 1999; Yang *et al.*, 2007). The most common sources of chlorinated phenols in the environment include production of chlorine from bleaching of pulp, combustion of organic matter, partial transformation of phenoxy pesticides such as 2,4-dichlorophenoxy acetic acid and 2,4,6-trichlorophenoxyacetic acid, treatment of wood against fungi and insects and preservation of raw hides in leather tanning industries (Shukla *et al.*, 2001). The toxicity of these compounds tends to increase with relative degree of chlorination (Reineke and Knackmuss, 1988; Fetzner and Lingens, 1994).

Among chlorinated phenols; PCP and its sodium salt have been widely used as wood and leather preservative owing to their toxic effect on bacteria, mould, algae and fungi (Kaoa *et al.*, 2004). PCP is toxic to all forms of life since it is an inhibitor of oxidative phosphorylation (Shen *et al.*, 2005; Yang *et al.*, 2006). The US

Environmental Protection Agency (EPA) has listed PCP as a priority contaminant because of its proven carcinogenicity and toxicity (Bock *et al.*, 1996). PCP may be washed into streams and lakes due to surface runoff or may infiltrate and contaminate groundwater. Its large amount finally gets deposited onto sediments thus persisting in the environment (Shiu *et al.*, 1994; Thakur *et al.*, 2001). Despite widespread pollution observed, few indigenous bacterial strains capable of degrading PCP have been isolated (Chanama and Crawford, 1997). Moreover, PCP is recalcitrant to degradation because of its stable aromatic ring system and high chloride content, thus persisting in the environment (Saber and Crawford, 1985; Okeke *et al.*, 1997; Copley, 2000).

In case of environmental contamination by PCP, traditional clean-up methods have not been proved successful, due to their higher treatment costs and possibilities of causing secondary pollution. However, in solving serious problem of PCP contamination, it is important to assess the potential of bacterial strains indigenous to PCP contaminated sites for its degradation. Bacterial isolates obtained from nature have not been proved effective in complete degradation of PCP and other related metabolites at contaminated sites. Consequently a microbial consortium is usually required to provide all the metabolic capabilities for complete degradation of PCP. In light of these facts, it is important to elucidate such biological interactions in a continuously operating system such as chemostat in order to develop a potential microbial consortium for the degradation of PCP. Therefore objectives of present study at initial stage are to develop a stable bacterial consortium by continuous enrichment in the chemostat, characterize the members of the bacterial consortium, identification of products released during degradation of PCP and evaluation of utilization potential of PCP in the presence of intermediary metabolites. The study could help in developing

strategies for enhancing degradation and large scale removal of PCP from the environment.

3.2 Materials and methods

3.2.1 Sediment sampling sites

Two different industries were selected for collection of sediment samples and effluent for degradation studies. One of the sites was cluster of leather processing (tanneries) units situated at Jajmau, Kanpur (UP). The sediment sample together with liquid effluent (1:10 w/v) were collected from three sited of main channel of tanneries located at Jajmau rural area towards Lucknow road. The tanneries were located on the either side of the road, spread over an area of 3×3 km^2. The individual tannery unit releases their effluent into the underground sub-channels. These sub-channels drain their effluent into the main channel, which ultimately joins the river Ganga after passing through a common effluent treatment plant.

The other sampling site was the main effluent discharging canal and nearby areas of M/s Century pulp and paper mill, Nainital, Uttarakhand state, India located at foothills of the Himalayas. Both the sites were having a history of chlorophenols contamination. The effluent and sediment samples were collected in September, 2004 and the collected samples were stored in a refrigerator for analysis work.

3.2.2 Microorganisms and culture conditions

Sediment core containing microorganisms were collected from the above mentioned sites and bacterial cells were extracted by centrifugation at 900 rpm for 10 min. The supernatant was plated on nutrient agar (NA) plates. The microbial diversity was enumerated and characterized by morphological observation. The colors were partially purified on nutrient agar plate and streaked on mineral salts medium

(MSM) containing PCP (100 mg/l) as a sole source of carbon and bromothymol blue (0.1%) (Thakur, 1995). The formation of yellow colored colonies indicated the utilization of chlorinated phenol by the bacteria. Colonies were partially purified by streaking and re-streaking alternatively on MSM agar plates and nutrient agar plates. The microbial diversity was enumerated and characterized by morphological observation. The nutrient agar medium used for morphological characterization of bacteria contained the following components at the specified concentrations (g/l): Peptone, 5.0; Beef extract, 3.0; Agar, 20.0. The MSM contained the following components at the specified concentrations (in mg/l): Na_2HPO_4, 780; KH_2PO_4, 680; NH_4Cl, 500; $MgSO_4 \cdot 7H_2O$, 200; $CaCl_2 \cdot 2H_2O$, 10; and trace elements solution, 1 ml/l (Thakur, 1995).

3.2.3 Selection of bacterial consortium

Bacterial consortium of both tannery and pulp and paper mill effluent was tested for the removal of chromium and PCP in batch culture and bioreactor study. 100 mg/l of PCP and 100 mg/l of potassium chromate solution, supplemented with MSM was used, pH and temperature was maintained at 7 ± 0.2 and 30°C respectively. Batch culture study was done in 500 ml Erlenmeyer flask and sampling was done at an interval of 0, 1, 3, 5, 7 and 15 days.

In order to see the effect of different metabolites on the growth of bacterial isolates of tannery and paper mill consortium, the chemical compounds (50 mg/l) of pentachlorophenol, tetrachlorohydroquinone, dichlorohydroquinone and monochlorohydroquinone were studied. 50 mg/l concentration of these compounds were taken and added to the growth media (MSM) before plating on the pre-sterilized Petri dishes. The different isolates were then spot inoculated and incubated for 3 days at 30°C. The growths of the isolates on these compounds were then studied by observing the growth development on the plates.

3.2.4 Chemostat culture

The mixed bacterial population indicating utilization of PCP was enriched in 2 liter (2000 ml) chemostat; effective volume was around 1000 ml. The chemostat was fabricated by taking 2 liter glass vessel provided with an inlet, for the entry of fresh sterile medium and an outlet, for the removal of spent medium. The culture vessel was provided with another inlet for maintenance of pH (7±0.75) and air outlet to provide 100% saturation. MSM containing 100 mg/l PCP as carbon source was used for the enrichment of bacterial population. The pH and temperature was maintained between 7.0±7.5 and 28–30°C respectively throughout the course of enrichment. The culture vessel was equipped with aeration and was placed over magnetic stirrer (Figure 3.1). The bacterial growth was determined by measuring absorbance at 600 nm (A_{600}). The numbers of bacterial cells were counted as colony forming units (CFU) on nutrient agar plate (Thakur, 1995). The potency of bacterial isolates in degradation of PCP was monitored in terms of growth and utilization of PCP as substrate with spectrophotometer and gas chromatograph/mass spectrometer (GC/MS) analysis.

3.2.5 Characterization of bacterial strains

Bacterial diversity of stabilized consortium was enumerated on nutrient agar plates by spreading 0.1 ml of culture medium removed from chemostat under aseptic condition. The colonies were further purified by streaking and re-streaking on nutrient agar plates. The colonies appearing on nutrient agar plates were characterized by morphological examination; through various biochemical tests further confirmation was done using method of 16S rDNA sequence analysis of the 16S rRNA gene.

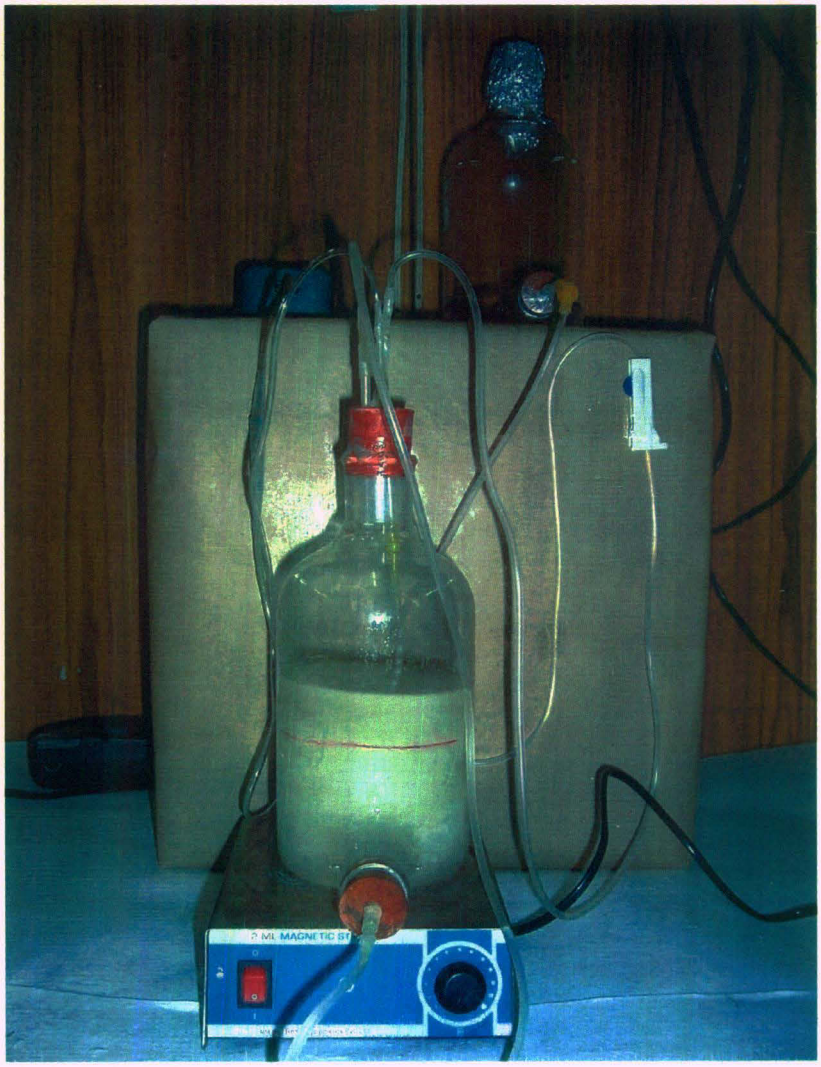

Figure 3.1 The chemostat set up used for enrichment of bacterial population

3.2.5.1 Morphological characterization

100 ml of liquid culture of purified bacterial strain was plated on nutrient agar plate and characterized by morphological examination depending upon shape, size, color, opacity, texture, elevation, and margin and spreading nature. The identification of PCP degrading bacterium was done according to Bergey's manual on determinative bacteriology (Holt *et al.*, 1994). Cell morphology of the isolated strains was observed by scanning electron microscopy (JEOL JSM–6360LV, Japan). Samples were picked from agar culture onto a glass cover slip and fixed with buffer containing glutaraldehyde. Fixed samples were dehydrated by passing through series of ethanol solutions with concentration 30%, 60% 90% of ethanol for 10 min each. Finally, cover slips containing bacteria were kept in absolute ethanol for 24 hr. Samples were vacuum dried followed by gold shadowing. The samples were observed under SEM with magnification at 15000×.

3.2.5.2 Biochemical characterization

For negative staining, a small crop of nigrosine was placed to one end of a clean slide. Loopfuls of inoculum was mixed with nigrosine. A smear was made with the help of another slide. Smear was dried and slides were observed under oil immersion in microscope. The strains were characterized depending upon shape, size and arrangement. Grams' staining was performed by modified method of Hucker (Collins and Lyne, 1989). A loop-full culture was heat fixed on a clean slide. The slide was stained with crystal violet for 30 sec, washed with water. Few drops of Gram's iodine was added for 30 sec, rinsed with distilled water and decolorized with 95% alcohol and again rinsed with water. Finally the slides were counter stained with safranin for 20–30 sec followed by rinsing with water and blot drying. The slide was observed under fluorescent microscope.

Starch hydrolysis test was performed as described by Collins and Lyne, (1989).The nutrient agar medium containing 0.5% soluble starch was used for this test. Culture was streaked on nutrient agar plate containing 0.5% soluble starch as carbon source followed by incubation at 30°C for 3 days and the formation of clear zone was then observed.

Gelatin hydrolysis test was performed as described by Collins and Lyne, (1989). The test culture was inoculated in gelatin tubes and allowed to incubate for 3–4 days at 30°C; the tubes were chilled in ice water for about 30 sec.

Oxidase test was performed by the method described by Collins and Lyne, (1989). The isolate were streaked on nutrient agar plates and incubated at 37°C for 24 hr. A few drops of oxidase reagent were poured over each plate and the change of color of colonies was observed.

Catalase test was performed as described by Collins and Lyne, (1989). Culture suspension was taken on a clean, grease free slide and gas bubble was observed.

Urease production test was performed by the method described by Collins and Lyne, (1989). Loopfuls of culture was inoculates in urea broth and the culture was incubated at 30°C for 24 hr. Change in color of broth was recorded for isolates.

Indole production test was performed by the Kovács method as described by Collins and Lyne, (1989). Sample containing 1% tryptone broth, was incubated at 30°C for 72 hr. 3 ml of Kovács reagent was added in to incubated tube. The tubes were shaken and the formation of red ring layer was recorded.

For methyl red test, the isolates were incubated in to glucose phosphate broth tubes at 37°C for 5 ml of absolute alcohol and 0.5 ml

methyl red reagent were added to the tubes. Change in color of medium was recorded (Collins and Lyne, 1989).

Voges Proskaver test was performed by Beritts method as described by Collins and Lyne, (1989). All the isolate were incubated in glucose phosphate broth at 37°C for 5 days. 3 ml of naphthol and 3 ml of potassium hydroxide (40%) were added in the culture tubes. Tubes were shaken well and allowed to stand for 3–5 min. Presence or absence of dark red ring formation was observed.

3.2.5.3 Bacterial fatty acid analysis

The only commercially available system for identification of microorganisms by fatty acid analysis is the Microbial Identification System (MIS) produced by Microbial ID (MIDI, Newark, DE, USA). The MIS requires isolates to be streaked onto agar plates, incubated for 24 hr, biomass transferred to test tubes, and derivatization reactions to be performed before gas chromatography. The software (Sherlock) provided with the MIS is used for qualitative and quantitative identification of fatty acid methyl esters, and pattern matching is then used to identify microorganisms based on their fatty acid profiles. The MIS is not very sensitive, and typically 40 mg of biomass (wet weight) is used in the analysis. This represents one quadrant from a well-grown quadrant-streaked plate. While slow-growing bacteria may be incubated for longer time periods, the method is not useful for bacteria that do not achieve high culture densities, and cannot be applied to primary isolation plates because single colonies do not contain enough biomass. This technique employs five sequential steps for analysis of fatty acids profile of the bacterial strains, viz. harvesting; removal of cells from culture media; saponificaion: lysis of cells to liberate fatty acids from the cellular lipids; methylation: formation of fatty acid methyl esters (FAMEs); extraction: transfer of FAMEs from the aqueous phase to the organic phase; base wash;

aqueous wash of the organic extract prior to chromatographic analysis.

An Agilent (Agilent Technologies, Palo Alto, CA, USA) 6890 Plus gas chromatograph, including a 7683 autoinjector, split-splitless inlet with Merlin microseal septum (Merlin Instrument, Half Moon Bay, CA, USA), flame ionization detector, and electronic pressure control, was used. The system was controlled with Chemstation (Agilent) and Sherlock (MIDI) software.

The procedure was a slight variation of the standard MIS protocol. Bacteria were streaked onto trypticase soy broth agar (TSBA) and incubated at 28°C for 48 hr. Single colonies were taken with a platinum inoculating loop and transferred to 10 ml Teflon centrifuge tubes equipped with Teflon screw caps (Nalge Nunc International, Rochester, NY, USA). Reagent I (1 ml) was added by pipet and the centrifuge tubes were capped and vortexed. After heating in boiling water bath for 5 min the tubes were again vortexed and returned to the bath for 25 min. Reagent II (2 ml) was added by pipet and the tubes were vortexed and heated at 80°C for 10 min. Reagent III (1.25 ml) was added by pipet and the tubes rotated again for 10 min. The bottom phases were removed with disposable pasteur pipets and discarded. Reagent 4 (3 ml) was added by pipet and the tubes rotated for 10 min. The top phases were transferred to high-recovery vials with disposable Pasteur pipets and evaporated down to approximately 100 ml under constant stream of nitrogen.

A DB–5 (Agilent, USA) capillary column (5% phenyl 95% methylpolysiloxane; 30 m length × 0.025 mm id × 0.25 μm film thickness) was used at a temperature programme of 65°C (1 min), then raised to 20°C/min to 170°C, again raising the temperature to 5°C/min to 260°C where it was held for 2 min. Hydrogen was used as the carrier gas (70 kPa) at a constant flow of 1.2 ml/min. Nitrogen was used as detector makeup gas, its flow rate was maintained at 30

ml/min. The samples were analyzed in splitless mode at an injection temperature of 250°C; detector temperature was set at 300°C.

3.2.5.4 16S rDNA sequence analysis

Further identification was performed using 16S rDNA sequence analysis. The genomic DNA of PCP-degrading bacterial strains were isolated from 1.5 ml of enrichment pure culture after pelleting by centrifugation and re-suspending the pellet in sterile water (150 µl). 10 µl of lysozyme (5 mg/ml) was added to break down cell walls; then 40 µl of SDS (10%) was added into the tube and incubated at 60°C for 30 min. After 30 min. 20 µl of protease K (10 mg/ml) was added to and incubated at 37°C for 4 hr; then 400 µl of STE buffer was added to the tube. The compositions of STE buffer were: NaCl, 0.1 M; Tris–HCl, 10 mM (pH 8.0); EDTA, 1mM. In order to extract total genomic DNA, equal volumes of phenol-chloroform were added to the tube and the tube was centrifuged at 12000 rpm for 5 min; then the supernatant was added to the new micro-tube. The procedure of extraction repeated three times until the impurity of material reduced mineral. After extraction, 50 µl of sodium acetate (3M, pH 5.2) and 1 ml of 95% ethanol were added into supernatant to precipitate for 10 min., and then tip was used to roll up the bacterial genomic DNA from the tube. Finally, the bacterial genomic DNA on the tip was transferred into 70 µl of H_2O and used in PCR reaction. The 16S ribosomal genes were amplified from the bacterial total genomic DNA by using the polymerase chain reaction (PCR).

The fragment of rDNA was amplified using a GeneAmp 2400 PCR System (PE, USA). A 5 µl purified extracted DNA served as a template in a PCR reaction mixture with the modified amplification primer 27f (5′–GAGAGTTTGATCCTGGCTCAG–3′) and 1495r (5′–CTACGGCTACCTTGTTACGA–3′). Each 200 µl microtube contained 5 µl purified extracted DNA; 2 µl of dNTP at 2.5 mM; 2.5 µl of 10× *Taq* DNA polymerase buffer; 0.2 µl of 2.5 unit *Taq* DNA polymerase; 11.3

µl of sterile water and 2 µl of modified amplification primers (total amount: 10 pmol). The tubes were then subjected to the following thermal cycling programme: hot starting performed at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, followed by a final extension performed at 72°C for 10 min. The 16S rDNA sequence was compared against the GenBank (http://www.ncbi.nlm.nih.gov/) database using National Centre for Biotechnology Information (NCBI) BLAST program. The sequences were aligned using multiple sequence alignment software CLUSTALW (version 1.81). A phylogenetic tree was then constructed using MEGA software (version 3.1) based on 16S rDNA sequences of closely related bacterial strains (Kumar *et al.*, 2004)

3.2.6 Batch culture studies

Batch culture studies were performed in a 500 ml flask containing 250 ml MSM containing 50 mg/l PCP as a sole source of carbon and energy. Bacterial consortium of both tannery and pulp and paper mill and bacterial strains were incubated at 30°C for 5 days. 10 ml samples from the culture were drawn at 0, 1, 3, 5 and 7th day.

Culture sample was removed from the chemostat under aseptic conditions and its absorbance was measured at 600 nm using MSM as a blank.

The chloride release was determined as described by Bergman and Sanik, (1957). The culture medium (3 ml) was mixed with 0.25M ferrous ammonium sulfate (0.3 ml), followed by addition of saturated solution of mercury thiocyanate in ethanol (0.3 ml). A blank was prepared in the sample. Development of color was noted and absorbance was taken at 460 nm against blank. Standard curve was prepared using different concentration of sodium chloride. Utilization of PCP by bacterial consortium was estimated as described earlier.

Rothera test was performed with some modification as described by Holding and Collee, (1997) for detection of *ortho* or *meta* ring cleavage of aromatic compounds. 4 ml of cell suspension was dissolved on 0.02 M Tris buffer (0.1 ml) and EDTX (0.1 ml) for lysis of bacterial cells and pH was adjusted to 7.8 The mixture was treated with little tolerance of and 0.1 M catechol (4.0 ml). The development of color was noticed. Not appearance of yellow color suggested absence of *meta* cleavage. Mixture was shaken for 1 hr at 170 rpm and tested for formation of β-ketoadipic acid (Rothera Reaction), which indicates the presence of ortho fission. In this procedure 0.10 ml culture fluid was acidified with 2 ml HCl followed by addition of 1 ml $NaNO_3$ (1%). After 2 minute, concentrated ammonia (15 ml) and ferrous sulfate solution (10%) were added. The development of reddish brown color indicates a typical Rothera reaction, and the presence of *ortho* cleavage.

3.2.7 Denaturing gradient gel electrophoresis

MSM was collected from the outlet of the chemostat for analysis of the microbial community structure. The genomic DNA of each sample was isolated as described above. V3 region of 16S rDNA or small sub unit sequence of ribosomal gene was amplified using primers. Two sets of universal primers were used for amplification of genomic DNA: P2 as forward primer and P3 as reverse primer. Forward primer was attached with a 40-bp GC clamp (in italics) to enhance separation and to avoid early denaturation of double stranded DNA in denaturing gradient gel electrophoresis (DGGE) (5′–*CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC*–3′).

DGGE was performed with the Bio–Rad Protean II system by applying amplified product of PCR on to poly-acrylamide gel (8% w/v in 1× TAE, 20 mM Tris acetate, pH 7.4) according to method of Muyzer *et al.*, (1993). The amplified products from PCR were loaded on 1 mm thick 8% (w/v) polyacrylamide (37.5:1) gels containing a linear 30% to

60% denatured gradient; 100% denaturant were urea (7 M) and formaldehyde (40% v/v). After polymerization, top portion of the gradient gel was overlaid with a 3% acrylamide cap solution into which a 12-tooth comb was inserted. A total of 25 µl of PCR products were loaded onto the gel. Electrophoresis was performed on a 30–60% denatured agent gradient at 60°C and 250 V for 180 min. After electrophoresis, the gels were incubated for 15 min in MQ water containing ethidium bromide (0.5 mg/l), ringed for 10 min with MQ water, and photographed with UV trans-illuminator. Individual DGGE bands of interest were excised from the gel, cloned, sequenced and phylogenetic analysis was performed to determine the closest relatives to chlorinated compounds degrading bacteria (Segev *et al.*, 2007).

3.3 Results

3.3.1 Bacterial population from effluent discharge sites

The bacterial population capable of utilizing PCP as sole carbon source were enumerated and characterized. Bacterial cells were extracted from sediments of tannery and pulping mill effluent discharge sites. Cells were cultured on MSM-PCP agar plates (100 mg/l). Colonies that appeared on the plate changed blue color of bromothymol into yellow after 72 hr due to dechlorination and change in pH. Bacterial isolated were removed from MSM-PCP agar plates and restreaked on nutrient agar plates.

Sediment core along with liquid effluent from sampling sites of effluent discharge sites of tannery and pulp and paper mill initially yielded 8 and 18 bacterial isolates, respectively. These isolates were able to survive on MSM containing PCP (10 mg/l) chromium (50 mg/l). Five morphologically distinct bacterial isolates from tannery sediments and three distinct bacterial isolates from paper mill

sediment were observed on MSM agar plates containing PCP (100 mg/l) and chromium (50 mg/l).

3.3.2 Characterization of bacterial consortia

A mixed bacterial population that had the potentiality to utilize PCP in the paper mill effluent was enriched in chemostat using MSM containing PCP (100 mg/l) as the sole source of carbon and energy. Initially, there was an increase in turbidity of culture medium, but after 3 day, it declined. The declination in turbidity persisted up to day 20. In order to maintain constant growth, the rate of nutrient supply in culture vessel was decreased from 10 ml/hr to 5 ml/hr.

The utilization of chlorinated organic compound by bacterial consortium of paper mill and tannery effluent was used for the selection of strain for the treatment of tannery effluent. Both pulp and paper mill consortium (PE) and tannery consortium (TE) was tested in MSM-PCP medium containing potassium chromate (as source of chromium) for the degradation of PCP at 7^{th} day. In batch culture, the study showed 67% and 44% removal of PCP by paper mill consortium and tannery consortium respectively (Figure 3.2). Chromium reduction (%) and uptake of chromium by tannery and paper mill consortium was observed during the period of seven days. Paper mill consortium and tannery consortium showed to remove 68% and 43% chromium from MSM-potassium chromate solution respectively (Figure 3.3).

Preliminary findings on the basis of utilization and effective degradation of PCP as sole carbon source by consortia obtained from both the industries sediments reveals, consortia obtained from pulp and paper mill effluent discharge site (PE) was relatively efficient in comparison to the consortia obtained from tannery effluent discharge site (TE). PE was selected for further studies and experiments for degradation of chlorinated phenols in tannery effluent. Morphological

characterization of the stabilized PE and TE consortium are shown in Table 3.1 and Table 3.2.

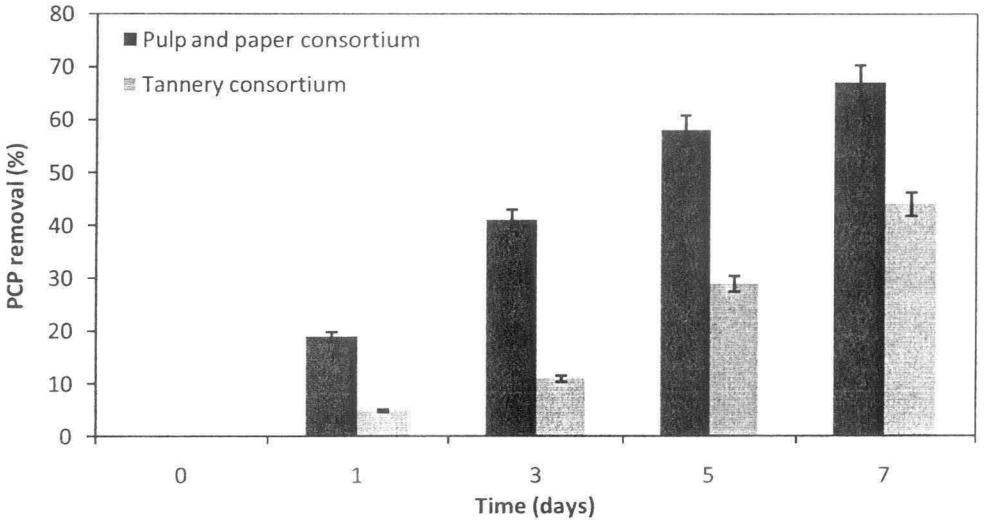

Figure 3.2 PCP removal by bacterial consortia isolated from pulp and paper mill and tannery sediments

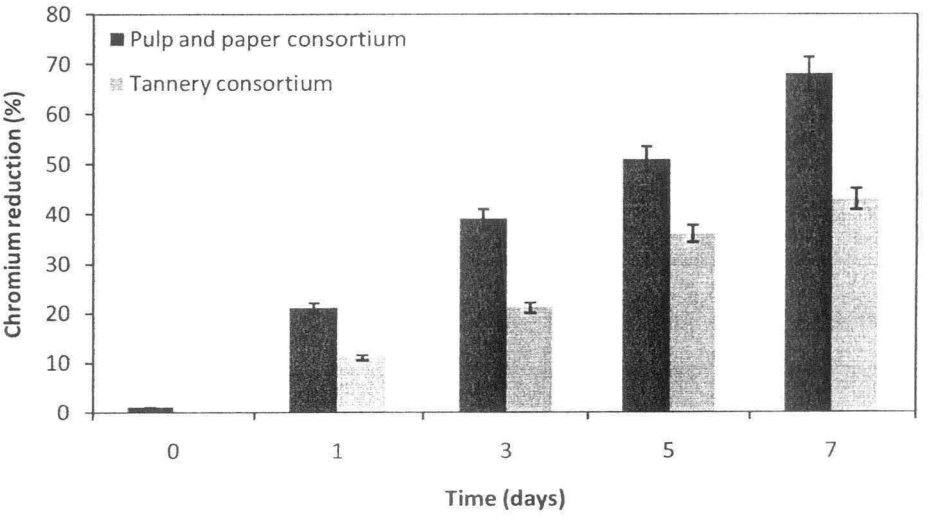

Figure 3.3 Chromium reduction and growth of bacterial consortia isolated from pulp and paper mill and tannery sediments

Table 3.1 Morphological characterization of bacterial strains belonging to tannery consortium

Characters	Bacterial strains and their responses				
	TE-1	**TE-2**	**TE-3**	**TE-4**	**TE-5**
Shape	I	C	T	I	C
Size	2 µm	2 µm	1 µm	1 µm	1 µm
Color	W	Y	LY	W	YF
Opacity	O	T	T	T	T
Colony elevation	R	F	R	F	R
Colony margin	S	E	S	S	E
Texture	V	V	N	V	N
Spreading nature	N	Y	Y	Y	Y

Table 3.2 Morphological characterization of bacterial strains belonging to pulp and paper consortium

Characters	Bacterial strains and their responses		
	PE-1	**PE-2**	**PE-3**
Shape	C	I	SR
Size	1 µm	1 µm	2 µm
Color	W	M	LY
Opacity	O	O	O
Colony elevation	F	R	R
Colony margin	E	S	S
Texture	N	V	V
Spreading nature	N	Y	Y

Where,

C: Circular, I: Irregular; YF: Yellow fluorescent; W = white; LY: Light Yellow; O: Opaque; T: Transparent; R: Raised; F: Flat; E: Entire; S: Smooth, V: Viscous; Y: Yes; N: No; SR: Short Rod; M: Metallic Grey

3.3.3 Enrichment of selected bacterial consortium

A mixed bacterial population had potentiality to utilize PCP was enriched in the chemostat using MSM containing PCP (100 mg/l) as sole source of carbon and energy.

Initially, there was an increase in the turbidity of culture medium, but after day 3, there was a sudden decrease in the turbidity, which persisted up to day 20. In order to maintain constant growth, the rate of nutrient supply in culture vessel was decreased from 10 ml/hr to 5 ml/hr. The fluctuation in turbidity continued till day 140, with almost constant growth rate. After 140 days, there was again an increase in turbidity, which persisted up to day 220. After that the growth of the culture became almost constant even after increasing the flow rate (10 ml/hr) until the end of the run (Figure 3.4).

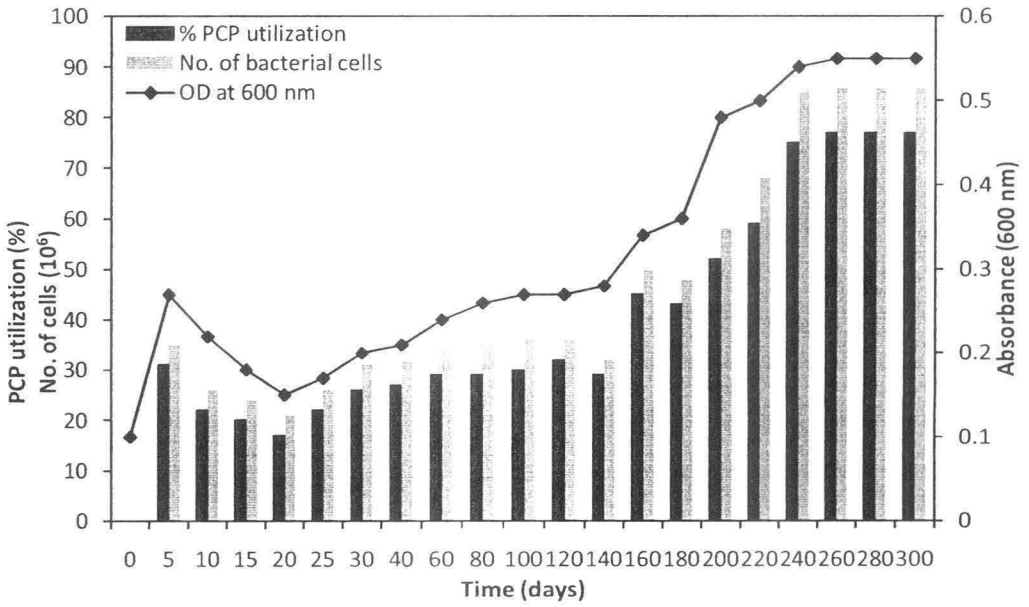

Figure 3.4 Growth pattern of stabilized bacterial consortium in the chemostat

3.3.4 Growth of bacterial cells in the chemostat

The culture medium removed under aseptic condition at different time interval during enrichment from chemostat was diluted in ten fold and plated (0.1 ml/plate) on nutrient agar. The total number of bacterial cells was determined by colony forming unit showed the number cells were almost constant from day 240 to the end of the run (Figure 3.4). Initially the bacterial population was able to utilized 30% of PCP. But after day 3 the utilization decreased which reached up to 19% at the day 20. The utilization was almost constant similar to the number of the bacterial cells up today 140. Data on this aspect showed that the significant amount of PCP utilization was observed from day 160 (45%), which increased, and approached to 79% at the end of the run i.e. 240 day (Figure 3.4).

The initial fluctuations in the turbidity of the medium may be due to inability of the bacterial strains to utilize PCP, formation of intermediary metabolites released during its degradation and also because cells were not well acclimatized to the new environment containing PCP. The growth of bacterial cells in the chemostat was constant as the cell became physiologically adapted to PCP, and was probably due to nutritional interaction between the members of the community. Results of the study indicated a significant increase in utilization of carbon source from 41% on day 140 to 82.5% at the end of the run i.e. day 280.

The individual members of the enriched bacterial consortium of pulp and paper mill were tested for their ability to utilize chromium (50 mg/l) in batch culture (Figure 3.5). The individual members showed varied response towards reduction in chromium percentage. PE-1 was able to remove only 21% chromium at day 7. PE-2 was able to remove almost 40% at that time. However, the strain PE-3 showed maximum chromium reduction ability, with almost 70% reduction at day 7. Uptake of chromate in cell biomass was 16.64 mg/g, 9.6 mg/g

and 2.2 mg/g of dry weight of cells for PE-3, PE-2 and PE-1, respectively.

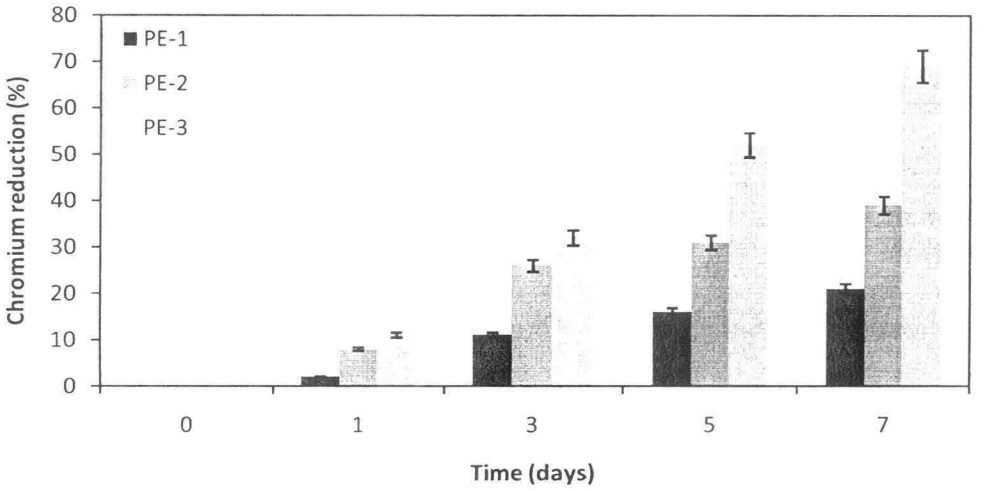

Figure 3.5 Chromium reduction by individual members of the bacterial community isolated from pulp and paper mill consortium

3.3.5 Identification of PCP degrading bacterial strains

Colonies appeared on nutrients agar on 24 hr at 28°C. The size of the colonies reached to diameter of 1 to 2 μm in a week. Colonies appeared on PCP agar containing 0.1% bromothymol blue reached a diameter of only 0.1 to 0.5 mm in one week. On this selective growth medium colonies were yellow in color. No colonies formed on carbon free agar. Three different type of colonies appeared on MSM agar plates after the stabilization of growth were named as PE-1, PE-2 and PE-3 (Figure 3.6). Size and shape of the cells were determined from scanning electron micrographs of the strains (Figure 3.7).

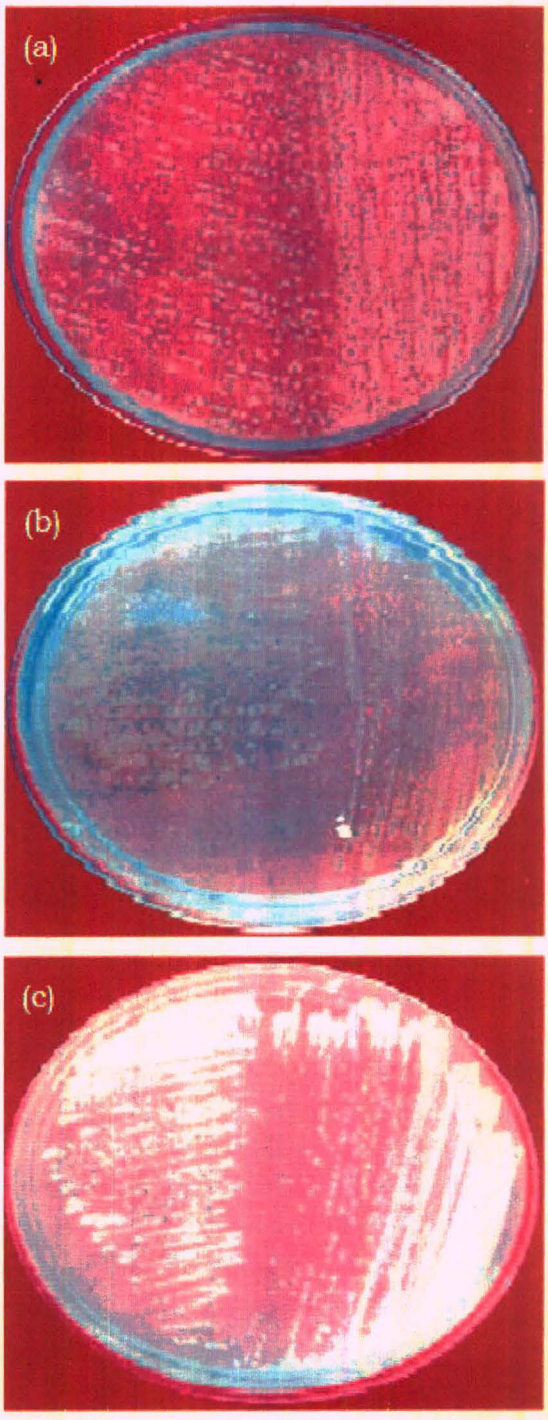

Figure 3.6 Pictograph of enriched bacterial strains isolated from the chemostat; (a), PE-1; (b), PE-2; (c), PE-3

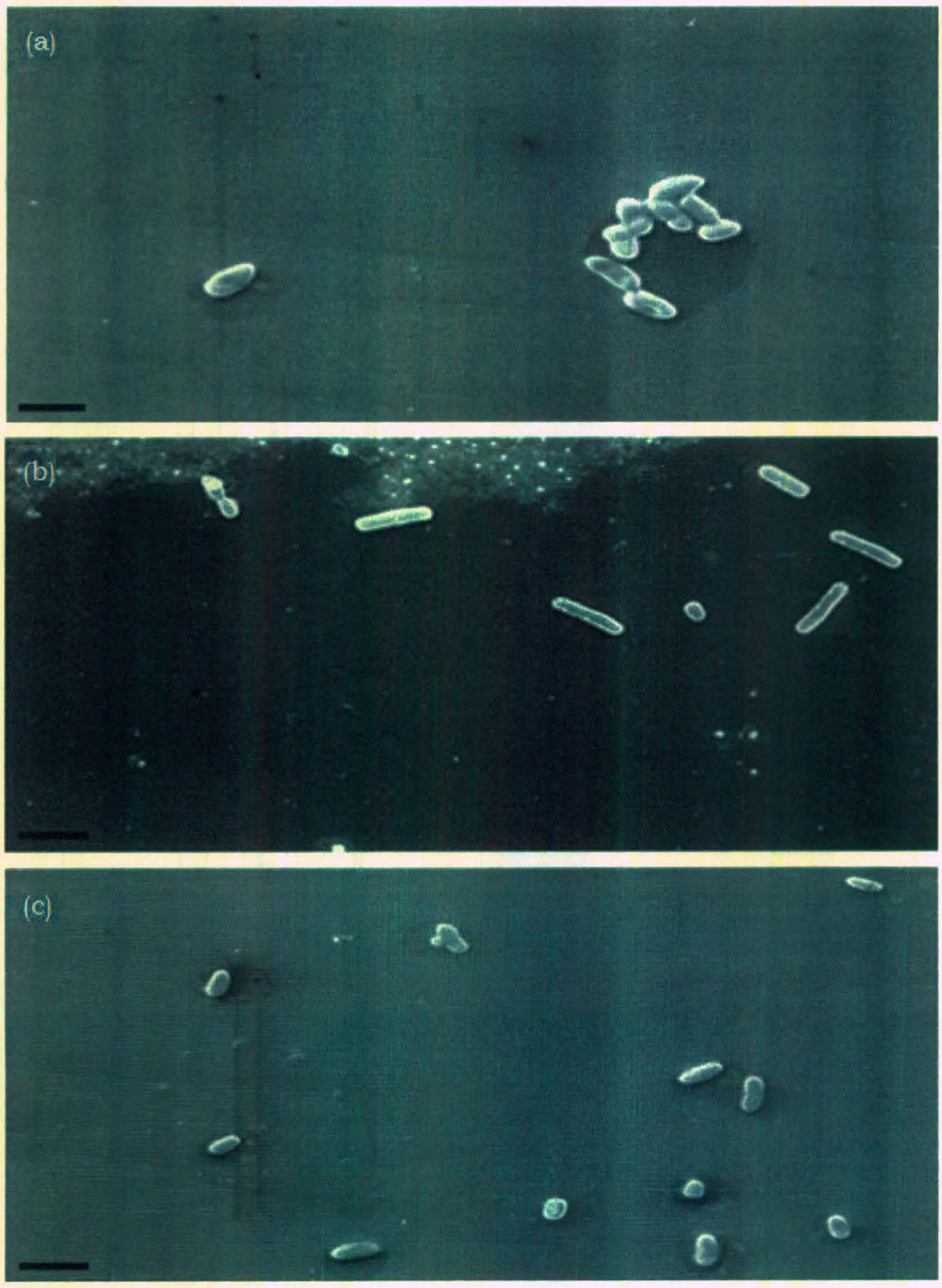

Figure 3.7 Scanning electron micrograph of isolated bacterial strains; (a), PE-1; (b), PE-2; (c), PE-3, viewed at 15000× (*Bar* represents 1µm)

3.3.5.1 Biochemical characterization

Among the isolated 8 strains, only three bacterial strains (PE-1, PE-2 and PE-3) with relatively high degradation ability were selected and discussed in this work. Bacterial isolates were subjected to different biochemical tests.

Negative results were obtained in the starch hydrolysis, urease and nitrate reduction test for all the three isolates of PE consortium. Positive results were obtained in catalase, methyl red and indole production test for all the three isolates. Oxidase test was positive for PE-2 and negative for PE1 and PE3, Gelatin was solidified by PE-2 and PE-3 while negative for PE-1. All the three strains showed negative results with Grams staining. The bacterial strains were identified morphologically and biochemically as species belonging to genus of *Enterobacter* sp. (PE-1), *Pseudomonas* sp. (PE-2) and *Acinetobacter* sp. (PE-3). The biochemical characteristics of selected bacterial strains have been shown in Table3.3.

Table 3.3 Biochemical characteristics of bacterial strains

Characteristics	**Bacterial strains**		
	PE-1	**PE-2**	**PE-3**
Oxidase test	–	+	–
Indole production test	+	+	+
Starch hydrolysis	–	–	–
Catalase production	+	+	+
Methyl red test	+	+	+
Voges Proskaver	–	–	+
Citrate test	–	+	+
Urease production	–	–	–
Gelatin hydrolysis	–	+	+
Nitrate reduction test	–	–	–
Sodium chloride tolerance	>10%	>18%	>22%

+, positive; –, negative

3.3.5.2 Fatty acid based profiling

Fatty acid profiling of bacterial total lipids gives precise results for identification of bacterial cells at genus level. A calibration mix was run in a GC with splitless mode, in order to have complete profiling of fatty acid composition in a cell. Calibration mix chromatograms are presented in Figure 3.8 for the standard (TSBA40). The MIS identifies peaks based on relative retention times, and those were generally unchanged. The sensitivity of the method was limited by the quality of the blanks. As peak area decreased in the samples, the percentage of peak area contributed by contaminants increased, and the quality of the library match declined. The minimum colony size necessary to provide enough peak area to get a good match varied with species.

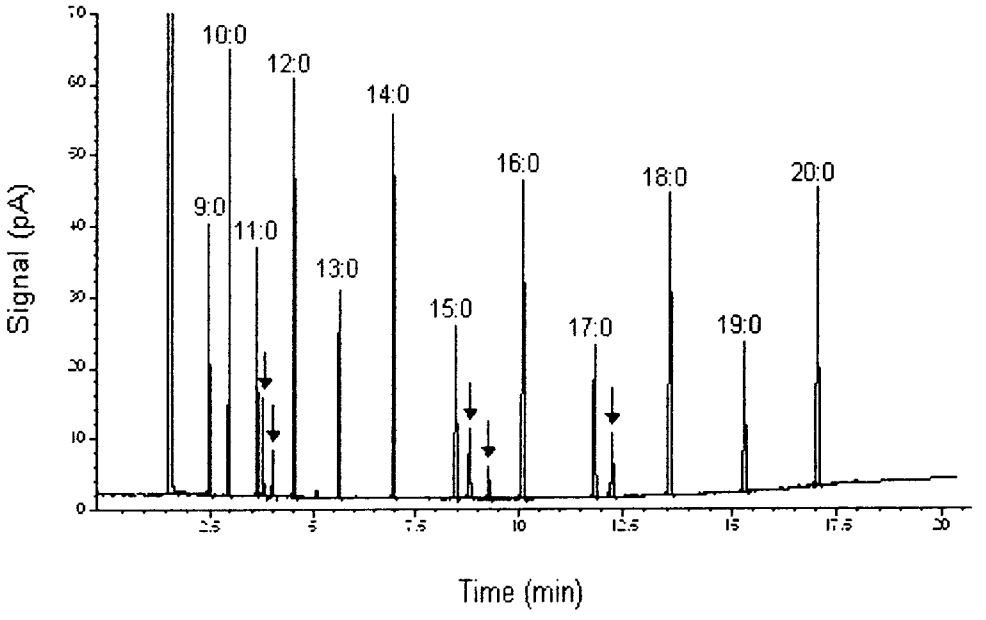

Figure 3.8 Chromatogram of MIS calibration mix run by standard (TSBA40) method. Hydroxy fatty acids present in calibration mix are designated with arrows

The bacterial cells were first harvested from the media. The cells were then liaised to liberate fatty acids from the cellular lipids. The released fatty acids were then methylated followed by their extraction from the aqueous phase to the organic phase so that these may be separated in a GC. The bacterial cells were identified on the basis of score, in the range of 0 to 1 (MIDI, Sherlock). A score of 0.6 or above is considered suitable for identification of cells at genus level. The analysis of bacterial fatty acid profile confirmed the strain PE-1 as *Enterobacter* sp.; PE-2 as *Pseudomonas* sp; and PE-3 as *Acinetobacter* sp. The results obtained from FAME were slightly different from preliminary morphological and biochemical characterization of the bacterial strains (Figure 3.9).

3.3.5.3 DGGE based 16S rDNA sequence analysis

The results of the conventional identification techniques were further verified by method of phylogenetic analysis. Hagstrom *et al.*, (2000) found that 16S rDNA sequence analysis having similarity of ≥97% is a reasonable for grouping bacteria into species. Using universal primers, around 1,4 kb of 16S rDNA sequences of isolated bacterial strains (PE-1, PE-2 and PE-3) was amplified, sequenced and submitted to GenBank and following accession numbers were obtained, PE-1 (EF432789; PE-2 (EF432790); EF-3 (EF432791). The nucleotide BLAST searches of PE-1, PE-2 and PE-3 showed 99.9%, 99.6% and 99.8% sequence homology with *Enterobacter* sp., *Pseudomonas aeruginosa* and *Acinetobacter* sp., respectively. The isolates were designated as *Enterobacter* sp. ISTPCP-1 (PE-1), *Pseudomonas aeruginosa* ISTPCP-2 (PE-2) and *Acinetobacter* sp. ISTPCP-3 (PE-3). Phylogenetic trees were constructed based on 16S rDNA sequences of individual bacterial strains; ISTPCP-1 (Figure 3.10), ISTPCP-2 (Figure 3.11) and ISTPCP-3 (Figure 3.12) using MEGA software (version 3.1).

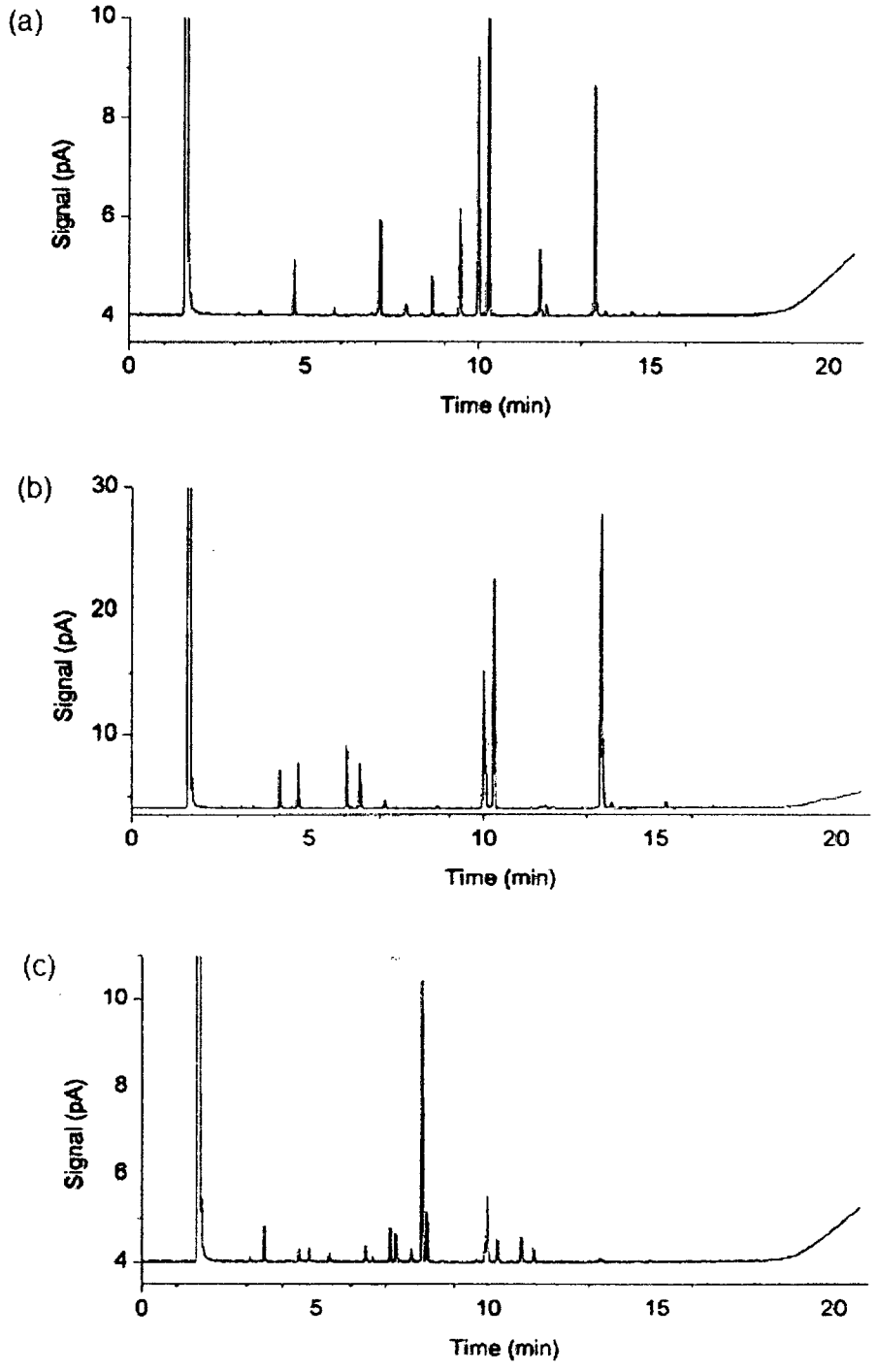

Figure 3.9 Total fatty acid profiles of isolated bacterial strains; (a), *Enterobacter* sp.; (b), *Pseudomonas* sp.; (c) *Acinetobacter* sp.

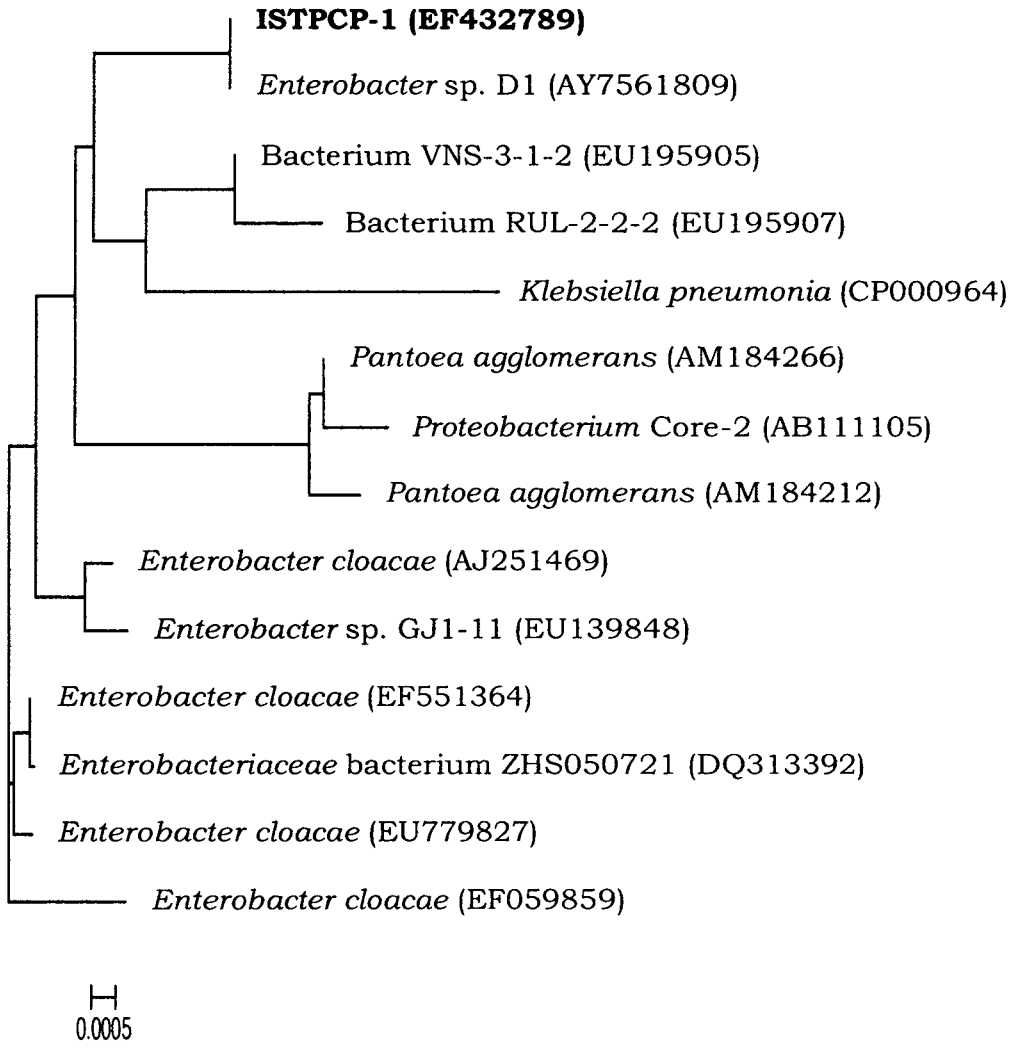

Figure 3.10 Phylogenetic tree based on 16S rDNA sequence of *Enterobacter* sp. ISTPCP-1 strain and related bacteria

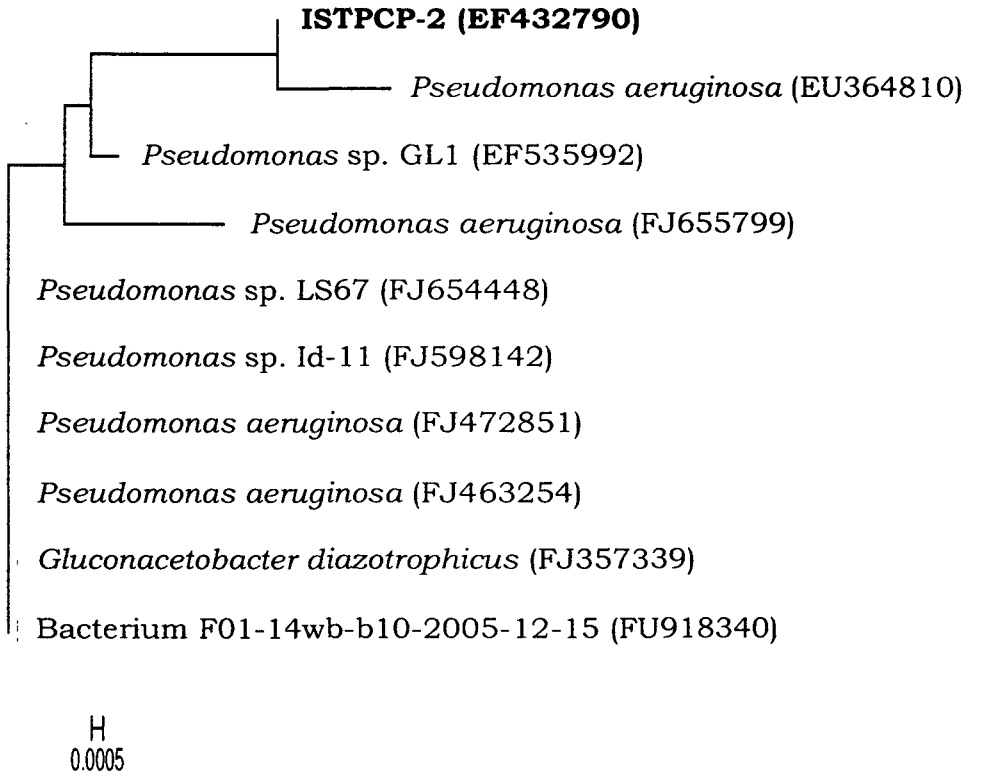

Figure 3.11 Phylogenetic tree based on 16S rDNA sequence of *Pseudomonas aeruginosa* ISTPCP-2 strain and related bacteria

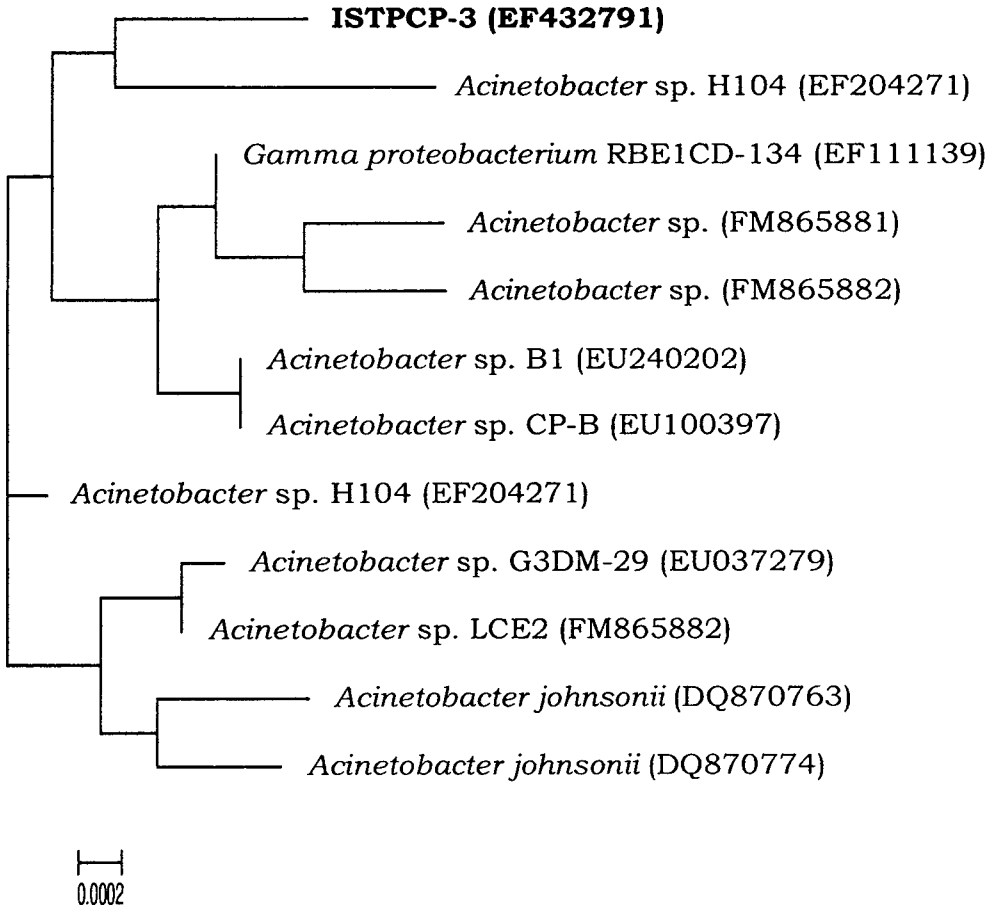

Figure 3.12 Phylogenetic tree based on 16S rDNA sequence of *Acinetobacter* sp. ISTPCP-3 strain and related bacteria

To understand the community dynamics, V3 region of 16S rDNA was amplified based on polymerase chain reaction. DGGE was performed from amplified DNA product of soil and sediment, bacteria of soil and sediment, and members of bacterial community enriched in the chemostat in presence of PCP. The partial 16S rRNA fragments of three bands of bacterial isolated appeared on DGGE (Figure 3.13). The DGGE fingerprint analysis indicated that the bacterial community structure was significantly changed after enrichment culture. The result of the study indicated presence of three bands, representing individual bacterial strains.

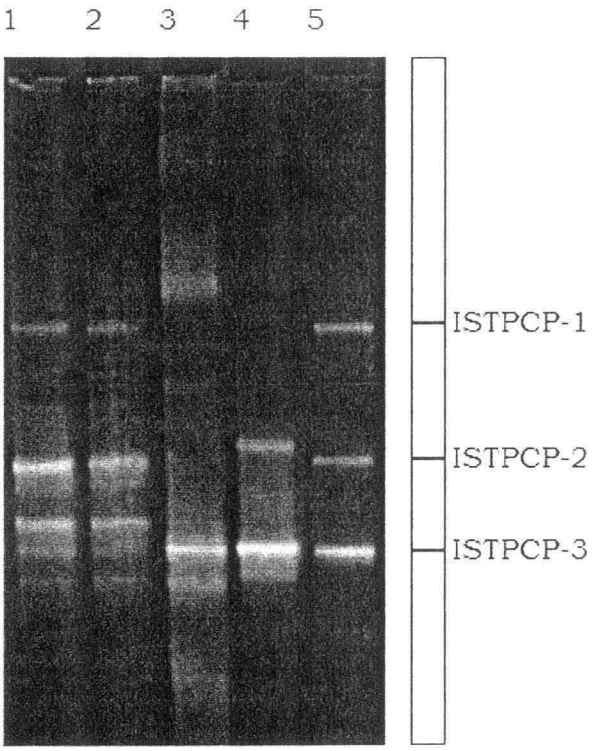

Figure 3.13 DGGE profiles of bacterial community extracted. DNA extracted from soil and sediment (1 and 2); total population enriched in the chemostat on day 90, (3); day 280 (4); bacterial strains isolated from the chemostat; ISTPCP-3, (5); ISTPCP-2, (6); ISTPCP-1 (7)

3.3.6 Degradation of PCP by bacterial strains

The degradation of PCP (100 mg/l) by bacterial strains was investigated in terms of growth, ring cleavage, chloride release and utilization of substrate concentration for a period of 96 hr.

Figures 3.14, 3.15 and 3.16 presents the growth of strains ISTPCP-1, ISTPCP-2 and ISTPCP-3 strain in MSM-PCP solution for a period of 96 hr. The data has indicated that the strains were able to grow effectively on PCP. In the entire bacterial strains growth rate was increased up to period of 72 hr and then declined.

The release of chloride was more by ISTPCP-3 strain (Figure 3.16) as compared to ISTPCP-1 and ISTPCP-2 strain (Figure 3.14, Figure 3.15). The strain ISTPCP-3 released high chloride indicating effective dehalogenation, which was followed by strain ISTPCP-2 and strain ISTPCP-1.

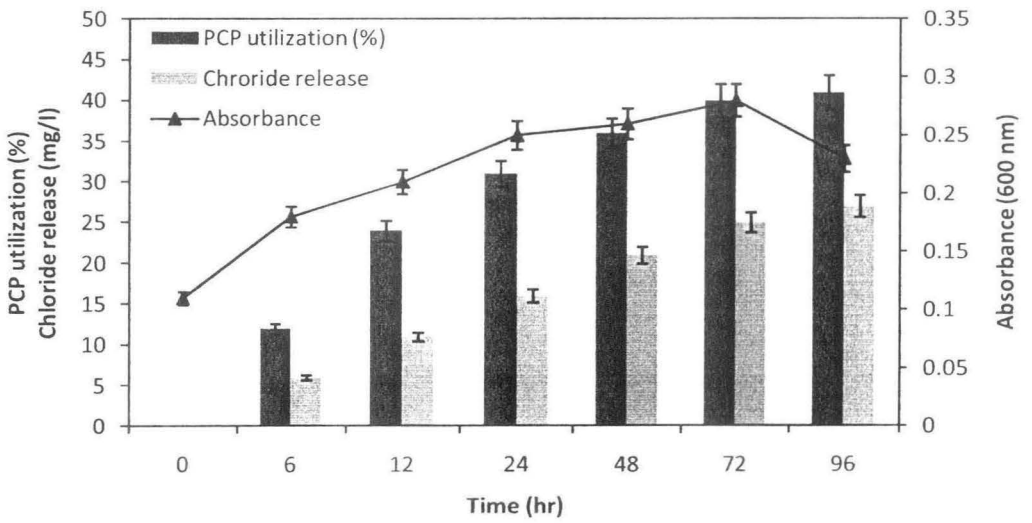

Figure 3.14 PCP utilization, chloride release and growth of strain ISTPCP-1 at different time intervals

The utilization of PCP by bacteria was analyzed by extracting the culture filtrate at different time interval up to 96 hr. As evident

from the figures, (Figure 3.14, Figure 3.15, Figure 3.16). The strain ISTPCP-3 was able to use 75% of PCP, while ISTPCP-1 and ISTPCP-2 utilized at 39% and 62.9% PCP respectively after a period of 96 hr. The results clearly suggest that strain ISTPCP-3 has better potential to degrade PCP than the strains ISTPCP-1 and ISTPCP-2.

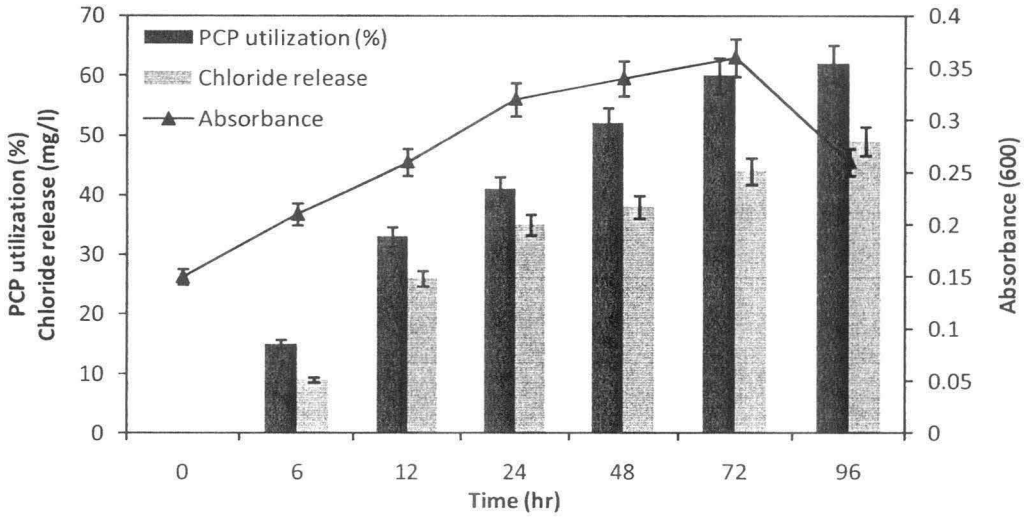

Figure 3.15 PCP utilization, chloride release and growth of strain ISTPCP-2 at different time intervals

Mode of ring fission of PCP by bacterial strains was assessed by observing *ortho* and *meta* ring cleavage. No yellow color appeared after mixing catechol in cell suspension, indicated absence of *meta* cleavage. However, positive Rothera test as violet color appeared due to β-ketoadipic acid formation for strains showed that ring cleavage was occurred at ortho position and confirmed dechlorination of PCP.

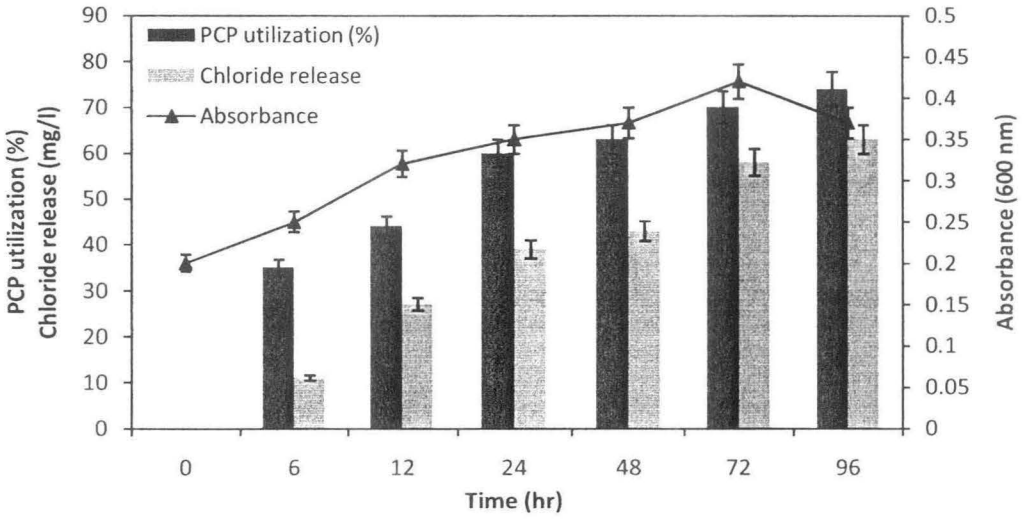

Figure 3.16 PCP utilization, chloride release and growth of strain ISTPCP-3 at different time intervals

The intensity of color produced in the solution was different for the strains ISTPCP-1, ISTPCP-2 and ISTPCP-3, indicating significantly different ring cleavage ability of the strains. ISTPCP-3 was most efficient in cleaving the benzene ring present in PCP.

3.4 Discussion

PCP is a major environmental pollutant discharged from tanneries, pulp and paper mills, distilleries and pharmaceutical industries. PCP is toxic to all life forms, since it is an inhibitor of oxidative phosphorylation (Shen *et al.*, 2005; Yang *et al.*, 2006). Moreover, PCP is recalcitrant to degradation because of its stable aromatic ring system and high chloride content, thus persisting in the environment (Okeke *et al.*, 1997). The USEPA and EU have listed PCP as a priority contaminant because of its proven toxicity and carcinogenicity (Bock

et al., 1996; Kovács *et al.*, 2008). Aerobic degradation of polychlorinated phenols has been studied extensively in the past (Häggblom, 1992). Several strains of bacteria that are able to degrade polychlorinated phenols have been isolated, characterized and applied for bioremediation of sites contaminated with PCP (Edgehill, 1996; Barbeau *et al.*, 1997; Valenzuela *et al.*, 1997). Numerous PCP degraders have also been isolated from the natural environment. Saber and Crawford, (1985) reported bacteria of genus *Flavobacterium* utilizing 100 mg/l of PCP as sole source of carbon and energy. Schenik, (1989) found that *Arthrobacter* sp. ATCC 33790 was capable of utilizing and degrading PCP. Nam *et al.*, (2003) reported isolation of a PCP-degrading *Pseudomonas veronii* strain PH-05 from a timber storage yard. Another strain, identified as *Pseudomonas fluorescens* was reported to degrade PCP (Shah and Thakur, 2003). In another study, Thakur *et al.*, (2002) isolated a bacterial strain identified as *Pseudomonas* sp. strain IST103 that was capable of degrading 100 mg/l of PCP. A PCP degrading bacterium was isolated from aquifer sediments and identified as *Pseudomonas mendocina* strain NYSU (Kao *et al.*, 2004). Yang *et al.*, (2006) reported isolation and physiological characterization of a PCP degrading bacterium identified as *Sphingomonas chlorophenolica*. Singh *et al.*, (2007) isolated and characterized a novel *Serratia marcescens* (AY927692) for PCP degradation from pulp and paper mill waste.

The present study describes enrichment and characterization of PCP-degrading bacterial consortium from the chemostat. Two stable consortia were developed by continuous enrichment of the bacterial population isolated from sediment core of tannery and pulp and paper mill effluents discharge sites. The consortia were enriched in the chemostat containing MSM, supplemented with PCP as sole source of carbon and energy. Sediment core along with liquid effluent from sampling sites of effluent discharge sites of tannery and pulp and paper mill initially yielded 8 and 18 bacterial isolates, respectively.

These isolates were able to survive on MSM containing PCP (10 mg/l) as sole carbon source. Five morphological distinct bacterial isolates from tannery sediments and three bacterial isolates of pulp and paper mill sediment were observed on MSM agar plates containing PCP (100 mg/l) as sole carbon and energy source. The members of the consortia were tested for their growth, removal of chromium and degradation of PCP in batch cultures at different time intervals. The bacterial consortium isolated from pulp and paper mill effluent discharge site (PE) was better in terms of growth, chromium reduction and degradation of PCP than the consortium isolated from tannery effluent discharge site (TE). The selected consortium showed significant degradation of PCP, when supplemented as sole source of carbon and energy. After preliminary morphological identification and biochemical characterization, the isolates were identified using methods based on total-cell fatty acid profiling technique. Further confirmation was done using 16S rDNA sequence analysis. The isolates were identified as *Enterobacter* sp. ISTPCP-1 (PE-1), *Pseudomonas aeruginosa* ISTPCP-2 (PE-2) and *Acinetobacter* sp. ISTPCP-3 (PE-3). Bacterial diversity of the strain was studied using DGGE technique. Three clear bands were observed, showing enrichment and stabilization of only three potent PCP degrading strains. Out of three acclimated bacterial isolates, ISTPCP-3 strain showed better PCP degradation capabilities than the other two strains (ISTPCP-1 and ISTPCP-2). The strain ISTPCP-3 was able to use 75% of PCP, while ISTPCP-1 and ISTPCP-2 utilized at 39% and 62.9% PCP respectively after a period of 96 hr. The results clearly suggest that strain ISTPCP-3 has better potential to degrade PCP than the strains ISTPCP-1 and ISTPCP-2. The strain ISPCP-3 also showed positive color formation in the Rothera test, indication ring cleavage at *ortho* position of benzene ring. The results highlight the potential of acclimated bacterial strains for remediation of sites contaminated with PCP.

OPTIMIZATION AND ELUCIDATION OF THE PATHWAYS FOR BIODEGRADATION OF PENTACHLOROPHENOL

CHAPTER 4

OPTIMIZATION AND ELUCIDATION OF THE PATHWAYS FOR BIODEGRADATION OF PENTACHLOROPHENOL

4.1 Introduction

A large variety of chemicals are being synthesized and produced each year. Compounds are finally discharged into the environment during their manufacturing and use of these chemicals. Over last decades, chlorophenolic compounds have been used extensively as wide spectrum biocides in industry and agriculture. These are among the most persistent environmental pollutants because of their physico-chemical characteristics (Annachhatre and Gheewala, 1996). The toxicity of these compounds tends to increase with relative degree of chlorination (Reineke and Knackmuss, 1988). Among chlorinated phenols; PCP and its sodium salt have been widely used as wood and leather preservative, owing to their toxic effect on bacteria, mould, fungi and algae (Kaoa *et al.*, 2004). PCP is toxic to all life forms as it is an inhibitor of oxidative phosphorylation (Shen *et al.*, 2005; Yang *et al.*, 2006). Extensive exposure to PCP could cause cancer, acute pancreatitis, immunodeficiency and neurological disorders (Sai *et al.*, 2001). Both the US Environmental Protection Agency (USEPA) and European Union (EU) have listed PCP as a priority contaminant (Bock *et al.*, 1996; Kovács *et al.*, 2008). PCP may be washed into streams and lakes due to surface runoff or may infiltrate and contaminate groundwater. Its large amount finally gets deposited onto sediments thus persisting in the environment (Shiu *et al.*, 1994). Moreover, PCP is recalcitrant to degradation because of its stable aromatic ring system and high chloride content, thus persisting in the environment (Saber and Crawford, 1985).

Polychlorinated phenols including PCP have been used extensively since the 1920s as preservatives to prevent fungal attack on wood (Colosio *et al.*, 1993). During this time, they have become serious environmental contaminants. PCP-degrading bacteria are present in soils worldwide (Saber and Crawford, 1985; Tiirola *et al.*, 2002a; Kao *et al.*, 2005; Yang *et al.*, 2006; Mahmood *et al.*, 2005). Although monochlorophenols and dichlorophenols are produced naturally by some fungi and insects (Gribble, 1996), natural sources of PCP are not known; therefore, the degradation pathway(s) employed by bacteria to degrade PCP likely evolved during the approximately 60 years since the human introduction of PCP into the environment (Copley, 2000). PCP-degrading pure cultures may be useful for bioaugmentation in the remediation of PCP-contaminated soils and waters (Saber and Crawford, 1985; Bielefeldt and Cort, 2005).

PCP from the pulp bleaching process is found both in free (hexane extractable) and bound (extractable with strong alkali) forms in dissolved organic matter and particles (Schnell *et al.*, 2000). High and low molecular weight compounds are produced by complex reactions between chlorine and lignin in the wood pulp (Yeber *et al.*, 2000). Effluents containing PCP have been identified as toxic according to the Canadian Environmental Protection Act (Schnell *et al.*, 2000). In addition, PCP is harmful to microorganisms since it destroys membrane function (Copley, 2000). It has been reported as mutagenic even at low levels of concentration. It has inherent toxicity to be bioaccumulated in various food chains of biological system. PCP is toxic to living organisms and has a high half-life in environment because of lack of biodegradative enzymes in the indigenous organisms (Bellinaso *et al.*, 2001).

The biodegradation of PCP has been studied in both aerobic and anaerobic systems. Anaerobic biodegradation of PCP in aquatic, sludge and soil environment has been studied by various researchers (McAllister *et al.*, 1996; Wang *et al.*, 1998; Vallecillo *et al.*, 1999;

Tartakovsky *et al.*, 2001; Thakur *et al.*, 2001). Reductive dechlorination has been suggested as the primary PCP biodegradation mechanism. The aromatic ring is thus totally dechlorinated prior to ring cleavage (Wang *et al.*, 1998; Tartakovsky *et al.*, 1999; Tartakovsky *et al.*, 2001). Aerobic degradation of PCP has also been studied extensively and several bacterial strains capable of degradation have been reported such as *Flavobacterium*, *Arthrobacter*, *Pseudomonas*, *Sphingomonas* and *Sphingobium* (Edgehill and Finn, 1983; Crawford and Mohn, 1985; Saber and Crawford, 1985; Xun and Orser, 1991c; Orser *et al.*, 1993; Edgehill, 1994; Miethling and Karlson, 1996; Chanama and Crawford, 1997; Leung *et al.*, 1999; Thakur *et al.*, 2002; Yang *et al.*, 2006; Dams *et al.*, 2007). To the best of my knowledge, the degradation pathways reported here by individual bacterial strains are different from previously characterized pathways (Figure 4.1).

In this study, three acclimated bacterial strains isolated from the chemostat were tested for their PCP degradation capabilities as a consortium and as individual bacteria. The strains were characterized for optimization of growth parameters such as temperature, pH, effect of initial PCP concentration and inoculum size. The strains were also tested for their utilization of various organic compounds as carbon source. The metabolic profile was also studied in order to deduce PCP degradation pathways. The highly potent bacterial strain could be applied for *in situ* or *ex situ* bioremediation of PCP contaminated soil and sediments.

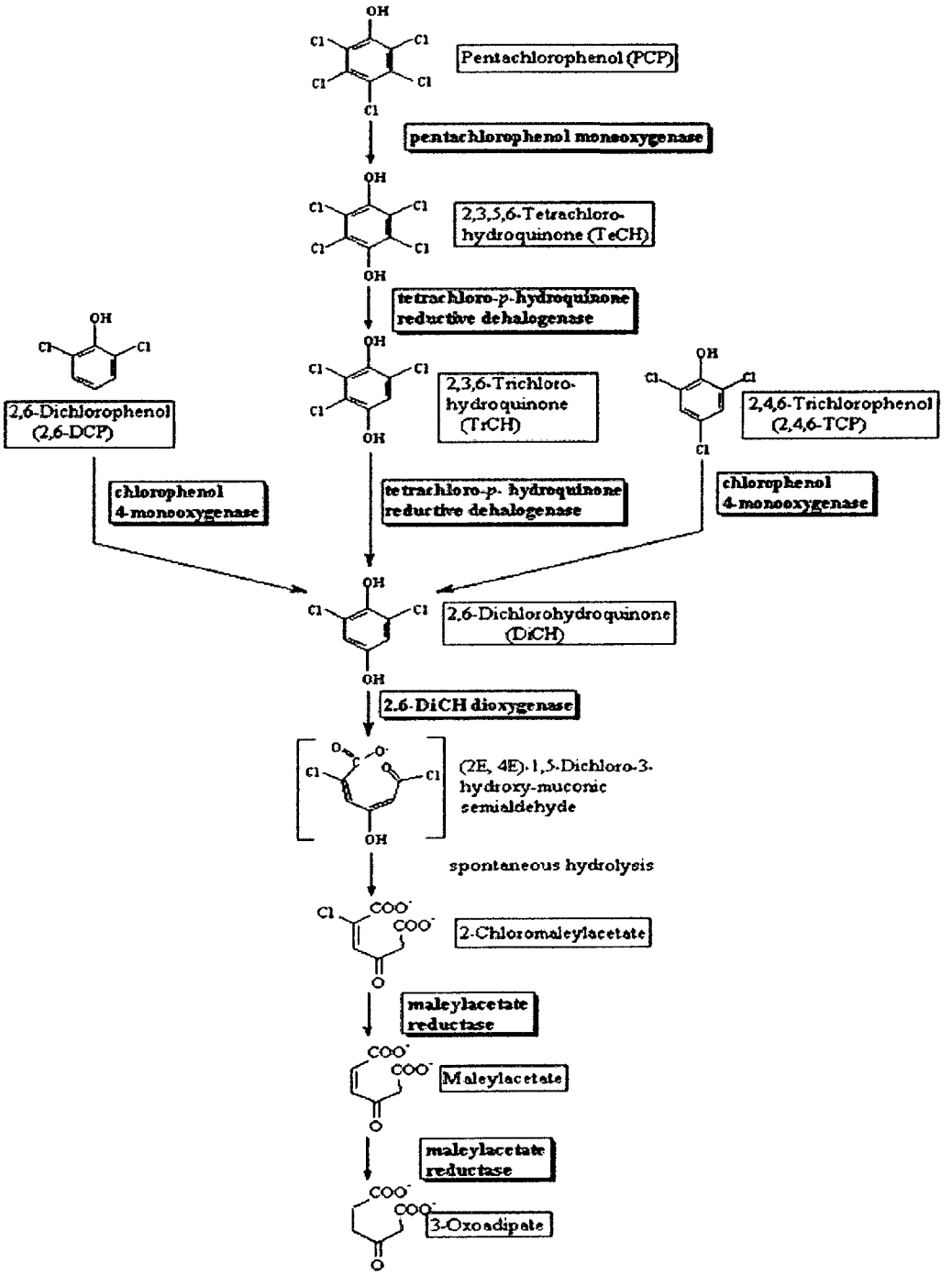

Figure 4.1 Pentachlorophenol biodegradation pathways and various products of its degradation

4.2 Materials and methods

4.2.1 Chemicals and growth medium

The following chemicals were purchased from Sigma-Aldrich (USA): PCP (FW 266.30), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), anhydrous sodium sulfate (AR). Organic solvents (GC/MS grade) ethyl acetate, hexane and acetone were obtained from Merck, India. All other inorganic chemicals were of analytical grade obtained from Qualigens fine chemicals, GSK, India. Anhydrous sodium sulfate was heated at 130°C for 24 h prior to its use.

Mineral salts medium (MSM) was used in enrichment culture and degradation studies. The medium contained the following components at the specified concentrations (in mg/l): The MSM contained the following components at the specified concentrations (in mg/l): Na_2HPO_4, 780; KH_2PO_4, 680; NH_4Cl, 500; $MgSO_4 \cdot 7H_2O$, 200; $CaCl_2 \cdot 2H_2O$, 10; plus 1 ml/l of trace metal solution which included $FeSO_4 \cdot 7H_2O$, 5; $ZnSO_4 \cdot 7H_2O$, 4; $MnSO_4 \cdot 4H_2O$, 0.2; $NiCl \cdot 6H_2O$, 0.1; H_3BO_3, 0.15; $CoCl_2 \cdot 6H_2O$, 0.5; $ZnCl_2$, 0.25; and EDTA, 2.5 (Thakur, 1995).

4.2.2 Bacterial strains and culture conditions

After enrichment of previously isolated bacterial strains *Enterobacter* sp. ISTPCP-1, *Pseudomonas aeruginosa* ISTPCP-2 and *Acinetobacter* sp. ISTPCP-3 were inoculated in 5% (v/v) LB broth medium having OD 1.0 at 600 nm. The culture were centrifuged at 8000 rpm for 8 min at 4°C the bacterial pellets were transferred to MSM containing PCP (100 mg/l) at pH 7.0. The mixture culture was placed on a shaker (120 rpm) in the dark at 30°C. The biodegradation of PCP was monitored periodically at 0, 6, 12, 24, 36, 48 hr. At specified time intervals, samples were collected and OD was measured at 600 nm (OD_{600}). MSM cultures were centrifuged at 8000 rpm for 8 min; the

supernatant was separated for biodegradation studies and pathway prediction.

Unless otherwise stated, all aerobic batch cultivations were carried out in 250 ml Erlenmeyer flasks containing 100 ml of liquid culture. The bacterial strains were grown of MSM containing PCP (100 mg/l) as sole carbon source for 24 hr. The cells were pelleted by centrifugation at 8000 rpm for 8 min, cells pellets were washed twice with fresh MSM. Cell density was monitored by spectrophotometer OD_{600} (Shimadzu UV2410, Japan). For all experiments 10^6 CFU/ml were used and samples were incubated at 30°C and shaking at 120 rpm in dark.

4.2.3 Substrate utilization by bacterial strains

In order to understand the ability of acclimated mixed culture to degrade PCP, the experiments were conducted with a series of 250 ml batch reactors. Each reactor contained 40 ml of MSM with mixed culture of bacteria. The initial amount of mixed culture of bacteria started at a cell concentration yielding 0.1 OD_{600} units. Different concentrations of PCP (10–180 mg/l) were added to the different batch reactor and sealed with cotton stoppers. The reactors were shaken at 120 rpm in dark at 30°C to measure variation of PCP concentration with spectrophotometer and gas chromatograph–mass spectrometer under aerobic conditions. The PCP removal by pure culture of bacteria was investigated using the same setup with the exception that mixed culture of bacteria was replaced by individual bacterial strains.

Utilization of various chlorinated compounds by bacterial strains was assessed on MSM agar plates with the test compound. Growth of bacterial strains at 30°C was monitored daily by visual inspection for 3 days. The bacterial cells were then washed from the plate with saline and absorbance (A_{600}) values of the suspension were determined. The bacterial strains were inoculated in Erlenmeyer flasks containing mineral salts medium supplemented with test

compounds (0.5 mM) as sole source of carbon and energy, and incubated at 30°C on an orbital shaker at 120 rpm in dark. The samples were removed after 0, 6 and 12 h, and growth of bacterial strains and utilization of carbon source was determined.

4.2.4 Optimization of growth parameters

The optimization of growth parameters of bacterial strains for degradation of PCP (50 mg/l) as sole source of carbon and energy was monitored in terms of temperature, pH, initial PCP concentration and inoculum size. The optimum temperature (20, 25, 30, 35/37, 40°C) for growth of the bacterial strains was determined following the modified procedure of Lee and Wang, (2004). In order to find out the optimum pH for PCP removal various pHs of MSM (5, 6, 7, 8, and 9) were prepared. The pHs of the MSM were adjusted with NaOH/HCl. In order to find out effect of initial PCP concentration (20, 50, 70, 100, 200 mg/l) on growth of bacterial strains, experiments were designed with supplementing required concentration of PCP in MSM. Effect of inoculum size were determined using various inoculum concentrations (0.5, 1, 5, 7, 10 v/v). All experiments were performed in triplicates and data represents are a mean of values obtained.

4.2.5 Elucidation of PCP biodegradation pathways

Phenolic compounds are most often determined by gas chromatography (GC) with various detectors among which mass spectrometry (MS) offers unrivalled resolution and selectivity. The degradation ability of the isolated strain ISTPCP-3 was analyzed in MSM containing PCP. Degradation efficiency was determined and estimated by loss of PCP from culture medium. In extraction of metabolites, the cell suspension (50 ml) was clarified by centrifugation at 8000 rpm for 8 min. The cell free supernatant fractions were extracted thrice with an equal volume of ethyl acetate by shaking for 45 min. The organic layer was dried with anhydrous sodium sulfate, and the solvent was removed by gently blowing under a stream of N_2.

Although several derivatization methods have been proposed, including acetic anhydride, diazomethane, benzoyl-chloride, and pentafluorobenzoyl-chloride or pentafluorobenzyl-bromide for electron capture detection (ECD), the derivatization of phenols and chlorophenols is not straightforward. Acylation requires alkaline pH, the optimum being between 9 and 11, but catechol and resorcinol are prone to oxidative degradation at pH above 8. Diazomethane is highly explosive and carcinogenic and needs to be generated in situ. The most widely applicable method is silylation with *N*-(*t*-butyldimethylsilyl)-*N*-methyltrifluoracetamide. The reaction requires 80°C and 1 hr to take place.

In another experimental set up, the residues obtained were derivatized in 300 µl of ethyl acetate with 100 µl of BSTFA at 80°C for 55 min and analyzed immediately on a GC–MS. Derivatization of metabolites was done in order to increase the chances for detection of metabolites. Therefore, the proton of hydroxyl group was replaced by silyl group after derivatization. If underivatized, phenolic compounds are prone to irreversible adsorption on active centers of the separation system, resulting in peak tailing and substance loss.

The GC–MS analyses were performed in electron ionization (EI) mode (70 eV) with an Agilent 6890N gas chromatograph, equipped with 5973 MSD (Agilent Technologies, Palo Alto, CA, USA). A HP–MS (Agilent, USA) capillary column (5% phenyl 95% methylpolysiloxane; 30 m length × 0.025 mm id × 0.25 µm film thickness) column was used at a temperature programme of 45°C for 1.5 min, increased to 100°C at 10°C/min, increased to 180°C at 4°C/min and finally increased to 300°C at 40°C/min and held at 300°C for 5 min. Helium was used as the carrier gas at a constant flow of 1.2 ml/min. The samples were analyzed in split mode (1:10) at an injection temperature of 250°C, an EI source temperature of 230°C and a quadrupole analyzer temperature of 150°C was maintained, unit mass resolution and scan range m/z 35–500, was selected with a scan cycle

of 3 scans/sec. The injected volume was 0.5 µl. Solvent delay was set under 5 min. Proton–Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR) of PCP and its metabolites were obtained in resonating frequency of 60 MHz instrument (Varian EM–368L) operated under continuous wave mode. The samples were dissolved in deuterochloroform and tetramethyl-silane was used as internal standard.

4.3 Results

4.3.1 Utilization of PCP by bacterial strains

The utilization of chlorinated phenols and intermediary metabolite of PCP by the bacterial strains; *Enterobacter* sp. ISTPCP-1, *Pseudomonas aeruginosa* ISTPCP-2 and *Acinetobacter* sp. ISTPCP-3, was tested on MSM containing various carbon sources under aerobic conditions (Table 4.1). The strain ISTPCP-3 was able to grow well on plates containing pentachlorophenol, tetrachlorohydroquinone, chlorohydroquinone and catechol. The growth was observed minimum when grown on 2,4,6-trichlorophenol. The utilization potential of ISTPCP-2 and ISTPCP-1 was good on catechol. Normal growth of strain ISTPCP-2 was observed on substrates such as pentachlorophenol, tetrachlorohydroquinone, trichlorophenol and dichlorophenol. None of the isolated strains were able to grown on dibenzofuran as substrate. An apparent lag in PCP utilization was greatly reduced by supplementation of readily degradable carbon source present along with PCP in MSM. Dextrose, sodium acetate and sodium citrate lead to significant growth. However, cellobiose and inulin were proved poor substrates compared to dextrose. Nitrogen source supplements included yeast extract, glutamate and aspartate as organic nitrogen and ammonium chloride and ammonium nitrate as inorganic nitrogen in MSM containing PCP. No PCP utilization was

observed in the presence of readily available carbon source and yeast extract.

Table 4.1 Growth of bacterial strains on various carbon sources (0.5 mM) on MSM agar plate

Carbon source	**Bacterial strains**		
	ISTPCP-1	**ISTPCP-2**	**ISTPCP-3**
Pentachlorophenol	++	++	+++
2,4,6-trichlorophenol	+	++	+
2,4-dichlorophenol	+	++	++
p-chlorophenol	+	+	++
Tetrachlorohydroquinone	–	++	+++
Chlorohydroquinone	–	+	+++
Catechol	+++	+++	+++
4-chlorosalicylic acid	–	+	++
4-chlorobenzoic acid	–	–	++
Dibenzofuran	–	–	–

+++, good growth; ++, normal growth; +, minimum growth; –, no growth

4.3.2 The PCP removal by bacterial consortium

The degradation and removal of different concentrations of PCP by acclimated mixed culture of bacteria was studied in a batch culture (250 ml flasks) at different time intervals (Figure 4.2). The result indicated that PCP concentrations up to 50 mg/l were completely degraded by acclimated mixed culture.

When the initial PCP concentration were 10 mg/l, 25 mg/l, 50 mg/l, 100 mg/l, 150 mg/l and 180 mg/l, the mixed culture could only remove PCP at low concentration of about 10 mg/l and 25 mg/l and 50 mg/l. However, when the PCP concentration was above 50 mg/l,

the removal of mixed culture had longer lag period. Only 60% of PCP could be removed by consortia at 96 hr. The bacterial consortia were not able to significantly remove PCP concentrations of 100 mg/l, 150mg/l and 180 mg/l. The aerobic pathway of PCP degradation is $C_6Cl_5OH + 4.5O_2 + 2H_2O \rightarrow 6O_2 + 5HCl$ (Crawford and Crawford, 1996). The equation showed that PCP degradation leads to a decrease in pH. However, there was no significant decrease in pH values during the experiment.

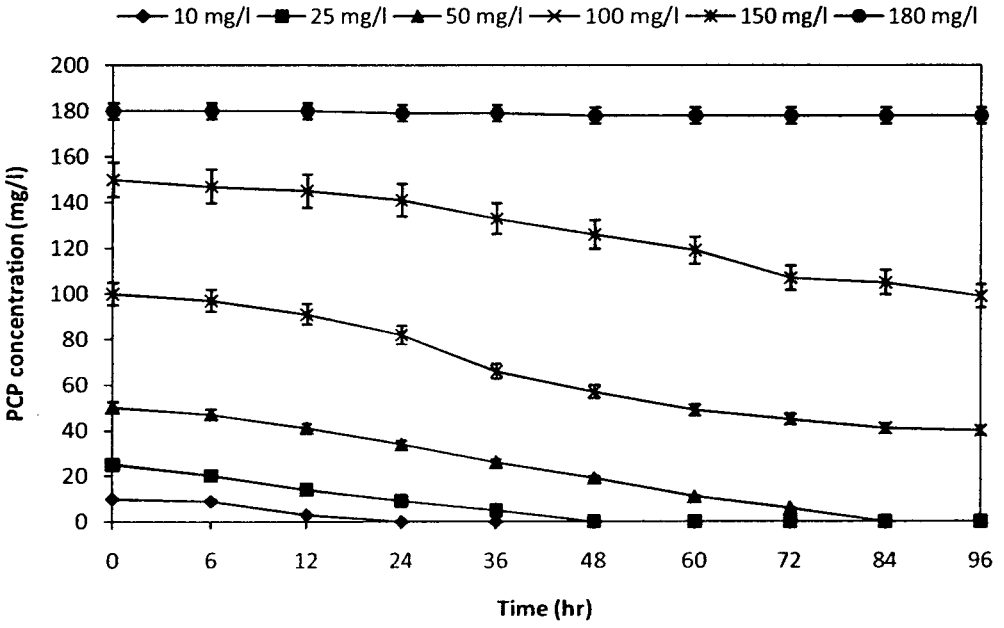

Figure 4.2 The removal of different concentrations of PCP by acclimated bacterial consortium in a batch reactor

4.3.3 Parameter optimization for PCP degradation

The growth of bacterial strains *Enterobacter* sp. ISTPCP-1, *Pseudomonas aeruginosa* ISTPCP-2 and *Acinetobacter* sp. ISTPCP-3 and effect of PCP degradation by strains were observed under different culture condition, such as temperature, pH, initial PCP concentration and inoculum amounts. The MSM was supplemented with PCP (50 mg/l) as sole source of carbon. The growth and degradation were studied by analyzing the samples at 6 hr interval. Figure 4.3 shows the degradation of PCP at different temperatures by *Enterobacter* sp. ISTPCP-1 strain. The result reveals that ISTPCP-1 is a mesophilic bacterium. The optimum growth temperature was 37°C; the strain was able to grow well in temperature range of 35°C–40°C, the degradation was weak at temperatures 20°C and 25°C. At optimum temperature the strain was able to degrade around 80% of PCP during 24 hr.

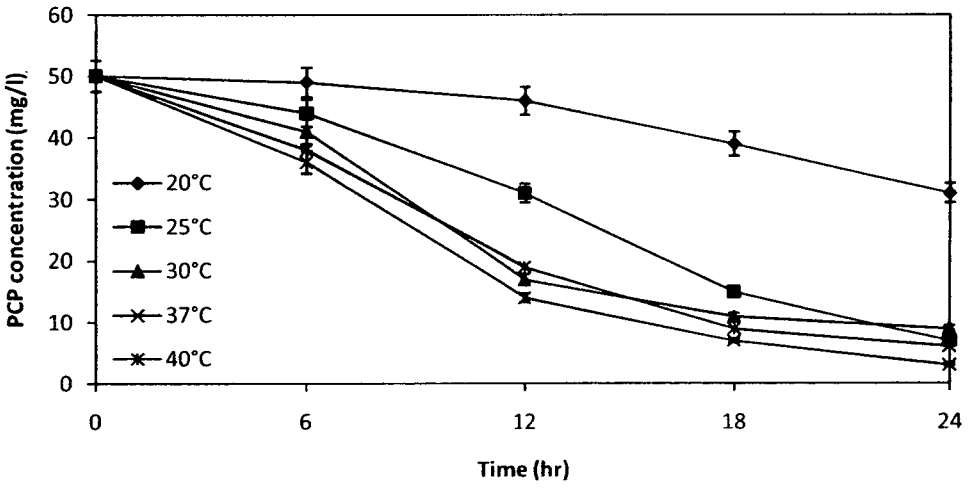

Figure 4.3 Effect of temperature changes on degradation of PCP by *Enterobacter* sp. ISTPCP-1

Figure 4.4 shows the degradation of PCP at different temperatures by *Pseudomonas aeruginosa* ISTPCP-2 strain. The optimum growth temperature was 30°C; the strain was able to grow well in temperature range of 30°C–35°C, the degradation was weak at temperatures 20°C and 40°C. At optimum temperature the strain was able to degrade around 85% of PCP during 24 hr.

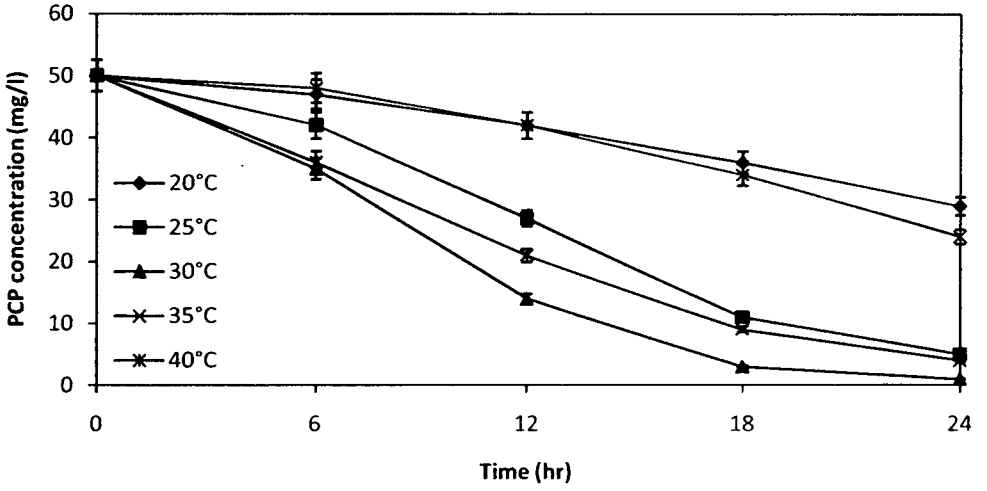

Figure 4.4 Effect of temperature changes on degradation of PCP by *Pseudomonas aeruginosa* ISTPCP-2

Figure 4.5 shows the degradation of PCP at different temperatures by *Acinetobacter* sp. ISTPCP-3 strain. The optimum growth temperature was 30°C; the strain was able to grow well in temperature range of 30°C–35°C, the degradation was weak at temperatures 20°C and 40°C. At optimum temperature the strain was able to degrade around 95% of PCP during 24 hr.

The strain ISTPCP-3 showed maximum PCP degradative capability at mesophilic temperature than strains ISTPCP-2 and ISTPCP-1.

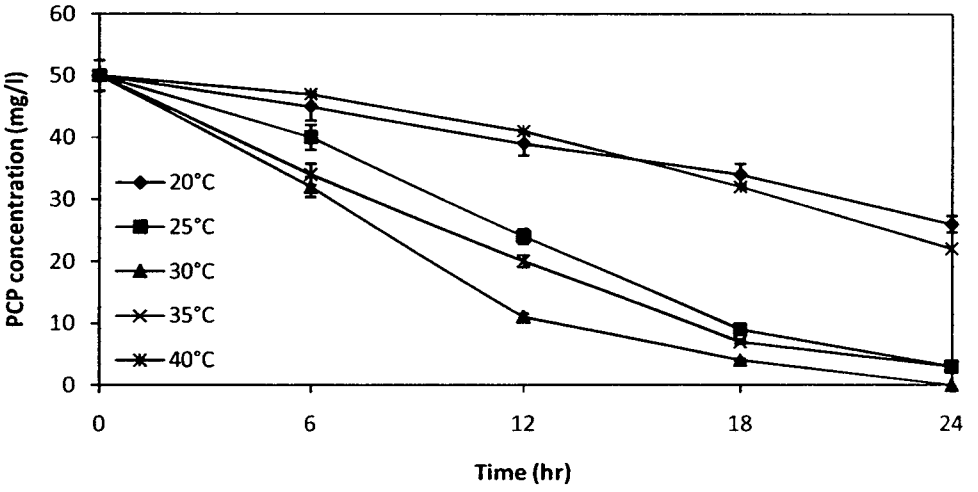

Figure 4.5 Effect of temperature changes on degradation of PCP by *Acinetobacter* sp. ISTPCP-3

The removal of 50 mg/l of PCP by *Enterobacter* sp. ISTPCP-1 with different initial pH value is shown in Figure 4.6. When the initial pH value was 5, 6, 7, 8 and 9, the strain ISTPCP-1 could remove 2%, 40%, 94%, 48% and 8% of PCP respectively. No significant reduction of PCP was observed by the strain at pH 5 and pH 9. Therefore, the optimum pH for PCP removal by strain ISTPCP-1 was at pH 7.0. No growth of the strain was observed below pH 4.0, showing that a neutral to alkaline pH may be required for degradation of PCP.

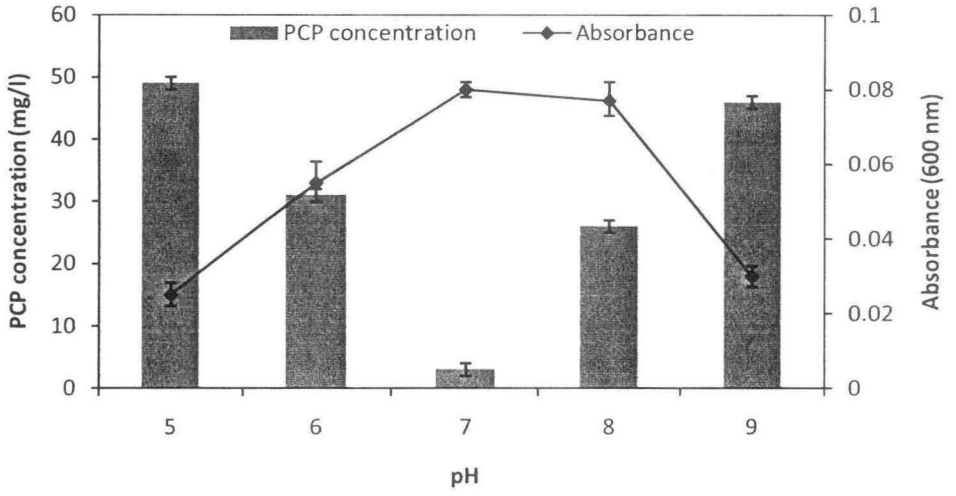

Figure 4.6 Effect of initial pH changes on degradation of PCP by *Enterobacter* sp. ISTPCP-1

The removal of 50 mg/l of PCP by *Pseudomonas aeruginosa* ISTPCP-2 with different initial pH value is shown in Figure 4.7. When the initial pH value was 5, 6, 7, 8 and 9, the strain ISTPCP-2 could remove 4%, 48%, 96%, 52% and 10% of PCP respectively. No significant reduction of PCP was observed by the strain at pH 5 and pH 9. Therefore, the optimum pH for PCP removal by strain ISTPCP-2 was at pH 7.0. No growth of the strain was observed below pH 4.0, showing that a neutral to alkaline pH may be required for degradation of PCP.

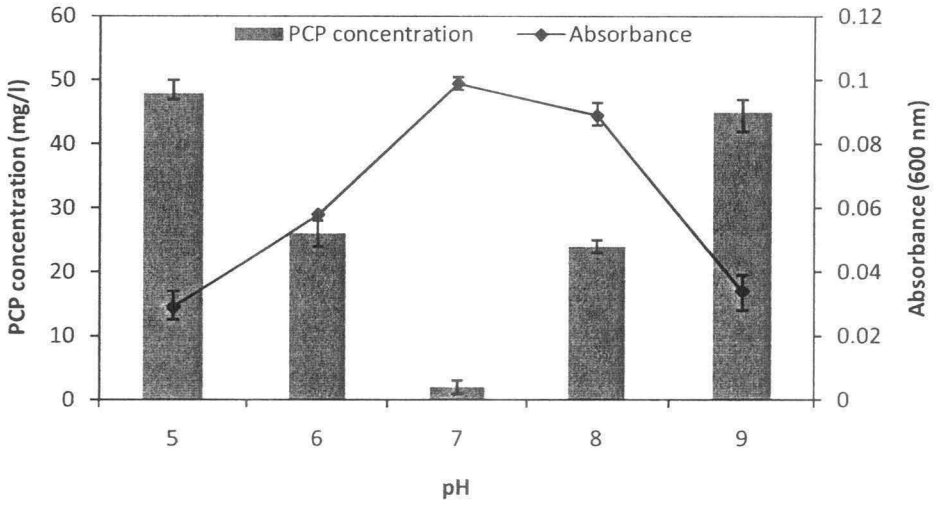

Figure 4.7 Effect of initial pH changes on degradation of PCP by *Pseudomonas aeruginosa* ISTPCP-2

The removal of 50 mg/l of PCP by *Acinetobacter* sp. ISTPCP-3 with different initial pH value is shown in Figure 4.8. When the initial pH value was 5, 6, 7, 8 and 9, the strain ISTPCP-3 could remove 4%, 54%, 98%, 62% and 10% of PCP respectively. No significant reduction of PCP was observed by the strain at pH 5 and pH 9. Therefore, the optimum pH for PCP removal by strain ISTPCP-3 was at pH 7.0. No growth of the strain was observed below pH 4.0, showing that a neutral to alkaline pH may be required for degradation of PCP.

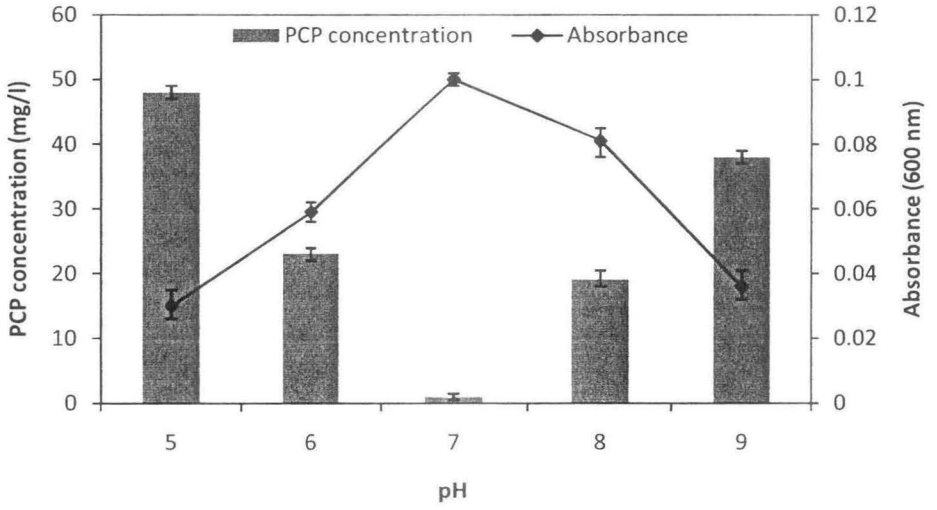

Figure 4.8 Effect of initial pH changes on degradation of PCP by *Acinetobacter* sp. ISTPCP-3

Figure 4.9 shows the removal of different concentrations of PCP by *Enterobacter* sp. ISTPCP-1 in 250 ml flasks. In batch culture, strain ISTPCP-1 was capable of completely degrading PCP at concentration lower than 70 mg/l. When the initial PCP concentration was 100 mg/l; the strain could only remove almost 40% of PCP at 96 hr. The complete degradation of PCP was observed after a period of 150 hr. At PCP concentration of 200 mg/l, no degradation was observed even after 150 hr, with curve showing an extended lag phase.

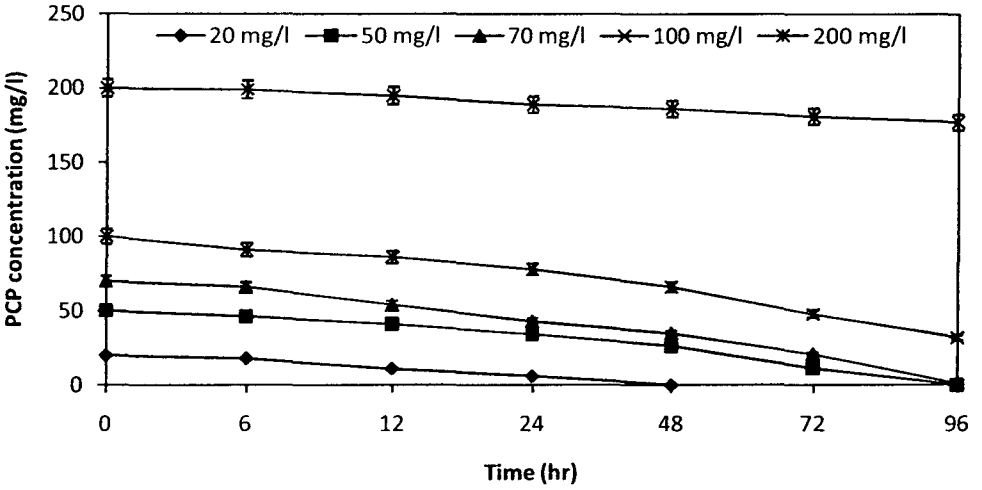

Figure 4.9 The removal of different concentrations of PCP by *Enterobacter* sp. ISTPCP-1

Figure 4.10 shows the removal of different concentrations of PCP by *Pseudomonas aeruginosa* ISTPCP-2 in 250 ml flasks. In batch culture, strain ISTPCP-2 was capable of completely degrading PCP at concentration lower than 70 mg/l. When the initial PCP concentration was 100 mg/l; the strain could only remove almost 63% of PCP at only 96 hr. The complete degradation of PCP was observed after a period of 120 hr. At PCP concentration of 200 mg/l, degradation was incomplete with about only 30% of PCP degraded at 96 hr with curve showing an extended lag phase.

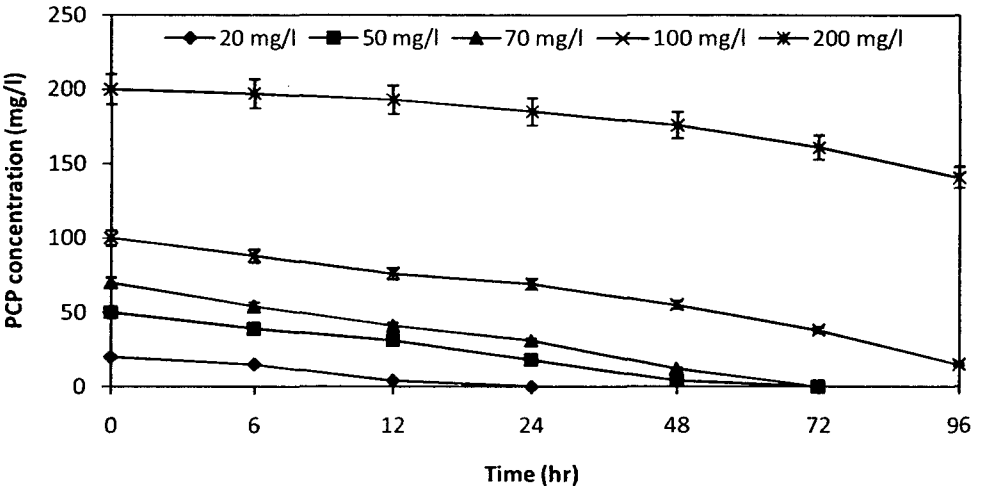

Figure 4.10 The removal of different concentrations of PCP by *Pseudomonas aeruginosa* ISTPCP-2

Figure 4.11 shows the removal of different concentrations of PCP by *Acinetobacter* sp. ISTPCP-3 in 250 ml flasks. In batch culture, strain ISTPCP-3 was capable of completely degrading PCP at concentration lower than 70 mg/l. When the initial PCP concentration was 100 mg/l; the strain could remove almost 75% of PCP at only 96 hr. The complete degradation of PCP was observed after a period of 110 hr. At PCP concentration of 200 mg/l, degradation was incomplete with about only 50% of PCP degraded at 96 hr.

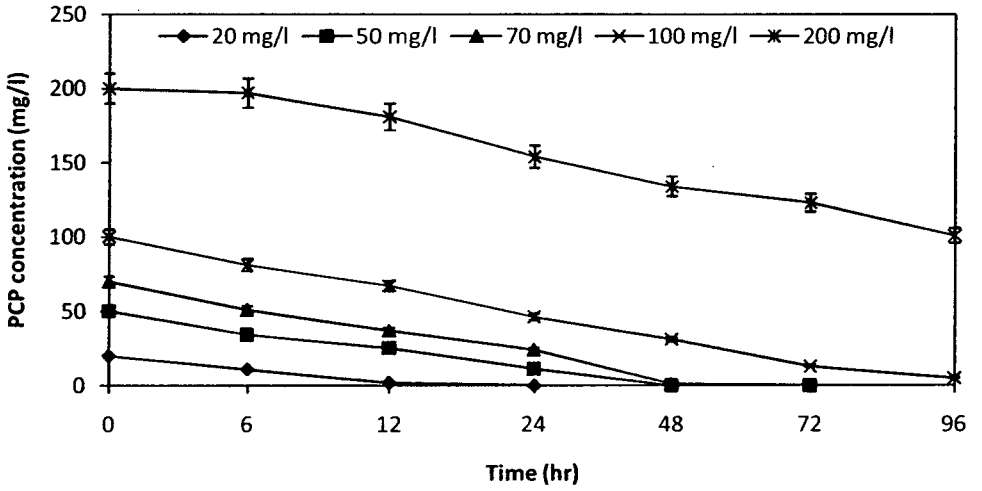

Figure 4.11 The removal of different concentrations of PCP by *Acinetobacter* sp. ISTPCP-3

The possible reason for the observed incomplete degradation of PCP by bacterial strains at a high concentration of 200 mg/l could be that the lower pH inhibited the activity of the organism. When initial PCP concentration was 200 mg/l, pH decreased from 7.0 to 5.1. The possible reason for an extended lag phase in degradation of high concentration of PCP by strains was that the amount of PCP-degrading enzyme needed more time to produce. The results also indicated that at experimental concentration levels, the degradation and growth of bacterial strains could partly be inhibited by high concentration of PCP; however, when the strains are acclimated with such high concentration in the chemostat, significant PCP degradation could be observed. It has also been observed that strain ISTPCP-3 was observed to grow and possessed the capability for significantly removing high concentration of PCP.

Figure 4.12 shows the effect of inoculum changes on degradation of PCP by *Enterobacter* sp. ISTPCP-1. At high inoculum density of 7% and 10%; PCP was degraded completely at 15 hr and 12 hr respectively. The degradation was slower at low inoculum density.

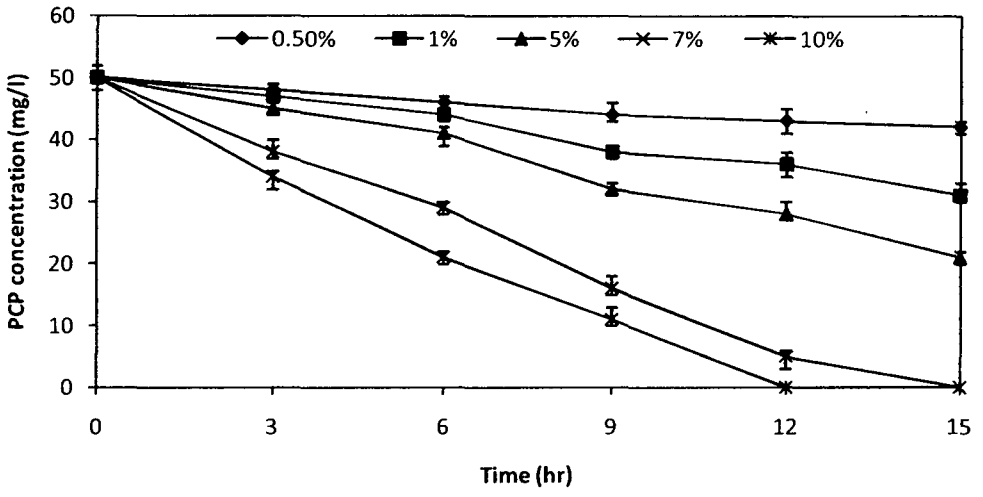

Figure 4.12 Effect of inoculum changes on degradation of PCP by *Enterobacter* sp. ISTPCP-1

Figure 4.13 shows the effect of inoculum changes on degradation of PCP by *Pseudomonas aeruginosa* ISTPCP-2. At high inoculum density of 7% and 10%; PCP was degraded completely at 15 hr and 12 hr respectively. The degradation was slower at low inoculum density.

Figure 4.14 shows the effect of inoculum changes on degradation of PCP by *Acinetobacter* sp. ISTPCP-3. At high inoculum density of 7% and 10%; PCP was degraded completely at 12 hr and 9 hr respectively. The degradation was slower at low inoculum density.

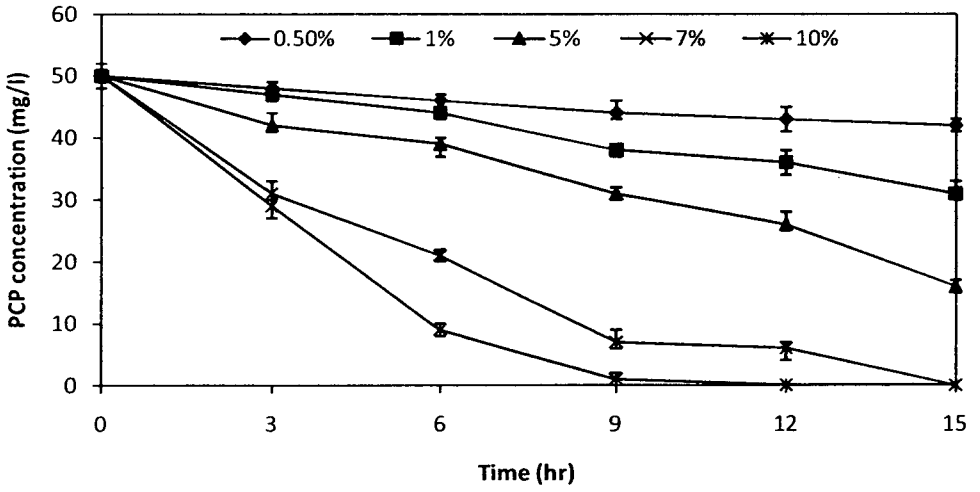

Figure 4.13 Effect of inoculum changes on degradation of PCP by *Pseudomonas aeruginosa* ISTPCP-2

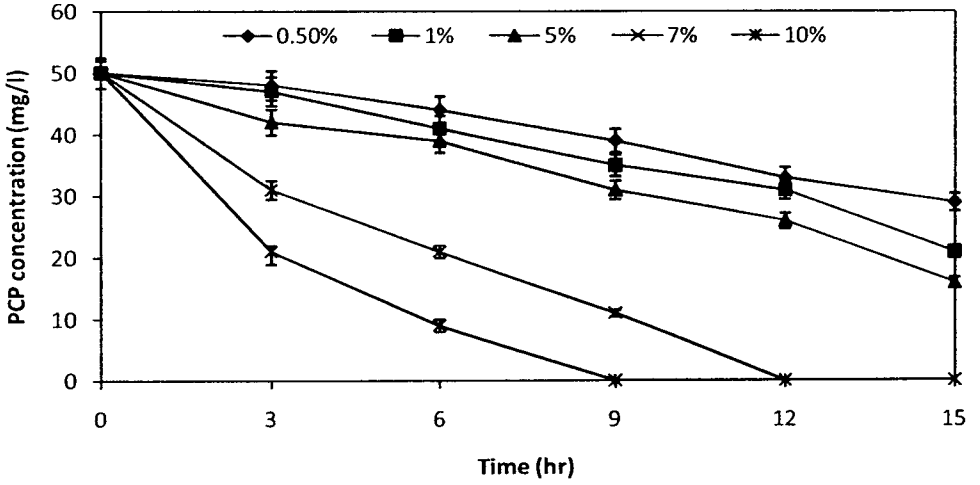

Figure 4.14 Effect of inoculum changes on degradation of PCP by *Acinetobacter* sp. ISTPCP-3

The study related to degradation of PCP by acclimated bacterial consortium and with individual bacterial strain clearly revealed that the removal rate of PCP by individual strains was better than consortium. The consortium was only able to completely degrade low concentration of PCP (up to 50 mg/l) during 96 hr. The consortium was not able to completely remove high concentration of PCP. No growth was observed at concentration of 180 mg/l of PCP. The performance of individual strains was relatively better than mixed bacterial consortium. Of the three predominant strains present in the mixed culture, the most efficient PCP degrader was *Acinetobacter* sp. ISTPCP-3.

Radehaus and Schmidt, (1992) isolated a PCP-degrading bacterium from polluted soil and identified it as *Pseudomonas* sp. strain RA2. When the initial PCP concentrations were 40 mg/l and 150 mg/l, the removal efficiency of PCP were 95% at 96 hr and 97% at 192 hr, respectively. At a PCP concentration of 200 mg/l, cell growth was completely inhibited and PCP was not degraded. Yu and Ward, (1996) investigated the abilities of the three bacterial strains (*Flavobacterium gleum*, *Agrobacterium radiobacter* and *Pseudomonas* sp.) to degrade PCP individually and in combination. Of the individual strains, *Flavobacterium gleum* manifested highest PCP degradation ability. At PCP concentration of 100 mg/l, degradation was incomplete with about 60% of PCP degraded after 4 days. *Pseudomonas* sp. exhibited poorest ability to degrade 100 mg/l PCP. Webb *et al.*, (2001) isolated eight of the *Saccharomonospora viridis* strains having the ability of degrading PCP. When PCP concentration was 10 mg/l, all eight strains were able to remove up to 90% of chlorophenol within 14 days. However, PCP concentrations greater than 20 mg/l were toxic to all the *S. viridis* strains, completely inhibiting growth in liquid culture. Wittmann *et al.*, (1998) tested the maximum degrading concentrations of PCP by PCP degrading bacteria *S. chlorophenolica* RA2 and *Mycobacterium chlorophenolicum* PCP-1. Strain RA2 showed a higher

activity concerning growth and PCP degradation than PCP-1 under optimum conditions, and Strain RA2 was capable of degrading PCP up to 850 mM (226.5 mg/l).

4.3.4 Degradation products and pathways for PCP

PCP degradation pathways followed by individual bacterial strains were studied using GC coupled with mass spectrometry and ^{1}H NMR spectroscopy. The peaks of metabolites were identified using documented data from National Institute of Standards and Technology (NIST) library database. The library used was NBS75K/NIST. It was constantly updated through live feeds from NIST server. The results of the study indicated degradation of 100 mg/l of PCP. Analysis of metabolites from culture filtrate was done at 0 hr, 24 hr and 48 hr. Heat killed cells were taken as negative control. The experiment was performed in two batches, one set of samples were derivatized using TMS and samples of other set were extracted without final derivatization.

Figures 4.15, 4.16 and 4.17 shows PCP control, degradation products and peaks of metabolites produced by *Enterobacter* sp. ISTPCP-1. In control sample (0 hr) single peak of PCP (RT = 17.2 min) was observed (Figure 4.15). No other peaks of metabolites were observed. Single new peak was observed at 24 hr, the peak was identified as 2,3,4,6-tetrachlorophenol (RT = 14.4 min). The peak area (concentration) of PCP was observed to be decreased (Figure 4.16). At 48 hr another peak of metabolism was observed, the peak was identified as 2,6-dimethoxyphenol (RT = 19 min). The PCP peak area was further reduced at 48 hr (Figure 4.17). No other peaks of metabolites were observed at these time intervals. Heat killed cells did not show any decrease in PCP concentration and no degradation products were observed in the chromatogram.

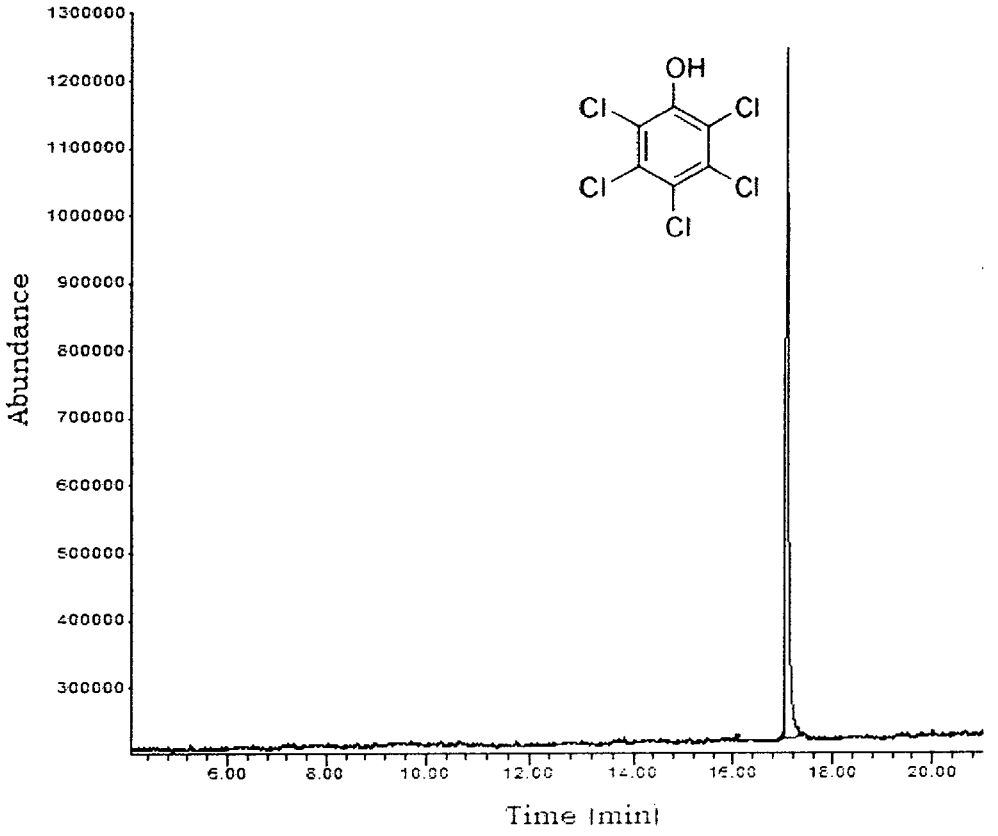

Figure 4.15 Gas chromatogram of extract obtained from the liquid culture at 0 hr of incubation with strain ISTPCP-1

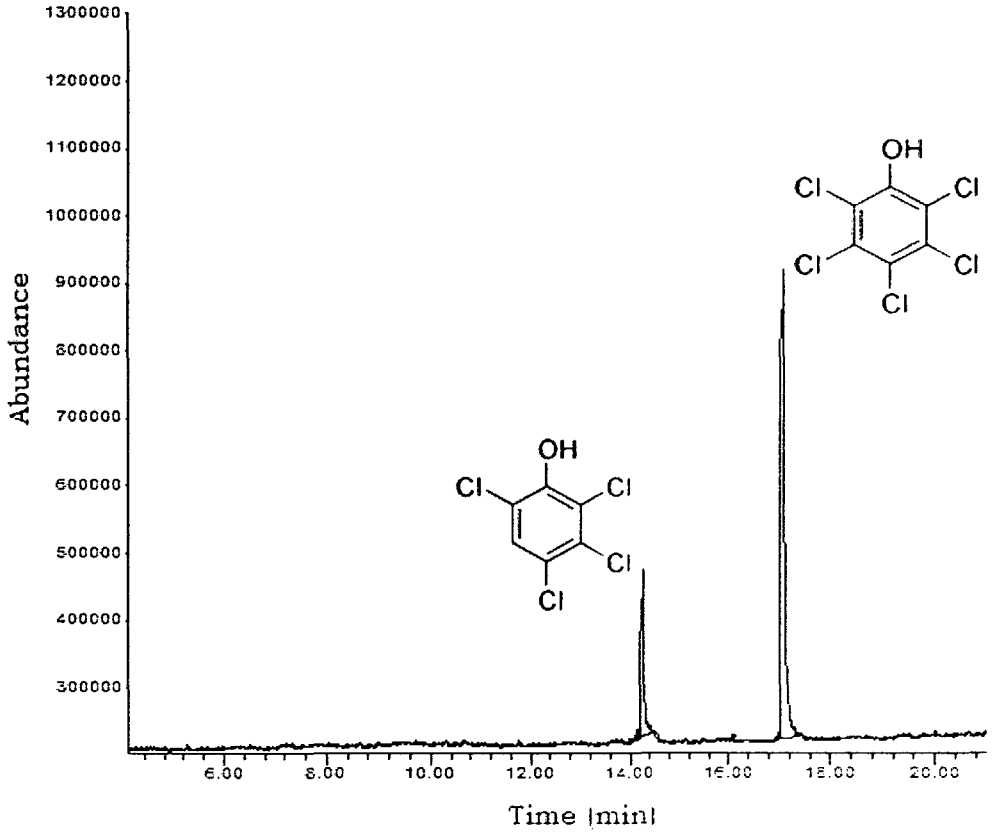

Figure 4.16 Gas chromatogram of extract obtained from the liquid culture after 24 hr of incubation with strain ISTPCP-1

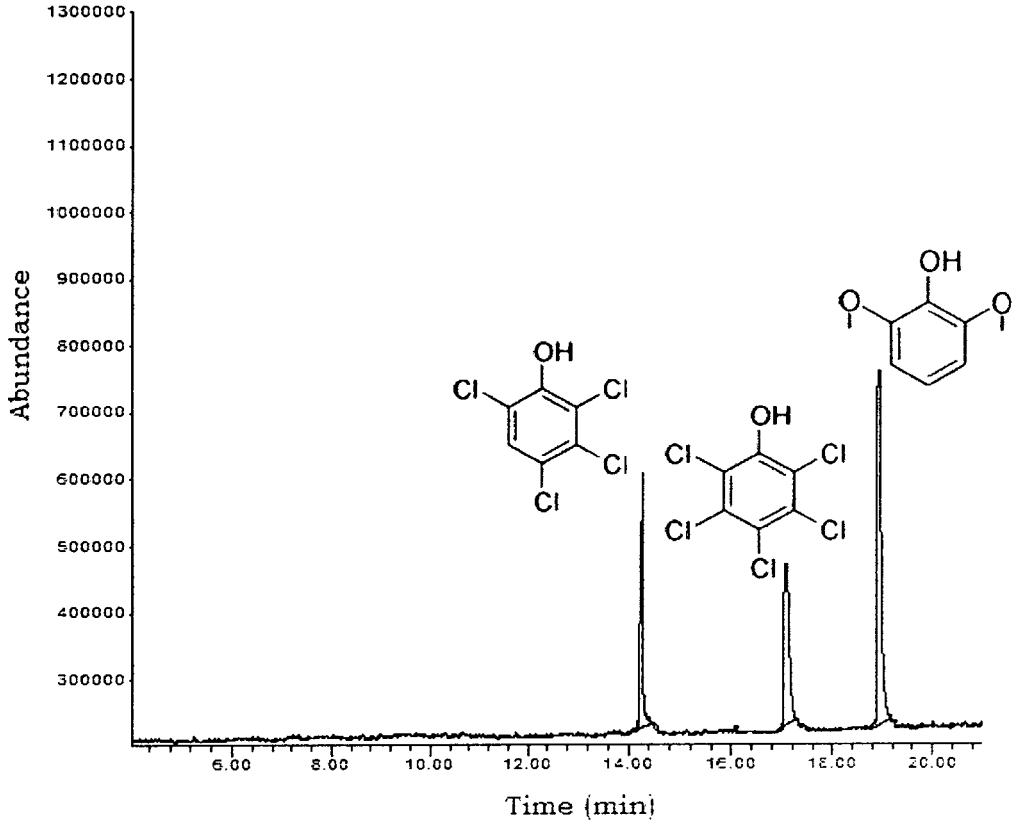

Figure 4.17 Gas chromatogram of extract obtained from the liquid culture after 48 hr of incubation with strain ISTPCP-1

Figures 4.18, 4.19 and 4.20 shows PCP control, degradation products and peaks of metabolites produced by *Pseudomonas aeruginosa* ISTPCP-2. In control sample (0 hr) single peak of PCP (RT = 17.2 min) was observed (Figure 4.18). No other peaks of metabolites were observed. Single new peak was observed at 24 hr, the peak was identified as 2,3,5,6-tetrachlorohydroquinone (RT = 18.5 min). The peak area (concentration) of PCP was observed to be decreased (Figure 4.19). At 48 hr another peak of metabolism was observed, the peak was identified as 2,3,5-trichlorophenol (RT = 11.1 min). The PCP peak area was further reduced at 48 hr (Figure 4.20). No other peaks of metabolites were observed at these time intervals. Heat killed cells did not show any decrease in PCP concentration and no degradation products were observed in the chromatogram.

Figures 4.21, 4.22 and 4.23 shows PCP control, degradation products and peaks of metabolites produced by *Acinetobacter* sp. ISTPCP-3. In control sample (0 hr) single peak of PCP (RT = 17.2 min) was observed (Figure 4.21). No other peaks of metabolites were observed. Single new peak was observed at 24 hr, the peak was identified as 2,3,5,6-tetrachlorohydroquinone (RT = 18.5 min). The peak area (concentration) of PCP was observed to be decreased (Figure 4.22). At 48 hr another peak of metabolism was observed, the peak was identified as 2-chloro-1,4-benzenediol (RT = 9.8 min). The PCP peak area was further reduced at 48 hr (Figure 4.23). No other peaks of metabolites were observed at these time intervals. Heat killed cells did not show any decrease in PCP concentration and no degradation products were observed in the chromatogram. At 48 hr about 95% of PCP was degraded indicating transformation of PCP into low molecular weight products. The selected mass-to-charge fragmentation pattern and relative intensity of various peaks of metabolism are shown in Table 4.2 (underivatized) and Table 4.3 (derivatized).

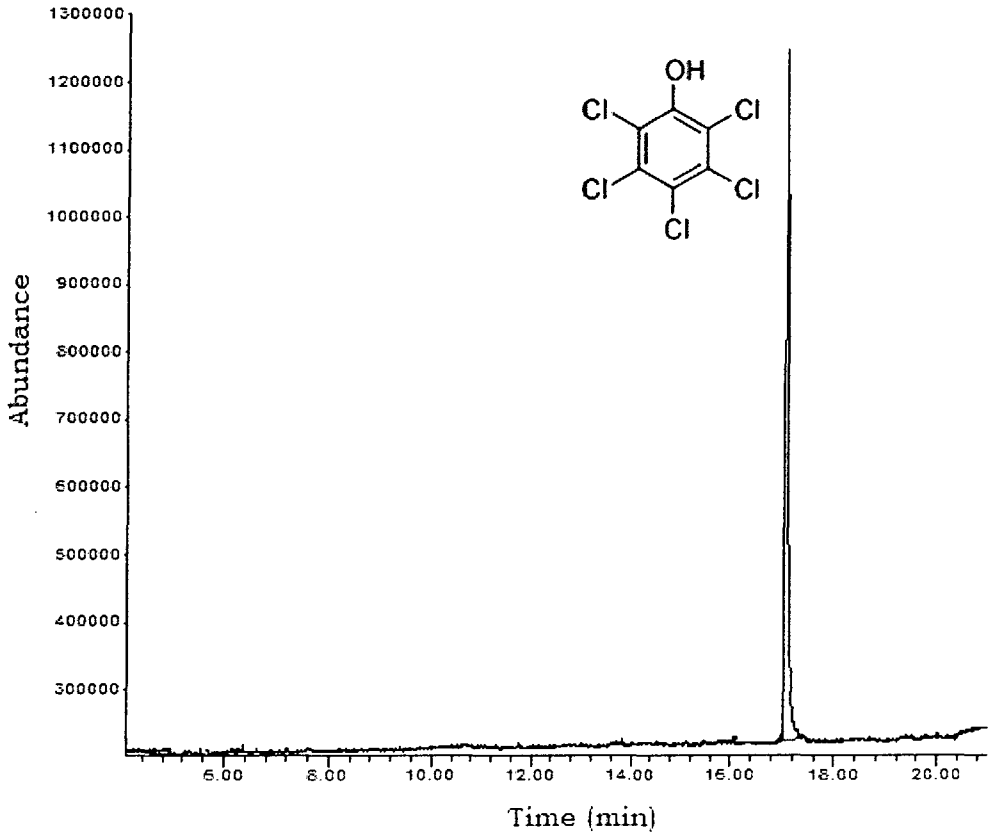

Figure 4.18 Gas chromatogram of extract obtained from the liquid culture at 0 hr of incubation with strain ISTPCP-2

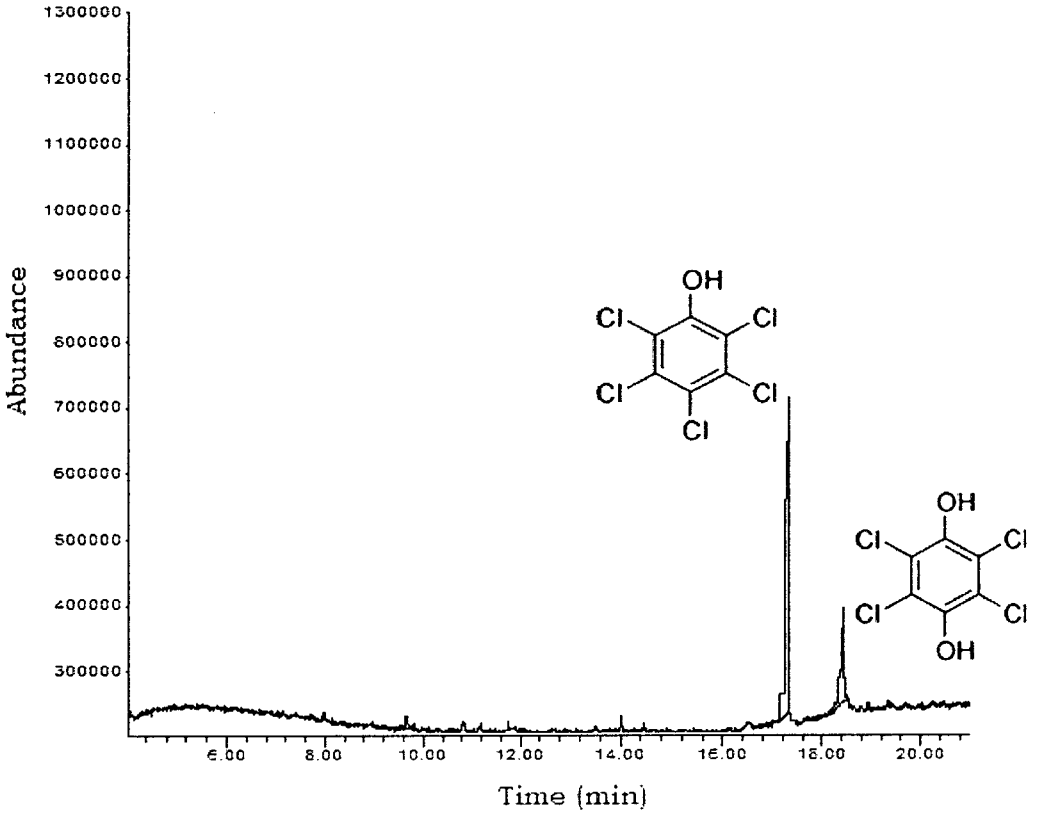

Figure 4.19 Gas chromatogram of extract obtained from the liquid culture after 24 hr of incubation with strain ISTPCP-2

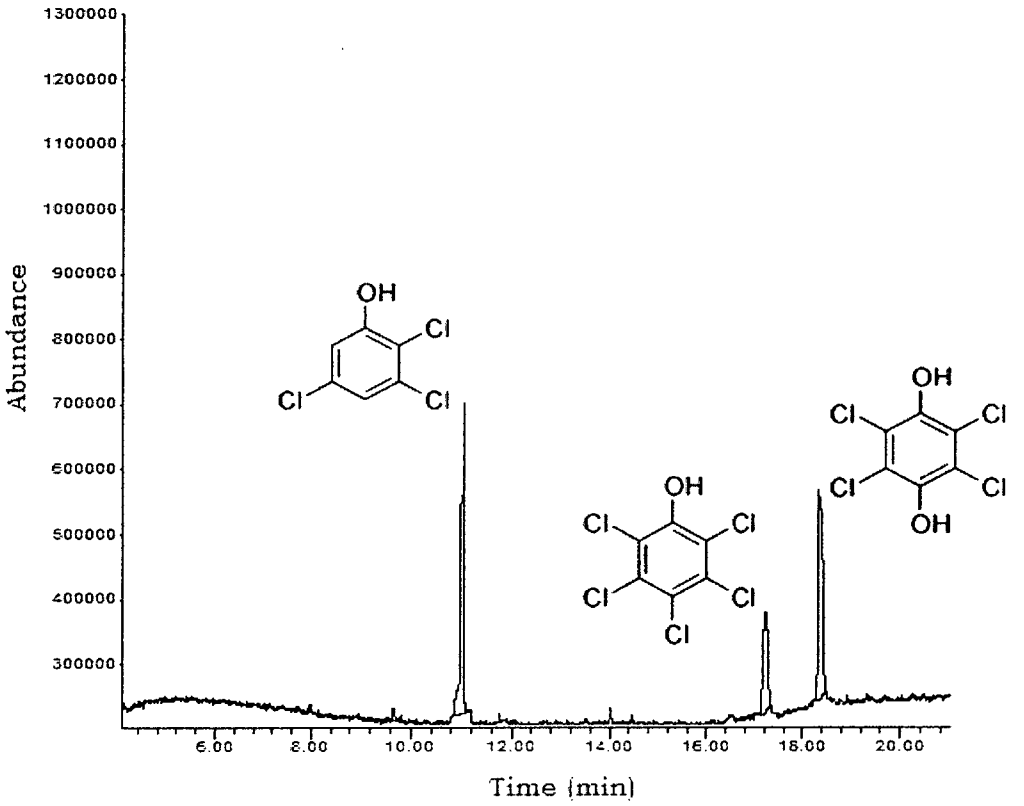

Figure 4.20 Gas chromatogram of extract obtained from the liquid culture after 48 hr of incubation with strain ISTPCP-2

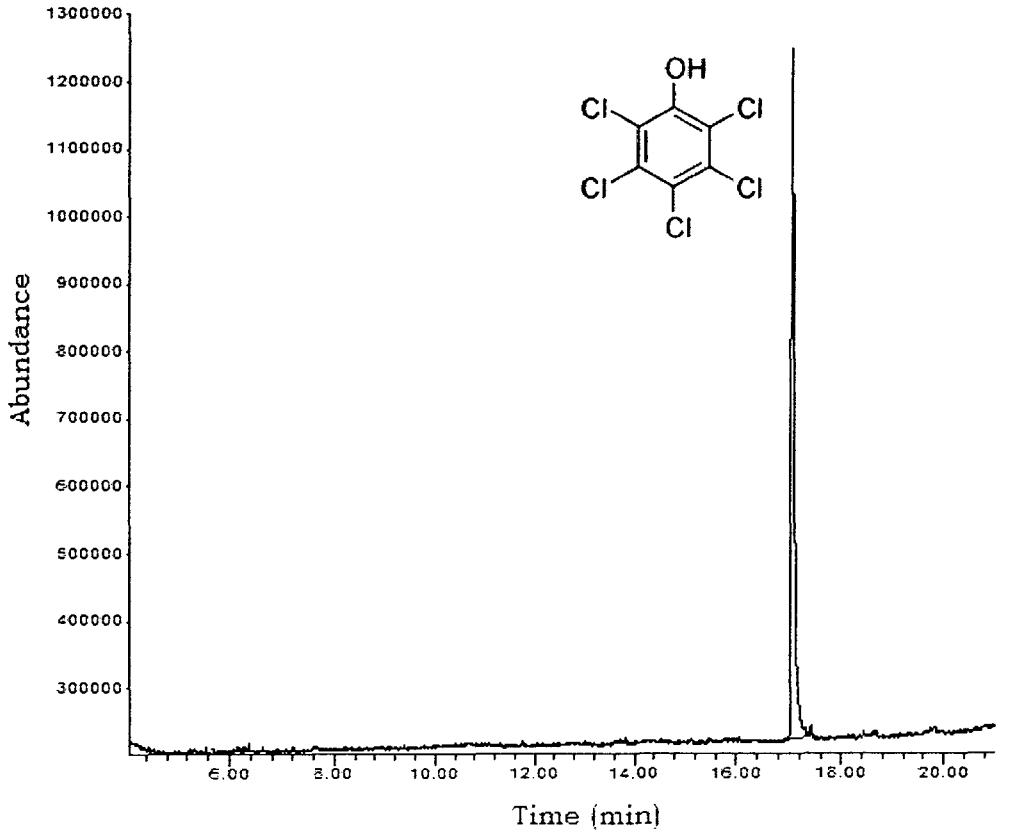

Figure 4.21 Gas chromatogram of extract obtained from the liquid culture at 0 hr of incubation with strain ISTPCP-3

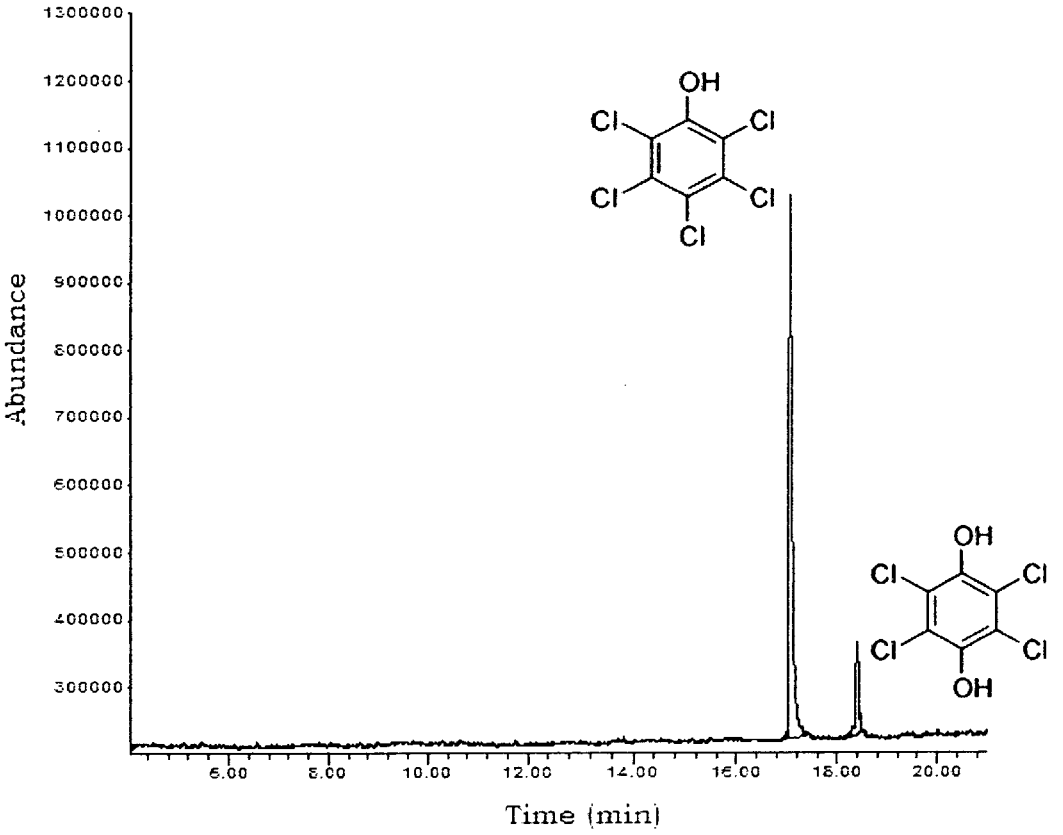

Figure 4.22 Gas chromatogram of extract obtained from the liquid culture after 24 hr of incubation with strain ISTPCP-3

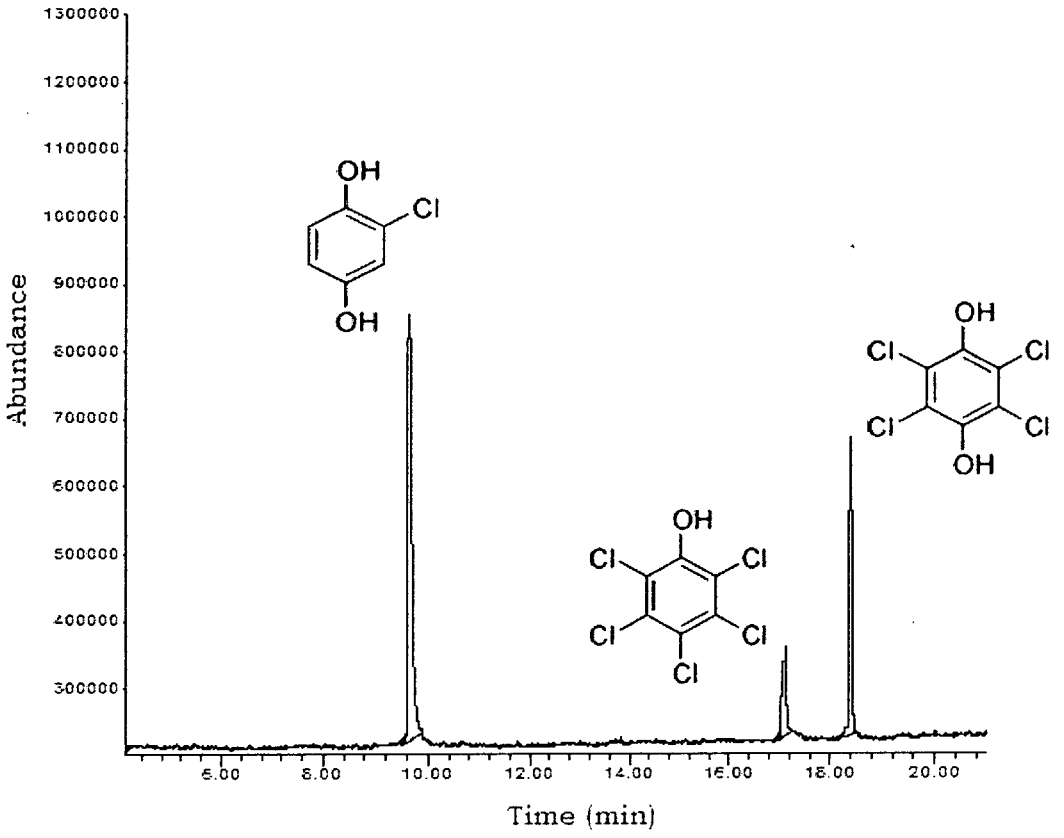

Figure 4.23 Gas chromatogram of extract obtained from the liquid culture after 48 hr of incubation with strain ISTPCP-3

Table 4.2 Molecular weight and mass spectral characteristic ion of major compounds

Compound	MW	Selected fragments m/z (relative intensity)
2-chloro-1,4-benzenediol	144	144 (100) 80 (39) 52 (43)
2,3,5-trichlorophenol	196	196 (100) 160 (20) 132 (57) 97 (65) 73 (17) 62 (40) 48 (36) 37 (22)
2,3,4,6-tetrachlorophenol	232	232 (100) 194 (21) 166 (43) 131 (62) 96 (24) 83 (21) 65 (24) 48 (11)
2,3,4,5,6-pentachlorophenol	266	266 (100) 230 (21) 202 (29) 165 (52) 130 (30) 95 (35)
2,3,5,6-tetrachlorohydroquinone	248	248 (100) 222 (18) 182 (16) 147 (31) 87 (50) 53 (12)
2,6-dimethoxy phenol	154	154 (100) 125 (48) 111 (12) 94 (18) 69 (21)

Table 4.3 Molecular weight and mass spectral characteristic fragmentation of trimethylsilyl derivative compounds

Compound	MW	Selected fragments m/z (relative intensity)
2-chloro-1,4-benzenediol	288	257 (100) 147 (52) 123 (10) 95 (18) 73 (52)
2,3,5-trichlorophenol	268	268(11) 253(49) 255(49) 253(49) 268(11)
2,3,4,6-tetrachlorophenol	302	287(78) 289(100) 287(78) 93(98)
2,3,4,5,6-pentachlorophenol	336	321(46) 323(72) 325(47) 93(100)
2,3,5,6-tetrachlorohydroquinone	390	392 (100) 390 (61) 340 (42) 267 (30)
2,6-dimethoxy phenol	286	271 (53) 169 (49) 153 (26) 147 (37) 143 (41) 73 (100)

Structures of metabolites produced were further confirmed by ^{1}H NMR spectroscopy. The NMR results attested the results obtained by GC-MS. Standard ^{1}H NMR proton shift of PCP and its major degradation products are given in Table 4.4. We report here; novel degradation pathways followed by individual isolated bacterial strains (Figure 4.24). In case of pathway followed by strain ISTPCP-3, PCP was initially converted to 2,3,5,6-tetrachlorohydroquinone, which was further converted to 2-chloro-1,4-benzenediol, this acts as a ring cleavage precursor for the enzyme. Strain ISTPCP-2, converted PCP to 2,3,5,6-tetrachlorohydroquinone, subsequent chlorophenol such as trichlorophenol was obtained, clearly indicating degradation through chlorophenol pathway. However, in case of degradation with strain ISTPCP-1, PCP was initially converted to 2,3,4,6-tetrachlorophenol, which was converted to 2,6-dimethoxy phenol, no intermediary products of degradation were detected in-between the two metabolites.

Table 4.4 ^{1}H NMR of pentachlorophenol and its major degradation products

Compound	Node	Shift	Base	Comment
chlorohydroquinone	CH	6.57	7.26	1–benzene
(2-chloro-1,4-benzenediol)			-0.17	1–O
			0.01	1–Cl
			-0.53	1–O
	CH	6.44	7.26	1–benzene
			-0.17	1–O
			-0.12	1–Cl
			-0.53	1–O
	CH	6.50	7.26	1–benzene
			-0.53	1–O
			-0.06	1–Cl
			-0.17	1–O
	OH	5.0	5.00	aromatic C–OH
	OH	5.0	5.00	aromatic C–OH
trichlorophenol	CH	6.92	7.26	1–benzene
			-0.17	1–O
			-0.12	1–Cl
			-0.06	1–Cl
			0.01	1–Cl
	CH	6.49	7.26	1–benzene
			-0.53	1–O
			-0.06	1–Cl
			-0.12	1–Cl
			-0.06	1–Cl
	OH	5.0	5.00	aromatic C–OH
tetrachlorophenol	CH	6.50	7.26	1–benzene
			-0.53	1–O
			-0.06	1–Cl
			-0.12	1–Cl
			-0.06	1–Cl
			0.01	1–Cl
	OH	5.0	5.00	aromatic C–OH
pentachlorophenol	OH	5.0	5.00	aromatic C–OH
tetrachlorohydroquinone	OH	5.0	5.00	aromatic C–OH
	OH	5.0	5.00	aromatic C–OH

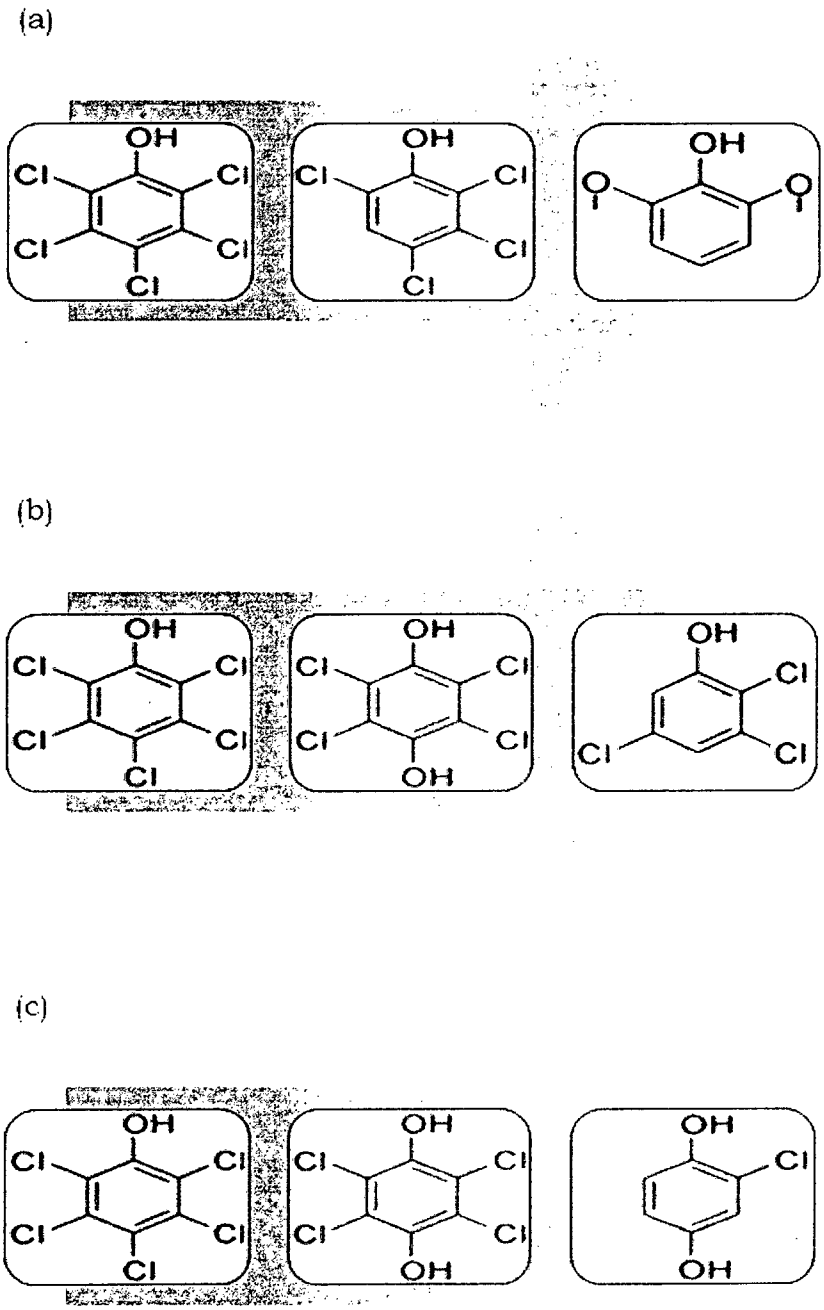

Figure 4.24 Proposed metabolic pathways for PCP degradation by bacterial strains; (a), ISTPCP-1; (b), ISTPCP-2; (c), ISTPCP-3

4.4 Discussion

The PCP biodegradation pathway has been studied extensively using soil and aquatic bacteria such as *Sphingobium chlorophenolicum*, *Rhodococcus chlorophenolicus*, *Arthrobacter* spp., *Pseudomonas* spp. by various researchers in anaerobic and aerobic environments (Edgehill and Finn, 1983; Crawford and Mohn, 1985; Saber and Crawford, 1985; Xun and Orser, 1991c; Orser *et al.*, 1993; Edgehill, 1994; McAllister *et al.*, 1996; Miethling and Karlson, 1996; Chanama and Crawford, 1997; Wang *et al.*, 1998; Leung *et al.*, 1999; Vallecillo *et al.*, 1999; Tartakovsky *et al.*, 2001; Thakur *et al.*, 2001; Thakur *et al.*, 2002; Yang *et al.*, 2006; Dams *et al.*, 2007). These bacteria have evolved pathways to degrade PCP and use the ring-cleavage products of PCP as their source of carbon and energy. So far, two types of pathways for aerobic degradation of PCP have been described; one is through formation of chloro-catechols and other is through formation of subsequent hydroquinone. In pathway via chloro-catechols, the subsequent chlorophenols formed are further metabolized via *ortho* or modified-*ortho* ring cleavage pathways (Thakur *et al.*, 2002). In the hydroquinone pathway, subsequent dechlorination leads to formation of hydroquinone, which is subsequently cleaved by *ortho* ring cleavage enzyme (Chanama and Crawford, 1997; Hu *et al.*, 2006).

According to earlier reports of PCP degradation pathway in bacteria (Crawford and Mohn, 1985; Xun and Orser, 1991c; Ohtsubo *et al.*, 1999; Cai and Xun, 2002), contains five catalytic enzymes, which are responsible for its mineralization. The bacterial enzyme PCP 4-monooxygenase catalyzes the oxygenolytic removal of the first chlorine from PCP to tetrachlorohydroquinone using nicotinamide adenine dinucleotide phosphate (NADPH) as a co-substrate. However recent reports showed the enzyme converted PCP to tetrachlorobenzoquinone rather than tetrachlorohydroquinone (Chen and Yang, 2008). In the present study, no such product of metabolism

(tetrachlorobenzoquinone) from PCP was detected. It could be possible that the gene encoding for enzyme responsible for tetrachlorobenzoquinone production, is present in specific bacterium such as *Sphingobium* spp. Tetrachlorohydroquinone is further converted trichlorohydroquinone by reductive dechlorination and subsequently to dichlorohydroquinone was formed by reductive dehalogenase enzyme. Dichlorohydroquinone acts as a precursor for ring-cleavage enzyme, which converts it to chloromaleylacetate, an open ring structure.

The purposes of this study were to optimize the physiological growth characteristics and elucidation of pathways followed by individual bacterial strains for the degradation of PCP. This study has shown transformation of PCP in a batch culture by microbial population isolated from effluent discharge site. The members of the bacterial consortium were previously identified as *Enterobacter* sp. ISTPCP-1, *Pseudomonas aeruginosa* ISTPCP-2 and *Acinetobacter* sp. ISTPCP-3. Strain ISTPCP-3 showed good growth on various carbon sources, strains ISTPCP-2 and ISTPCP-1 were able to show only satisfactory growth. No strain was able to grow on highly toxic dibenzofuran compound. The results demonstrated that, the individual pure strains have higher potential for PCP degradation compared to the acclimated mixed culture of bacteria. However, at very high PCP concentration the strains showed an extended lag phase in their growth. The results related to optimization of growth parameters for degradation of PCP revealed that all the three strains were mesophilic and showed maximum degradation at neutral pH. The strain ISTPCP-3 was able grow well at high initial PCP concentration, followed by ISTPCP-2 and ISTPCP-1. Of the three isolated bacterial strain, *Acinetobacter* sp. ISTPCP-3 manifested highest PCP degradation capability followed by *Pseudomonas aeruginosa* ISTPCP-2 and *Enterobacter* sp. ISTPCP-1. Strain ISTPCP-1 exhibited decreased ability to degrade PCP. All three acclimated

bacterial strains followed novel pathways for degradation of PCP. The reported pathways are different than previously characterized pathways for PCP degradation. The results of the study clearly indicate the nature of pathway followed by individual bacterial strains. ISTPCP-2 and ISTPCP-3 were able to degrade PCP via hydroquinone pathway. However, detection of tetrachlorophenol as a metabolite of ISTPCP-1 also indicates a novel degradation pathway followed by the strain in aerobic condition. Results clearly indicated significance of strains ISTPCP-3 and ISTPCP-2 in degradation of PCP and acted as a potential member of the consortium, however, the strain ISTPCP-1 may have played as associated members of the consortium in degradation of PCP. The relationship between the structural and functional properties of bacterial strains in PCP transformation provided in this study may provide insight about potential enrichment of *in situ* microbial population.

PURIFICATION AND CHARACTERIZATION OF THE MONOOXYGENASE ENZYME INVOLVED IN THE BIODEGRADATION OF PENTACHLOROPHENOL

CHAPTER 5

PURIFICATION AND CHARACTERIZATION OF THE MONOOXYGENASE ENZYME INVOLVED IN THE BIODEGRADATION OF PENTACHLOROPHENOL

5.1 Introduction

Polychlorinated phenols such as the trichlorophenols, the tetrachlorophenols, and pentachlorophenol have been used extensively since the 1920s as preservatives to prevent fungal attack on timber and wood (Colosio *et al.*, 1993). PCP was first introduced as a pesticide in 1936 (Cline *et al.*, 1989) and is not known to be a natural product. It has been listed as a priority contaminant by USEPA and EU (Wild *et al.*, 1992; Bock *et al.*, 1996; Kovács *et al.*, 2008). It is still being used in many countries including India, as a biocide for prevention of fungal and slime deterioration of timber and leather. PCP may be washed into streams and lakes due to surface runoff; its large amount is finally deposited in sediments and soil. It is highly toxic to both humans and animals. Extensive exposure to PCP could cause cancer, acute pancreatitis, immunodeficiency and neurological disorders (Sai *et al.*, 2001).

Despite its recent introduction into the environment, its high toxicity and its resistance to its biodegradation due to high and obstructive halogenation, a number of soil and aquatic bacteria such as *Sphingobium chlorophenolicum* (previously *Sphingomonas chlorophenolica*), have evolved pathways to degrade PCP and use the ring-cleavage products of PCP as their carbon source (Xun and Orser, 1991c; Xun *et al.*, 1992a; Xun *et al.*, 1992c; McCarthy *et al.*, 1997;

Miyauchi *et al.*, 1999; Copley, 2000). The best studied of these are strains ATCC 39723 (Orser and Lange, 1994), RA-2 (Radehaus and Schmidt, 1992) and UG30 (Cassidy *et al.*, 1999). It appears that *S. chlorophenolicum* has assembled a new metabolic pathway capable of converting this anthropogenic compound into recognizable metabolites. Previous studies of Copley, 2000 have suggested that the PCP degradation pathway has been assembled by patching together enzymes from at least two different metabolic pathways.

Despite the isolation of many PCP degrading bacteria, a pathway for degradation of PCP is known in detail only for *S. chlorophenolica* ATCC 39723. This strain was originally isolated by Saber and Crawford before the era of 16S rDNA phylogeny and was identified at the time as a strain of *Flavobacterium* (Saber and Crawford, 1985). In the years since the original isolations of ATCC 39723 and other closely related PCP-degrading bacterial strains and adoption of 16S rDNA phylogeny for bacterial classification, the genus *Sphingomonas* has been divided into multiple genera (Takeuchi *et al.*, 2001). *Sphingomonas chlorophenolica* (Karlson *et al.*, 1996; Ederer *et al.*, 1997) is now classified as a member of the genus *Sphingobium*. The rate-limiting step for PCP degradation in *Sphingobium chlorophenolicum* ATCC 39723 appears to be the *para*-hydroxylation of PCP to tetrachlorohydroquinone (McCarthy *et al.*, 1997). This step is catalyzed by the enzyme PCP 4-monooxygenase (EC 1.14.13.50), encoded by the *pcpB* gene (Orser *et al.*, 1993) and works in concert with a tetrachlorobenzoquinone reductase encoded by *pcpD* gene (Dai *et al.*, 2003). In addition to PCP, the PCP 4-monooxygenase can use trichlorophenols, tetrachlorophenols, and several other halogenated phenols as substrates (Xun *et al.*, 1992a, b, c). The PCP catabolic pathway of *Sphingobium chlorophenolicum* ATCC 39723 is shown in Figure 5.1. In addition to PcpB, several of the other enzymes in the PCP pathway have now been characterized. Tetrachlorohydroquinone reductive dehalogenase (PcpC; Figure 5.1) has been isolated and

characterized from *Sphingobium chlorophenolicum* ATCC 39723 (Xun *et al.*, 1992c; Anandarajah *et al.*, 2000) and *Sphingomonas* sp. UG30 (Habash *et al.*, 2002).

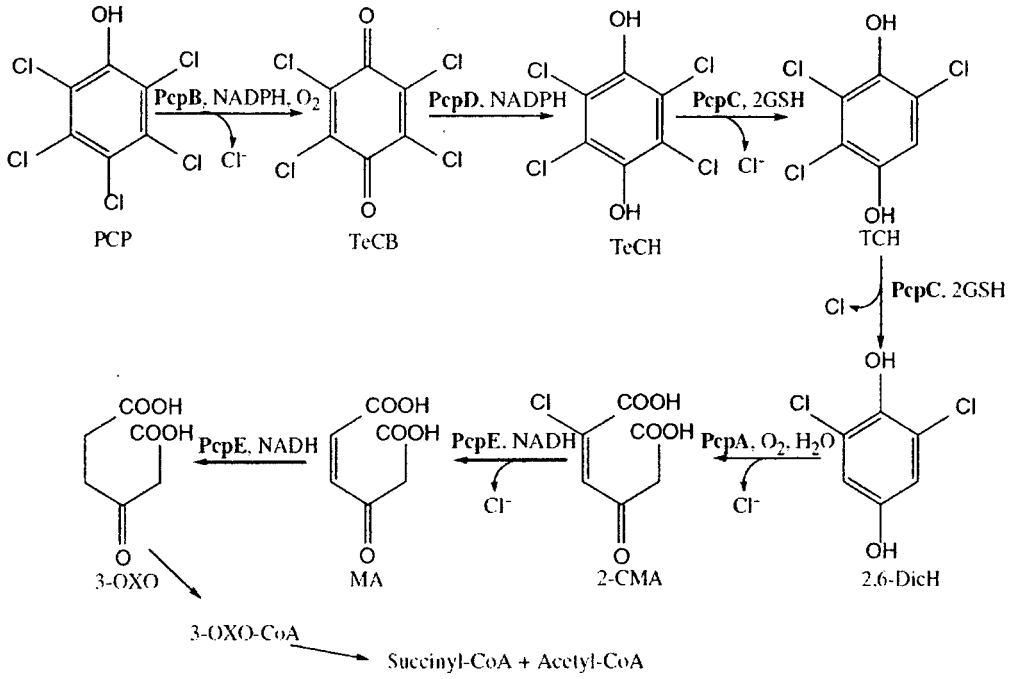

Figure 5.1 The PCP biodegradation pathway in *S. chlorophenolicum* ATCC 39723. PcpB, PCP hydroxylase; PcpD, TCBQ reductase; PcpC, TCHQ dehalogenase; GSH, glutathione; PcpA, 2,6-DCHQ dioxygenase; PcpE, MA reductase; PCP, pentachlorophenol; TeCB, tetrachloro-*p*-benzoquinone; TeCH, tetrachloro-*p*-hydroquinone; TCH, 2,3,6-trichloro-*p*-hydroquinone; 2,6- DiCH, 2,6-dichloro-*p*-hydroquinone; 2-ClMA, 2-chloromaleylacetic acid; MA, maleylacetic acid; 3-OXO, 3-oxoadipic acid (Cai and Xun, 2002; Dai and Copley, 2004)

PcpC is a member of the glutathione transferases that are widely found in both prokaryotes and eukaryotes. Though the UG30 PcpC shares 94% primary sequence identity with the PcpC from *S. chlorophenolicum* ATCC 39723, there are significant differences between the two enzymes in some of their functional and kinetic properties (Habash *et al.*, 2002). 2,6-Dichloro-*p*-hydroquinone 1,2-dioxygenase (PcpA) has been purified from *Sphingobium* (formerly *Sphingomonas*) *chlorophenolicum* ATCC 39723 (Xun and Orser, 1991b; Ohtsubo *et al.*, 1999; Xun *et al.*, 1999). PcpA shows novel Fe^{2+} and O_2-dependent ring-cleavage dioxygenase activity against hydroquinone derivatives and within the PCP pathway converts 2,6-DCHQ to 2-chloromaleylacetate (Figure 5.1). In this study, purification and characterization of PCP 4-monooxygenase, that catalyses the conversion of PCP to TeCH from a novel and highly efficient PCP degrading bacterium *Acinetobacter* sp. ISTPCP-3 has been reported.

5.2 Materials and methods

5.2.1 Bacterium and culture conditions

Out of previously enriched and characterized three bacterial strains (ISTPCP-1, ISTPCP-2 and ISTPCP-3), *Acinetobacter* sp. ISTPCP-3 that manifested high PCP-degrading capability was selected for this work. The strain was cultured in MSM containing PCP (100 mg/l) as sole source of carbon and energy. PCP degradation was induced by the addition of PCP at early log phase of growth as previously described. All chemicals were purchased from Sigma–Aldrich Chemicals. The stock solution of PCP (10 mg/ml) was prepared in 0.1 M NaOH and stored in dark at room temperature. TeCH stock solution (10 mg/ml) was prepared in absolute ethanol and stored at 4°C. The NADPH solution (10 mM) was freshly prepared and contained 1 mM dithiothrietol in 1 mM tris–base (pH 13).

5.2.2 Enzyme purification

All operations were performed at 4°C, and the buffer was 20 mM Tris hydrochloride (pH 8.0) unless mentioned otherwise. When ammonium sulfate was added, the buffer also contained 1 mM EDTA. Ammonium sulfate saturation levels referred to a temperature of 25°C. The major steps included are extraction of cells, fractionations of supernatants followed by chromatographic separations.

5.2.2.1 Extraction of cells

The bacterial strain ISTPCP-3 was inoculated in MSM containing PCP as carbon source. After 12 hr, growth culture (50 ml) was removed from the growth medium. The extracted culture was centrifuged at 12, 000*g* (4°C) for 10 min. The cell pellet so obtained was suspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 25% sucrose, 0.5 mM PMSF and 40 mg lysozyme) and incubated with gentle rotation for 20 min. The cells were disrupted in an ultrasonic bath (sonication) using a Sonifer™ 150 sonicator. The cell lysate was centrifuged at 24, 000*g* for 20 min, and the supernatant was saved. The precipitate was resuspended in the buffer, was sonicated twice, and centrifuged as described above. The supernatant was combined with the previous supernatant, and the precipitate was discarded. The supernatant was used as the crude enzyme source for all the assays.

5.2.2.2 Fractionation

For protamine sulfate fractionation, 2% solution of protamine sulfate in 20 mM Tris buffer (pH 8.0) was added to the supernatant slowly with constant stirring to a final concentration of 0.1 mg/ml. After 5 min of stirring, the mixture was centrifuged at 17, 000*g* for 10 min, and the precipitate was discarded. The preserved supernatant was further fractionated with ammonium sulfate. Solid ammonium sulfate was added to the supernatant to 40% saturation with constant stirring. The pH of the solution was not adjusted. After 10 min of stirring, the mixture was centrifuged (17,000*g* for 10 min), and the precipitate was discarded. Additional solid ammonium sulfate was

added to 60% saturation with constant stirring. After 10 min of stirring, the mixture was centrifuged (17,000*g* for 10 min). The precipitate was saved, and the supernatant was discarded.

5.2.2.3 Chromatography

5.2.2.3.1 Phenyl agarose

Phenyl agarose chromatography was performed by suspending the precipitate with an equal volume of buffer. The suspension was centrifuged (17,000*g* for 10 min), and the precipitate was discarded. The supernatant was loaded onto a phenyl agarose (Sigma) column (30 cm × 1.5 cm) previously equilibrated with buffer containing 10% ammonium sulfate. The enzyme was eluted with 300 ml of a 10-to-0% ammonium sulfate gradient. The enzyme eluted at around 5% ammonium sulfate. The fractions containing enzyme activity were pooled and precipitated by adding ammonium sulfate to 70% saturation. The precipitate was collected by centrifugation (17,000*g* for 10 min).

5.2.2.3.2 Mono Q

For Mono Q chromatography, the proteins were resuspended in 3 ml of 20 mM Tris–HCl buffer (Ph 8.0) and dialyzed against 1 liter of the same buffer for 1 h. The protein solution was injected onto a Mono Q HR 5/5 column (Pharmacia) equilibrated with 20 mM Tris buffer. Proteins were eluted with a step-and-linear gradient of sodium chloride (percentage of 1 M NaCl in volume of 20 mM Tris: 0%, 2 ml; 35%, 4 ml; 35 to 55%, 9-ml linear gradient; 100%, 4 ml; 0%, 2 ml) by a Pharmacia fast protein liquid chromatography system (Pharmacia LKB Programmer GP-250 Plus and P-500 pump). A single PCP hydroxylase activity peak was associated with a single protein peak around a NaCl concentration of 0.45 M. The fractions containing enzyme activity were pooled and precipitated by adding ammonium sulfate to 75% saturation. The precipitate was collected by centrifugation (17,000*g* for 10 min).

5.2.2.3.3 Gel filtration

The protein was further purified using gel filtration chromatography. The protein precipitate was dissolved with an equal volume of buffer and injected onto a Superose–6 column (Pharmacia) equilibrated with 20 mM Tris buffer containing 100 mM NaCl. The enzyme was eluted with the same buffer by the Pharmacia fast protein liquid chromatography (FPLC) system. PCP 4-monooxygenase activity was associated with a major protein peak with a retention volume of 19 ml. The fractions containing the enzymatic activity were pooled and precipitated by adding ammonium sulfate to 75% saturation. The precipitate was collected by centrifugation (17,000*g* for 10 min).

5.2.2.4 Optimization of pH and temperature

PCP 4-monooxygenase activity was measured at various pH values within the range of 6.5 to 8.5 by using 100 mM potassium phosphate (KP_i) buffer (pH 6.5 to 8.5) in a total volume of 100 µl. The enzyme reaction mixture was otherwise the same as defined above for the enzyme assay. The temperature optimum for the enzyme activity was determined in a similar way in KP_i buffer (pH 7.5). The reaction mixture without NADPH was incubated at the corresponding temperature for 4 min, and then 1 µl of 10 mM NADPH solution was added to the reaction mixture to start the reaction.

5.2.3 Analytical methods

Decrease in the concentration of PCP and increase in the concentration of TeCH was measured using mass spectrometric analysis.

5.2.3.1 SDS–PAGE

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed by the method of Laemmli, (1970). In this method acrylamide gel (10%) was used, and electrophoresis was performed. Gels were stained for protein with Coomassie Brilliant blue R-250. Total protein was measured using Bradford method (Bradford, 1976).

5.2.3.2 MALDI–TOF and MS

The protein sample was further characterized using matrix assisted LASER desorption ionization–time of flight (MALDI–TOF) mass spectrometric analysis. The protein spots of SDS–PAGE were excised from the gel and digested with trypsin. In this case samples were digested with 200 ng trypsin in 50 mM NH_4HCO_3 at 37°C overnight, vacuum dried in a Savant SVC100 Speed Vac, desalted using C_{18} Omix microextraction column tips (Varian, Palo Alto, CA), and mixed with matrix solution (~1.5 µl) consisting of 10 mg/ml of alpha-cyano-4-hydroxy-cinnamic acid (CHCA) in 50% acetonitrile and 0.1% trifluoroacetic acid. The samples were mixed well and spotted onto an AnchorChip target plate (Bruker Daltonics Ltd, Coventry, UK) keeping the droplet centered on the anchor spot (400 µmol/l target selected). This was allowed to dry, and MALDI mass spectra were obtained using a Biflex IV MALDI–TOF MS (Brucker Daltonics) with a nitrogen laser at 337 nm following routine calibration. The singly charged peptide fingerprint was assigned mono-isotopic peptide masses using Biotools software (Brucker Daltonics). These data were then used to search the NCBI non-identical protein sequence database using MASCOT software (Matrix Science), and statistically significant hits were recorded together with the number of peptides and percentage coverage of the protein. Finally, each of the peptides was used to BLAST search to confirm that the protein 10 identified by MASCOT was the only relevant match in the non-redundant protein database for a particular peptide sequence. Peptide sequences of MALDI–TOF MS spectra were analyzed using the above program compared with the NCBI data base for placement of enzyme with known sequences (Altschul *et al.*, 1997; Thompson *et al.*, 1997).

5.2.4 Enzyme assay

All experiments in the current study were carried out in triplicate. PCP 4-monooxygenase activity was assayed spectrophotometrically by monitoring the decrease of UV absorption by following the substrate-

dependent oxidation of NADPH at 340 nm. The enzyme activity was estimated at 25°C. The standard assay system contained (final concentration) 50 mM KP_i or Tris acetate buffer (pH 7.5), 4 µM FAD, 0.2 mM NADPH solution and 5–50 µl enzyme/ml. The reaction was initiated by the addition of PCP to give a final concentration of 0.1 mM. The UV absorption was taken at 0.5 min intervals for 5 min (Varian, USA). The UV path length was measured to be 0.71 cm. One unit of enzyme activity was defined as the substrate-dependent oxidation of 1 µmol of NAD(P)H per min. The extinction coefficient of NADPH (6220/M/cm) was used to calculate the specific activity of the enzyme. The products of the reaction were also extracted and analyzed GC–MS (details previously described).

5.2.5 Enzyme kinetics

The *Michaelis–Menten* or allosteric kinetics parameters were determined in 80 mM KP_i buffer, (pH 7.5) with 0.5% Tween 20. The final volume of the reaction mixture was 50 µl with 15 µg of purified enzyme. The values of K_m and V_{max} for PCP were determined with a fixed concentration of NADPH (100 µM) and a variable concentration of PCP (from 20 to 50 µM). The values of K_m and V_{max} for NADPH and NADH were determined with a fixed concentration of PCP (100 µM) and a variable concentration of NADPH (from 20 to 80 µM) or NADH (from 40 to 100 µM). The initial velocity of the reactions remained constant for the first 5 min of the reaction over the range of substrate concentrations tested.

5.3 Results

5.3.1 Enzyme purification

The results of typical enzyme purification are summarized in Table 5.1. The purification scheme, consisting of six steps, resulted in a 115-fold purification of PCP 4-monooxygenase relative to the crude cell extract, the specific activity increased from 0.086 to 14.56 U/mg of protein. Approximately 38.4% of the activity of PCP 4-

monooxygenase in the crude extract was recovered. The maximum absorption was at 281 for TeCH, from UV–visible spectra. No interference of TeCH was observed at the characteristic absorption of 340 nm. Therefore, the enzyme activity was assayed by monitoring the UV absorption of the reaction system at 340 nm.

The elusion profile of various proteins eluted from FPLC Mono Q column is shown in Figure 5.2. The enzymatic assay studied suggested, the nature of activity found in fraction 8 to be a reductase. The activity of PCP 4-monooxygenase was found only in large eluted protein peak located in fractions 18 and 19. The peak of PCP 4-monooxygenase peak was not present when proteins from PCP-uninduced cells were chromatographed using FPLC, indicating the inductive nature of the enzyme by PCP.

Table 5.1 Fractionation and purification of PCP 4-monooxygenase enzyme from *Acinetobacter* sp. ISTPCP-3

Purification step	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity (U/ml)	Total activity (U)	Specific activity (U/mg)	Recovery (%)
Crude extract	52.00	23.80	1,569	8.145	136.53	0.086	100
Protamine sulfate fractionation	54.00	16.42	1,328	6.078	131.60	0.097	100
Ammonium sulfate fractionation	21.00	13.51	860	5.904	120.56	1.985	97.7
Phenyl agarose chromatography	18.50	7.34	110	4.756	118.27	2.67	88
Mono Q chromatography	11.22	4.00	78	3.780	97.52	11.07	51.6
Gel filtration chromatography	8.50	2.80	34	1.980	63.48	14.56	38.4

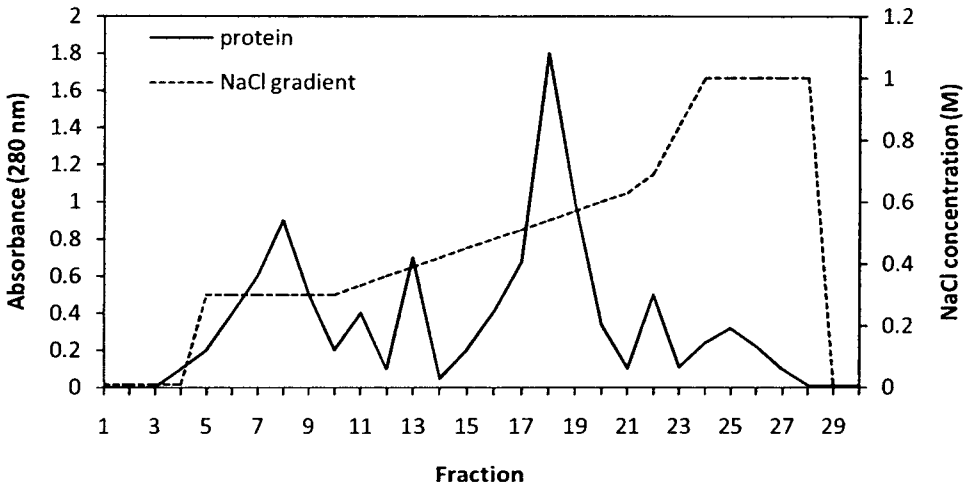

Figure 5.2 Chromatography of PCP 4-monooxygenase on a mono Q column with NaCl linear gradient

5.3.2 Enzyme activity

The enzyme activity of PCP 4-monooxygenase is shown in Figure 5.3. The enzyme completely converted 37 μM PCP to TeCH in 100 mM KP_i buffer (pH 7.0) within 20 min in the presence of 100 μM NADPH. The reaction was confirmed to be enzymatic because controls without enzyme or with boiled enzymes exhibited no change in PCP concentration.

The enzyme used PCP and NADPH as substrates. The results strongly suggest that O_2 also served as a substrate for the enzyme. Without NADPH, PCP was not degraded. Without PCP, NADPH remained virtually unoxidized. When the reaction was conducted in an anaerobic chamber which contained a very small amount of oxygen (<1 ppm [<1 μl/liter]), the rate of TeCH production was slowed significantly. In this case, the reaction was stopped by adding acetic acid to 20%. Only 8 of 37 nmol of PCP was quantitatively converted to TeCH within 1 hr, and no further PCP consumption occurred for another 24 hr; however, when a sample was taken from the anaerobic

chamber after 1 hr and incubated with air, the remaining 29 nmol of PCP was completely converted to TeCH within 25 min. The results indicated that the enzyme was a monooxygenase with a relatively low K_m for oxygen. The quantities of PCP and NADPH utilized by the enzyme were estimated by using various amounts of PCP and NADPH in 80 mM KP_i buffer (pH 7.0). NADPH was used as the limiting substrate and was determined to completely disappear at the end of the reaction time. The NADPH-to-PCP consumption ratio was determined to be exactly 2 under various initial concentrations of NADPH and PCP. The overall reaction was proposed to be the following:

$$2NADPH + 2H^+ + O_2 + PCP \rightarrow 2NADP^+ + H_20 + HCl + TeCH$$

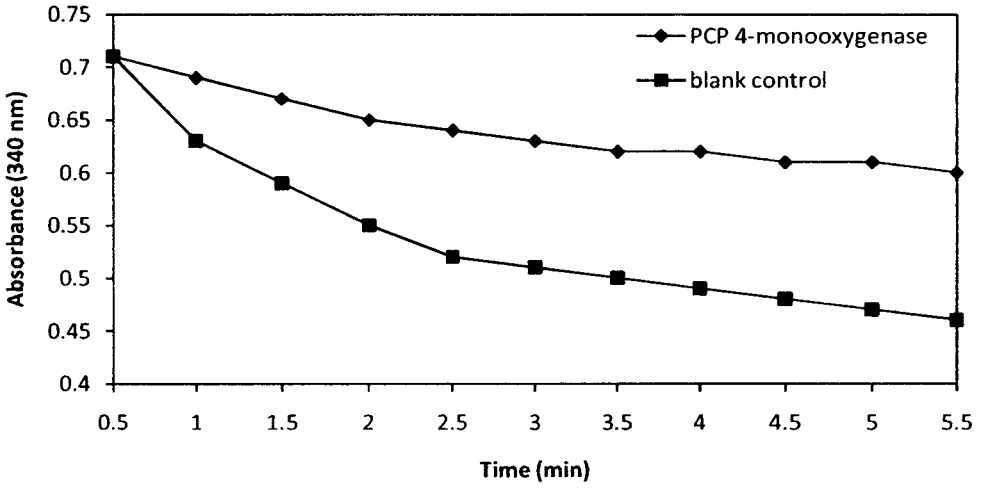

Figure 5.3 Enzyme assay of PCP 4-monooxygenase. As a blank control, boiled enzyme sample was added

5.3.3 Enzyme properties

The molecular weight of the protein was determined using BSA, ovalbumin, chymotrypsin and ribonuclease was found to be 60 kDa. The protein was again pooled and SDS–PAGE was performed. The molecular weight of PCP 4-monooxygenase was estimated to be 30,000 (Figure 5.4). Appearance of a minor band of molecular weight 64,000 along with the major band of molecular weight 30,000 on native gradient page indicated the existence of the protein as a monomer, but also could associate as dimers under native PAGE conditions.

The PCP 4-monooxygenase of bacterial strain, *Acinetobacter* sp. ISTPCP-3, was characterized after excision of SDS-PAGE protein band and data of MALDI–TOF which indicated presence of 14 target peptides (Figure 5.5). Overall, the results of this study demonstrated that the hydrolase enzyme is most easily detectable by peptide mass fingerprinting (PMF) in digested whole extract. The sensitivity of PMF analysis was further investigated in that matrix. Again, the ions corresponding to peptides of the hydrolase were among the most prominent in the mass spectra (Figure 5.5). In MALDI–TOF MS, only a finite number of molecules are actually analyzed by the detector. The mass spectra and Mascot search results presented in the figure illustrated that the simple and inexpensive sample preparation techniques employed-sonication of cells, followed by several purification steps and C_{18} micro-extraction cleanup of digested soluble proteins-were highly effective in reducing baseline noise and improving the overall results of PMF analysis of microorganisms by MALDI–TOF MS.

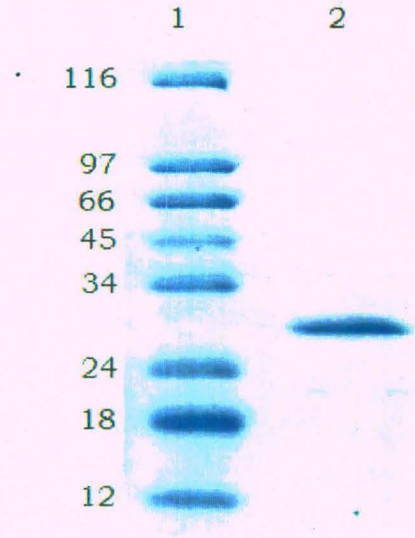

Figure 5.4 SDS–PAGE of purified PCP 4-monooxygenase. Lanes; 1, molecular size markers (kDa); 2, purified enzyme

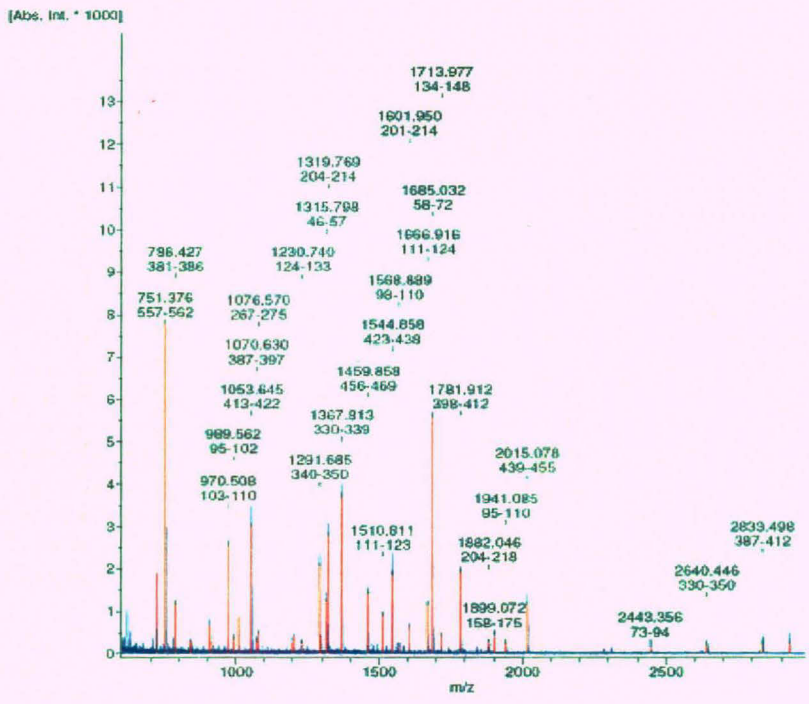

Figure 5.5 MALDI–TOF MS analysis of purified protein of SDS-PAGE obtained by peptide mass fingerprinting

The result suggested that PMF of SDS-PAGE extracts may be a promising technique for the identification and phenotypic characterization of certain microbial pure cultures offering high-abundance proteins composed of peptides with favorable ionization behavior. The result of the amino acids sequence data was compared with known sequences of data bank indicated the homology of peptides of bacterial monooxygenase family.

5.3.4 Optimum growth conditions for enzymatic activity

The growth and biological function of bacteria are significantly influenced by its surrounding pH and temperature. Previous studies have shown the optimum growth parameters for *Acinetobacter* spp. are between 7.0 and 7.4 (pH) and 30°C temperature (Zhao *et al.*, 2009). The reaction mixture (1 ml) contained 30 µg/ml PCP 4-monooxygenase, 1 µmol, 50 µmol PCP and 100 µmol NADPH in 20 mM of the following buffers: acetate buffer, pH 4.0; acetate buffer, pH, 5.0; phosphate buffer, pH 6.0; Tris–HCl buffer, pH 7.0; Tris–HCl buffer, pH 8.0; and TAPS buffer, pH 9.0. Boiled enzyme sample was used as a blank control under each pH. As expected, the optimal pH for PCP 4-monooxygenase activity was indeed around 7.0 (Figure 5.6). The enzyme was less sensitive towards acidic pH than basic pH, maintaining more than 70% of its optimal activity even at pH 5.0. However, the enzyme lost its activity quickly in basic solutions, retaining less than 20% of its optimal activity at pH 8.0.

The optimal temperature of the enzymatic reaction was between 30°C and 35°C. Greater than 70% of the maximum activity was retained at 35°C. The enzymatic activity was completely absent at 50°C. For the purified enzyme, $MgSO_4$ was not required for activity because the addition of $MgSO_4$ or EDTA to 5 mM did not influence the enzymatic activity. The experiments with $MgSO_4$ or EDTA indicate that the enzyme does not require divalent cations for enzymatic activity. Non-ionizing detergents were not required for enzymatic activity for the purified enzyme, as measured by TeCH production.

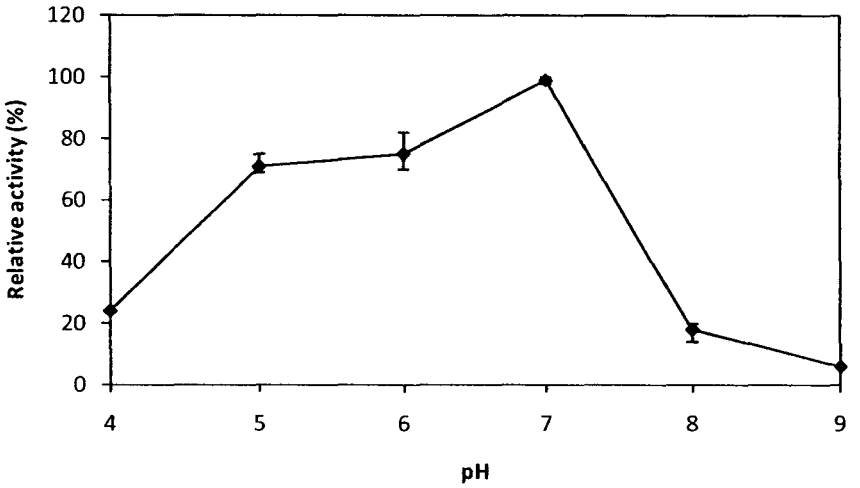

Figure 5.6 The effect of pH on PCP 4-monooxygenase activity

5.3.5 Enzyme kinetics

When PCP was used as substrate, the PCP hydroxylase followed *Michaelis–Menten* kinetics. Values for the *Michaelis–Menten*-type saturation kinetics were determined for PCP, NADPH, and NADP by measuring the initial velocities of TeCH production in 80 mM KP_i (pH 7.0) at 2 min after the start of the reaction at different concentrations of PCP, NADPH, and NADH. K_m and V_{max} values for PCP were 30 ± 7 μM and 15 ± 5 μmol/min/mg of protein, respectively. For NADPH, the K_m was 81 ± 5 μM and the V_{max} was 20 ± 4 μmol/min/mg of protein. NADH, but not dithiothrietol, could replace NADPH as the reducing power, and, for NADH, the K_m was determined to be 215 ± 2 μM and the V_{max} was 30 ± 1 μmol/min/mg of protein. The correlation coefficients (r) for PCP, NADPH, and NADH for the best fit to a straight line were 0.95, 0.97 and 0.99, respectively.

Since product inhibition is a common regulating mechanism observed in enzymes, the activity of PCP 4-monooxygerase by increasing concentration of its catalyzed product (TeCH) was examined. The reaction mixture (200 μl) contained 50 mM KP_i buffer

(pH 7.0), 100 µmol PCP, 1 µmol FAD, 100 µmol NADPH and 7 µg enzyme. The concentration of TeCH was 0 µmol, 50 µmol, 100 µmol and 200 µmol, respectively. Boiled enzyme sample was used as a blank control. Activity of the enzyme under different TeCH concentrations was compared to the activity in the absence of TeCH, which was assigned a relative activity of 100%. Surprisingly, the activity of PCP 4-monooxygenase was stimulated by TeCH at low concentrations but inhibited at high concentrations (Figure 5.7). The activity of enzyme was increased by 40% and 85%, respectively, when TeCH concentration was 50 µmol and 100 µmol in reaction mixture. However, the enzyme was inhibited by almost 65% when the consumption of TeCH reached 200 µmol. The stimulation of PCP 4-monooxygenase by TeCH at low concentrations was possibly through the positive cooperative binding of the substrate.

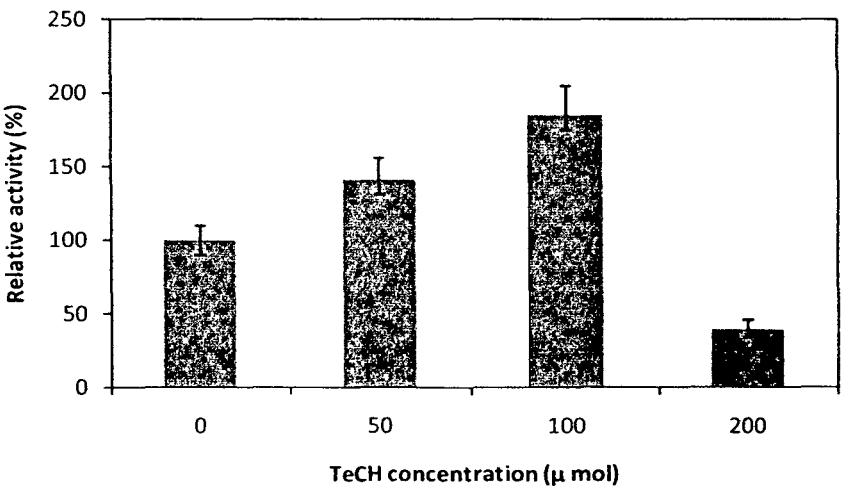

Figure 5.7 The stimulation effect of TeCH on PCP 4-monooxygenase

Oxidative dehalogenation is one mechanism for the elimination of halogens from aromatic compounds. Monooxygenase types of dehalogenase have been shown to be able to remove chlorines from chlorophenols such as PCP (Xun and Orser, 1991a; Ohtsubo *et al.*,

1999; Dai *et al.*, 2003; Shah and Thakur, 2003; Chen and Yang, 2008). Dehalogenation requires 2 mol of NADPH per mol of halogenated substrate hydroxylated by these monooxygenases.

5.4 Discussion

The PCP biodegradation pathway in one of the most extensively studied soil bacterium *S. chlorophenolicum* strain ATCC 39723 contains five catalytic enzymes, which are evolved from at least two different metabolic pathways (Xun and Orser, 1991c; Xun *et al.*, 1992a; Dai *et al.*, 2003). The pathway reported is inefficient both *in vitro* and *in vivo* (Xun *et al.*, 1992a; Dai *et al.*, 2003), mainly due to the low catalytic activity and substrate specificity of PCP 4-monooxygenase (PcpB), which is the first and rate-limiting enzyme in the pathway (Xun and Orser, 1991c; Xun *et al.*, 1992a; Dai *et al.*, 2003; Cai and Xun, 2002). PCP 4-monooxygenase is a FAD binding, NADPH requiring oxygenase with similar functional domains as other bacterial flavoprotein monooxygenase specific for phenolic substrate (Chen and Yang, 2008). Early studies of Xun *et al.*, (1992a; 1992b) showed that PcpB catalyzed the conversion of PCP to TeCH using NADPH as a co-substrate. However, recent studies on PcpB enzyme have reported the conversion of PCP into tetrachlorobenzoquinone (TeCB) rather than TeCH and the reduction of TeCB to TeCH was catalyzed by PcpD (Dai *et al.*, 2003; Chen and Yang, 2008).

Some recent reports suggests, PcpD, which was originally deposited as a monooxygenase reductase in GenBank, is encoded by gene *pcpD*, which is immediately downstream of gene *pcpB* encoding enzyme PcpB (Cai and Xun, 2002; Dai *et al.*, 2003; Chen and Yang, 2008). It contains 324 amino acid residues and consists of three predominant domains: an N-terminal NADPH-binding domain, a FAD binding domain and a C-terminal Rieske-type iron-sulfur cluster (2Fe2S) domain (Dai *et al.*, 2003; Chen and Yang, 2008). The presence of TeCB reductase encoding *pcpD* gene has been reported in *S.*

chlorophenolicum strain ATCC 39723. To the best of my knowledge, no such gene has been reported till date in *Acinetobacter* sp. group.

According to arguments by Dai *et al.*, (2003) on the evolution of PCP biodegradation pathways in bacteria, the pathway has evolved recently in response to the introduction of PCP into the environment, and then it would not be expected to perform at the high level characteristic of pathways that have evolved over periods of millions or billions of years. Indeed, the PCP degradation pathway shows signs of immaturity in several respects. First, PCP hydroxylase, the first enzyme in the pathway, is very inefficient both *in vitro* and *in vivo* (Xun *et al.*, 1992a; Dai *et al.*, 2003) and appears to severely limit the flux of PCP through the pathway in vivo (McCarthy *et al.*, 1997). Second, TCHQ dehalogenase is profoundly inhibited by its aromatic substrate. Third, TCHQ dehalogenase expression is not regulated in tandem with the other known enzymes in the pathway but is apparently constitutive (Orser *et al.*, 1993). All of these findings are consistent with the idea that the PCP degradation pathway has been patched together rather recently and has not been fine-tuned to perform as effectively as do most bacterial metabolic pathways.

Purification and characterization of PCP 4-monooxygenase that converted PCP to TeCH from *Pseudomonas fluorescens* have been reported by Shah and Thakur, (2003). In this study, identification, purification and characterization of PCP 4-monooxygenase enzyme that catalyses the conversion of PCP to TeCH from *Acinetobacter* sp. ISTPCP-3 has been reported. The enzyme was purified using phenyl agarose, mono Q and gel filtration chromatographic techniques. Almost 38% of the protein was recovered after the gel filtration chromatography. The optimum pH for PCP 4-monooxygenase activity was found to be at around 7.0. The enzyme was less sensitive towards acidic pH than basic pH, maintaining more than 70% of its optimal activity even at pH 5.0. However, the enzyme lost its activity quickly in basic solutions, retaining less than 20% of its optimal activity at pH

8.0. The optimal temperature of the enzymatic reaction was found to be lie between 30°C and 35°C. Greater than 70% of the maximum activity was retained at 35°C. The enzymatic activity was completely absent at 50°C. The enzyme completely converted 37 μM PCP to TeCH in 100 mM KP_i buffer. The NADPH-to-PCP consumption ratio was determined to be exactly 2 under various initial concentrations of NADPH and PCP. Gel filtration chromatography and SDS–PAGE confirmed the molecular weight of PCP 4-monooxygenase to be 30,000. The result of the amino acids sequence, obtained from MALDI–TOF MS data indicated the homology of peptides of bacterial monooxygenase family. K_m and V_{max} values for PCP were 30 ± 7 μM and 15 ± 5 μmol/min/mg of protein, respectively. For NADPH, the K_m was 81 ± 5 μM and the V_{max} was 20 ± 4 μmol/min/mg of protein.

The present study reported the direct conversion of PCP to TeCH, by *Acinetobacter* sp. ISTPCP-3, as concluded from mass spectrometric, NMR spectroscopy and during purification of the enzyme catalyzing its conversion. Although, a detailed study in-comprising of latest molecular biology techniques should be carried out to confirm the present hypothesis.

BIOLOGICAL TREATMENT OF TANNERY EFFLUENT IN AEROBIC SEQUENTIAL REACTORS

CHAPTER 6

BIOLOGICAL TREATMENT OF TANNERY EFFLUENT IN AEROBIC SEQUENTIAL REACTORS

6.1 Introduction

India occupies a key position on the worlds map with respect to number of leather tanning units (tanneries). Tanneries are significant in terms of Indian exports and employment opportunities for people of economically weaker populations. There are about 2,500 registered tanneries in India, which are mainly located in clusters at Jajmau–Kanpur (Uttar Pradesh), Kolkata (West Bengal) and Chennai (Tamil Nadu) and in some parts of Karnataka and Maharashtra (More *et al.*, 2001). Sustenance of tanneries, particularly of the smaller units, is becoming increasingly difficult because of alarming levels of environmental pollution caused by various tanning operations and practices. Annually about 80 million pieces of skins are processed by the tanning industries for manufacturing semi finished and finished leather (Wiegant *et al.*, 1999; Sreeram and Ramasami, 2003; Stoop, 2003). Approximately around 7 lakh tonne of hides and skins are processed each year in India that releases around 75,000 m^3 per day of toxic liquid effluents into nearby water bodies (Sahasranaman and Buljan, 2000; Tare *et al.*, 2003).

The leather processing requires a large quantity of chemicals and water (Chhonkar, 2000). Leather production involves tanning, which is a chemical process that converts the semi-soluble proteins known as the 'collagen,' present in the corium of animal skin and hides into tough, flexible, water resistant and highly durable finished leather. Different chemicals are used at different leather processing

stage since two types of tanning modes are followed in India. Consequently, two types of effluents are discharged from tanneries viz., effluents from vegetable tanning and chrome tanning (Babu *et al.*, 1994; Srivastava and Pathak, 1997). Chrome tanning is used for the production of thin leather, whereas vegetable tanning produces mainly heavy leathers. During chrome tanning, large amount of basic chromium sulfate is used, that makes leather tough and flexible. Large amount of wastewater is generated in this process to wash out the residual salts, which might interfere in the quality of finished leather products. However in vegetable tanning process, tannins are used, which are extracted from specific tree barks. These vegetable tannins are biodegradable, for producing plump leather, which is easily molded and are less affected by humidity (Srivastava and Pathak, 1997).

In Jajmau, around 375 tanneries operate with an average daily rawhide processing capacity of 320 tons. Approximately 315 tanneries convey 7.75 million liters per day (MLD) of effluent to the up-flow anaerobic sludge blanket (UASB) based CETP at Jajmau, which was designated to treat 9 MLD of tannery wastewater using the UASB process (Shukla *et al.*, 2001; Tare *et al.*, 2003). Preliminary treatment of wastewater for removal of grit and chromium is performed in the tannery itself. On special directives from the Government, the effluent generated from individual tannery, after passing through preliminary treatment, is conveyed to the CETP for further treatment.

The anaerobic and aerobic treatment methods applied for reducing the pollution load in industries have been proved successful up to some extent. Biological removal of chlorinated phenols and related major toxicants from the complex tannery effluent using sequential reactors has not been carried out so far. Therefore, the objectives of the present investigation are to develop a sequential bioreactor for the treatment of tannery wastewater contaminated with

chlorinated phenols and chromium with bacterial and fungal strains and treatment of other major contaminants present in the effluent.

6.2 Material and methods

6.2.1 Source of tannery wastewater

The study was conducted on the effluent released from tanneries located at Jajmau, Kanpur industrial area (UP). Effluent was collected from the main channel of tanneries located towards Unnao side of Lucknow road. The tanneries were located on the either side of the road, spread over an area of 3 km^2. The individual tannery releases their effluent in to the underground sub-channels. These sub-channels drain their effluent into the main channel, which ultimately joins the river Ganga after passing through a CETP. The effluent was collected from the main channel before the CETP. Untreated effluent was stored at 4°C in a refrigerated cold room.

6.2.2 Microorganisms and culture conditions

Sediment sample along with liquid effluent was collected from the effluent discharge site of tanneries located at Jajmau area. The sediment was serially diluted (10-fold), and diluted sample (0.1 ml) was spread on the potato dextrose agar (PDA) plates. The plates were incubated at 30°C for 4 days. The microbial colonies (fungi) appeared on the PDA plates were isolated, purified and characterized. Fungal inoculum was prepared in the form of pellets. Erlenmeyer flasks (250 ml) containing potato dextrose broth and streptopenicillin (100 ppm) was taken and inoculated by mycelial discs. These flasks were incubated at 30°C for 4 days with shaking in orbital shaker. The mycelium was filtered by cheesecloth and placed on Petri plates.

The fungal isolates were screened for their chromium removal potentiality with MSM containing (mg/l): KH_2PO_4, 800; Na_2HPO_4, 800; $MgSO_4 \cdot 7H_2O$, 200; $CaCl_2 \cdot 2H_2O$, 10; NH_4Cl, 500; plus 1 ml of trace metal solution which includes $FeSO_4 \cdot 7H_2O$, 5; $ZnSO_4 \cdot 7H_2O$, 4; $MnSO_4 \cdot 4H_2O$, 0.2; $NiCl \cdot 6H_2O$, 0.1; H_3BO_3, 0.15; $CoCl_2 \cdot 6H_2O$, 0.5;

$ZnCl_2$, 0.25; and EDTA, 2.5, pH was adjusted to 5.5 as described by Thakur, (1995). The salt of potassium chromate (500 ppm) was used as the source of hexavalent chromium. The effluent was inoculated in an Erlenmeyer flask with individual fungal isolates and incubated at 30°C in a rotary shaker for 7 days. Uptake of chromium was measured at an interval of 0, 1, 3, 5 and 7 days. On the basis of commiserative analysis percentage reduction in parameter was studied by the individual isolates along with the control and the most potential strains were selected for further analysis. The most potent chromium tolerant fungal strain *Aspergillus niger* FK1, was used for bio-sorption of chromium from tannery effluent in the bioreactor.

The bacterial strain used for degradation of chlorinated phenols including PCP was isolated from effluent discharge site of a major pulp and paper industrial plant in Nainital, located at foothills of Himalayas. The site was having a history of PCP contamination. The samples were taken into sterilized tube and preserved at 4°C. Sediment sample (2 g) was added to MSM (100 ml) with PCP (10 mg/l) as sole source of carbon and energy and incubated at 30°C for 5 days in a rotary shaker at 150 rpm. Enriched cultures, 5 ml, showing degradation of PCP were transferred to 100 ml fresh MSM with PCP (50 mg/l). Three phenotypically different colonies were picked and purified by repeated streaking on the same medium. Of the three isolated bacterial strains, one pure isolate with highest PCP-degrading capability was characterized as *Acinetobacter* sp. ISTPCP-3, and was selected for treatment of tannery effluent.

6.2.3 Physico-chemical parameters of tannery effluent

Total dissolved solids (TDS), total suspended solids (TSS), conductivity, dissolved oxygen (DO), biochemical oxygen demand (BOD), chemical oxygen demand (COD), total phenols, pentachlorophenol (PCP), total Kjeldahl nitrogen (TKN), sulfide, chromium, temperature, color and pH were analyzed following APHA,

(1998). Quality control was ensured using standards as well as duplicates.

6.2.4 Fabrication of bioreactors

The experiment related to biological treatment of tannery wastewater was conducted in two phases; fungal treatment in the first phase (stage I) followed by bacterial treatment in the second (stage II), in bioreactors of different capacity (2 l, 10 l, and 100 l). The aerobic sequential reactors (ASR) were operated for 90 days to remove, mainly high concentrations of chromium and PCP along with an intention of removing overall pollutant load from the untreated tannery effluent. The lab-scale ASR, illustrated in Figure 6.1, had a volume of 10 l. Silicone tubes were used for connecting individual bioreactor and also for filling of the untreated and semi-treated effluent using peristaltic pumps. The reactors were equipped with stirring and aeration facility, constant flow rate of air at 2 l/min was maintained in both reactors by electric air compressor. Online pH and temperature monitoring instrument was installed. The ambient temperature of influent and effluent in the reactors were maintained at 30°C. The pH was maintained around 7.0, using HCl/NaOH, throughout the course of treatment.

The effluent was initially passed through a muslin cloth, for removal of large impurities, which otherwise would choked the reactor. The influent was delivered to the bioreactor at a flow-rate of 1 l/day until 30, then at a rate of 2 l/day between 30 and 10 and finally 3 l/day until the end of the experiment. Consequently, the hydraulic retention time (HRT) was 3 days in initial phase and was reduced to 2 days and finally to 1 day at the end of the experiment. The sludge was allowed to accumulate at the bottom of the reactors. However, in certain periods (between day 40 and 60), sludge withdrawal was done by stopping the entire set-up for collection process only. Only sludge was removed without removing the liquid effluent.

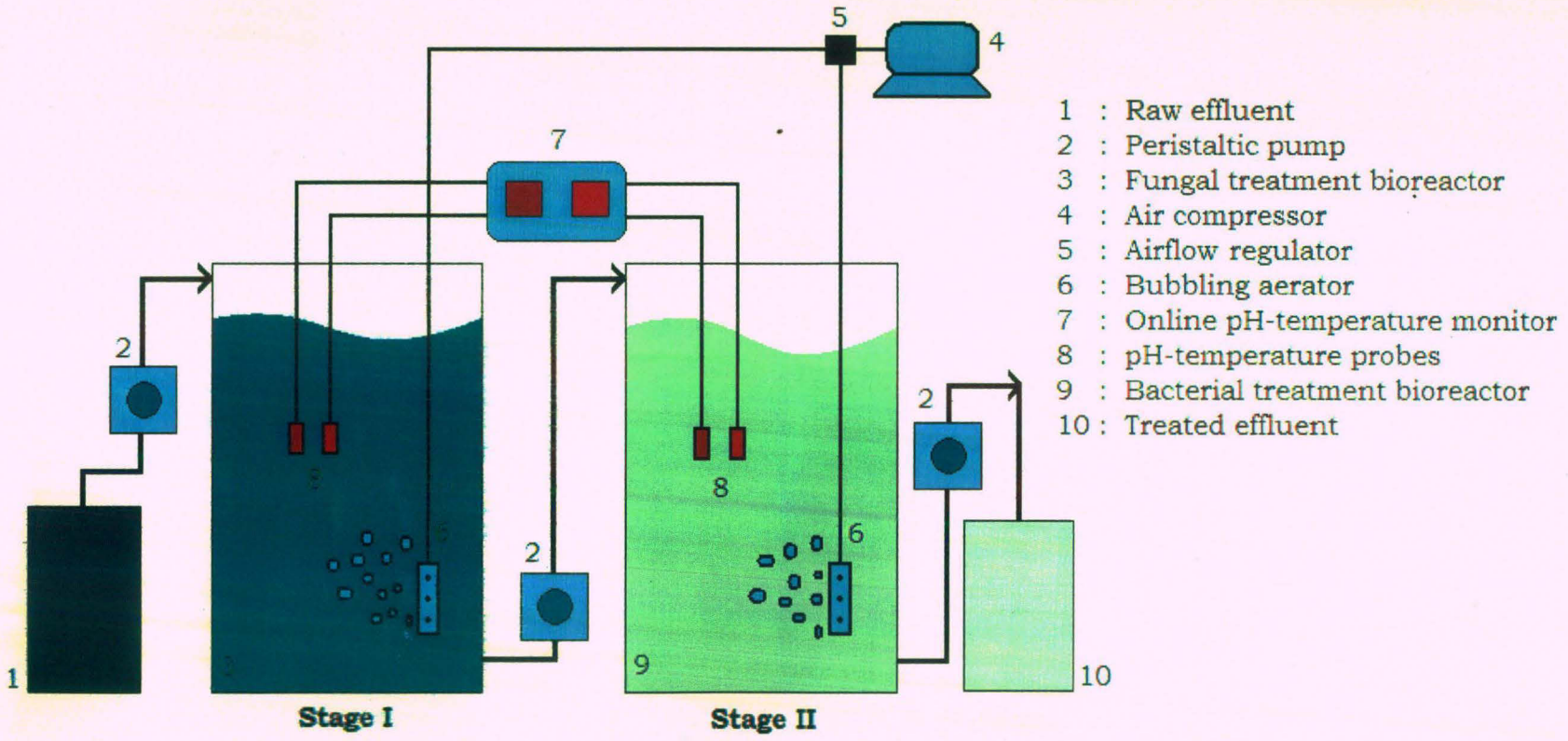

Figure 6.1 A schematic illustration of the lab-scale aerobic sequential bioreactors

Stage I of the sequential bioreactor consists of a reactor, where fungus was used as inoculum for the treatment. The untreated tannery effluent was delivered to the bioreactor at a constant flow-rate and inoculated with fungal strain FK1 (10% [w/v]). The bioreactor was then supplemented with sucrose (0.2%) and urea (0.1%) as carbon and nitrogen source. Stage II was filled with same quantity of the effluent and was inoculated with bacterial strain ISTPCP-3 at the rate of 10% (v/v), along with other supplements (MSM). The treated effluent from ASR, stage I and stage II was removed after, 0, 6, 18 and 27 days from the reactor and changes in pollution load parameters were determined.

Pilot-scale bioreactor (100 l) was fabricated following the procedures as described for 10 l sequential bioreactor. The scaling-up of bioreactor was done for studying and verifying the actual potential of isolated strains for their efficacy towards *ex situ* large scale treatment of tannery effluent in bioreactors.

Each time the sample was collected from the bioreactors, the same volume of the inoculum and nutrient sodium acetate as a carbon source and yeast extract as a nitrogen source for the fungal strain (FK1) and dextrose as a carbon source and sodium nitrate as a nitrogen source for the bacterial strain (ISTPCP-3) was added to the effluent in the bioreactor to maintain uniformity in culture and nutrient supplement in the treatment process. Samples of treated tannery effluent were taken at different sampling interval, samples were centrifuged, and both supernatant and pellets was used for the determination of chromium. In supernatant percentage reduction of chromium was estimated while in fungal and bacterial pellets biosorption of chromium was estimated. Chlorinated phenols in the effluent were extracted by liquid-liquid extraction and analyzed on GC–MS.

In extraction of metabolites, the effluents samples were taken out at specified time intervals to study degradation of chlorophenols and other relevant compounds. The samples were clarified by centrifugation at 8000 rpm for 3 min. The supernatant fractions were extracted three times with an equal volume of n-hexane by shaking vigorously for 15 min in a standard separating funnel. The organic layer was dried with anhydrous sodium sulfate, and the solvent was removed by gently blowing under a steam of N_2. The residue was finally dissolved in 50 µl mixture of n-hexane: ethyl acetate (10:1) and analyzed immediately on a GC–MS.

6.3 Results

6.3.1 Characterization of the tannery effluent

The influent wastewater (untreated effluent) was characterized with common parameters (pH, temperature, salts, COD, BOD, TKN, phenols, chromium, sulfide etc.). The physico-chemical characteristics of the composite effluent collected from tanneries located at Jajmau, Kanpur (UP) is given in Table 6.1. Each value represented in the table is the mean of three replicates ± standard error of mean. The effluent collected was acidic in nature (pH 4.0–4.1 ± 0.1), dark green in color, deficient in dissolved oxygen level, it contained large amount of solids (TSS: 4876 ± 8.5 mg/l, TDS: 14500 ± 4.2 mg/l) and high values of COD (5700 ± 10.4 mg/l) and BOD (2350 ± 11.5 mg/l). The effluent emanating from the tanneries was also characterized with high concentration of TKN (277 ± 5.8 mg/l), sulfate (245 ± 11.3 mg/l), chloride (4500 mg/l), sulfide (35 ± 2.2 mg/l) and total chromium (860 ± 5.8 mg/l). In addition, 330 ± 10 mg/l of phenol and 62 ± 4 mg/l of PCP were detected in the effluent.

Table 6.1 Physico-chemical characteristics of the tannery effluent

Parameters	Values
pH	4.0–4.1 ± 0.1
Temperature (°C)	29.7 ± 0.3
Color	Dark green
Total dissolved solids (mg/l)	14500 ± 4.2
Total suspended solids (mg/l)	4876 ± 8.5
Electrical conductivity (dS/m)	22.30 ± 0.2
Chemical oxygen demand (mg/l)	5700 ± 10.4
Biochemical oxygen demand (mg/l)	2350 ± 11.5
Total Kjeldahl nitrogen (mg/l)	277 ± 5.8
Sulfate (mg/l)	245 ± 11.3
Chloride (mg/l)	4500
Sulfide (mg/l)	35 ± 2.2
Chromium (mg/l)	860 ± 5.8
Phenols (mg/l)	330 ± 10
Pentachlorophenol (mg/l)	62 ± 4

Useful relationships between parameters were calculated based on characterization of the effluent. According to it soluble COD averaged 40% of total COD, which indicated less than half of the total COD was soluble. The BOD_5/COD ratio was 0.4, which was low in comparison to domestic wastewater (i.e. 0.5). This indicated low biodegradability of the influent, according to the criteria of Ahn *et al.*, (1999). However, BOD_5 is a controversial parameter, when it is applied to tannery wastewater, since it contains many inhibitors of BOD_5 (Ates *et al.*, 1997; Lefebvre *et al.*, 2005). The VSS/(total COD-soluble COD) ratio averaged 1.5 ± 0.5, indicating that every kg of VSS contributed to 1.5 kg of particulate COD. Finally, the COD/N ratio averaged 20/9 showed that wastewater contained significant amounts of nitrogen. It could well be estimated that parameters in the influent wastewater enabled the bioreactor to be operated without the much addition of external nutrient source such as nitrogen and carbon.

6.3.2 Treatment of tannery wastewater in bioreactors

The study related to biological treatment of tannery effluent was performed in ASR. The treatment was performed in two steps (stage I and stage II). In stage I, the influent was treated with fungal strain FK1, followed by treatment with bacterial strain ISTPCP-3, in stage II. The choice of treatment in stage I with fungal strain was based on series of experiments performed on treatability study of tannery influent under various combinations, in-order to achieve maximum sorption of chromium and biodegradation of chlorinated phenols (PCP) and various other pollution parameters (data not shown).

In-order to verify the results obtained from treatment of small volume of influent in the bench-scale batch reactors (500 ml), lab-scale (2l and 10 l) and pilot-scale (100 l) sequential bioreactors were fabricated and operated under specified conditions. The idea behind scaling-up process was to develop a suitable technology and generation of various pollution parameter data for large scale treatment of effluent for biological removal of chromium and recalcitrant chlorinated phenols.

Changes in various pollution parameters (COD, TKN, sulfate, sulfide, chromium, phenols and PCP by subsequent treatment of tannery influent in sequential bioreactors by fungal strain (FK1), followed by bacterial strain (ISTPCP-3) are given in Tables (6.2, 6.3 and 6.4). Data recorded on this aspect clearly indicated significant reduction of above mentioned pollution parameters and overall treatment of tannery wastewater.

The results of reduction in various pollution parameters in a 2 l ARS are presented in Table 6.2. The results clearly indicated significant reduction, in terms of percentage pollutants/contaminants removal from treated tannery effluent. More than 50% removal was observed for chromium (81.6%), phenols (69.6%) and PCP (65%) at day 27. Almost 44% removal of COD was observed at the same day.

No significant reduction (% removal) was observed in control reactors at day 27.

The results of reduction in various pollution parameters in a 2 l ARS are presented in Table 6.3. In almost the same case with 2 l ASR, the results for 10 l ASR also indicated significant reduction, in terms of percentage pollutants/contaminants removal from treated tannery effluent. In this case, more than 50% removal was observed for COD (56.4), sulfide (61.5%), chromium (95.2%), phenols (75.0%) and PCP (81.6%) at day 27. The performance of 10 l ASR was better as compared to 2 l ASR. The possible reason could be due to physico-chemical transformation of various parameters due to constant flow of air and periodic removal of sludge from the reactors. No significant reduction (% removal) was observed in control reactors at day 27.

The results of reduction in various pollution parameters in a pilot-scale ARS are presented in Table 6.4. The results clearly indicated significant reduction, in terms of percentage pollutants/contaminants removal from treated tannery effluent. More than 50% removal was observed for COD (57.0%), sulfide (65.3%), chromium (95.8%), phenols (77.5%) and PCP (83.3%) at day 27. No significant reduction (% removal) was observed in control reactors.

Table 6.2 Changes in pollution parameters by subsequent treatment of tannery influent in a 2 liter sequential bioreactor by fungal strain FK1 (stage I) and bacterial strain ISTPCP-3 (stage II)

Parameters	Day 0	Day 6	Day 18	Day 27
COD	5600 ± 11.8	5100 ± 8.5	3900 ± 10.7	3140 ± 11.6
% COD removal	0	8.9	30.3	43.9
TKN	270 ± 6.8	251 ± 5.8	211 ± 7.3	193 ± 6.5
% TKN removal	0	7.0	21.8	28.5
Sulfate	230 ± 12.5	221 ± 10.1	200 ± 5.8	187 ± 6.7
% sulfate removal	0	3.9	13.0	18.6
Sulfide	32 ± 3.2	28.3 ± 1.2	21.3 ± 1.3	18.7 ± 1.2
% sulfide removal	0	11.5	33.4	41.5
Chromium	857 ± 6.8	701 ± 5.5	415 ± 6.5	58 ± 7.2
% chromium removal	0	18.2	51.5	81.6
Phenols	325 ± 10	228 ± 6.5	157 ± 7.3	98.6 ± 6.8
% phenols removal	0	29.8	51.6	69.6
PCP	60 ± 4	51.5 ± 2.6	39 ± 3.5	21 ± 3.2
% PCP removal	0	14.1	35.0	65.0

Table 6.3 Changes in pollution parameters by subsequent treatment of tannery influent in a 10 liter sequential bioreactor by fungal strain FK1 (stage I) and bacterial strain ISTPCP-3 (stage II)

Parameters	**Day 0**	**Day 6**	**Day 18**	**Day 27**
COD	5600 ± 11.8	5000 ± 7.5	3720 ± 6.3	2440 ± 9.5
% COD removal	0	10.7	33.5	56.4
TKN	270 ± 6.8	236 ± 4.5	198 ± 5.7	173 ± 5.7
% TKN removal	0	12.5	26.6	35.9
Sulfate	230 ± 12.5	211 ± 7.1	188 ± 6.1	169 ± 5.8
% sulfate removal	0	8.2	18.2	26.5
Sulfide	32 ± 3.2	22 ± 2.0	18.1 ± 1.5	12.3 ± 1.0
% sulfide removal	0	31.2	43.4	61.5
Chromium	857 ± 6.8	681 ± 4.5	401 ± 5.6	41 ± 5.2
% chromium removal	0	20.5	53.2	95.2
Phenols	325 ± 10	211 ± 5.5	147 ± 6.6	81 ± 7.1
% phenols removal	0	35.0	54.7	75.0
PCP	60 ± 4	43.7 ± 1.8	31 ± 2.5	11 ± 3.0
% PCP removal	0	27.1	48.3	81.6

Table 6.4 Changes in pollution parameters by subsequent treatment of tannery influent in a 100 liter sequential bioreactor by fungal strain FK1 (stage I) and bacterial strain ISTPCP-3 (stage II)

Parameters	**Day 0**	**Day 6**	**Day 18**	**Day 27**
COD	5600 ± 11.8	5050 ± 9.5	3710 ± 5.6	2408 ± 6.5
% COD removal	0	9.8	33.7	57.0
TKN	270 ± 6.8	245 ± 5.1	195 ± 5.5	163 ± 3.7
% TKN removal	0	9.2	27.7	39.6
Sulfate	230 ± 12.5	201.5 ± 6.4	177 ± 6.5	158 ± 3.9
% sulfate removal	0	12.6	19.6	31.3
Sulfide	32 ± 3.2	30 ± 3.0	16.1 ± 2.3	11.1 ± 1.0
% sulfide removal	0	6.6	49.6	65.3
Chromium	857 ± 6.8	671.5 ± 4.5	391 ± 4.5	35.7 ± 3.3
% chromium removal	0	21.7	54.3	95.8
Phenols	325 ± 10	201 ± 6.5	138.7 ± 6.6	73.1 ± 6.8
% phenols removal	0	38.1	57.3	77.5
PCP	60 ± 4	40.1 ± 1.8	26.5 ± 2.5	10 ± 2.3
% PCP removal	0	33.1	55.8	83.3

The presences of organic compounds, including chlorinated aromatic compounds were detected from the GC–MS profiling of the untreated tannery effluent (Figure 6.2). The peaks were identified using documented data from NIST (NBS75K) and Wiley 7 libraries. The effluent was characterized with varied spectrum of detectable organic compounds. The effluent mainly contained phenols, including chlorinated phenols. The peaks were identified as: phenol (RT = 4.43) [m/z (% relative intensity)]: [166(25) 151(100) 151(100) 166(25) 152 (14)]; *o*-cresol (RT = 4.83): [180(63) 165(100) 165(100) 135(55) 180(63)]; 2-chlorophenol (RT = 5.29): [200(28) 185(100) 185(100) 149(98) 200(28)]; 3-chlorophenol (RT = 5.31): [200(27) 185(100) 185(100) 187(36) 200(27)]; 4-chlorophenol (RT = 5.44): [200(33) 185(100) 185(100) 200(33) 187(36)]; catechol (RT = 5.77): [254(12) 239(4) 73(100) 254(12) 151(5)]; 3,5-dichlorophenol (RT = 6.27): [234(29) 219(100) 219(100) 221(70) 234(29)]; 2,4,6-trichlorophenol (RT = 6.91): [268(25) 253(100) 255(97) 253(100) 268(25)]; 2,3,5-trichlorophenol (RT = 7.02): [268(11) 253(49) 255(49) 253(49) 268(11)]; 2,4,5-trichlorophenol (RT = 7.19): [268(13) 253(45) 255(44) 253(45) 268(13)]; 2,3,6-trichlorophenol (RT = 7.73): [268(20) 253(99) 255(99) 253(99) 268(20)]; 2,3,4-trichlorophenol (RT = 7.77): [268(14) 253(49) 255(49) 253(49) 268(14)]; 2,3,4,6-tetrachlorophenol (RT = 9.52): [287(78) 289(100) 287(78) 93(98)]; 2,3,4,5-tetrachlorophenol (RT 9.58); [287(35) 289(47) 287(35) 93(100)]; PCP (RT = 10.50): [321(46) 323(72) 325(47) 93(100)]; and dibenzofuran (RT = 14.21): [194(15) 168(20) 139(45) 84(21)]. Low molecular weight aliphatic compounds, esters of long chain fatty acids and sulfur containing compounds were also detected. Figure 6.3 shows the chromatogram of the effluent after treatment with fungal strain FK1 (stage I). Figure 6.4 shows the GC profile of treated effluent for degradation of chlorinated aromatic compounds, including chlorinated phenols by bacterial strain ISTPCP-3 (stage II).

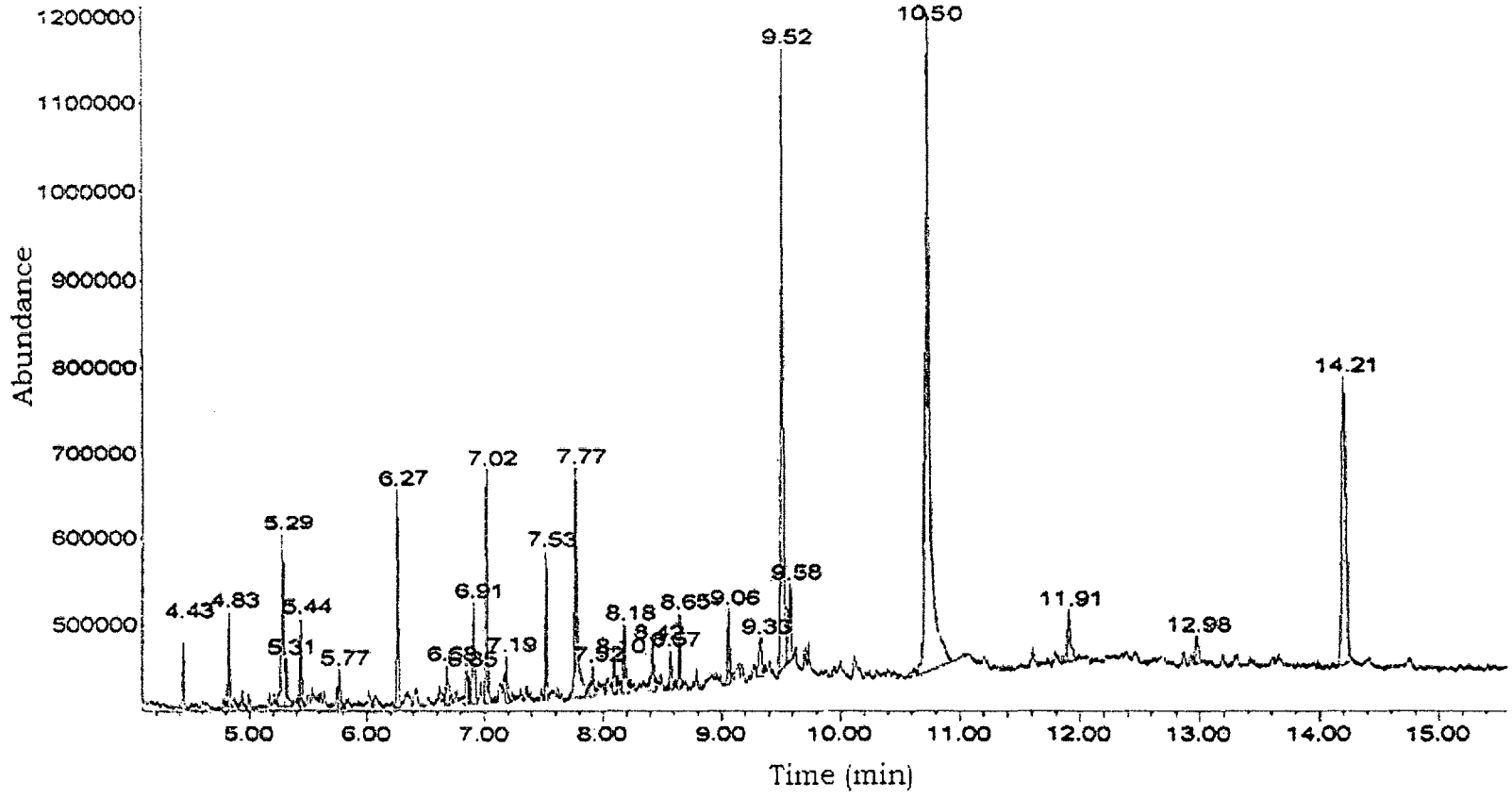

Figure 6.2 Gas chromatographic profile of untreated tannery wastewater

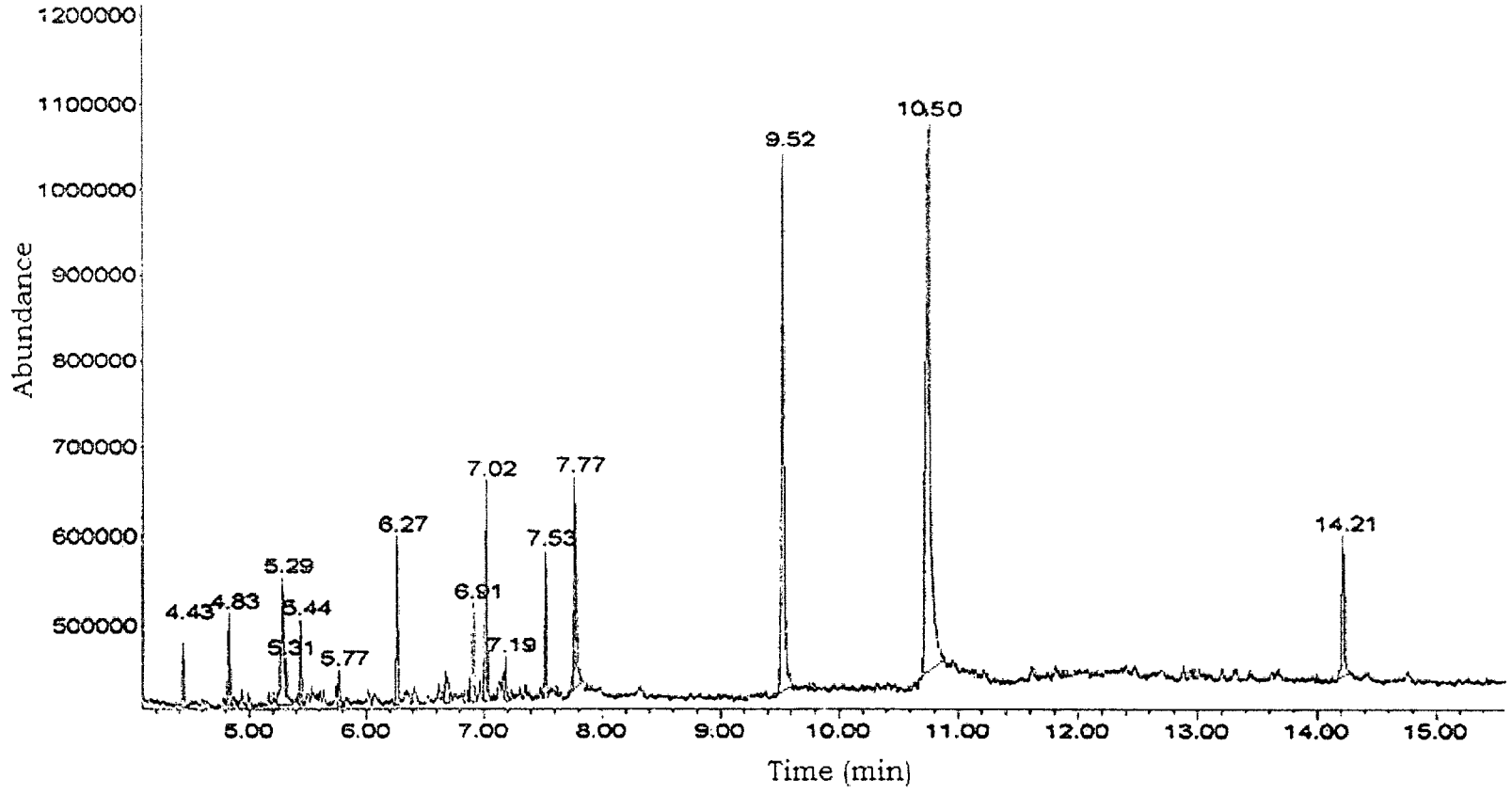

Figure 6.3 Gas chromatographic profile of effluent after fungal treatment (stage I) of ASR

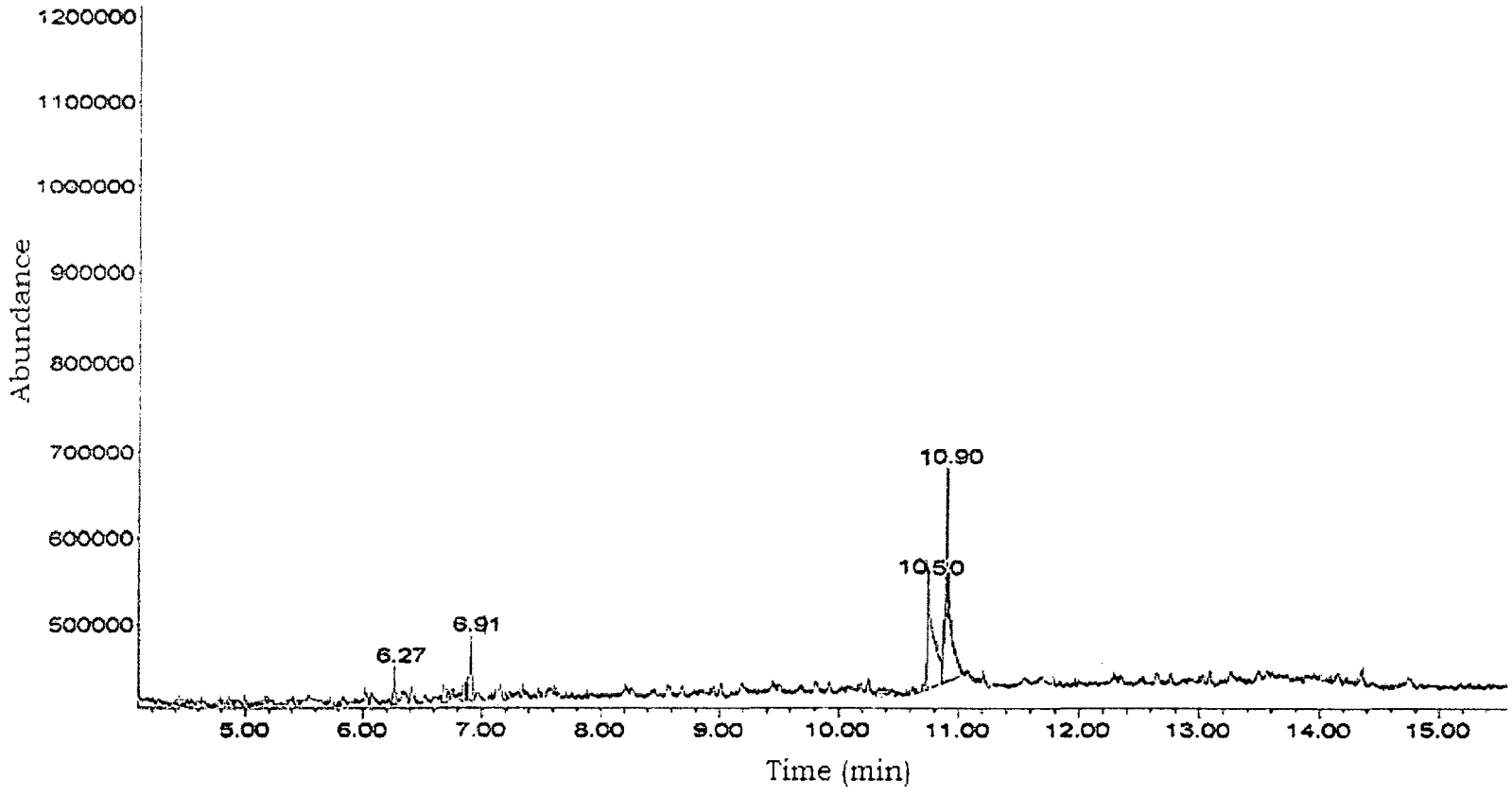

Figure 6.4 Gas chromatographic profile of effluent after bacterial treatment (stage II) of ASR

The gas chromatographic profile of fungal treated effluent (Figure 6.3) indicated no significant removal of recalcitrant chlorinated phenols, although there was a slight decrease in the concentration of few chlorophenols, but this could be due to physical adsorption of these compounds on fungal hyphae. Whereas, in the case of treatment of effluent by the bacterial strain (Figure 6.4), significant removal of chlorinated aromatic compounds including chlorinated phenols was observed. Almost 90% of PCP was removed at that stage. The emergence of the peak of TeCH clearly indicated the standardized pathway followed by bacterial strain ISTPCP-3. In fact, one of the most toxic recalcitrant compounds (dibenzofuran) was also shown to be degraded by the bacterial strain.

6.4 Discussion

Leather tanning is almost wholly a wet process from which a large volume of liquid waste is continuously generated. Due to variety of chemicals added at different stages of processing of hides and skin, the wastewater has complex characteristics. Variety of chemicals, such as; sodium chloride in curing and preservation of raw hides, chlorinated phenols, including PCP as biocides for preventing fungal and mould deterioration of raw hides, ammonium chloride in deliming, sodium sulfide in unhearing and deliming, alkyl-phenyl ethoxylate in degreasing, synthetic fat liquors and surfactants in fat liquoring including formaldehyde and benzidine in finishing of leather are being used in the entire process of leather tanning. The chemicals used causes severe pollution and pose a serious threat to flora and fauna.

As mentioned earlier in the text, the problem of high concentration of chlorinated phenols, chromium and high COD are major problems associated with tannery effluents. Various attempts have been made by workers to remove major contaminants from the

tannery effluent using biological methods, chemical methods or combination of both the methods (Guiot *et al.*, 1993; Kaul *et al.*, 1993; Nandy *et al.*, 1993; Singram, 1994; Vajpayee *et al.*, 1995; Rose *et al.*, 1996; Chandra *et al.*, 1997; Shukla *et al.*, 2001; Tare *et al.*, 2003; Farabegoli *et al.*, 2004; Lefebvre *et al.*, 2005; Barrera and Urbina, 2008).

Various bioreactors have been designed for the treatment of chlorinated phenols at bench-scale or lab-scale. Bench-scale continuous flow activated sludge reactors were used to study the removal of PCP and other toxic materials. Bioreactors have been designed and applied by the workers for the treatment of toxicants by the microorganism Nyholm *et al.*, 1992; Venkatadri *et al.*,1992; Hendriksen and Ahring, 1992; Makinen *et al.*, 1993; Jacobsen *et al.*, 1993; Middaugh *et al.*, 1994; Hu *et al.*, 1994; Kang and Stevens, 1994; Alleman *et al.*, 1995; Puhakka *et al.*, 1995; Bae *et al.*, 1995; Melin *et al.*, 1997; Lo *et al.*, 1998; Karmanev and Samson, 1998; Taseli and Gokcay, 2005; Zilouei *et al.*, 2006.

The study proved the feasibility of treating tannery wastewater using fungus and bacteria in order to remove high concentrations of chromium and chlorinated phenols including PCP present in the effluent. Two stage sequential bioreactors were fabricated and operated under aerobic conditions. The ASR were operated for 90 days on untreated tannery influent for removal of chromium, chlorinated phenols and other pollution parameters (COD, TKN etc). The experiment related to biological treatment of tannery wastewater was conducted in two phases; fungal treatment in the first phase (stage I) followed by bacterial treatment in the second (stage II), in bioreactors of different capacity (2 l, 10 l and 100 l). Almost similar conditions were maintained for operating the ASR for bench-scale, lab-scale and pilot-scale and the results obtained were compared to evaluate relative performances of each volume setup. Fungus was used in the first stage of the bioreactor for maximum removal to chromium by fungal

hyphae. The semi-treated wastewater was then treated with bacteria for effective removal of recalcitrant chlorinated aromatic compounds including chlorinated phenols. More than 50% removal of chromium, phenols and PCP was observed in all the scales of bioreactors. The GC–MS results revealed presence of different chlorinated phenols along with their isomeric forms including PCP. Complex aromatic structures formed by reactions between different low molecular weight aromatic compounds and low molecular weight sulfur compounds were also detected. Long-chain fatty acids were also detected. Significant removal of chlorinated aromatic compounds including PCP was observed after bacterial treatment of the influent wastewater. Chromium could also be economically removed and recovered from wastewater by various physico-chemical processes including precipitation with MgO. Whereas, in the case with phenols, its large scale removal from wastewater through physico-chemical process, even at quaternary level of treatment is difficult are not economical. Biological methods for removal of such contaminants from the industrial wastewater are much more economical and environment friendly. Also, if clubbed together with conventional wastewater treatment process, could help in achieving the required discharge standards of the pollutants present in the industrial wastewater.

DETOXIFICATION ANALYSIS OF CHLORINATED PHENOLS AND ITS METABOLITES IN THE TANNERY EFFLUENT

CHAPTER 7

DETOXIFICATION ANALYSIS OF CHLORINATED PHENOLS AND ITS METABOLITES IN THE TANNERY EFFLUENT

7.1 Introduction

Over the recent decades, significant quantities of industrial, agricultural and domestic chemicals have been released into the environment. Halogenated aromatic compounds constitute one of the largest groups of chemicals used in industrial application and preservation of biological materials (Yang *et al.*, 2007). Chlorinated phenols in general are noted for exhibiting strong biological effects: like 2,4-dinitrophenol, the standard un-coupler of oxidative phosphorylation, they intervene in the oxidative pathways of metabolism. A clinical manifestation of this property is the very rapid onset of rigor mortis in victims of PCP poisoning. Not all of the 19 different chlorinated phenols are commercially important; of those that are, most are not important in their own right, but only as intermediates in chemical syntheses, e.g., in the production of herbicidal phenoxy acids like 2,4-D or 2,4,5-T. Chlorinated phenols and their derivatives are inert, hydrophobic, stays longer in the environment and cause toxicity to flora and fauna (Chu *et al.*, 2008). Many chlorinated xenobiotics are known to have carcinogenic and/or genotoxic properties, and this fact has elicited a number of studies concerning the induction of such effects by PCP. PCP is a general cytotoxic agent, its efficacy due to its inhibitory properties upon oxidative phosphorylation. It is also a specific inhibitor of sulfotransferase, a phase II metabolizing enzyme.

This unspecific and strong cytotoxic activity of PCP has led to its use in a great variety of biocidal applications. Among the minor applications are its use as a molluscicide (against snails as vectors of schistosomiasis), as an insecticide (in termite control), as a herbicide (in the pre-harvest defoliation and desiccation of cotton, or for algae control in rice paddy fields), and at one time even as a preservative for soy sauce (Bevenue and Beckman, 1967). The introduction of more specific pesticides, better suited to the different single tasks, has rendered such uses of PCP largely obsolete, and registrations have been cancelled in many countries. Its cheapness and broad spectrum of action might, however, be seen as an inducement for its continued use in some parts of the world. The main, and recently disputed, use of PCP, however, has been as a protectant in the tannery (fungicide), wood (fungicide) and paper (slimicide) industry.

In contrast to other polychlorinated aromatic compounds, PCP can be metabolized to a certain extent in the environment by microbial degradation, leading to an estimated half-life in soil of about 20 days (Hattemer-Frey and Travis, 1989). However, the main metabolic pathway is not only the reductive dechlorination and eventual total degradation, but also conversion to pentachloroanisole could take place, which again is a very stable molecule (Ahlborg and Thunberg, 1980). Due to its low volatility the dissipation of PCP within the environment is mainly (i.e., >95%) to the soil. Exposure of the general population to this compound has therefore been estimated to originate from food and to amount to 15–20 μg per day from this source (Hattemer-Frey and Travis, 1989). Human exposure to PCP can also, albeit to a minor part, originate through the metabolic formation from hexachlorobenzene (Stewart and Smith, 1986) or from hexachlorocyclohexane (Munir *et al.*, 1984), both compounds again being ubiquitous environmental contaminants.

The microbial metabolite pentachloroanisole was shown to induce mutations in *S. typhimurium* TA1537 and TA98 in the absence of exogenous metabolic activation at concentrations leading to

precipitation of the substance on the plates, i.e., at 3–10/μg/plate (Mortelmans *et al.*, 1986). In the absence of metabolic activation pentachloroanisole demonstrated no clear mutagenic response in the L5178Y mouse lymphoma test, but in the presence of rat liver S9 the number and frequency of TK mutants was strongly increased, starting at a lowest effective concentration of about 50/μg/ml (McGregor *et al.*, 1987).

Ostling and Johanson, (1984) were the first to quantify DNA damage in cells using a microgel electrophoresis technique known as "single cell gel electrophoresis or comet assay". However, the neutral conditions they used, allowed the detection of only DNA doublestrand breaks. Later, the assay was adapted under alkaline conditions by Singh *et al.*, (1988), which led to a sensitive version of the assay that could assess both double- and single-strand DNA breaks as well as the alkali labile sites expressed as frank strand breaks in the DNA. Since its inception, however, the assay has been modified at various steps (lysis, electrophoresis) to make it suitable for assessing various kinds of damage in different cells (Collins, 2004; Speit and Hartmann, 2005). The assay is now a well-established, simple, versatile, rapid, visual, and a sensitive, extensively used tool to assess DNA damage and repair quantitatively as well as qualitatively in individual cell populations (Olive and Banath, 2006). Some other lesions of DNA damage such as DNA cross-links (e.g., thymidine dimers) and oxidative DNA damage may also be assessed using lesion-specific antibodies or specific DNA repair enzymes in the comet assay. It has gained wide acceptance as a valuable tool in fundamental DNA damage and repair studies (Speit and Hartmann, 2005), genotoxicity testing (Moller, 2005), and human biomonitoring (Kassie *et al.*, 2000; Moller 2006).

Relative to other genotoxicity tests, such as chromosomal aberrations, sister chromatids exchanges, alkaline elution, and micronucleus assay, the advantages of the comet assay include its demonstrated sensitivity for detecting low levels of DNA damage (one

break per 10^{10} Da of DNA; Gedik *et al.*, 1992), requirement for small number of cells (~10,000) per sample, flexibility to use proliferating as well as non-proliferating cells, low cost, ease of application, and the short time needed to complete a study (Dhawan *et al.*, 2009). The alkaline version of the method is a very sensitive assay for detection of single-strand breaks in DNA, alkali-labile sites, and any other damage that generates DNA breaks (Fairbairn *et al.*, 1995). SCGE is rapid and relatively easy to perform and may be used in different test programs, such as biomonitoring studies on a wide range of mutagenic compounds and ionizing radiation (Moller *et al.*, 2000; Tice *et al.*, 2000). Untill now, the comet assay has been applied to animal cells, plant cells and bacteria. However, it would be advantageous to apply it to *Saccharomyces cerevisiae*, which is a fast growing organism and its cultivation is cheap and easy to handle. Higher eukaryotes and yeast exhibit striking similarities in the molecular mechanisms of fundamental cellular processes, such as transcription, translation, replication, and DNA repair. This simple eukaryote possesses homologues or functional analogues of almost all factors involved in these processes. *S. cerevisiae* is one of the most thoroughly studied model systems whose full genome sequence is now available (Terziyska *et al.*, 2000).

Tanning is one of the major industries in India and the effluent which is discharged from this industry is highly complex and causes severe pollution. The tannery effluent contains large amounts of organic and inorganic nutrients including PCP and chromium. Tannery effluent with its high total dissolved solids load may pose a severe inhibitory effect on seed germination of various economical crops. Nevertheless, different crop species may have differential tolerance/sensitivity towards the effluent. For the present study we used untreated effluent discharged from the tannery units containing high concentrations of chromium and PCP. This study was undertaken to investigate the toxicity and bacterial detoxification of untreated and treated wastewater in the bioreactor. Seed germination

test was performed for evaluating the effects of different concentrations of tannery effluent on germination of some vegetable seeds. Comet assay was performed for evaluation of bacterial detoxification of major chlorinated phenols and their metabolites present in treated tannery wastewater.

7.2 Materials and methods

7.2.1 Source of tannery effluent

The tannery effluent used in the present study was collected from the main discharge site of tannery effluent from tannery cluster near Jajmau, Kanpur (UP). The samples were collected from the main drainage pipe towards CETP. The collected effluent was analyzed for chromium and PCP concentration following the methods outlined by APHA, (1998). Treated samples were collected from the outlet of sequential batch reactors.

7.2.2 Seeds and cell cultures

Seeds of some vegetable crops; bottle gourd (*Lagenaria siceraria*), chilli (*Capsicum annuum*), cucumber (*Cucumis sativus L.*), onion (*Allium cepa*) and tomato (*Lycopersicon esculentum*) were kindly provided by the Head, Division of Seed Science and Technology, Indian Agricultural Research Institute (IARI), New Delhi.

Saccharomyces cerevisiae strain MTCC 36 was purchased from Institute of Microbial Technology (IMTECH), Chandigarh. In this study yeast was chosen as model organism for evaluation of toxicity. Yeast cells were cultivated in MYPG media (0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 1% glucose, pH 5.0) at 30°C to middle logarithmic phase of growth (5×10^7 cells/ml).

7.2.3 Seed germination studies

A laboratory experiment was conducted to find out the effect of different concentrations (0–100%) of tannery effluent in above mentioned vegetable crops. Seeds germination studies were carried out in Petri dishes. For the germination test, 25 seeds of each species were placed in sterilized glass Petri dishes of uniform size lined with

two filter paper discs. Five replicates were maintained for each test species and for each treatment. The seeds that germinated were counted and removed from the Petri dishes at the time of first count on each day until there was no further germination. A portion (5 ml) of different concentrations (5%, 10%, 15%, 20%, 25%, 50%, 75% and 100%) of raw effluent was added to the Petri plates on alternate days and the controls received same amount of distilled water. The Petri plates containing seeds were kept at 25°C in an incubator. The criterion of germination was the visible protrusion of radical from the seed coat and it was expressed in percentage. From the data recorded on germination percentage, speed of germination (Maguire, 1962), peak value, germination value (Czabator, 1962) and relative germination percentage were computed as below:

$$\text{speed of germination} = \frac{\text{number of seeds germinated}}{\text{days of first count}} + \cdots + \frac{\text{number of seeds germinated}}{\text{days of final count}}$$

$$\text{peak value} = \frac{\text{cumulative percent germination on each day}}{\text{number of days elapsed since initial imbibition}}$$

$$\text{germination value} = \text{peak value} \times \text{germination (\%)}$$

The data thus obtained were arcsine and logarithmic transformed for percentage values and other values, respectively, and the statistical analysis was carried out using two-way ANOVA.

7.2.4 Yeast comet assay

The alkaline comet assay was performed essentially as described by Miloshev *et al.*, (2002). Up to 200 µl of 0.5% agarose (Sigma) was spread on each slide and this supportive agarose layer was air dried before the application of the cell suspension on slides. Yeast cells were

collected by centrifugation in an Eppendorf micro-centrifuge for 5 min, washed with water, and resuspended in S-buffer (1 M sorbitol, 25 mM KH_2PO_4 pH 6.5). Aliquots of approximately 5×10^4 cells were mixed with 0.7% low-melting agarose containing 2 mg/ml of zymolyase 20T and spread over the slides, covered with coverslips and incubated for 20 min at 30°C to disintegrate the yeast cell wall and obtain spheroplasts. In order to minimize the activity of the endogenous cell enzymes, all further procedures were done in a cold room at 8–10°C. Coverslips were removed and the slides were incubated in 30 mM NaOH, 1M NaCl, 0.1% laurylsarcosine, 50 mM EDTA, pH 12.3 for 1 h to lyse the spheroplasts. The slides were rinsed three times for 20 min each in 30 mM NaOH, 10 mM EDTA, pH 12.4 to unwind DNA and then subjected to electrophoresis in the same buffer. The electrophoresis was carried out for 20 min at 0.5 V/cm, 24 mA. After electrophoresis the gels were neutralized by submerging the slides in 10 mM Tris–HCl pH 7.5 for 10 min followed by consecutive 10 min incubations in 76 and 96% ethanol. Finally, the slides were left to air dry, stained with ethidium bromide (1 mg/ml) for 5 min. The slides were rinsed in cold water to remove the excess ethidium bromide and covered with coverslip.

Comets were analyzed using the fluorescence microscope with an excitation filter of 355 nm and a barrier filter of 450 nm. The fluorescence microscope was fitted with 100× oil immersion lens. A computerized image analysis system (Komet version 5.5, Kinetic Imaging Ltd., Andor technology, Nottingham, UK) was employed. Standard deviation was calculated on the basis of five independently cultivated yeast cell cultures from which aliquots were treated with the indicated above drug concentrations. The tail lengths (μm) and percentage change in DNA present in the tail of 50 comets per treatment of test sample were measured in each experiment.

Yeast cell viability was tested by growing cells to middle logarithmic phase and were treated with raw effluent, fungal treated followed by bacterial treated effluent from the bioreactor. The cells

were collected, washed with water, diluted with rich MYPG medium and approximately 10^3 cells per dish were spread on Petri dishes containing MYPG medium. After 3 days of incubation at 30°C, the colony forming units (CFU) were counted and the percentage of survived cells was calculated. The CFU of control, mock-treated yeast cells was accepted as 100%.

7.3 Results

7.3.1 Seed germination test

In the present study the physico-chemical characteristics of the tannery effluent showed that it was highly acidic (pH 4.0–4.1) and saline (EC 22.30 dS/m) in nature, dark green in color and was rich in organic matter. The concentrations of BOD, COD and tannins were very high. The value to total phenol ranged from 300 mg/l to 350 mg/l. The concentration of PCP was 60 mg/l and that of chromium was 857 mg/l. As mentioned above, the effluent employed in the present study was highly acidic; this could be due to the use of sulfuric acid during tanning process.

The vegetable crop species differed widely in response to different concentrations and treatments of tannery effluent with respect to germination percentage, speed of germination, peak value and germination value. At lower concentrations, the tannery effluent did not inhibit sees germination in crops except tomato. Among the five crops studied, tomato recorded the lowest (29.6%) and chilli the highest (52%) germination (Table 7.1). In general the germination percentage decreased with an increase in concentration of the effluent. The germination was inhibited in all the different vegetable seeds studied with concentration exceeding 50%. Growth of tomato seeds were inhibited even at 5%, it appears to be highly sensitive among all the five crops. In contrast, the effluent at 10% concentration showed a positive effect on seed germination in onion and chilli.

Significant differences were also observed in speed of germination, peak value and germination value (Table 7.2). Speed of

germination was highest in cucumber followed by onion, bottle gourd, chilli and tomato. Onion recorded significantly higher peak value and germination value followed by bottle gourd, whereas tomato showed significantly the lowest value. Similar to germination percentage, these values decreased significantly with the increase in concentration of the effluent. In the present study, it was found that a concentration of 5% was the critical value for tomato and bottle gourd and 25% for the rest of vegetable seeds.

The tannery effluent used in the present study had a pH of about 4.1 irrespective of the degree of dilution (Table 7.3). The similarity of pH values across various concentrations suggests that the observed variation in seed germination of crops is not a reflection of the pH effect. However, Justice and Reece, (1954) reported that germination can occur over a wide range of hydrogen ion concentrations and the germination of almost all species occurs between pH values 4.0 and 7.6. The reason for the reduction in germination percent at higher concentration in all the crops and, even at lower concentrations in tomato and bottle gourd could therefore be attributed to excessive quantities of inorganic salts, and consequently its higher EC values (Table 7.3).

Table 7.1 Effect of different concentrations (v/v %) of tannery effluent on germination percentage

Treatment concentration	Germination (%)					
	Bottle gourd	**Chilli**	**Cucumber**	**Onion**	**Tomato**	**Mean**
0 (DW)	89	81	56	65	85	75.2
5	88	77	56	69	70	72.0
10	62	81	56	78	52	65.8
15	64	77	57	66	47	62.2
20	56	71	45	61	11	48.8
25	21	70	45	53	2	38.2
50	0	11	9	22	0	8.4
75	0	0	0	0	0	0
100	0	0	0	0	0	0
Mean	42.2	52.0	36.0	46.0	29.6	

CD calculated from the data on transformed values. CD (0.05); crops, 3.39; treatments, 2.59; interaction, 7.80

DW, distilled water

Table 7.2 Effect of different concentrations (v/v %) of tannery effluent on speed of germination, peak value and germination value

Treatment	Speed of germination	Peak value	Germination value
Crop			
Bottle gourd	14.23	3.57	256
Chilli	13.01	2.11	153
Cucumber	22.00	2.14	139
Onion	17.08	4.89	376
Tomato	6.32	1.16	65
CD (0.05)	0.05	0.03	0.09
Concentration of tannery effluent (%)			
0 (DW)	29.45	6.35	455
5	27.34	6.01	432
10	26.11	4.89	324
15	21.45	4.02	251
20	17.06	3.43	199
25	14.45	2.76	128
50	2.32	0.21	9
75	0.00	0.00	0.00
100	0.00	0.00	0.00
CD (0.05)	0.04	0.03	0.06

CD calculated from the data on transformed values

DW, distilled water

Table 7.3 Characteristics of different concentrations (v/v %) of the tannery effluent

Treatment	pH	EC (dS/m)	Op (atm)
5	4.0	1.72	0.62
10	4.0	3.32	1.19
15	4.1	5.01	1.80
20	4.0	5.24	1.88
25	4.1	7.87	2.83
50	4.0	12.08	4.34
75	4.1	17.12	6.16
100	4.1	26.30	9.46

$Op = ECe \times 10^3 \times 0.36$

The EC of the effluent increased with the increase in the concentration. The results were consistent with findings of earlier workers (Neelam and Sahai, 1988; Karunyal *et al.*, 1994) who also observed a decrease in seed germination with an increase in the concentration of the effluent. Further, the osmotic pressure of the effluent also increased at higher concentrations. Rodger *et al.*, (1957) reported that high osmotic pressures of the germination solution make imbibition more difficult and retard germination, while the ability of seeds to germinate under high osmotic pressure differs with variety as well as species (Ungar, 1987). Richards (1968) on the other hand observed that it is the total concentration of the solute particles in the solution rather than their chemical nature that is mainly responsible for the inhibitory effects of saline solutions on the growth of crops, mainly by decreased imbibition.

7.3.2 Comet assay

In order to evaluate the toxicity and bacterial detoxification of untreated and treated tannery effluent, SCGE assay was performed on yeast cells. Two types of control were used in the experiment, untreated effluent and distilled water. Distilled water was used to observe the effect of the process of comet formation on yeast nuclei. In all experimental setup, distilled water control did not show any formation of the comet. Formation of the comets with untreated tannery effluent clearly indicated the nature of effluent i.e. highly genotoxic. Figure 7.1 shows the effect of treated effluent in relation to the untreated effluent on yeast cells, sphaeroplasted and subjected to comet assay for results obtained in a 2 liter sequential bioreactor. The parameter measured was percentage of DNA (% of DNA in comet tail). The average DNA in tail was 88%, in untreated effluent, followed by 64% in fungal treatment, which reduced to about 10% in final bacterial treated effluent, the treatment was significant at $p \leq 0.05$ (Figure 7.2). The percentage of DNA in tail was 95% less in final treated effluent when compared to untreated raw effluent.

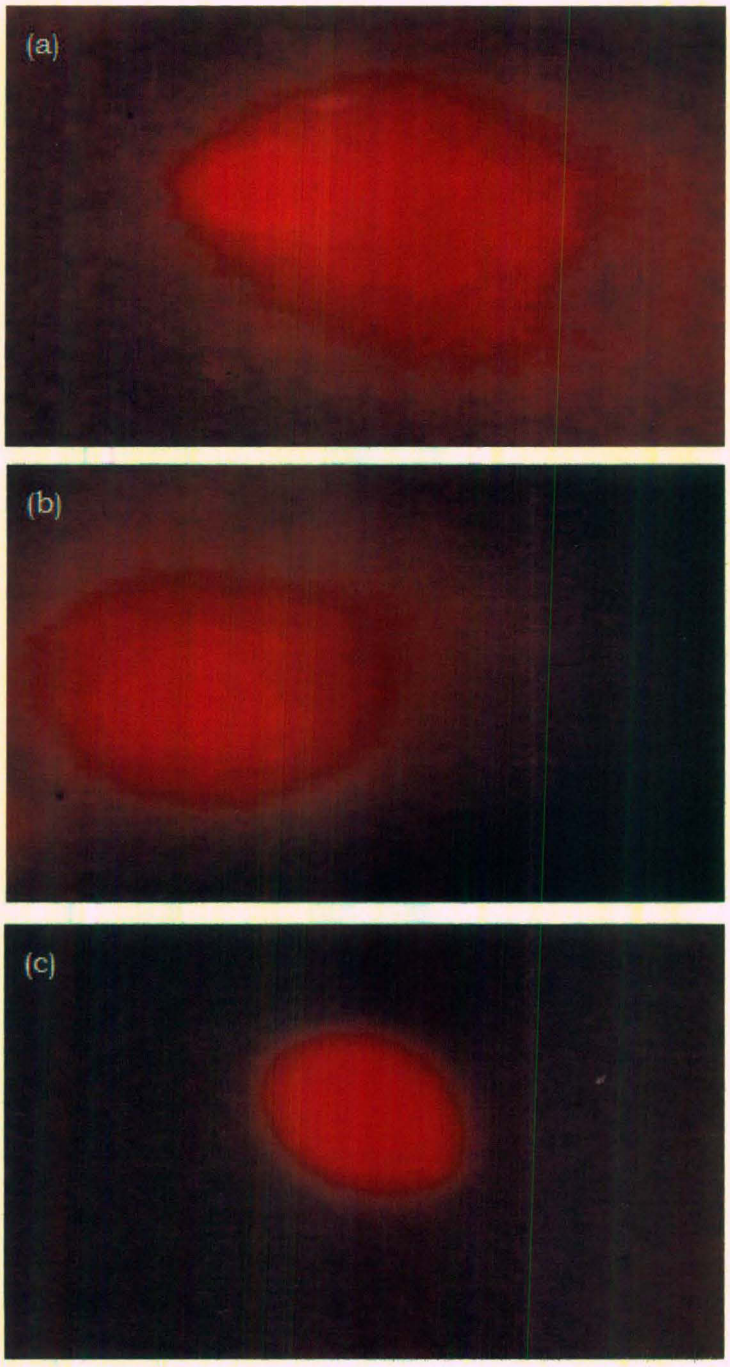

Figure 7.1 SGGE of yeast cells in a 2 liter sequential bioreactor; (a), untreated effluent; (b), after fungal treatment; (c), after bacterial treatment. Slides were viewed using fluorescent microscope (250×)

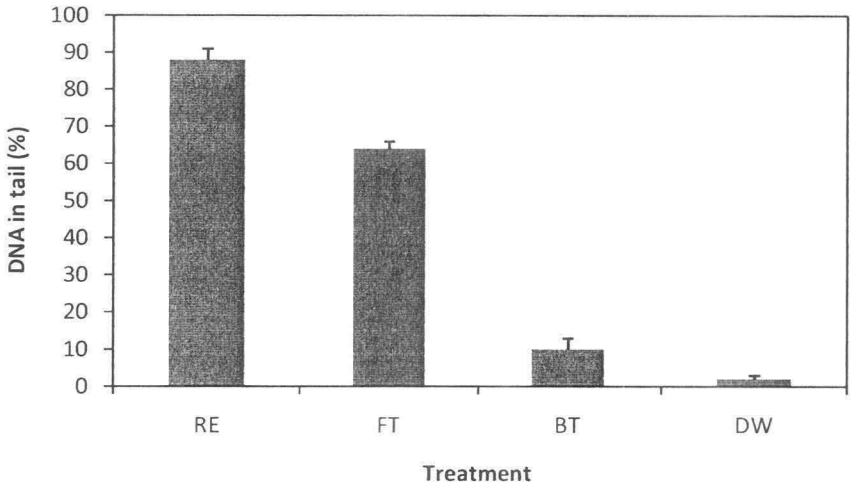

Figure 7.2 Effect of various treatments on percentage change in DNA present in the comets tails for 2 l bioreactor; RE, untreated raw effluent; FT, after fungal treatment; BT, after bacterial treatment; DW, distilled water. Results are mean of five independent experiments. In all, 50 comets per treatment were measured in each experiment. *Error bars* show the standard deviation from the mean for the 150 comets measured

Figure 7.3 shows the effect of treated effluent in relation to the untreated effluent on yeast cells, sphaeroplasted and subjected to comet assay for results obtained in a 10 liter sequential bioreactor. The parameter measured was percentage of DNA (% of DNA in comet tail). The average DNA in tail was 85%, in untreated effluent, followed by 66% in fungal treatment, which reduced to about 10% in final bacterial treated effluent, the treatment was significant at $p \leq 0.05$ (Figure 7.4). The percentage of DNA in tail was 92% less in final treated effluent when compared to untreated raw effluent. Results of the study suggest significant reduction in toxicity of the effluent after bacterial treatment. No significant detoxification was observed in case of fungal treated effluent.

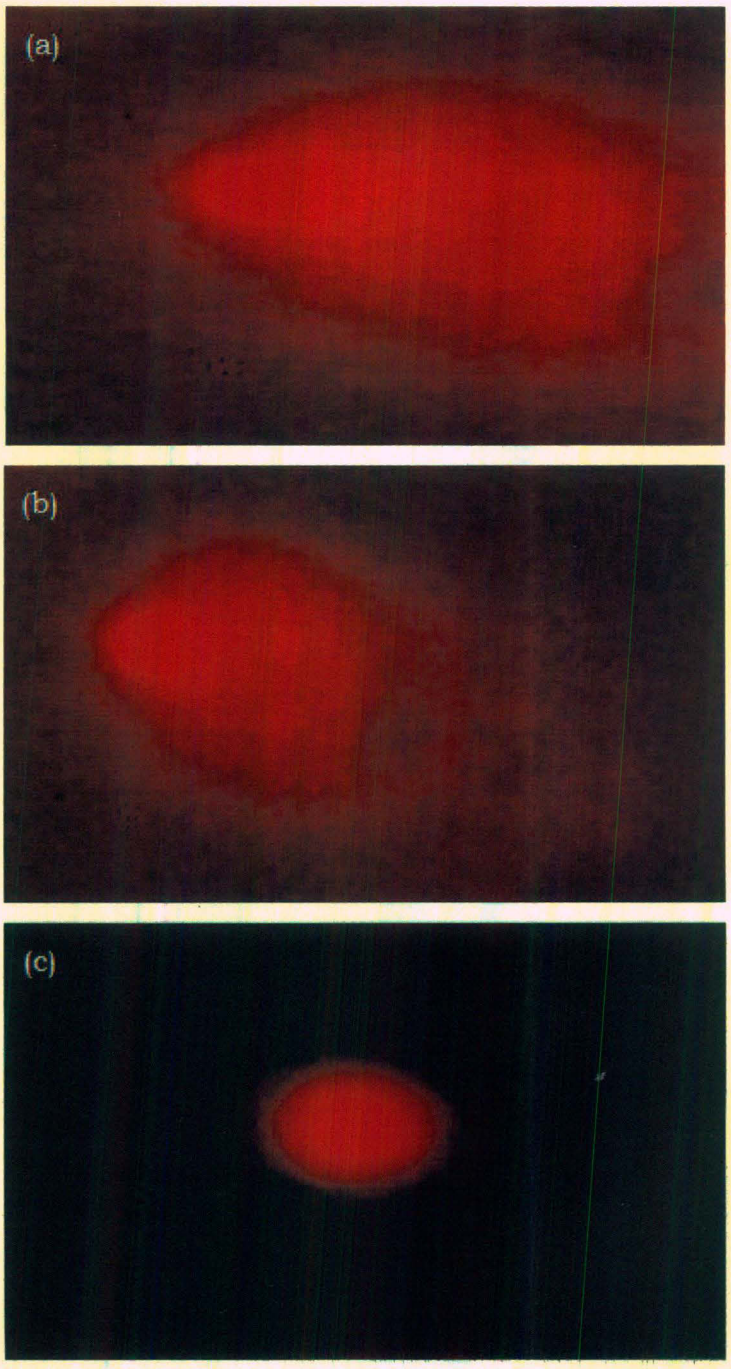

Figure 7.3 SGGE of yeast cells in a 10 liter sequential bioreactor; (a), untreated effluent; (b), after fungal treatment; (c), after bacterial treatment. Slides were viewed using fluorescent microscope (250×)

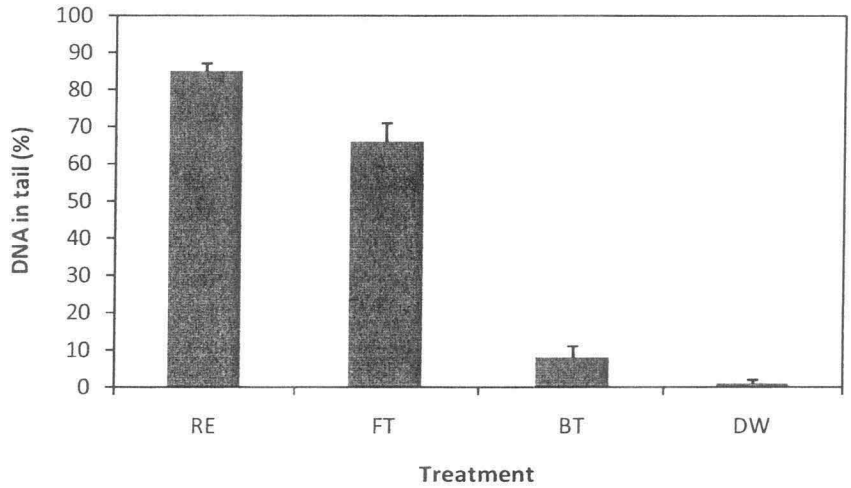

Figure 7.4 Effect of various treatments on percentage change in DNA present in the comets tails for 10 l bioreactor; RE, untreated raw effluent; FT, after fungal treatment; BT, after bacterial treatment; DW, distilled water. Results are mean of five independent experiments. In all, 50 comets per treatment were measured in each experiment. *Error bars* show the standard deviation from the mean for the 150 comets measured

Figure 7.5 shows the effect of treated effluent in relation to the untreated effluent on yeast cells, sphaeroplasted and subjected to comet assay for results obtained in a 100 liter sequential bioreactor. The parameter measured was percentage of DNA (% of DNA in comet tail). The average DNA in tail was 86%, in untreated effluent, followed by 67% in fungal treatment, which reduced to about 10% in final bacterial treated effluent, the treatment was significant at $p \leq 0.05$ (Figure 7.6). The percentage of DNA in tail was 94% less in final treated effluent when compared to untreated raw effluent. Results of the study suggest significant reduction in toxicity of the effluent after bacterial treatment. No significant detoxification was observed in case of fungal treated effluent.

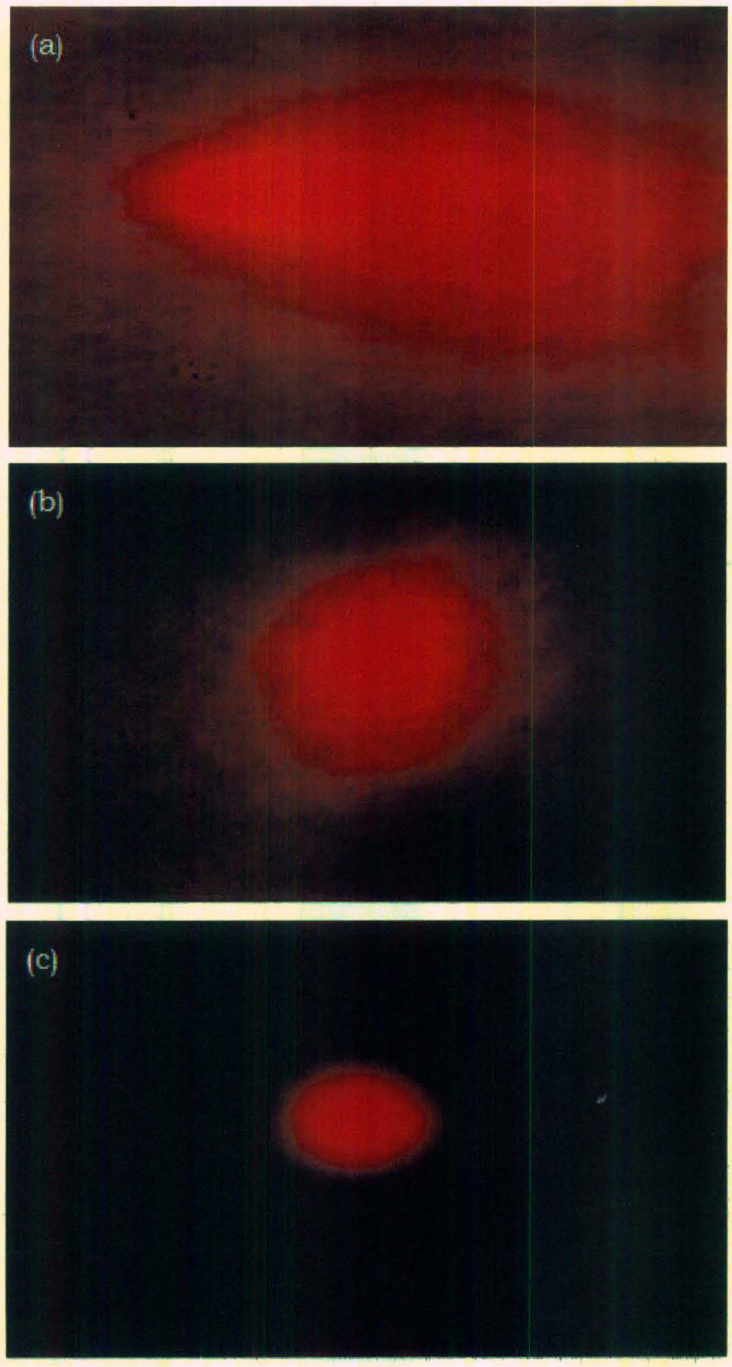

Figure 7.5 SGGE of yeast cells in a 100 liter sequential bioreactor; (a), untreated effluent; (b), after fungal treatment; (c), after bacterial treatment. Slides were viewed using fluorescent microscope (250×)

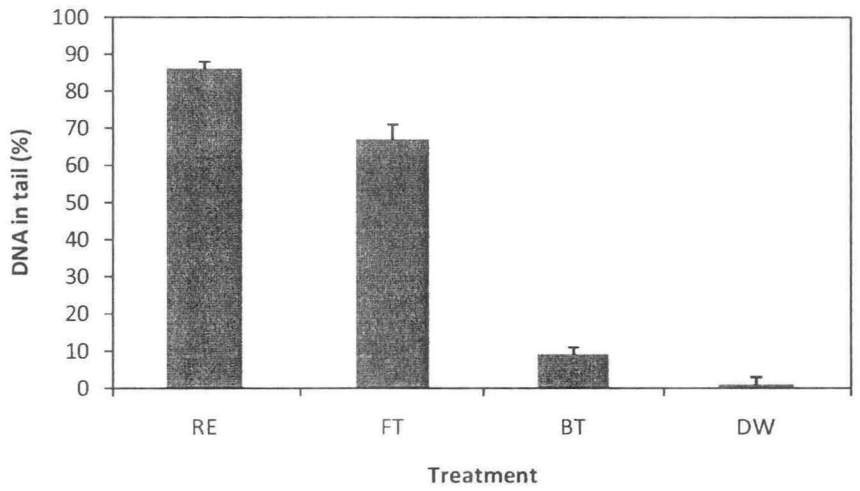

Figure 7.6 Effect of various treatments on percentage change in DNA present in the comets tails for 100 l bioreactor; RE, untreated raw effluent; FT, after fungal treatment; BT, after bacterial treatment; DW, distilled water. Results are mean of five independent experiments. In all, 50 comets per treatment were measured in each experiment. *Error bars* show the standard deviation from the mean for the 150 comets measured

7.4 Discussion

The Indian leather industry has earned a special status in the national economy as the fourth largest foreign exchange earner with a share of around 7% in the total country's exports. The tanning sector is the basic skeleton on which the entire leather industry depends and it is also one of the largest polluters. The leather processing capacity in India is more than 2500 tanning units out of which 75% are in the small scale sector. The inherent nature of the tanning process is such, that large quantity of water and chemicals are consumed, which are mostly discharged without its prior treatment, into major river systems such as the Ganga in the northern part of India. Untreated tannery wastewater was treated in different scales of aerobic sequential reactors, with fungal and bacterial strain, in order to

achieve reduced pollution load and maximum detoxification of the treated effluent.

Various attempts have been made by workers to remove major contaminants of the tannery effluent using biological methods, chemical methods or combination of both the methods (Vivekanandan, 1984; Guiot *et al.*, 1993; Kaul *et al.*, 1993; Nandy *et al.*, 1993; Singram, 1994; Vajpayee *et al.*, 1995; Rose *et al.*, 1996; Chandra *et al.*, 1997; Shukla *et al.*, 2001; Tare *et al.*, 2003; Farabegoli *et al.*, 2004; Lefebvre *et al.*, 2005; Barrera and Urbina, 2008).

The results of the study clearly indicated the detoxification of tannery effluent in a sequential bioreactor. No significant difference was obtained for detoxification at increasing volume of the wastewater (scaling up). In other words, increase in volume of wastewater has no effect on detoxification capability of the isolated bacterial strain. The results show that the length of the comet tails was dependent on ability of the fungal and bacterial strains to detoxify the effluent. Five independent experiments were performed and there was good reproducibility from cell culture to cell culture (Figures 7.2, 7.4 and 7.6). In case of fungal treatment results, more than 50% of DNA was reported in comet tails. The possible reason for appearance of this kind of comets could be because of the presence of PCP still in the fungal treated effluent, as effluent was treated with fungal strain for sorption (adsorption and absorption) of high concentration of chromium. No DNA significant DNA tailing was observed in case of bacterial treatment, because of the ability of the strain to degrade highly toxic chlorinated phenols including PCP into less toxic metabolites and open-ring compounds. The results also attested the genotoxic effects of both chromium and PCP at high concentration, as reported in the literature.

Easy to identify sources of environmental contamination are effluents or process waste from industrial plants. Since PCP is used in leather preservation and in paper mills, the mutagenic activity of the waste products originating from such sources has been investigated.

On the one hand, such effluents or wastes have been analyzed and the main components tested for their activity (Nestmann and Lee, 1983). On the other hand, environmental samples have been analyzed for total mutagenicity thus obtaining an as complete as possible picture of the various chemical interactions (additive, synergistic, inhibitory effects). In view of these possible interactions within the wild spectrum of chemicals present in an environmental sample it is rather difficult, if not impossible, to ascribe any mutagenic activity to one or more specific compounds. Therefore, although the presence of PCP may be detected by chemical analysis in environmental samples exhibiting mutagenic activity, this would by no means constitute proof of a genotoxic activity of PCP itself. PCP has been found to be a potent inhibitor of the enzyme arylsulfotransferase (Meerman *et al.*, 1980). Since a number of mutagens and carcinogens are activated to their ultimate form by a sulfotransferase-mediated reaction, the inhibition of this enzyme should influence the frequency of such effects, otherwise induced by these mutagens or carcinogens. This has indeed been found to be true in a number of cases, and PCP has even been used as a tool in the investigations on the mode of action of certain compounds.

In conclusion, vegetable seed germination test and yeast single cell gel electrophoresis assay were performed for evaluation of toxicity analysis and bio-detoxification of chlorinated phenols in tannery effluent. The results of the present study demonstrate that the effect of tannery effluent on seed germination is governed by its concentration and is crop-specific. Out of five crops selected, tomato was found to be very sensitive against any toxic substances found in water, whereas chilly showed some resilience. The percentage of germination of seeds decreased with an increase in concentration of the effluent. Significant differences were also observed in speed of germination, peak value and germination value. Therefore, caution should be exercised while using the tannery effluent for pre-sowing irrigation purposes. It was also showed that the comet assay could be

applied on yeast cells for evaluating the genotoxic effects of tannery effluent. However, SCGE should also be used in combination with other toxicological tests. A good correlation between the percentage of DNA in the comet tail and various treatments in the bioreactor was observed. Maximum percentage of DNA was observed in the tails of comet when exposed to untreated raw effluent, due to the presence of chromium and PCP. Significantly reduced percentage of DNA was observed in the tails of comet when exposed to bacterial treated effluent. A reasonable percentage of DNA was also observed in the tails of comet when the yeast cells treated with fungal treated effluent suggesting the presence of toxic compounds (phenols) in the effluent. At final stage of treatment, successful detoxification was observed in effluent. The comet assay of *S. cerevisiae*, therefore, being straightforward, rapid and inexpensive, could be used together with other tests to detect damages to cells at DNA level in biomonitoring studies for effluents containing genotoxic agents.

SUMMARY

Synthetic organic substances were produced throughout the world due to their widespread use as agricultural chemicals, detergents and plastics. Disposal of industrial wastes, leakage and volatilization has led to the release of these xenobiotics (synthetic products not formed by natural biosynthetic processes) into the environment. These organic pollutants can lead to damage of plants and animals in polluted areas and they can cause health problems and genetical defects in humans. Waste disposal sites and chemical accidents are sources for the accumulation of pollutants in food chains and for air and drinking water pollution. These sites have to be cleaned up to reduce the negative influences of the pollutants upon the environment and the human beings. Conventional technologies involve removal, alteration, or isolation of the pollutant. These technologies typically consist of excavation followed by incineration or containment. Such conventional treatment is cost extensive and often results in the transfer of the contaminating compounds from one environment or form to another.

Bioremediation is the result of the biological breakdown, or biodegradation, of contaminating compounds. Biodegradation can be defined as the breakdown of organic compounds, usually by microorganisms, into more cell biomass and less complex compounds. Some organic pollutants can be mineralized to CO_2, H_2O and other inorganic components, whereas others are recalcitrant (poorly biodegradable) and persist in the environment. Bioremediation is an attractive alternative to physicochemical methods or disposal, as it is cheap, can result in mineralization of organic pollutants and can be used *in situ* for the cleanup of low, but environmentally relevant concentrations of pollutants. The mineralization of complex materials into simple inorganic constituents is important for the recycling of carbon, hydrogen, oxygen, nitrogen and sulphur.

Xenobiotics, such as the chlorinated phenols have been used extensively since early 1900s as wide spectrum biocides in industries and agriculture. Among chlorinated phenols, PCP and its sodium salts are being used to prevent deterioration of raw hides in tanneries and commercial wood by bacteria and fungus. A large amount of PCP is discharged along with liquid effluent from various tanneries, located in different parts of the country. Its large amount finally gets deposited into soils and sediments, thus contaminating the environment. Moreover, PCP is recalcitrant to degradation due to its stable aromatic ring system and high chloride content. PCP is toxic to all life forms since it is an inhibitor of oxidative phosphorylation and its excessive exposure could cause cancer, immunodeficiency and neurological disorders. Most of the countries have imposed a complete ban on the manufacturing and use of PCP. Some of the countries have listed it as one of the major environmental contaminant. Despite a major ban on the use of PCP in India, its sodium salt is still being used in various industrial applications. The sodium salt of PCP is almost equally toxic to the biota. In case of environmental contamination by PCP, traditional clean-up methods have not been proved successful due to their higher treatment costs and possibilities of causing secondary pollution. However, in solving serious problem of PCP contamination, it is important to assess the potential of bacterial strains indigenous to PCP contaminated sites, for its bioremediation.

Initially, a stable PCP-degrading bacterial consortium was isolated, characterized and enriched in the chemostat. Two stable consortia were developed by continuous enrichment of the bacterial population isolated from sediment core of tannery and pulp and paper mill effluents discharge sites. The consortia were enriched in the chemostat containing MSM, supplemented with PCP as sole source of carbon and energy. The members of the consortia were tested for their growth, removal of chromium and degradation of PCP in batch cultures at different time intervals. The bacterial consortium isolated

from pulp and paper mill effluent discharge site (PE) was better in terms of growth, chromium reduction and degradation of PCP than the consortium isolated from tannery effluent discharge site (TE). The isolates were identified as *Enterobacter* sp. ISTPCP-1 (PE-1), *Pseudomonas aeruginosa* ISTPCP-2 (PE-2) and *Acinetobacter* sp. ISTPCP-3 (PE-3). Out of three acclimated bacterial isolates, ISTPCP-3 strain showed better PCP degradation capabilities than the other two strains (ISTPCP-1 and ISTPCP-2).

The next part of the study focuses on optimization and elucidation of the pathways for biodegradation of PCP. This study has shown transformation of PCP in a batch culture by microbial population isolated from effluent discharge site. The results of the study demonstrated that, the individual pure strains have higher potential for PCP degradation compared to the acclimated mixed culture of bacteria. The results related to optimization of growth parameters for degradation of PCP revealed that all the three strains were mesophilic and showed maximum degradation at neutral pH. Of the three isolated bacterial strain, *Acinetobacter* sp. ISTPCP-3 manifested highest PCP degradation capability followed by *Pseudomonas aeruginosa* ISTPCP-2 and *Enterobacter* sp. ISTPCP-1. Strain ISTPCP-1 exhibited decreased ability to degrade PCP. All three acclimated bacterial strains followed novel pathways for degradation of PCP. The reported pathways are different than previously characterized pathways for PCP degradation. The results of the study clearly indicate the nature of pathway followed by individual bacterial strains. ISTPCP-2 and ISTPCP-3 were able to degrade PCP via hydroquinone pathway. However, detection of tetrachlorophenol as a metabolite of ISTPCP-1 also indicates a novel degradation pathway followed by the strain in aerobic condition. Results clearly indicated significance of strains ISTPCP-3 and ISTPCP-2 in degradation of PCP and acted as a potential member of the consortium, however, the

strain ISTPCP-1 may have played as associated members of the consortium in degradation of PCP.

This part of the research aimed to purify and characterize PCP 4-monooxygenase enzyme involved in catalyzing the conversion of PCP to TeCH by *Acinetobacter* sp. ISTPCP-3 strain. The enzyme was purified using phenyl agarose, mono Q and gel filtration chromatographic techniques. Almost 38% of the protein was recovered after the gel filtration chromatography. The optimum pH for PCP 4-monooxygenase activity was found to be at around 7.0. The enzyme was less sensitive towards acidic pH than basic pH, maintaining more than 70% of its optimal activity even at pH 5.0. Gel filtration chromatography and SDS–PAGE confirmed the molecular weight of PCP 4-monooxygenase to be 30,000. The result of the amino acids sequence, obtained from MALDI–TOF MS data indicated the homology of peptides of bacterial monooxygenase family. K_m and V_{max} values for PCP were 30 ± 7 μM and 15 ± 5 μmol/min/mg of protein, respectively. For NADPH, the K_m was 81 ± 5 μM and the V_{max} was 20 ± 4 μmol/min/mg of protein. The present study reported the direct conversion of PCP to TeCH, by *Acinetobacter* sp. ISTPCP-3, as concluded from mass spectrometric, NMR spectroscopy and during purification of the enzyme catalyzing its conversion.

In this part of the research, treatment of tannery wastewater in aerobic sequential reactors using fungal and bacterial strains was proposed. The study proved the feasibility of treating tannery wastewater using fungus and bacteria in order to remove high concentrations of chromium and chlorinated phenols including PCP present in the effluent. The experiment related to biological treatment of tannery wastewater was conducted in two phases; fungal treatment in the first phase (stage I) followed by bacterial treatment in the second (stage II), in bioreactors of different capacity (2 l, 10 l and 100 l). Almost similar conditions were maintained for operating the ASR for bench-scale, lab-scale and pilot-scale and the results obtained were

compared to evaluate relative performances of each volume setup. Fungus was used in the first stage of the bioreactor for maximum removal to chromium by fungal hyphae. The semi-treated wastewater was then treated with bacteria for effective removal of recalcitrant chlorinated aromatic compounds including chlorinated phenols. More than 50% removal of chromium, phenols and PCP was observed in all the scales of bioreactors.

This final part of the doctoral research focuses on studying the detoxification analysis of chlorinated phenols and its metabolites in tannery effluent. Vegetable seed germination test and yeast single cell gel electrophoresis assay were performed for evaluation of effluent toxicity, under different dilutions and bio-detoxification of tannery effluent. The results of the present study demonstrate that the effect of tannery effluent on seed germination is governed by its concentration and is crop-specific. Significant differences were also observed in speed of germination, peak value and germination value. It was also showed that the comet assay could be applied on yeast cells for evaluating the genotoxic effects of tannery effluent. A good correlation between the percentage of DNA in the comet tail and various treatments in the bioreactor was observed. The comet assay of *S. cerevisiae*, therefore, being straightforward, rapid and inexpensive, could be used together with other tests to detect damages to cells at DNA level in biomonitoring studies for effluents containing genotoxic agents.

In conclusion, an indigenous bacterial population capable of degrading PCP was enriched and characterized. The individual bacterial strains degraded PCP through novel and modified pathways. Out of three acclimated strains, *Acinetobacter* sp. ISTPCP-3, manifested highest chromium tolerance and PCP-degrading capability. The strain could thus be applied for bioremediation and detoxification of tannery wastewater contaminated with chlorinated phenols including PCP.

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CURRICULUM VITAE

RESEARCH INTERESTS

My research interests' focus on understanding molecular mechanisms involved in degradation of xenobiotics by microbes and their diversity, with the aim of improving our understanding the complex interactions and relationships for their application in bioremediation of contaminated sites. My current research interests focus on isolation and molecular characterization of novel bacterial consortium for treatment of chlorinated phenols in industrial effluent, optimization of process parameters for up-scaling of industrial effluent treatment. I am also interested in policy related environmental management solutions and applicability of environmental research.

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ACHIEVEMENTS, AWARDS & FELLOWSHIPS

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PUBLICATIONS

Journal Papers

1. **Sharma, A.**, and I.S. Thakur "Biological treatment of tannery effluent in aerobic sequential reactors," (communicated)
2. **Sharma, A.**, and I.S. Thakur "Enzymatic dehalogenation of pentachlorophenol by *Acinetobacter* sp. ISTPCP-3," (communicated)
3. **Sharma, A.**, I.S. Thakur, and P. Dureja (2009) Enrichment, isolation and characterization of pentachlorophenol degrading bacterium *Acinetobacter* sp. ISTPCP-3 from effluent discharge site," *Biodegradation*, doi: 10.1007/s10532-009-9251-5
4. **Sharma, A.**, and I.S. Thakur (2009) "Identification and characterization of integron mediated antibiotic resistance in pentachlorophenol degrading bacterium isolated from the chemostat," *Journal of Environmental Sciences*, 21(6), 858–864
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Book Chapters

1. Thakur, I.S., P. Jaiswal, and **A. Sharma** (2007) "Bioremediation and detoxification of dioxin-like compounds in food by-products by *Serratia marcescens*," in *Current Topics on Bioprocesses in Food Industry* (Volume II), pp. 91–101, edited by Athanasios Koutinas, Ashok Pandey and Christian Larroche, Asiatech Publishers, New Delhi
2. **Sharma, A.**, and I.S. Thakur (2006) "Pesticide Industry," in *Industrial Biotechnology: Problems and Remedies*, pp. 183–212, IK International Pvt. Ltd, New Delhi

Conference Proceedings

1. **Sharma, A.**, and I.S. Thakur (2009) "Biological treatment of tannery wastewater for removal of chromium and pentachlorophenol in a sequential batch reactor," in Proceedings of *Recent Advances in Waste Management* (RAWM 2009), pp. 206–210, edited by RS Singh *et al.*, Varanasi, India
2. **Sharma, A.,** C. Sharma, and I.S. Thakur (2008) "Biodegradation of 2,4,6-trichlorophenol by *Pseudomonas oleovorans* isolated from contaminated sediment," in Proceedings of the *Sixteenth National Symposium on Environment* (NSE–16), pp. 411–416, edited by VD Puranik and VK Garg *et al.*, Hisar, India

Presentations and Published Abstracts

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1. **Sharma, A.**, and I.S. Thakur, "Biological treatment of tannery wastewater for removal of chromium and pentachlorophenol in a sequential batch reactor," in national conference on *Recent Advances in Waste Management* (RAWM–09), Banaras Hindu University, Varanasi, India, 20–21 February, 2009 (see also conference proceedings)

2. **Sharma, A.**, and I.S. Thakur, "Optimization of process parameters for up-scaling of tannery effluent treatment by microorganism," in national conference on *Recent Advances in Waste Management* (RAWM–09), Banaras Hindu University, Varanasi, India, 20–21 February, 2009

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1. **Sharma, A.**, A. Mishra, and I.S. Thakur, "Isolation and characterization of intradiol ring cleavage chlorohydrolase enzyme in *Acinetobacter* sp. IST3 for degradation of pentachlorophenol," in *3rd International Congress on Bioprocesses in Food Industries* (ICBF) and *5th Convention of Biotech Research Society of India* (BRSI), Osmania University, Hyderabad, India, 6–8 November, 2008
2. Srivastava, S., **A. Sharma,** and I.S. Thakur, "Bioremediation potentiality of microorganisms for removal of chromium and pentachlorophenol in tannery effluent," in *14th International Biodeterioration and Biodegradation Symposium* (IBBS–14), International Biodeterioration and Biodegradation Society, Taormina-Naxos (Messina), Italy, 6–11 October, 2008
3. **Sharma, A.**, C. Sharma, and I.S. Thakur, "Biodegradation of 2,4,6-trichlorophenol by *Pseudomonas oleovorans* isolated from contaminated sediment," in *Sixteenth National Symposium on Environment* (NSE–16), Guru Jambheshwar University of Science and Technology and Bhabha Atomic Research Centre, Hisar, Haryana, India, 16–18 July, 2008 (see also conference proceedings)
4. **Sharma, A.**, and A.K. Sharma, "Decentralized composting as an option for the management of municipal solid waste in NCT of Delhi, India," in *23rd International Conference on Solid Waste Technology and Management*, Philadelphia, PA, USA, March 30–2 April, 2008
5. **Sharma, A.**, A. Mishra and I.S. Thakur, "Characterization of pentachlorophenol monooxygenase, tetrachloro-*p*-hydroquinone reductive dehalogenase of *Acinetobacter* sp. IST3," in symposium on *Contemporary Environmental Problems and Biotechnological applications in their management*, Jawaharlal Nehru University, New Delhi, India, 7–8 March, 2008
6. **Sharma, A.**, and I.S. Thakur, "Biodegradation of pentachlorophenol by *Acinetobacter* sp. IST3 isolated from mixed bacterial consortia of the chemostat," in IUPAC sponsored *First International Conference on Agrochemicals Protecting Crop,*

Health and Natural Environment, Indian Agricultural Research Institute, New Delhi, India, 8–11 January, 2008

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7. **Sharma, A.,** and I.S. Thakur, "Isolation and physiological characterization of the pentachlorophenol degrading bacterium *Serratia marcescens*," in *International Congress of Environmental Research* (ICER–2007), Government PG College, Bhopal, India, 28–30 December 2007
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Characterization of Pentachlorophenol Degrading Bacterial Consortium from Chemostat

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Abstract A microbial consortium was developed by continuous enrichment of bacterial population isolated from sediment core of pulp and paper mill effluent in mineral salts medium (MSM) supplemented with pentachlorophenol (PCP) as sole source of carbon and energy in the chemostat. The enriched consortium contained three bacterial strains identified as *Escherichia coli* (PCP1), *Pseudomonas aeruginosa* (PCP2) and *Acinetobacter* sp. (PCP3) by morphological and biochemical tests, further confirmation was done using 16S rDNA sequence analysis. The potency of bacterial isolates in degradation of PCP was monitored in terms of growth and utilization of PCP as substrate with spectrophotometer and gas chromatograph-mass spectrometer (GC-MS) analysis. The strains were tested for their utilization of various organic compounds. The strain PCP3, showed higher potency to utilize PCP as sole source of carbon and energy than PCP1 and PCP2. The bacterial strain were able to utilize PCP through an oxidative and reductive route as indicated with the formation of tetrachloro-*p*-hydroquinone (TeCH), 2-chloro-1,4-benzenediol and 2,3,4,6-tetrachlorophenol, respectively.

Keywords Biodegradation · Chemostat · Consortia · GC-MS · Pentachlorophenol

The chlorophenolic compounds are major environmental contaminants giving global concern mainly due to use of these compounds as wide spectrum biocides in industry and agriculture (Vallecillo et al. 1999). The most common sources of chlorinated phenols in the environment include production of chlorine from bleaching of pulp, combustion of organic matter, partial transformation of phenoxy pesticides such as 2,4-dichlorophenoxy acetic acid and 2,4,6-trichlorophenoxyacetic acid, treatment of wood against fungi and insects and preservation of raw hides in leather tanning industries (Shukla et al. 2001). The toxicity of these compounds tends to increase with relative degree of chlorination (Reineke and Knackmuss 1988; Fetzner and Lingens 1994). Among chlorinated phenols pentachlorophenol (PCP) and its sodium salt have been widely used as wood and leather preservative owing to their toxic effect on bacteria, mould, algae and fungi (Kaoa et al. 2004). PCP is toxic to all forms of life since it is an inhibitor of oxidative phosphorylation (Shen et al. 2005; Yang et al. 2006). The US Environmental protection agency (EPA) has listed PCP as a priority contaminant because of its proven carcinogenicity and toxicity (Bock et al. 1996). PCP may be washed into streams and lakes due to surface runoff or may infiltrate and contaminate groundwater. Its large amount finally gets deposited onto sediments thus persisting in the environment (Shiu et al. 1994; Thakur et al. 2001). Despite widespread pollution observed, few indigenous bacterial strains capable of degrading PCP have been isolated (Chanama and Crawford 1997). Moreover, PCP is recalcitrant to degradation because of its stable aromatic ring system and high chloride content, thus persisting in the environment (Saber and Crawford 1985; Okeke et al. 1997; Copley 2000).

In case of environmental contamination by PCP, traditional clean-up methods have not been proved successful due to their higher treatment costs and possibilities of causing secondary pollution. The biodegradation of PCP has been studied in both aerobic and anaerobic systems. Anaerobic biodegradation of PCP in aquatic, sludge and

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ORIGINAL PAPER

Enrichment, isolation and characterization of pentachlorophenol degrading bacterium *Acinetobacter* sp. ISTPCP-3 from effluent discharge site

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Received: 14 January 2009 / Accepted: 28 January 2009

Abstract Three pentachlorophenol (PCP) degrading bacterial strains were isolated from sediment core of pulp and paper mill effluent discharge site. The strains were continuously enriched in mineral salts medium supplemented with PCP as sole source of carbon and energy. One of the acclimated strains with relatively high PCP degradation capability was selected and characterized in this study. Based on morphology, biochemical tests, 16S rDNA sequence analysis and phylogenetic characteristics, the strains showed greatest similarity with *Acinetobacter* spp. The strain was identified as *Acinetobacter* sp. ISTPCP-3. The physiological characteristics and optimum growth conditions of the bacterial strain were investigated. The results of optimum growth temperature revealed that it was a mesophile. The optimum growth temperature for the strain was 30°C. The preferential initial pH for the strain was ranging at 6.5–7.5, the optimum pH was 7. The bacterium was able to tolerate and degrade PCP up to a concentration of 200 mg/l. Increase in PCP concentration had a negative effect on biodegradation rate and PCP concentration above 250 mg/l was inhibitory to its growth. *Acinetobacter* sp. ISTPCP-3 was able to utilize PCP through an oxidative route with *ortho* ring-cleavage with the formation of 2,3,5,6-tetrachlorohydroquinone and 2-chloro-1,4-benzenediol, identified using gas chromatograph–mass spectrometric (GC–MS) analysis. The degradation pathway followed by isolated bacterium is different from previously characterized pathway.

Keywords *Acinetobacter* sp. ISTPCP-3 · Biodegradation · Characterization · Gas chromatograph–mass spectrometer · Pentachlorophenol

Introduction

A large variety of chemicals are being synthesized and produced each year. Compounds are finally discharged into the environment during their manufacturing and use of these chemicals. Over last decades, chlorophenolic compounds have been used extensively as wide spectrum biocides in industry and agriculture. These are among the most persistent environmental pollutants because of their physico-chemical characteristics (Annachhatre and Gheewala 1996). The toxicity of these compounds tends to increase with relative degree of chlorination (Reineke

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Identification and characterization of integron mediated antibiotic resistance in pentachlorophenol degrading bacterium isolated from the chemostat

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Abstract

A bacterial consortium was developed by continuous enrichment of microbial population isolated from sediment core of pulp and paper mill effluent in mineral salts medium (MSM) supplemented with pentachlorophenol (PCP) as sole source of carbon and energy in the chemostat. The consortia contained three bacterial strains. They were identified as *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter* sp. by 16S rRNA gene sequence analysis. *Acinetobacter* sp. readily degraded PCP through the formation of tetrachloro-*p*-hydroquinone (TecH), 2-chloro-1,4-benzenediol and products of *ortho* ring cleavage detected by gas chromatograph/mass spectrometer (GC-MS). Out of the three acclimated PCP degrading bacterial strains only one strain, *Acinetobacter* sp. showed the presence of integron gene cassette as a marker of its stability and antibiotic resistance. The strain possessed a 4.17 kb amplicon with 22 ORF's. The plasmid isolated from the *Acinetobacter* sp. was subjected to shotgun cloning through restriction digestion by *Bam*HI, *Hin*dIII and *Sal*I, ligated to pUC19 vector and transformed into *E. coli* XLBlue1α, and finally selected on MSM containing PCP as sole source of carbon and energy with ampicillin as antibiotic marker. DNA sequence analysis of recombinant clones indicated homology with integron gene cassette and multiple antibiotic resistance genes.

Key words: *Acinetobacter* sp.; antibiotic resistance gene; chemostat; integron gene cassette; pentachlorophenol; recombinant clone

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Introduction

Over recent decades, significant quantities of industrial, agricultural and domestic chemicals have been released into the environment. Halogenated aromatic compounds constitute one of the largest groups of chemicals used in industrial application and preservation of biological materials (Yang *et al.*, 2007). Chlorinated phenols and their derivatives are inert, hydrophobic, stay longer in the environment and cause toxicity to flora and fauna (Chu *et al.*, 2008). The toxicity of these compounds tends to increase with their degree of chlorination (Fetzner and Lingens, 1994; Reineke and Knackmuss, 1988). Pentachlorophenol (PCP) is general biocide used primarily as preservative of wood, leather, textile and related commercial products (Chanama and Crawford, 1997). Preservative materials containing PCP may be washed into streams and lakes by surface runoff or may infiltrate to contaminate ground water. In case of environmental contamination by PCP, traditional clean-up methods have not been proved successful. The major difficulties encountered in biological treatment methods are paucity of knowledge concerning PCP-degrading bacterial population and adverse environmental conditions (Thakur *et al.*, 2001; Kaoa *et al.*, 2004). Bacteria with the ability to degrade PCP are widespread in soil and sediment, however, most studies of PCP biodegradation have been carried out under laboratory conditions with arbitrarily selected PCP-degrading bacteria (Xun and Orser, 1991; Orser *et al.*, 1993; McAllister *et al.*, 1996; Thakur *et al.*, 2002; Kaoa *et al.*, 2004; Suegara *et al.*, 2005).

Microbes are often found in consortia bound to surfaces, such as in biofilms or granules. Under these conditions bacteria are positioned in a heterogeneous environment with gradients of nutrients and waste products as a consequence of diffusion and mass transport processes, and it is therefore to be expected that this heterogeneity be reflected in the physiology of individual cells. The development of structurally organized communities may argue for the presence of overall regulatory elements, which control the formation of the community structures (Senior *et al.*, 1976). An important factor in understanding community level processes is the relationship between structure and function in microbial communities, and its genetic stability which may be governed by antibiotic resistance (Hochhut *et al.*, 2001; Holmes *et al.*, 2003).

The genomics era has clearly indicated that a large proportion of bacterial genes have been acquired by horizontal gene transfer. Horizontal gene transfer is facilitated

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