Studies on the Biodegradation of some Chlorophenolics in Secondary Sludge of Pulp and Paper Industry

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Submitted in fulfillment of the requirements
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CERTIFICATE

Certified that the thesis **"Studies on the Biodegradation of some Chlorophenolics in Secondary Sludge of Pulp and Paper Industry"** which is submitted by **Mr. Santosh Kumar Karn**, in fulfillment of the requirement for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is a record of the candidate's own independent and original research work carried out by him under our supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

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DECLARATION

I hereby declare that the work which is being presented in this thesis **"Studies on the Biodegradation of some Chlorophenolics in Secondary Sludge of Pulp and Paper Industry"** submitted by me for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of Dr. M. Sudhakara Reddy, Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, and Dr. S. K. Chakrabarti, Dy. Director, TCIRD Yamunanagar, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

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- **Santosh Kr. Karn,** S. K. Chakrabarti and M. S. Reddy **(2010)**. Pentachlorophenol degradation by *Pseudomonas stutzeri* CL7 in the secondary sludge of pulp and paper mill. ***Journal of Environmental Sciences***. Vol. 22(10):1608-1612. Elsevier Publication.

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- **Santosh Kr. Karn** S. K. Chakrabarti and M. S. Reddy **(2011)**. Degradation of pentachlorophenol by *Kocuria* sp. CL2 isolated from secondary sludge of pulp and paper mill. ***Biodegradation.*** Vol. 22(1): 63-69. Springer Publication.

- **Santosh Kr. Karn,** S. K. Chakrabarti and M. S. Reddy **(2011)**. Biodegradation of 2,4,6-Trichlorophenol by bacterial isolates from secondary sludge of pulp and paper industry. ***Indian Journal of Microbiology.*** Springer Publication *(In Press).*

Conferences Presentations

- **Santosh Kumar Karn**, S. K. Chakrabarti and M. S. Reddy **(2008)** Degradation of chlorophenolic compounds by *Bacillus sp.* isolated from secondary sludge of pulp and paper Industry in **AMI-2008**, International symposium on Microbial Biotechnology: Diversity, Genomics and Metagenomics organized by University of Delhi, India.

- **Santosh Kumar Karn,** S. K. Chakravarti and M. S. Reddy **(2010)** 2,4,5-trichlorophenol degradation by bacterial isolates from industrial sludge. In **ABP-2010** National conference on emerging trends in biopharmaceuticals: Relevance to human health organized by TIFAC-CORE, Department of Biotechnology & Environmental Science, Thapar University, Patiala, India.

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LIST OF ABBREVIATION

%	Percent
°C	Degree centigrade
2,4,5-T	Ttrichlorophenoxyacetic acid
AOX	Absorbable organic halogen
ARDRA	Amplified ribosomal DNA restriction analysis
BCF	Bio concentration factor
BOD	Biochemical oxygen demand
BOX	Repetitive DNA sequences of the BOX element
bp	Base pair
CC	Chlorocatechols
CFU	Colony forming units
CHNO	Carbon hydrogen nitrogen oxygen
CHQ	Chloroquoinone
Cl^-	Chloride ion
Cl_2	Chlorine gas
ClO_2	Chlorine dioxide
CMA	Chloromaleylacetate
CO_2	Carbon dioxide
COD	Chemical oxygen demand
CP	Chlorophenol
DCHQ	Dichlorohydroxyquionine
DCP	Dichlorophenol
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside-5'-triphosphate
EC	Electrical conductivity
EDTA	Ethylenediamine-tetra acetic acid
EOX	Extractable organic halogen
ERIC	Enterobacterial Repetitive Intergenic Consensus
EtBr	Ethidium bromide
FISH	Fluorescence *in situ* hybridisation

g	Gram
H_2O	Water
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
HQDO	Chlorohydroxyquinol dioxygenase
IPTG	Isopropyl-β-thiogalactoside
kb	Kilo base
M	Molar
MAR	Maleylacetate reductase
mg	Milligram
mg/l	Milligram per liter
ml	Milliliter
mM	Mill molar
MO	Monooxygenase
MSM	Mineral salt medium
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOCl	Sodium hypochlorite
NO_2	Nitrite
NO_3	Nitrate
OD	Optical density
P	Phosphorus
PCB	Polychlorinated bipenyls
PCP	Pentachlorophenol
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
rDNA	Ribosomal DNA
RDP	Ribosomal data base project
REP	Repetitive Extragenic Pallindrome sequence
SDS	Sodium dodecyl sulphate
SE	Standard error
SO_4^{-2}	Sulphate

TCP	Trichlorophenol
TeCHQ	Tetrachlorohydroquinone
TeCHQ	Tetrachlorohydroxyquionine
TeCP	Tetrachlorophenol
Tris	Tris-(hydroxymethyl-) aminomethane
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
w/v	Weight by volume
w/v	Weight by volume
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
µg	Microgram
µl	Microlitre

CHAPTER-1

Chapter-1

1. Introduction

1.1 Pulp and Paper Mill and Chlorophenol

Advances in industrialization bring with it unpleasant partners; pollution and degradation of the environment. The effects on the environment connected with industrial activities are mainly related with the production of industrial wastes. Over recent decades significant quantities of organochemicals are released by industrial, agricultural, and domestic activities into the environment. Microorganisms rapidly degrade a few chemicals and eliminate them from the environment, but there are other chemicals that are degraded slowly, accumulate in the environment and occasionally exhibit toxicity (Alexander, 1981). Pulp and paper mills are categorized as a core industrial sector which is the fifth largest contributor to water pollution. Globally pulp and paper production has increased significantly and will increase further.

The pulp and paper industry is the world's second largest chlorine consumer, using about 3 million tones each year to bleach the wood pulp (Martin et al., 1993). Chlorine is used in a number of different forms; as elemental chlorine gas (Cl_2), chlorine dioxide (ClO_2) or sodium hypochlorite (NaOCl). Because chlorine is extremely reactive, it combines quickly with the organic matter in the pulp to generate a large number of compounds called organochlorines. Over 300 organochlorines have been identified in the discharge of bleached pulp mills, including dioxins, dibenzofurans, chlorinated phenols and acids, and many others (Bonsor, 1989 and Suntio et al., 1988). These identified compounds account for less than 10 percent of all the organochlorines in the effluent; the majority remain "mystery" chemicals that have not been specifically identified or assessed.

The wood pulping and production of the paper products generate a considerable amount of pollutants characterized by biochemical oxygen demand (BOD), chemical oxygen demand (COD), chloroorganics and color when untreated or poorly treated effluents are discharged to receiving waters (Pokhrel and Viraraghvan, 2004). Each pulping process utilizes large amounts of water, which reappears in the form of an effluent. The most significant sources of pollution among various process stages are wood preparation, pulping, pulp washing, screening, bleaching, and paper machine and coating operations. Figure 1 represents flow chart of generation of pollutants during pulping and paper making process in pulp and paper industry.

Among the processes, pulping generates a high-strength wastewater especially by chemical pulping. This wastewater contains wood debris and soluble wood materials. Pulp bleaching generates most toxic substances as it utilizes chlorine for brightening the pulp. Pulp fibers can be prepared from a vast majority of plants in nature such as woods, bamboos, straws and grasses or canes and reeds. Wood is the most abundant source of paper making fiber. Wood consists of various compounds (carbohydrate, lignin and extractives) the parts of which are difficult to biodegrade, and these derivatives are washed away from the fibers during the washing, dewatering, and screening processes. Depending upon the type of the pulping process, various toxic chemicals such as resin acids, unsaturated fatty acids, diterpene alcohols, juvaniones, chlorinated resin acids, and others are generated in the pulp and papermaking process (Pokhrel and Viraraghavan, 2004).

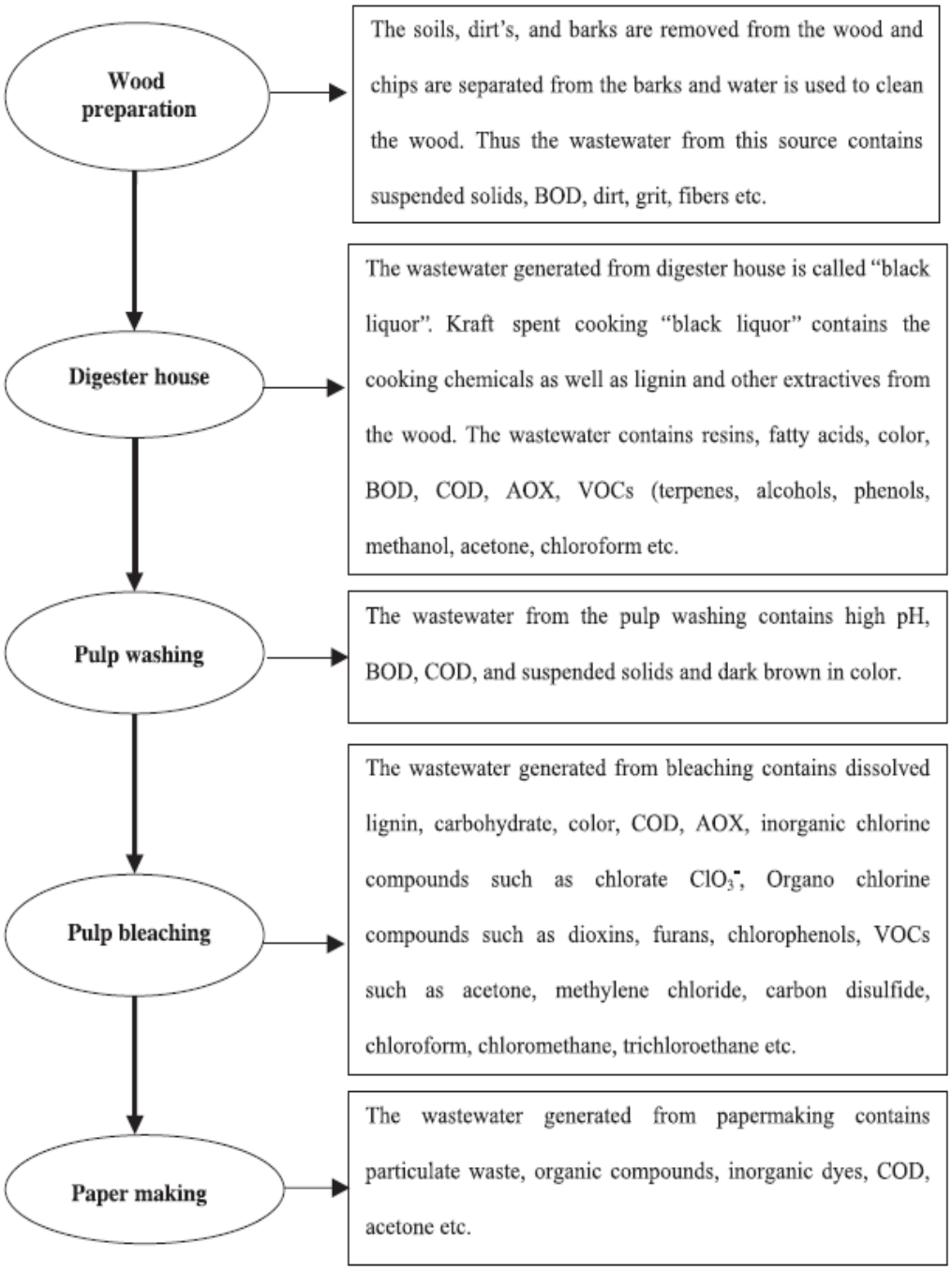


Figure 1: Pollutants from various sources of pulping and papermaking process (EPA, 1995).

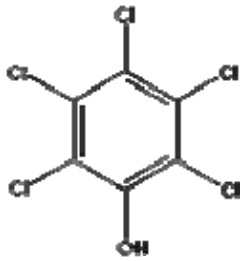
Organochlorines are also found in the sludge generated while treating as the wastewaters. Accounting for as much as four percent of the total weight of the material, contaminated sludge is spread on the land, buried in landfills, or incinerated, releasing chlorinated by-products into the air, including polychlorinated bipenyls (PCB) and dioxins (Mantykoski, 1989).

The pulp and paper industry is also a highly energy intensive industry. In recent times, the high cost of input energy and increased environmental concerns are forcing the pulp and paper industry to look for cost-effective and environment friendly alternatives. Application of biotechnology in the pulp and paper sector is one such alternative to achieve both energy and cleaner production.

The United States Environmental Protection Agency (USEPA) regulates chlorophenolic substances in the wastewater to less than detection level. These chlorophenolics are twelve in number viz, 2,4,5-trichlorophenol; 2,4,6-trichlorophenol; 3,4,5-trichlorocatecol; 3,4,6- trichlorocatecol; 3,4,5-trichloroguaiacol; 3,4,6,trichloroguaciacol; 4,5,6-trichloroguaiacol; Trichlorosyringol; Tetrachlorocatecol; Tetrachloroguaiacol; 2,3,4,6-tetrachlorophenol and Pentachlorophenol. Out of these twelve chlorophenols, present study deals with the degradation of four different chlorophenols, Pentachlorophenol (PCP), 2,3,4,6-tetrachlorophenol (TeCP), 2,4,5-trichlorophenol (2,4,5-TCP) and 2,4,6-trichlorophenol (2,4,6-TCP).

1.2 Pentachlorophenol

C_6Cl_5OH (266.34)



Pentachlorophenol (PCP) is a man-made chlorinated hydrocarbon compound, pure PCP exists as a colorless crystal. Impure PCP, usually found at hazardous waste sites, is dark grey to brown dust, beads or flakes. PCP is a non-flammable solid, and does not evaporate easily. It exists in two forms: the non polar one dissolves easily in water, and the other one does not dissolve. The polar form of PCP is soluble in alcohol and benzene, and slightly soluble in cold petroleum ether (EHC, 1997).

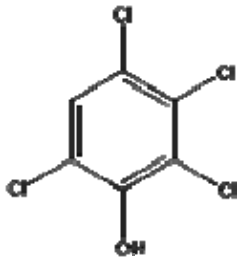
PCP is used as preservative for timber from fungal rot and wood-boring insects, intermediate for pesticide and fungicide, cooling tower additive of electric plant, as additive to adhesive, additive in shingle, brick wall, concrete block, insulation, pipe sealant compound, photographic solution, and textile and in drilling mud in the petroleum industry (EPA, 1984).

Pentachlorophenol is released in the air by evaporation from treated wood surfaces and factory (chemical manufacturing plants and wood preservation plants) waste disposal. It enters surface water and groundwater from factories, wood-treatment facilities, and hazardous waste sites. It also enters the soil as a result of spills, disposal at hazardous waste sites, and its use as a pesticide. The physical and chemical properties of the compound suggest that not much will evaporate into the atmosphere and that most of it

will move with water and generally stick to soil particles. The compound can be present in fish or other species used for food. PCP is expected to be recalcitrant to aerobic biodegradation because it is highly chlorinated and, in general, aromatic compounds with higher amounts of chlorine are more resistant to biodegradation (Anandarajah et al., 2000). In addition, this compound is very harmful to microorganisms because it destroys membrane function due to its ability to uncouple oxidative phosphorylation (Copley, 2000; Ito and Ohnishi, 1982). Humans are generally exposed to pentachlorophenol, which usually contains such toxic impurities as polychlorinated dibenzo-p-dioxins and dibenzofurans. Studies have shown that exposure to high levels of PCP can cause the cells in the body to produce excess heat. When this occurs, a person may experience a very high fever, refuse sweating, and difficulty in breathing (ATSDR, 1992). The body temperature can increase to dangerous levels, causing injury to various organs and tissues, and even to death. Liver effects and damage to the immune system have also been reported in humans subjected to chronic exposure of high PCP levels (Gasiewicz, 1991). Damage to the thyroid and reproductive system has been observed in laboratory animals exposed to high doses of PCP (Gasiewicz, 1991). Some of the harmful effects of PCP are actually caused by the other chemicals present in pentachlorophenol (U.S. Public Health Service, 1994). Increase in liver, adrenal gland, and nasal tumors have been found in laboratory animal and fish exposed to high PCP dose (Hill and Camardese, 1986). Other studies have determined that PCP is a probable human carcinogen (Apajalahti and Salkinoja-Salonen, 1984).

1.3 2,3,4,6-tetrachlorophenol

$C_6HCl_4(OH)$ (231.89)



2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP) has been used as herbicide, algaecide, germicide and fungicide, defoliant, wood preservative for wood, leather and latex, sap stain (sodium salt form). 2,3,4,6-TeCP is regarded as toxic compound and the presence of the compound in textile products are regulated under USEPA regulation (EPA, 1987).

2,3,4,6-TeCP enters the environment primarily in wastewater during its production and use as a wood preservative. It may also be released from the use of pentachlorophenol since it is a major impurity and degradation product of that chemical. If spilled on soil, 2,3,4,6-TeCP is adsorbed fairly strongly under acidic condition, but since it exists principally as a phenolate ion at neutral and alkaline pH, it would be expected to leach in soil. If released in water, it is adsorbed in sediment and particulate matter and photo degrade in surface waters (EPA, 1999). Human exposure takes place through contact with penta- or tetrachlorophenol treated products. The skin, lung and gastrointestinal lining absorb these compounds, causing contact dermatitis, diffuse urticaria and chlorance (EPA, 1987). 2,3,4,6-TeCP biodegrade very slowly with acclimated microbial populations (EPA, 1987).

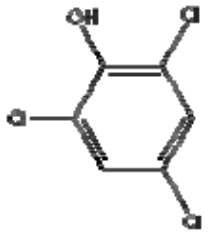
1.4 2,4,5-trichlorophenol

$C_6H_2Cl_3$ (OH) (197.45)

Among the chlorinated phenols 2,4,5-trichlorophenol (2,4,5-TCP) comes in the environment when it is used in the production of a variety of biocide (Hazardous Substances Data Bank) ingredients of germicidal soaps and germicide for preservation of plywood. 2,4,5-TCP has been used as a biocide itself and as an intermediate in the production of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), one of the most widely used herbicides in the 20th century. It is included in the USEPA's list of priority pollutants (Maltseva and Oriel, 1997). Furthermore, TCP is the first intermediate in microbial degradation of 2,4,5-T (Daubaras et al., 1995). On exposure, 2,4,5-TCP causes hypertension, myocardial failure, pulmonary edema, neurological changes, liver and renal toxicity, methemoglobinemia and hemolysis (EPA, 1984). As very few work has been done for 2,4,5-TCP degradation relative to other chlorophenols, it seems that 2,4,5-TCP is remarkably more resistant to biodegradation than other trichlorophenols (Maltseva and Oriel, 1997).

1.5 2,4,6-trichlorophenol

$C_6H_2Cl_3(OH)$ (197.45)



Chlorophenols, which have been produced industrially on a large scale, constitute a significant class of environmental pollutants. 2,4,6-trichlorophenol (2,4,6-TCP), widely used as a biocide and preservative, is considered to be priority environmental pollutant worldwide (Huynh et al., 1985). 2,4,6-TCP is used for the preservation of leather and textile goods, as a glue and wood preservative, and as an antimildew agent (Sittig, 1981). Furthermore, 2,4,6-TCP is a precursor for the synthesis of 2,3,4,6- tetrachlorophenol, pentachlorophenol, and herbicides 2,4-dichloro and 2,4,5-trichlorophenoxyacetic acids (Rappe, 1980). A major component of chlorinated phenols in kraft paper mill effluent is is 2,4,6-TCP was determined to be a principal contaminant, occurring at concentrations of 0.15 to 0.33 ppm in bleaching mill effluent (Huynh et al., 1985). From the fungicide KY-5, containing 5 to 15% 2,4,6-TCP, considerable amounts of 2,4,6-TCP enters the environment (Lampi, 1990). It is carcinogenic in animals, causing lymphomas, leukemia, and liver cancer via oral exposure. From the six isomers of trichlorophenol, the 2,4,5- and 2,4,6-isomers have been placed on the U.S. Environmental Protection Agency's list of priority pollutants (Sittig, 1981).

1.6 Fate and effect on the environment

The pollutants discharged from the pulp and paper industry affect the environment consisting of water, air soil, flora and fauna. Many authors reported the presence of toxic pollutants in fish and toxic effects on fish such as respiratory stress, mixed function oxygenase activity, mutagenicity, liver damage, or genotoxic effects, and lethal effects while exposed to pulp and paper mill wastewater (Erisction and Larsson, 2000). Baruah (1997) reported adverse effect on the surface plankton population in Elengabeel's wetland ecosystem in India due to untreated paper mill effluent discharged into the system. Yen et al. (1996) reported on the possibility of the sub-lethal effects to the aquatic organisms in the Dong Nai River in Vietnam due to the effluent discharged from a pulp and paper mill. Howe and Michael (1998) studied the effects of the treated pulp mill effluent on irrigated soil in northern Arizona, which showed change in soil chemistry. Dutta (1999) investigated the toxic effect of the paper mill effluent (treated) applied to a paddy field in Assam. Gupta (1997) and Singh et al. (1996) reported high loads of organic pollutants derived from the paper mill wastewater in Tamilnadu, and Punjab, respectively. Singh et al. (1996) also indicated high level of coliform bacteria in the effluent too. However, Archibald (2000) indicated that the presence of coliform bacteria in the pulp and paper mill effluent did not necessarily mean a health hazard to the environment unless pathogens were observed. Skipperud et al. (1998) and Holmbom et al. (1994) reported the presence of various trace metals in the pulp and paper mill effluents at low levels. King et al. (1999) reported elevated levels of Mn accumulation in the Crayfish exposed to the paper mill wastewater. Mandal and Bandana (1996) reported on health impacts such as diarrhoea, vomiting, headaches, nausea, and eye irritation on

children and workers due to the pulp and paper mill wastewater discharged to the environment. Use of chlorophenols was restricted to that of a wood preservative since these are probable human carcinogen. Short term ingestion or inhalation results in neurological disorders, Leukemia, liver damage, and eye irritation. Long-term exposure damages the respiratory tract, blood, kidneys, liver, immune system, eyes, and skin (Pepper et al., 1999).

1.7 Remediation of chlorophenols (PCP, 2,3,4,6-TeCP, 2,4,5-TCP, 2,4,6-TCP)

Although different physical, chemical and electrochemical methods like adsorption, ultrafilteration, precipitation, coagulation, and electrodylysis are available for treatment of pulp and paper mill effluent, these are less desirable than biological treatment due to generation of secondary pollutant and cost-ineffectiveness. Biological treatment is known to be effective in reducing the organic load and toxic effects of pulp and paper mill effluent. There have been several attempts to use biological methods to decontaminate effluent from Kraft mills because of their ability to degrade lignin by several microorganisms (Haggblom and Salonen, 1992).

Biological treatment of chlorophenols attracts more attention than physical and chemical methods, because a variety of microorganisms such as *Rhodococcus chlorophenolicum*, *Flavobacterium* sp., *Novosphingobium lentum, Sphingomonas chlorophenolica* and *Pseudomonas* sp., (Field and Sierra-Alvarez 2008, and references therein) are known to utilise chlorophenols as the sole carbon or energy source and the reaction products are Cl^- ions, CO_2 and biomass. There are still many unknown bacteria that have tremendous degradation capacity for chlorophenols present in nature and it is important to asses the potential of bacterial isolates indigenous to sites contaminated with the chlorophenols.

In the present investigation the diversity of bacteria capable of degrading some of chlorophenols (PCP, 2,3,4,6-TeCP, 2,4,5-TCP and 2,4,6-TCP) were studied. The efficient bacteria which degrade different chlorophenols were identified and tested their efficacy in degradation of these chlorephenols under *in vitro* and *in situ* conditions.

CHAPTER-2

Chapter- 2

2. Review of literature

2.1 Isolation and enrichment of microorganism

Microbial diversity offers immense environment friendly options for mineralization of contaminants or their transformation into less harmful non-hazardous compounds. There is a general interest in studying the diversity of indigenous microorganisms capable of degrading different pollutants due to their varied effects on the environment. Efforts have been made to characterize bacterial communities, isolate potential degraders, and identify the genes involved in degradation processes (Watanabe et al., 2002).

Microorganisms with specific degradation capabilities may be isolated by adding the compound of interest to enrich those cells from environmental samples. Isolates can then be obtained on culture medium containing the compound as a carbon source. In this manner one may select for those microorganisms that are able to metabolize or at least survive in the presence of pollutant compounds, such as aliphatics, aromatics and their chlorinated derivates e.g. polychlorophenol (3 to 5 chlorine content). Many bacteria possess the ability to degrade simple intermediates of chloroaromatics such as chlorocatechol (Chakrabarty, 1996).

Enrichment and isolation of suitable microorganisms facilitate studies of the potential for biodegradation of chlorophenols. Several aerobic PCP-degrading bacteria have been isolated from contaminated soils such as *Flavobacterium* sp., *Arthrobacter*, *Sphingomonas chlorophenolica* (Edgehill and Finn, 1983; Crawford and Mohn, 1985; Saber and Crawford, 1985; Miethling and Karlson, 1996; Yang et al., 2006).

2.2 Molecular characterization

Early traditional characterization of the microorganism depended upon phenotype, biochemical and serological tests. But these are not fully reliable as mutation and environmental conditions can affect the physiological traits. Ribosomal RNA (particularly 16S rRNA) is considered to be most reliable candidate molecule for identification-classification study. The analysis of 16S rRNA genes, aided by using PCR to amplify target sequences in environmental samples, has enabled microbial ecologists to identify and characterize microorganisms in a natural community like activated sludge. The taxonomic position of an organism can be determined by comparing the sequence with those of other bacteria (Amann et al., 1995).

Analysis of 16S rRNA gene is now widely used for analysis of bacterial population. The macromolecules that are most suitable for this purpose would require the following prerequisites: (i) generally present, (ii) functionally homologous in all organisms, and iii) the sequence in the molecule should equally change during evolutionary process. Ribosomal RNA is one of the best candidates and it has been used for the studies on bacterial evolution. Major properties of rRNA are: (1) present as old molecules in the ribosomes, (2) functionally constant, (3) wide distribution, (4) well conserved over large phylogenetic distances, and (5) occurence in large number in cells (10^4-10^5/cells). In bacteria three types of rRNA molecules are present with different chain length and sedimentation rate(s): 5S rRNA (about 120 nucleotides), 16S rRNA (~ 1600 nucleotides) and 23S rRNA (~3000 nucleotides). The 5S molecule is too small and only suitable to distinguish major phylogenetic groups. 23S rRNA is an excellent candidate very few

phylogenetic studies are available. 16S rRNA has been given most attention (Woese et al., 1990).

Since rDNA-based fingerprinting lays too much emphasis on a single locus for deciphering phylogenetic affinities, isolates are subjected to multi-locus analysis like BOX-PCR, ERIC-PCR and random amplification of polymorphic DNA (RAPD). The BOX-PCR, in contrast to ARDRA, is the multi-locus analysis and produces higher degree of resolution among the isolates. The repetitive sequences in the form of BOX elements are randomly located within the whole genome and the BOX primers amplify genomic regions between the two BOX elements. The distribution of these repetitive sequences (BOX and ERIC) is nearly a true reflection of genomic structure and amplification of inter-REP elements often detects similarities in a given group of bacteria. It is anticipated that REP- and ERIC-like sequences are virtually ubiquitous in bacteria and facilitate a rapid molecular characterization by PCR-based fingerprinting (Selenska-Pobell et al., 1995).

Recently, the development of culture-independent molecular techniques, like fluorescence *in situ* hybridization (FISH), polymerase chain reaction (PCR) or denaturing gradient gel electrophoresis (DGGE) improved the analysis of environmental samples. Beside fluorescent probes for the major groups of bacteria species-specific probes were applied for filamentous bacteria (Eschenhagen et al., 2003).

Although 16S rRNA analysis represents a very useful technique for culture independent analysis of complex microbial communities, the clone frequencies in the clone libraries do not reflect the *in situ* quantities of the respective microorganisms. Possible reasons are

differences in the numbers of rRNA operons, efficacies of cell lyses and DNA extraction or shifts due to PCR amplification (Eschenhagen et al., 2003).

2.3 Microbiology and biochemistry of chlorophenol degradation

Chlorophenols are subject to both aerobic and anaerobic metabolism. Under aerobic conditions, both lower and higher chlorinated phenols can serve as sole electron and carbon sources required for the growth and in some cases chlorophenols are aerobically cometabolized. Under anaerobic conditions, chlorinated phenols are subject to reductive dechlorination when suitable electron-donating substrates are available. Halorespiring bacteria are known, which can use chlorophenols as electron acceptors to support growth. Lower chlorinated phenols are also used as a carbon and energy source by anaerobic microbial consortia (Field and Sierra-Alvarez, 2008).

2.4 Aerobic bacterial cometabolism of chlorophenols

Several examples of aerobic cometabolism of chlorinated phenols are reported. A *Nocardia* strain and three *Pseudomonas* strains isolated from soil with benzene as a sole carbon source were observed to cometabolize chlorophenols (Haider et al., 1974) when precultivated on benzene. ^{14}C-Labelled 2-CP and 4-CP were extensively mineralized to $^{14}CO_2$ by 60 to 85% within 7 days. Under the same conditions, two of the *Pseudomonas* partially mineralized 2,4-DCP and 2,4,5-TCP supplied as isomer mixtures. Toluene-grown cells of *Pseudomonas putida* were also shown to effectively oxidize 2-CP, 3-CP, 4-CP, 2,4-DCP, 2,3-DCP, 2,5-DCP, 3,4-DCP and 2,4,5-TCP generating, in most cases, chlorocatechols as intermediates (Spain and Gibson, 1988). Methylstyrene served as primary substrate in the oxidation of 2-CP and 4-CP by *P. putida* (Bestetti et al., 1992).

O-Cresol served as a primary substrate for the oxidation of 2,4-DCP by *Alcaligenes eutrophus* JMP222 to 3,5-dichlorocatechol (Koh et al., 1997). Benzoate-induced cells of *Rhodococcus erythropolis* M1 cometabolized 2-CP, 4-CP and 2,4-DCP. In several studies the analogue substrate, phenol, has been utilized by various bacterial strains as the primary substrate to support the cometabolism of 2-CP, 4-CP (Cobos-Vasconcelos et al., 2006; Loh and Wu, 2006), or 2,4-DCP (Beltrame et al., 1982; Cobos-Vasconcelos et al., 2006). There are also examples of chlorophenols serving as primary substrates allowing for the cometabolism of other chlorophenols. *Pseudomonas* strain B13 cometabolized 3-CP, and 2-CP and 4-CP as growth substrate (Knackmuss and Hellwig, 1978). 3-Chlorophenol served as a primary substrate supporting 3,5-DCP cometabolism (Liu et al., 1991) and PCP served as a primary substrate supporting 3,4,5-TCP and 2,3,5,6-TeCP cometabolism (Liu et al., 1991). 2,4,6-TCP served as a growth substrate of either *Azobacter* sp., *Streptomyces rochei* or *Pseudomonas pickettii* to support the cooxidation of a large number of other chlorophenols (Li et al., 1991). Dichloro or trichlorophenoxyacetic acid-grown cells of *Arthrobacter* or *Pseudomonas cepacia* also supported the degradation of numerous chlorophenols (Kilbane et al., 1982). Finally, ammonia in a nitrifying bioreactor served as a primary substrate supporting the cooxidation of 2,4,6-TCP (Nevalainen et al., 1993). PCP-grown cells of the PCP-degrading bacterium, *Sphingomonas chlorophenolica*, have the ability to mineralize 2,3,6-TCP, 2,4,6-TCP and 2,3,4,6-TeCP as evidenced by chloride release (Yang et al., 2005).

The primary substrates considered above are all expected to induce either dioxygenases or monooxygenases facilitating the co-oxidation of the cometabolized chlorophenols.

However, primary substrates such as sugar, which do not require oxygenases for metabolism, have also been implicated in the cometabolism of 2 and 4-CP (Haider et al., 1974), 2,4-DCP (Beltrame et al., 1982) and PCP (Yang et al., 2005). Sugars as primary substrate may support cometabolism by generating NADH required by oxygenases (Wang and Loh, 1999).

2.5 Aerobic bacterial growth on chlorophenols as a sole source of carbon and energy

A large variety of bacteria are known which can utilize chlorophenols as a carbon and energy source under aerobic conditions. The earlier reports of bacterial utilization of chlorophenols include those of Chu and Kirsch (1972) and Tyler and Finn (1974). Chu and Kirsch (1972)) described a bacterial strain (KC 3) capable of mineralizing [^{14}C] PCP to $^{14}CO_2$ when supplied as a sole carbon source. PCP mineralization was shown to be linked to cell growth. Tyler and Finn (1974) described *Pseudomonas* sp. strain NCIB9340 that grow on 2,4-DCP. Since then a wide variety of bacterial strains have been shown to utilize chlorophenols (Table 2.1).

Table 2.1: Aerobic bacterial strains capable of growing on chlorinated phenols as a sole source of carbon and energy.

Bacterial Strain	Chlorophenol	Reference
Pseudomonas spp. UG25 and UG30	PCP	(Leung et al., 1997)
Pseudomonas sp. RA2	PCP	(Radehaus & Schmidt, 1992)
Pseudomonas sp. strain SR3	PCP	(Resnick & Chapman, 1994)
Pseudomonas sp. strain IST103	PCP	(Thakur et al., 2001; 2002)
Pseudomonas mendocina NSYSU	PCP	(Kao et al., 2005)
Mycobacterium sp. strain CG-2	PCP	(Haggblom et al., 1988b)
Mycobacterium chlorophenolicum PCP-1	PCP	(Wittmann et al., 1998)
Sphingomonas sp. strain P5	PCP	(Rutgers et al., 1997)
Sphingomonas chlorophenolica RA2	PCP	(Nohynek et al., 1995; Ederer et al., 1997; Wittmann et al., 1998)
Novosphingobium lentum MT1	PCP	(Tiirola et al., 2005)

Sphingomonas chlorophenolica	PCP	(Yang et al., 2006)
Rhodococcus chlorophenolicus PCP-1	PCP	(Apajalahti & Salkinoja Salonen, 1986; 1987b)
Rhodococcus sp. CP-2 and CG1	PCP	(Haggblom et al., 1988b)
Strain KC-3	PCP	(Chu and Kirsch, 1972)
Flavobacterium sp ATCC39723	PCP	(Hu et al., 1994; Orser & Lange, 1994)
Flavobacterium sp.	PCP	(Gonzalez & Hu, 1991)
Flavobacterium strains	PCP	(Saber & Crawford, 1985; Steiert & Crawford, 1986)
Arthrobacter strain NC	PCP	(Stanlake & Finn, 1982)
Pseudomonas saccharophila	2346-TeCP	(Puhakka et al., 1995)
Rhodopseudomonas sp. K13	2346-TeCP	(Mannisto et al., 1999)
Nocardioides sp. strain K44	2346-TeCP	(Mannisto et al., 1999; 2001)
Unidentified strain K112	2346-TeCP	(Mannisto et al., 1999)
Sphingomonas K74 and MT1	2346-TeCP	(Mannisto et al., 2001)
Sphingomonas sp. strain P5	2346-TeCP	(Rutgers et al., 1997)
Rhodococcus chlorophenolicus	2346-TeCP	(Apajalahti & Salkinoja Salonen, 1986; 1987b)
Novosphingobium lentum MT1	2346-TeCP	(Tiirola et al., 2005)
Pseudomonas cepacea AC1100	245-TCP	(Kilbane et al, 1982)
Nocardioides simplex 3E	245-TCP	(Golovleva et al, 1990)
Arthrobacter strain NC	246-TCP	(Stanlake & Finn, 1982)
Pseudomonas saccharophila	246-TCP	(Puhakka et al., 1995)
Pseudomonas pickettii	246-TCP	(Kiyohara et al., 1992)
Rhodopseudomonas sp. strain K13	246-TCP	(Mannisto et al., 1999)
Nocardioides sp. strain K44	246-TCP	(Mannisto et al., 1999; Mannisto et al., 2001)
Unidentified strain K112	246-TCP	(Mannisto et al., 1999)
Sphingomonas strains K74 and MT1	246-TCP	(Mannisto et al., 2001)
Alcaligenes eutrophus JMP134(pJP4)	246-TCP	(Valenzuela et al., 1997)
Ralstonia eutropha JMP134(pJP4)	246-TCP	(Matus et al., 2003; Xun & Webster, 2004)
Azotobacter sp strain Gp1	246-TCP	(Li et al., 1991)
Streptomyces rochei 303	246-TCP	(Golovleva et al., 1992)
Novosphingobium lentum strain MT1	246-TCP	(Tiirola et al., 2005)

There is an extensive evidence that chlorophenols are mineralized by bacteria that utilize the compounds as a carbon and energy source. The evidence is based on the

stoichiometric release of inorganic chloride (Tyler and Finn, 1974; Saber and Crawford, 1985; Radehaus and Schmidt, 1992; Mannisto et al., 1999; Yang et al., 2005), the concomitant production of biomass linked to chlorophenol utilization (Tyler and Finn, 1974; Edgehill and Finn, 1982; Radehaus and Schmidt, 1992; Hu et al., 1994; Resnick and Chapman, 1994; Rutgers et al., 1997) or the conversion of ^{14}C-labeled chlorophenols to $^{14}CO_2$ (Chu and Kirsch, 1972; Saber and Crawford, 1985; Apajalahti and Salkinoja Salonen, 1986; Haggblom et al., 1988b).

Pseudomonas sp. biotransformed [^{14}C]-pentachlorophenol rapidly and released radiolabeled carbon dioxide as well as the intermediate metabolites, tetrachlorophenol and tetrachlorohydroquinone (Radehaus and Schmidt, 1992). In another study, strains of *Pseudomonas putida* and *Acinetobacter calcoaceticus* were able to use pentachlorophenol as a sole carbon and energy source (Martins et al., 1997).

A little work has been done on 2,4,5-TCP biodegradation compared to other chlorophenols; it seems that 2,4,5-TCP is remarkably more resistant to biodegradation than other trichlorophenols. There are a few reports on bacteria that degrade 2,4,5-TCP. *Pseudomonas cepacea* AC1100 (Kilbane et al., 1982) and *Nocardioides simplex* 3E (Golovleva et al., 1990) grown on 2,4,5-TCP.

A microbial consortium that efficiently degrades 2,4,6-TCP, as the sole source of carbon and energy under aerobic conditions was selected from municipal activated sludge. Six bacterial strains, designated S1, S2, S3, S4, S5 and S6, were isolated from the selected consortium and five were identified as *Sphingomonas paucimobilis* (S2, S3), *Burkholderia cepacia* (S4), *Chryseomonas luteola* (S5) and *Vibrio metschnikovii* (S6). This consortium having specific degradation rate of 34 mg 2,4,6-TCP per gram dry

weight per hour (about 51 mg 2,4,6-TCP per gram cell protein per hour) (Kharoune et al., 2002).

Two main strategies are used to degrade chlorophenols by aerobic bacteria utilizing these compounds as a carbon and energy source (Solyanikova and Golovleva, 2004). Lower chlorinated phenols (1 to 2 chlorine substituents) are initially attacked by monooxygenases yielding chlorocatechols as the first intermediates (chlorocatechol pathway), which are subject to ring cleavage prior to dechlorination. On the other hand, polychlorinated phenols (3 to 5 chlorines) are converted to chlorohydroquinones as the initial intermediate (hydroquinone pathway). Subsequent reactions progressively remove chlorines from the ring prior to ring cleavage.

The chlorocatechol pathway was exemplified for the chlorophenol, 2,4-DCP, as shown in figure 2.1. 2,4-DCP attack was initiated by monooxygenases forming 3,5-dichlorocatechol (Bollag et al., 1968; Finkel'shtein et al., 2000). The chlorocatechol is *ortho* cleaved yielding 2,4-dichloromuconic acid (Bollag et al., 1968; Tiedje et al., 1969). The first dechlorination takes when a lactonizing enzyme converts the dichloromuconic acid to 2-chloro-4-carboxymethylene but-2-enolide (Bollag et al., 1968; Tiedje et al., 1969; Sharpee et al., 1973).

The butenolide is converted to 2-chloromaleylacetic acid which is further metabolized to succinate (Tiedje et al., 1969). The 2,4-dichlorophenol monooxygenases of *Ralstonia eutropha* JMP134 (formerly *Alcaligenes eutrophus*) and from an unidentified bacterial strain S1 were purified and characterized as a heterodimer and a homotetramer, respectively (Farhana and New, 1997; Makdessi and Lechner, 1997). Like other chlorophenol hydroxylases described in the literature (Solyanikova and Golovleva,

2004), 2,4-dichlorophenol hydroxylases lack heme groups and contain FAD as the prosthetic group.

In most strains studied, the conversion of chlorocatechols generally proceeds via ortho-cleavage. The alternative pathway via meta-cleavage is less common because the 2,3-dioxygenase is inactivated by 3-chlorocatechols (3-CC) and the cleavage product from 4-CC, 5-chloro-2-oxymuconic semialdehyde, is toxic to bacteria (Solyanikova and Golovleva, 2004). Nevertheless, a few bacterial strains are able to degrade lower chlorinated phenols by meta-cleavage (Bae et al., 1997; Koh et al., 1997). A 2,3-dioxygenase has been isolated from *Pseudomonas putida* GJ31 which can catalyzes the meta-cleavage of 3-CC without being inactivated and resulting in the release of the chloro-group as chloride (Kaschabek et al., 1998).

The other main pathway of chlorophenol degradation is via chlorohydroquinone intermediates. The chlorohydroquinone pathway has been exemplified with PCP, as shown in figure 2.2. The pathway begins with a hydroxylation in the para position resulting in the formation of p-tetrachlorohydroquinone (TeCHQ) (Apajalahti and Salkinoja-salonen, 1987b; Fetzner, 1998; Solyanikova and Golovleva, 2004). In *Sphingomonas chlorophenolica* ATCC 39723 (formerly *Flavobacterium* sp), the hydroxylation is carried out by PCP 4-monooxygenase which is a soluble flavoprotein requiring NADPH (Tiirola et al., 2002). PCP 4-monooxygenase requires oxygen from O_2 (Xun et al., 1992a). Similar monooxygenases are found in strains of other PCP-degrading aerobic bacteria (Orser and Lange, 1994; Leung et al., 1997; Thakur et al., 2002). Tetrachlorohydroquinone sequentially dechlorinated in two steps to 2,6-dichloro-1,4-hydroquinone (2,6-DCHQ) by a reductive dehalogenase, which is a glutathione S-

transferase (GST), known as Pcp C (Xun et al., 1992b). 2,6-DCHQ is oxidized by 2,6-DCHQ 1,2-dioxygenase (Pcp A) which requires O_2 and results in the formation 2-chloromaleylacetate as well as the liberation of one of the chlorogroups as chloride (Xun et al., 1999). Recently, a hydroxyquinone hydratase was discovered in *Sphingobium chlorophenolicum* which converts hydroxyl-1,4-quinone to 1,2,4,5-tetrahydroxybenzene, an intermediate that is subsequently auto-oxidized to 2,5-dihydroxyquinone in the presence of O_2 (Bohuslavek et al., 2005). The hydratase may provide an alternative pathway of metabolizing the aromatic ring.

In *Mycobacterium chlorophenolicum* PCP1 and *Mycobacterium fortuitum* CG-2 (formerly *Rhodoccocus* strains), PCP hydroxylation to TeCHQ is carried out by a membrane bound cytochrome P-450 type enzyme (Uotila et al., 1991). The hydroxylation reaction can use oxygen from H_2O based as evidenced from experiments with ^{18}O labelled H_2O (Apajalahti and Salkinoja Salonen, 1987b). Tetrachlorohydroxyquinone is subsequently converted to dichloro-1,2,4-trihydroxybenzene by a sequence of hydrolytic and reductive dechlorinations without any accumulation of a trichlorinated intermediate (Apajalahti and Salkinoja Salonen, 1987a). The dichloro-1,2,4-trihydroxybenzene metabolite is then subject to two reductive dehalogenation steps leading to the formation of the nonchlorinated metabolite 1,2,4-trihydroxybenzene (Apajalahti and Salkinoja Salonen, 1987a).

Several unique pathways of chlorophenol degradation by aerobic bacteria have come to light. 4-Chlorophenol degradation by *Arthrobacter ureafaciens* strain CPR706 and *Arthrobacter chlorophenolicus* strain A6, is initiated by a dechlorination yielding 1,4-hydroquinone as the intermediate (Bae et al., 1997; Nordin et al., 2005). The proposed

pathway involves the conversion of 4-CP to hydroquinone to hydroxyquinol to maleylacetate. A ring cleaving hydroxyquinol dioxygenase is a key enzyme in the pathway. When a gene coding for the dioxygenase is disrupted, *Arthrobacter chlorophenolicus* has negligible growth on 4-CP (Nordin et al., 2005). The monooxygenase from *Ralstonia eutropha* strain JMP134 catalyzes successive dechlorination reactions of 2,4,6-TCP to 2,6-dichlorohydroquinone and then to 6-chlorohydroxyquinol prior to ring cleavage by a hydroxyquinol dioxygenase (Matus et al., 2003). A unique property of the monooxygenase is that the second dechlorination reaction does not require O_2, instead the dechlorination of 2,6-dichlorohydroquinone is catalyzed by a hydrolytic activity associated with the monooxygenase (Xun and Webster, 2004).

A catabolic pathway for 2,4,6-TCP supported by biochemical evidence, has been proposed (Louie et al., 2002). The pathway is initiated by the conversion of 2,4,6-TCP to 2,6-dichloro-*p*-hydroquinone (2,6-DCHQ) and then to 6-chlorohydroxyquinol (6-CHQ), both steps catalyzed by 2,4,6-TCP monooxygenase (TCP-MO). 6-CHQ is transformed to 2-chloromaleylacetate (2-CMA) by 6-chlorohydroxyquinol 1,2-dioxygenase (HQDO), and 2-CMA is converted to β-ketoadipate by maleylacetate reductase (MAR). However, the genes involved in the 2,4,6-TCP catabolic pathway and their genetic organization are little known. *hadAB* and *hadC* genes, encoding TCP-MO and HQDO, respectively, have been found in *Ralstonia pickettii* DTP0602 (Hatta et al., 1999).

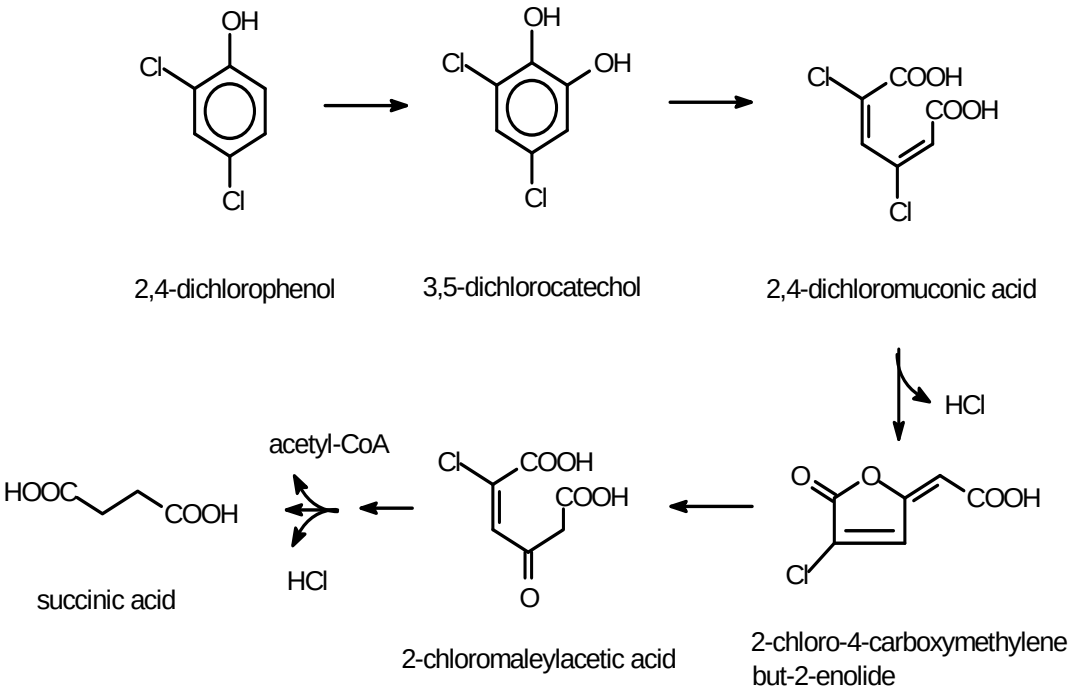


Figure 2.1: Proposed pathway of the aerobic degradation of 2,4-dichlorophenol by bacteria (Bollag et al., 1968; Tiedje et al., 1969; Sharpee et al., 1973; Farhana and New, 1997; Finkel'shtein et al., 2000).

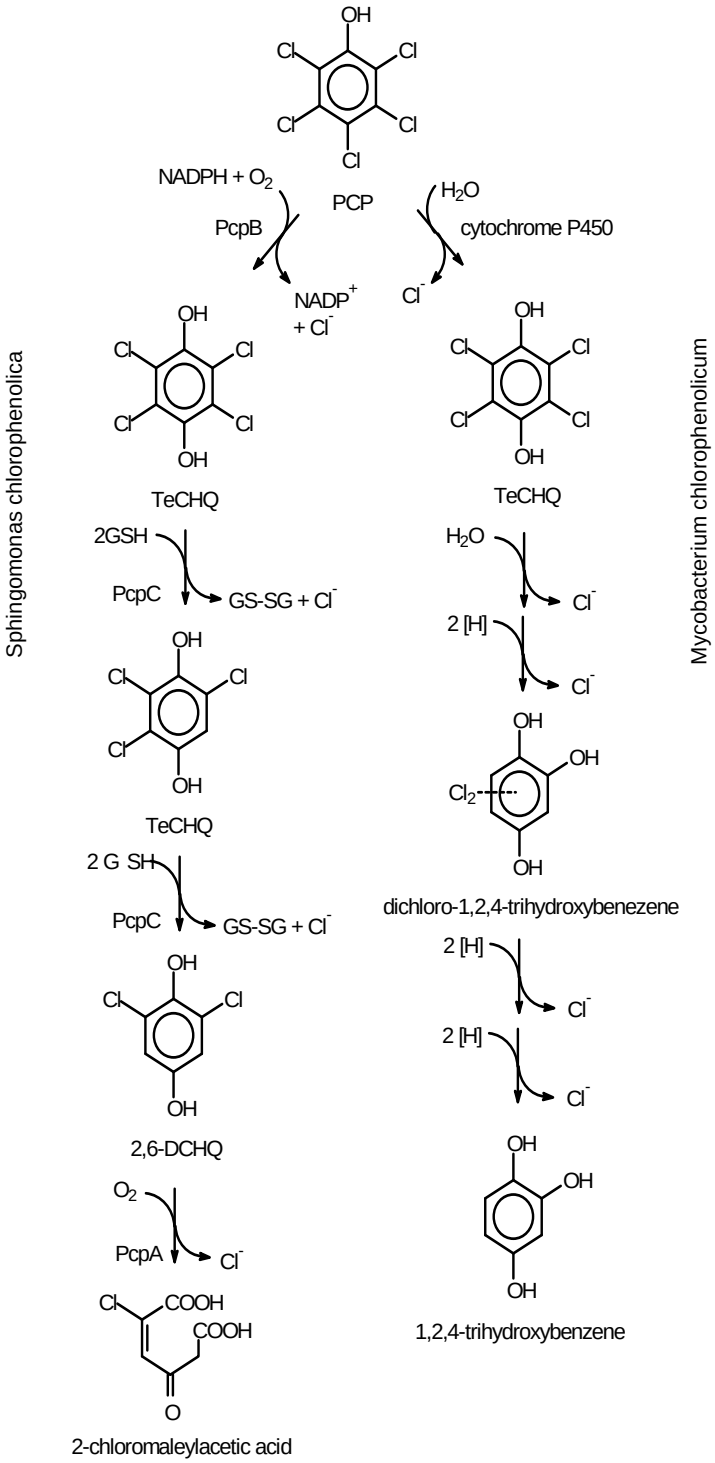


Figure 2.2: Proposed pathway of the aerobic degradation of pentachlorophenol by bacteria. Pathway on left is for *Sphingomonas* (Steiert and Crawford, 1986) and pathway on right is for *Mycobacterium* (Uotila et al., 1991).

Several aerobic bacteria implicated in the rapid degradation of higher chlorinated phenols are known to convert chlorophenols to methylated biotransformation products. *Mycobacterium* strains were shown to O-methylate a variety of chlorophenols (Haggblom et al., 1988b). The preferred substrates of the methylation were hydroxyl groups doubly flanked by chlorine groups. Chlorinated hydroquinones were readily O-methylated (Haggblom et al., 1988b), accounting for the occurrence of methylated chlorohydroquinone intermediates during PCP biodegradation (Suzuki, 1983). Direct methylation of PCP resulted in the formation of pentachloroanisole in soil (D'Angelo and Reddy, 2000).

2.6 Anaerobic degradation of chlorinated phenols

Chlorinated phenols are readily metabolized by bacteria under anaerobic conditions. Anaerobic conditions favour the reductive dechlorination, which results from the displacement of chloro-groups by hydrogen atoms. Reductive dechlorination generally requires the input of electron donating substrates. For most of the studies it is impossible to discern whether the reductive dechlorination is cometabolic or beneficial to microorganisms. In a few cases, reductive dechlorination is known to be linked to growth due to the use of the chlorinated phenols as electron acceptors (halorespiration). Likewise, there are a few examples in which chlorinated phenols are clearly the carbon and energy source (the electron donor) to microorganisms (Field and sierra-Alvarez, 2008).

2.6.1 Anaerobic metabolism of chlorophenols serving as carbon and energy source

Several examples are known in which chlorinated phenols are completely degraded when supplied as sole carbon and energy source to microbial enrichment cultures. Anaerobic

bioreactors have been operated under methanogenic conditions with monochlorinated phenols as the only substrate for extended periods of time ranging from 190 to 400 days. In these experiments, mineralization of 3-CP and 4-CP to CO_2 and CH_4 was demonstrated by mass balances or by the use of ^{14}C-labeled substrates (Krumme and Boyd, 1988). Sulfate-reducing consortia enriched from estuarine sediment were maintained on either 2-CP, 3-CP or 4-CP as the only source of carbon and energy for over 5 years (Haggblom and Young, 1995). Detailed studies on the stoichiometry of the 4-CP degrading enrichment culture established that sulfate reduction concomitant with 4-CP degradation accounted for 81% of the theoretical expected from the complete oxidation of the chlorophenol, clearly suggesting 4-CP oxidation was linked to sulfate reduction. Sulfate or other sulfoxy ions (sulfite or thiosulfate) was required as an electron acceptor for 4-CP degradation by the enrichment. Phenol was observed as a temporal intermediate during 4-CP degradation (Haggblom, 1998) and phenol is readily metabolized by the enrichment (Haggblom and Young, 1995). Sulfate-reducing enrichment cultures capable of degrading 2-CP or 4-CP which were derived from Hudson River sediments were also shown to couple the mineralization of the chlorophenols to sulfate reduction (Haggblom et al., 1993). Iron-reducing 2-CP, 3-CP or 4-CP enrichment cultures were developed from Hudson River sediments (Kazumi et al., 1995). Fe^{2+} recovered from Fe^{3+} reduction could be completely accounted for by the chlorophenol oxidation, providing strong evidence that the chlorophenol degradation was linked to iron reduction. Chlorophenol degradation linked to denitrification has been demonstrated with an enrichment culture utilizing 2-CP derived from activated sludge (Bae et al.,

2002). The consumption of nitrate was equivalent to the expected amount based on the stoichiometry of 2-CP denitrification. In the absence of nitrate, 2-CP was not degraded. Enrichment cultures from freshwater sediments degrading 2-CP or 3-CP under methanogenic conditions converted the chlorophenols to methane and CO_2 with temporal accumulation of phenol and benzoate as intermediates (Genthner et al., 1989). Similar methanogenic enrichment cultures developed from municipal digester sludge mineralized radiolabeled [^{14}C]4-CP, [^{14}C]2-CP and [^{14}C]2,4-DCP by greater than 90% to $^{14}CH_4$ and $^{14}CO_2$ (Boyd and Shelton, 1984). The studies indicate that chlorophenols utilized as a carbon and energy source are first reductively dechlorinated to phenol. Subsequent phenol mineralization provides the energy and carbon to support growth as well as electrons to support the initial dechlorination. The presence of benzoate as an intermediate is in keeping with the accepted route of anaerobic phenol degradation in which phenol is carboxylated in the para position and subsequently dehydroxylated to yield benzoate (Bisaillon et al., 1993; Letourneau et al., 1995). In fact, 2-chlorophenol is sometimes carboxylated and dehydroxylated prior to reductive dechlorination, resulting in the formation of 3-chlorobenzoate as an intermediate (Bisaillon et al., 1993).

2.7 Kinetics of chlorophenol degradation

High growth rates were observed for aerobic bacteria specialized in the utilization of chlorophenols as a carbon and energy source. Cell yields during aerobic growth on chlorinated phenols decreased with increasing chlorine number. This was partly due to the molecular weight of chlorine (which is not incorporated into biomass) and partly because of lower carbon conversion efficiencies. Aerobic cell yields with DCP and TCP were between 0.132 and 0.421 gram dry weight biomass per gram of chlorophenol

metabolized (Rutgers et al., 1997) whereas, the values ranged from 0.054 to 0.190 gram dry weight biomass per gram of chlorophenol metabolized for PCP (Karamanev and Samson, 1998). Cell yields observed during halorespiration are relatively low and range from 0.016 to 0.052 gram dry weight biomass per gram of chlorophenol biotransformed. Remarkably, high specific activities are noted for halorespiration of chlorophenols. Rates ranging from 213 to 178 mg chlorophenol biotransformed per gram dry weight biomass per day have been found (Stuart and Woods, 1998). The combination of relatively high growth rates and low cell yields accounts for the high specific activities that far exceed those of aerobic chlorophenol degrading bacteria. The specific activities of aerobic bacteria utilizing chlorophenols as growth substrate mostly ranged between 29 to 851, excluding two outliers for PCP of 7111 and 12280 mg PCP biotransformed per gram dry weight of biomass per day (Rutgers et al., 1993). Reductive dechlorination of chlorophenols in anaerobic reactor biofilms was observed between 0.37 to 12.9 mg chlorophenol biotransformed per gram dry weight of biomass per day. Half-velocity coefficients are generally low during chlorophenolic compound biodegradation indicating a high affinity. Most microorganisms had K_s and K_m values between 0.01 and 11.7 mg/l respectively, the only exception was a value of 112 mg/l for cometabolism by an aerobic bacterium. Microorganisms, which were previously enriched in bioreactors treating low concentrations of chlorinated phenols in groundwater, displayed remarkable affinities as judged from half-velocity coefficients as low as 0.014 to 0.016 mg/l under both aerobic (Melin et al., 1997) and anaerobic conditions (Magar et al., 1999).

2.8 Factors affecting chlorophenol degradation

Murialdo et al. (2003) studied with *Pseudomonas* sp. strain which was isolated from a consortium that degrades PCP. It was shown that glucose and glutamate have positive effects on its population density. This microorganism worked very effectively for *in situ* bioremediation in an environment, which is highly contaminated with PCP, other chlorinated phenols and hexadecane.

The success of bioremediation may depend on the availability of microbial strains that can mineralize high levels of PCP and withstand adverse conditions to compete under *in situ* conditions. An effective bacterial inoculum should be able to tolerate high levels of PCP while maintaining a level of activity to provide efficient mineralization (Shaw et al., 1997).

Bioremediation can be enhanced by the manipulation of environmental factors to create an optimum environment for microbial degradation. To achieve maximum degradation efficiency factors such as temperature and pH need to be optimized (Providenti et al., 1993).

Temperature is an important environmental factor which may influence the rates at which pollutants are degraded (Trevors, 1982) through altering both microbial activity and the physical and chemical properties of pollutants (Providenti et al., 1993; Miller et al., 2004). Some studies have been performed at different temperatures and usually without attempt to model the effect. As shown in table 2.2, bacteria can depict a different capability for PCP degradation at different temperatures. Usually, too low and too high temperature is not suitable for bacteria growth, although some degradation of PCP can be observed on some cases (Baker et al., 1980).

Different microorganisms require different scale of pH. For some bacteria, the optimal pH is very important. Wittmann et al. (1998) found that the *Sphingomonas chlorophenolica* RA2 cannot grow at pH 4.0. In contrast, *Mycobacterium chlorophenolicum* PCP-1 showed better tolerance for acidic pH. Barbeau et al. (1997) found that bacterial activity was apparently reduced when the pH was less than 6.0. Edgehill (1994) also found that the growth rate for *Arthrobacter sp*. at pH 7.4 was higher than at acidic conditions in the presence of PCP.

Table 2.2: Effect of temperature for PCP degradation

Bacterial type	Optimum temperature	Reference
Flavobacerium sp.	Showed significant removal of PCP between 24-35ºC, but is ineffective below 12ºC or above 40ºC	(Crawford and Mohn, 1985)
R.chlorophenolicus	PCP degradation rate increased as temperature up to 41 ºC. no PCP degradation at 44ºC	(Apajalahti and Salkinoja-Salonen, 1987b)
Three *Pseudomonas* strains	At 0ºC, no degradation was seen after 100days. At 4ºC strains 1, 2 and 3 degraded 50%, 23% and 11%, respectively after 100 days. At 20ºC, all three strains degraded about 50% of PCP	(Haggblom, 1988)
Pseudomonas sp.	PCP can be degraded at 10ºC and the rates increased linearly with increasing temperature to 30ºC	(Shaw et al., 1997)
Mixed culture	A 10ºC decrease in temperature generally results in over seven times slower degradation rates.	(Melin et al. 1998)
Mixed culture	Half-life of PCP (500 mg/kg) in soil increased from 60 to 179 days when the temperature was decreased from 25 to 5 ºC	(Trudell et al., 1994)

2.10 *In situ* remediation

Several techniques are available for the removal of contaminants from water, although not all are efficient enough to destroy the contaminant. The use of microorganisms for bioremediation of PCP-contaminated sites may prove to be a viable alternative to conventional clean-up methods. Biodegradation is a technique, which could potentially degrade these contaminants to innocuous products (mainly CO_2 and H_2O; also Cl^- in the case of chlorinated phenols). Biological treatment of chlorophenols attracts more attention than physical and chemical methods, because a variety of microorganisms are known to utilize chlorophenols as their sole carbon or energy source; reaction products are Cl^- ions, CO_2 and biomass. Many species of soil bacteria have been isolated from contaminated soil samples. PCP-degrading bacteria include species of *Arthrobacter* (Stanlake and Finn, 1982), *Flavobacterium* (Saber and Crawford, 1985), *Pseudomonas* (Radehaus and Schmidt, 1992), *Rhodococcus* (Apajalahti and Salkinoja-Salonen, 1986) and *Corynebactrium* (Chu and Kirsch, 1972).

Biodegradation is considered to be the major transformation mechanism for PCP in soil. PCP is metabolized rapidly by most acclimated microorganisms (Kaufman, 1978). In a study by Edgehill and Finn (1983) inocula of a strain of PCP-acclimated *Arthrobacter* bacteria was added to soils in laboratory and enclosed outdoor tests. The soils were amended with 120-150 mg PCP/L and 34 kg PCP per hectare, respectively. In the laboratory test conducted in the dark at electrical conductivity (EC) 30, the half-life of pentachlorophenol in inoculated samples was about one day, whereas the half-life in uninoculated samples was 12–14 days. PCP loss from uninoculated control plots in outdoor tests was 25% after 12 days at ambient temperatures and EC 8-16, while losses

from inoculated plots, were 50–85%.Biodegradation can occur in soil and water where acclimated microbial populations occur but its activity to be slow, based on a reported persistence of >72 days in a laboratory study using a soil inoculum.

Moderate bioconcentration in fish may occur based on a 24 hours log BCF of 2.30 in guppies in static bioconcentration tests. If released into the atmosphere, 2,3,4,6-TeCP would photodegrade, in a day. Human exposure will primarily be from coming into contact with penta or tetrachlorophenol treated products. Occupational exposure may occur via dermal or inhalation of contaminated dust (EPA, 1987).

In soil, 2,4,5-TCP is likely to biodegrade and its mobility is expected to vary from highly mobile in sandy soil and moderately mobile in clay and silt loams to slightly mobile in muck (due to adsorption to humic acids and other organic matter). 2,4,5-trichloroanisole is the primary degradation product of 2,4,5-TCP in soil. The anisole is apparently formed by microbial methylation of the phenol. The persistence of 2,4,5-TCP in soil is reported to vary from 14 to 300 days depending upon climatic conditions and population of soil microorganisms, but usually does not exceed one full growing season regardless of the application rate (EPA, 1984).

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 pentachlorophenol in soils is pH dependent. The compound has a pKa 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). Adsorption decreases in neutral and basic soils and is strongest in acidic soils. Therefore,

the compound is most mobile in neutral-to-basic mineral soils and least mobile in acidic organic soils. Volatilization and photolysis do not appear to be important transport and transformation processes for PCP in soils. Marine sediments collected off the coast of Sweden were observed to dechlorinate PCP, yielding 2,3,4,5-TeCP, 3,4,5-TCP and 3,5-DCP as products (Abrahamsson and Klick, 1991).

Anaerobic PCP degradation was observed in five of ten wetland soil samples incubated under methanogenic conditions (D'Angelo and Reddy, 2000). TeCP, TCP and DCP were observed as intermediates in the anaerobic degradation of PCP. Tetrachloro-1,4-hydroquinone was shown to be dehydroxylated and dechlorinated by a mixed culture derived from Baltimore harbor sediments to yield 2,3,5-TCP (Milliken et al., 2004).

The biodegradability of chlorinated phenolic compounds in the natural environment has been considered in a large number of studies. Indigenous microorganisms in forest soil were shown to degrade 2-CP (Lallai and Mura, 2004). Satisfactory phenol and chlorophenol degradation has been observed under natural aerobic environment such as soil and fresh water lakes. Research on the biodegradation of phenol and chlorophenol in the aerobic soil environment revealed that phenol, o-chlorophenol, 2,4-DCP, 2,6-DCP and 2,4,6-TCP could be readily degraded as compared to PCP, 2,4,5-TCP, 4-DCP and m-chlorophenol (Baker and Mayfield, 1980).

The biodegradation of chlorophenols with pure culture of microorganisms *Arthrobacter* sp. *Pseudomonas* sp. and *Nocardia* sp. precultivated in chlorophenols isolated from soil was reported by Bollag et al. (1968) and Haider et al. (1974); The incubation of 2-CP, 4-CP, DCP and TCP in a natural soil for one week degraded 13, 22.2, 31.4 and 35% of the respective original compounds to complete mineralization (Haider et al. 1974).

Alexender and Aleem (1961) observed that complete disappearance of chlorophenols in two natural soils took the following time period: 2-CP 14- 47 days; 3-CP 47 days to 72 days; 4-CP 3 to 9 days; 2,4-DCP 5 to 9 days; 2,5- DCP 72 days; 2,4,5-TCP 47 to 72 days. 2,4,6-TCP 5 to 13 days; 2,3,4,6-TeCP, >72 days. It concluded that some chlorophenols are easily biodegradable in soil, others are more resistant.

Several studies evaluated the anaerobic degradation of chlorinated phenols in marine and estuarine sediments. Anoxic estuarine sediments from Taiwan were found to degrade 2-CP, 3-CP, 4-CP. 2,5-DCP, 3,4-DCP, 3,5- DCP and PCP (Liu et al., 1996). Estuarine sediments from the lower Hudson River in New York were able to degrade 2-CP, 3-CP and 4-CP under sulfate reducing conditions (Haggblom et al., 1993). Estuarine sediments from the Chesapeake Bay biotransformed 2,4-DCP to 4-CP when incubated under methanogenic conditions (Warner et al., 2002). Similarly, 2,4-DCP and 3,4-DCP were dechlorinated by marine sediments to 4-CP and 3-CP, respectively (Boothe et al., 1997).

Municipal anaerobic digester sludge not previously exposed to chlorinated phenols also has been shown to degrade chlorophenols. All monochlorinated isomers were degraded by anaerobically-digested primary sewage sludge (Boyd and Shelton, 1984). Additionally, dichlorophenols (except 3,4-DCP and 3,5-DCP) were degraded via monochlorophenol intermediates. Municipal anaerobic digester sludge was shown to dechlorinate 2-CP to phenol and selective inhibitors demonstrated that the population responsible for the dechlorination reaction was associated with syntrophic acetogens (Basu et al., 2005). The ability of municipal anaerobic digester sludge to degrade 12 chlorinated phenolic compounds was tested and 10 of the compounds were shown to be degraded (Wang et al., 1998). The digester sludge culture did not degrade PCP or

2,3,4,6-TeCP. In another study, all nineteen possible isomers of chlorinated phenols were degraded by methanogenic sludge (Takeuchi et al., 2000). Dechlorination of ortho-chlorines occurred at the fastest rate whereas, dechlorination of para chlorines occurred at the slowest rate. First order rate constants were lower with decreasing number of chlorine substituents.

Soils not only have the capability to transform or degrade natural compounds, but recalcitrant xenobiotic compound as well by enriching microbial populations endowed with the ability to degrade organic compounds available in their milieu. Thus, soils have proved as a reliable source of microbes and genes for the degradation or transformation of various organic compounds (Zhang et al., 2004).

Chlorophenols may be present in soils by land application of biocides or chlorophenol and chloroguaiacol-containing sludge from pulp bleaching effluent treatment. After 60 days of incubation, soil mineralized 15- 45% of 2,4,5-TCP when it was exposed to 1, 10, or 100 ppm of this compound. Minimal half-lives for 2,4,5-TCP of 35-170 days were estimated. Less than 2% of mineralization was observed in soil exposed to 500 ppm or in incubations with sterile soil spiked with 10 or 100 ppm of 2,4,5-TCP. Thirty days of preincubation of soil with 10 or 100 ppm of 2,4,5-TCP increased the rate of mineralization of an additional amount of this pollutant (Matus et al., 2003).

Schwrzenbach and Westall (1985) studied the dependence of the sorption partition coefficients between water and organic carbon on the pH of the soils. At neutral soil pH, higher chlorinated phenols with lower pka value will exist primarily in the ionic state and will not be significantly absorbed onto soils, although sorption of soil particularly clay soil, may be due to ionic attraction. The sorption of chlorophenols on soil will also

increase with an increase of organic carbon content of soil. Thus, in a sandy aquifer, Sutton and Barker (1985) observed very little sorption of chlorophenol. Boyad (1982) measured the following K_{oc} values in natural soil at a neutral pH; 2-CP 51; 3-CP 66; 4-CP 70; 2,4-DCP 126; 2,4,5-TCP 363. It is concluded that chlorophenols are susceptible to leaching from soil to ground water particularly from sandy soil with pH >10.

Based on data regarding chlorophenol in water, photolysis, hydrolysis and evaporation will not be significant process in soils. The two important processes in soil are sorption and biodegradation. While 2-CP, 4-CP, and 2,4,6-TCP were found to be easily biodegradable in natural soil; 3-CP, 2,5-DCP and 2,4,5-TCP and 2,3,4,6-TeCP were persistent in soil (Alexender and Aleem, 1961). The sorption of chlorophenols in soil increases with decrease in soil pH and increase chlorine substitution. Chlorophenols are especially susceptible to leaching from sandy soil with pH >10 (Johnson et al., 1985).

Soil represents a complex ecosystem in which relationship and interactions between microorganisms are also complex. Microbes adapt to microhabitats and live together in consortia interacting with each other and their environment. Microbial diversity is critical to ecosystem functioning due to diversity of processes such as decomposition, nutrient cycling, soil aggregation and pathogenecity. According to a current estimate 1 g of soil may harbor up to 10 billion bacteria of possibly 4000–7000 different species and a biomass density of 300–30,000 kg /hectare (Rosello-Mora and Amann, 2001).

CHAPTER-3

Chapter- 3

3. Material and Methods

3.1 Collection of sludge sample

Secondary sludge samples of pulp mill and paper were collected from the effluent treatment plant of Ballarpur Industries Ltd., Unit- Shree Gopal, Yamunanagar, Haryana in sterile conical flasks and stored at 4°C. This mill adopts kraft process for pulping of raw materials mainly eucalyptus, poplar, bamboo. Unbleached pulp is bleached with multistage bleaching. The effluent generated by this process contains dissolved chloro-lignins, chlorophenols and other soluble components of raw material and chlorinated derivatives. The effluent is treated in activated sludge process for the biological removal of organics.

3.2 Physiochemical characterization of secondary sludge

3.2.1 pH

Secondary sludge samples were taken into conical flask and shaken properly onto gyratory shaker at 120 rpm for two hours. The pH of mixture was measured with a pH meter (Cyber Scan 510).

3.2.2 CHNO Analysis (Carbon, Hydrogen, Nitrogen and Oxygen)

One gram of sludge sample was taken and oven dried at 105°C for 24 hours. The sample was removed from oven and kept into desiccator and grinded in analytical mill to get particles having particle size of about 0.1mm size. 10 to 100 mg of the dried sludge sample was taken for the CHNO analysis using Elemental analyzer (Thermo Scientific USA).

3.2.3 Phosphorus (P)

1. The pH of sludge sample was adjusted to neutral with 0.5 N NaOH or 2.0 N CH_3COOH.
2. Prepared different concentration of standard PO_4^{3-} by KH_2PO_4 in 100 ml of volumetric flasks. Also taken appropriate amount of sample in 100 ml of volumetric flask.
3. Added 10 ml molybdate solution (Molybdate solution: Dissolved 2.5 g of sodium molybdate, $Na_2Mo_4.2H_2O$ in 10 N sulphuric acid and diluted to 100 ml with 10 N sulphuric acid) in each volumetric flask and mixed 4 ml of hydrazine sulphate reagent in it (Hydrazine sulphate solution: Dissolved 0.75 g of hydrazine sulphate in distilled water and diluted to 500 ml).
4. Adjusted the volume with distilled water and mixed well. Boiled the contents in water bath for 10 min. After cooling, the content in volumetric flask was mixed well.
5. The absorbance was measured at 830 nm against a reagent blank. The calibration curve was calibrated using the standard phosphate solution.

Calculation:

PO_4^{-3} content in the sample, (mg/l)

$$: \quad \frac{X(mg)}{Y(ml)} \times \frac{100 \times 10^{-3}}{Z(g)}$$

Where X is concentration in mg corresponding to Y ml of sample solution (100 ml of total sample volume)

Z is amount of sample in gm dissolved in 100 ml

3.2.4 Sulphate (SO_4^{-2})

pH of the sludge sample was adjusted to 4.5 - 5.0 by HCl using methyl red indicator, then 1-2 ml HCl in excess was added. Boiled with stirring and 10% $BaCl_2$ solution (warm) was added slowly until precipitation appears to be complete, then added 2 ml in excess, digested the precipitate at 80-90°C for at least 2 hours. Filtered the precipitate through filter paper (Whatman no. 42), washed it with small portion of warm distilled water until the washings are free of chloride ions as indicated by testing with $AgNO_3$-HNO_3 reagent. Dried the precipitate in a pre-weighed crucible and ignited at 800°C±50°C in muffle furnace for 1 hour.

Calculation

$$\text{Sulphate concentration mg/l} = \frac{\text{mg } BaSO_4 \text{ X } 411.5}{\text{ml of sample}}$$

3.2.5 Zeta potential (Surface Charge Analysis)

Zeta potential was analyzed by adjusting pH of the sludge sample to neutral. The pH was adjusted with NaOH or HCl for determination. The secondary sludge was placed in the zeta potential analyzer, Muteck SZP06 (BTG Mutek GmbH, Germany)

3.2.6 Absorbable Organic Halogen (AOX)

1. Sludge samples were taken and oven dried at 105± 2°C for 24 hours.
2. Sample was removed from the oven and kept in desiccator and ground in an analytical mill to get particles of about 0.1 mm size. Grinded material was again placed in desiccator.

3. 10 to 100 mg of the dried sludge sample was taken in 250 ml of conical flask and dispersed in 50 ml of dilution water (having pH 2 to 3 adjusted with nitric acid). Prepared blank by taking 50 ml dilution water.

4. Added 50 mg of activated carbon and 10 ml of nitrate stock solution (0.25 M $NaNO_3$). Placed on a shaker for 1 hour at 200 rpm.

5.) Diluted 10 ml working standard (P-chlorophenol) was used to 100 ml and similarly blank was prepared by taking 100 ml dilution water added with 50 mg activated carbon. 5 ml nitrate stock solution was added in standard and sample solution, shaken in an incubator shaker overnight.

6. After that suspension was filtered through quartz frit under nitrogen gas pressure.

7. Filter cake is washed with nitrate solution and finally with distilled water. After filtration, placed the quartz frit into quartz combustion boat of AOX analyzer, ECS 1200 (Thermo Fischer Scientific, UK).

8. The sample wt was fed into software for calculation.

Calculation:

For sample, instrument directly gives AOX content in mg/kg.

For standard check

AOX, µg/L = S- B

Where S: AOX content in standard, µg/l

B: AOX content of blank, µg/l

3.2.7 Extractable Organic Halogen (EOX)

Determination of EOX was done by EPA Method. Organic halide was extracted from sludge sample using ethyl acetate by sonication. Extraction procedure is mentioned below. A 25 µl aliquot of the extracted sample were injected into a pyrolysis furnace

using stream oxygen and argon gas, the hydrogen halide pyrolysis product was determined by microcolumetric titration.

Extraction

1. Taken one gram of sludge sample in 10 ml vial using a metal spatula. Then 1 ml of reagent water (75% Acetic acid) and 5 ml of ethyl acetate were added.
2. The sample was shaken vigorously for thirty second and agitated the suspension directly using a sonic probe for 5 minutes.
3. Suspension was allowed to settle for 10 minutes, and transferred the upper layer (ethyl acetate) to 15 ml conical centrifuge tube, capped the tube and centrifuged at 1000 rpm for five minutes.
4. Transferred ethyl acetate layer to 10 ml vial and stored in refrigerator.
5. For analysis 25 µl sample was taken through micro syringe and injected to the instrument.

Calculation:

EOX, mg/kg = (S-B) X 5X 1000/1000X W

Where:

S: EOX content of sample, mg/l

B: EOX content of blank, mg/l

W: OD weight of the sample, g

3.2.8 Total protein estimation (Folin-Lowry Method) (Lowry et al. 1951)

1. BSA (Bovine serum albumin) standard solutions were prepared in the range of concentrations 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 mg/ ml

2. From these solutions, 0.2 ml protein solutions were pipetted out to different test tubes. Simultaneously sample was also taken. Added 2 ml of alkaline copper sulphate reagent [prepared by (a) 50 ml of 2% sodium carbonate mixed with 50 ml of 0.1 N NaOH solution (0.4 gm in 100 ml distilled water) (b) 10 ml of 1.56% copper sulphate solution mixed with 10 ml of 2.37% sodium potassium tartarate solution. Prepared analytical reagents by mixing 2 ml of (b) with 100 ml of (a)]. Mixed the solutions well. The final volume in each of the test tubes was 5 ml.

3. This solution is incubated at room temperature for 10 min.

4. Then added 0.2 ml of reagent Folin Ciocalteau solution (1N) to each tube and incubated for 30 min and measured the absorbance at 660 nm.

5. Ploted the absorbance against protein concentration to get a standard calibration curve.

6. Checked the absorbance of sample and determined the concentration of the sample using the standard curve.

3.2.9 Anthrone test for carbohydrate

1. The glucose standard (0.1 mg/ml) was prepared in distilled water; in the range of 10 to 100 μg final volume for 1.0 ml. Included 1 ml distilled water as blank and sludge sample.

2. Placed all the tubes in the ice water bath.

3. Added 5.0 ml chilled anthrone reagent (Prepared by adding 200 mg anthrone to 5 ml ethanol: volume was made up to 100 ml with 75% sulfuric acid and stored at 4°C) to each tube; mixed thoroughly and kept into ice water bath.

4. Transferred all the tubes to boiling water bath for exactly 10 min.

5. Removed and placed in an ice water bath.

6. Absorbance was taken at 625 nm using the distilled water as a blank.
7. Prepared standard curve for the glucose standard by plotting absorbance verses glucose concentration.

3.3 Isolation and enrichment of chlorophenol degrading bacteria

The bacterial strains were isolated by the serial dilution technique, and purified by repeated streaking on nutrient agar plates. Colonies appearing after incubation at 37^{o}C for 48 hours were selected for further screening. Screening of chlorophenol tolerant bacterial strains were done by the nutrient enrichment technique in mineral salt medium (MSM) (Appendix I B, Dams et al., 2007) supplemented with 50 mg/l of pentachlorophenol (PCP), 2,3,4,6- tetrachlorophenol (TeCP), 2,4,5-trichlorophenol (2,4,5-TCP) and 2,4,6-trichlorophenol (2,4,6-TCP) separately as a sole source of carbon for energy. The final enriched cultures were spread on MSM agar plates.

3.3.1 Growth medium

The MSM (Appendix I B) contained the following components at the specified concentrations. The pH of the medium was adjusted to 7.5. PCP, TeCP, 2,4,5-TCP and 2,4,6-TCP was added separately to the medium after autoclaving. Solid MSM plates were prepared, if necessary, by adding 1.2 (w/v) bacteriological grade agar.

3.4 Morphological and biochemical characterization of bacterial isolates

3.4.1 Gram staining

Bacterial smear prepared from actively growing cells was spread on a glass slide and fixed by heat. Crystal violet was flooded for 10 sec. Briefly, washed in water to remove excess crystal violet. Gram iodine was flooded for ten seconds and washed briefly in water. Further, it was decolourised with ethyl alcohol until the moving dye front has passed the

lower edge of the section and washed immediately in tap water. Safranine was used to counter stain for 15 sec and washed with water to remove the excessive stain. The slides were visualized under microscope at different magnifications.

3.4.2 Capsule Staining

The capsule stain is a differential stain, which selectively stains external capsules surrounding bacterial cells. Capsules are highly ordered polymers of sugars and proteins that surround some bacterial cells, and can be easily dislodged by heat or water. The primary stain applied is crystal violet, which stains both the bacterial cell and the surrounding capsule. A 20% copper sulfate solution is then applied, which serves a dual function as both decolorizer and counterstain. It removes and replaces the crystal violet in the capsule only. At the end of the staining procedure, the capsule appears as a faint blue or white halo around a purple cell observed under microscope.

3.4.3 Catalase test

Small amount of bacterial cells were placed onto a clean microscope slide. A few drops of H_2O_2 (3%) were added onto the smear. Rapid evolution of O_2 bubble was considered as the positive result. Either no bubble or only a few scattered bubbles indicate negative result.

3.4.4 Oxidase test

Small amount of bacteria from an agar slant or plate was obtained with a sterile swab. One drop of N,N,N',N'-tetramethyl phenylenediamine dihydrochloride reagent was placed onto the culture on the swab. Positive reactions turned the bacteria violet to purple immediately or within 10 to 30 seconds.

3.4.5 Nitrate reduction test

Nitrate media (Appendix I D)) was used to determine the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2) using the enzyme nitrate reductase. It also tests the ability of organisms to perform nitrification of nitrate to nitrite. Nitrate broth contained potassium nitrate as a source of nitrate. Bacteria were grown in nitrate agar plate. After sufficient growth of bacteria, 2-3 drops of sulfanilic acid and α-naphthylamine were added. If the organism had reduced nitrate to nitrite, the nitrites in the medium will form nitrous acid. Sulfanilic acid reacts with the nitrous acid to produce diazotized sulfanilic acid. This reacts with the α-naphthylamine to form a red-colored compound. Therefore, if the medium turns red after the addition of the nitrate reagent, it was considered a positive result for nitrate reduction.

3.4.6 DNase Test

By making a single streak line on DNase Test Agar with methyl green containing dye on agar plate incubated at 37°C for 24-48 hours. Methyl green forms a complex with intact (polymerized) DNA to form the green color of the medium. DNase activity depolymerizes the DNA, breaking down the methyl green-DNA complex, which results in the formation of colorless zones around colonies of the test organism. A negative test is indicated by the absence of a colorless zone around the colonies.

3.4.7 Starch hydrolysis test

Starch hydrolysis was checked on starch agar plate by making a streak over the surface of agar plate, incubated at 37°C. After growth of the culture, iodine was added to the surface of the agar. Iodine turns blue-black in the presence of starch. Absence of the blue-black color indicates that starch is no longer present in the medium. Bacteria, which show a clear zone around the growth, produce the extracellular enzyme amylase.

3.4.8 Antibiotic profiling of bacterial isolates

Bacterial isolates were grown in nutrient broth until the absorbance reached to 1.0. Absorbance was taken by UV spectrophotometer. The grown bacterial cells were spread on nutrient agar and antibiotic discs were kept on it. These plates were incubated at 37^{o}C and the inhibition zone was noted. Ready precoated twenty antibiotic discs (Himedia Lab., India) were used to check the sensitivity of the bacterial isolates. These were: Norfloxacin (10 µg), Gentamicin (10 µg), Chloramphenicol (30 µg), Cefuroxime (30 µg), Ciprofloxacin (5 µg), Cefaperazone (75 µg), Ceftazidime (30 µg), Roxithromycin (30 µg), Calaritromycin (15 µg), Co-Trimoxazole (25 µg), Netillin (30 µg), Cefaclor (30 µg), Cephotaxime (30 µg), Cephadroxil (30 µg), Azithromycin (15 µg), Ampicillin/Cloxacillin (10/10 µg), Penicillin (10 units), Amikacin (30 µg), Sparfloxacin (5 µg) and Ampicillin/sublactam (10/10 µg).

3.4.9 Fermentation of carbon substrates

A total of 35 carbohydrate fermentation tests were performed with isolated bacterial species according to the manufacturer's direction (Hi-media Lab., Mumbai). Inocula were prepared by growing the bacterial cells in nutrient broth at 37^{o}C in shaking condition until the inoculum turbidity was ≥0.5 OD at 600 nm.

3.5 Molecular characterization

3.5.1 Isolation of genomic DNA

1. Picked a single colony of bacterial isolate from a freshly grown plate and transferred it into 20 ml nutrient broth in a 250 ml of flask. Incubated the culture for 16-24 hours at 37^{o}C with vigorous shaking (120 rpm/min in a rotary shaker).

2. Harvested the grown cells in 2.0 ml sterile microfuge tube. Decanted the media from the cell pellet, the tubes were kept in an inverted position for 1 min to allow the last traces of media to drain away (The cell pellet may be washed here using 10 mM Tris-HCl pH 8.0).
3. Resuspended each cell pellet in approximately 0.8 ml saline-EDTA buffer thoroughly. Added 50 µL freshly prepared lysozyme solution, mixed well. incubated at 37^{o}C for 20 min.
4. Added 0.2 ml 10% SDS, mixed well by inversion, and incubated in the water bath at 60^{o}C for 15 min.
5. Extracted once with phenol: chloroform: isoamyl alcohol (25:24:1).
6. Transferred the upper aqueous phase to a sterile microfuge tube. Precipitated DNA by adding equal volume of isopropanol.
7. After centrifugation, the supernatant was discarded and the pellet was air dried.
8. Dissolved the DNA in 50 µL TE buffer.
9. Added DNase free RNase solution to a final concentration of 40 µg/ml and incubated at 37^{o}C for 30 min, with occasional shaking.
10. Extracted once with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1)
11. Precipitated DNA from the upper aqueous layer with 2 volume of ethanol. Centrifuged and redissolved it in 50 µL Tris-EDTA (pH 8.0). Stored at 4^{o}C for further use.

3.5.2 Electrophoresis of nucleic acids on non-denaturing agarose gels

Nucleic acids were loaded on agarose gels (0.7%- 2 %(w/v)) prepared in 0.5X TBE buffer (pH 8.0) (Appendix I L) using a 6X loading buffer (Appendix I Q). Ethidium bromide (EtBr) (0.5 µg/ml) was added to stain the gel prior to pouring. The nucleic acids were then migrated and visualized on a U.V. transilluminator (312 nm).

3.5.3 Nucleic acid quantification

3.5.3.1 Ethidium bromide fluorescent quantification

DNA was migrated electrophoretically in an agarose gel containing ethidium bromide (0.5 µg/ ml). The quantity of DNA was visually determined with reference to a known λ phage DNA quantity by comparing the intensity of fluorescence after staining.

3.5.3.2 Spectrophotometric quantification

DNA was quantified by measuring the sample at O.D. of 260 nm. One absorbance unit corresponds to approximately 50 µg/ml of double stranded DNA (Sambrook et al., 1989). The purity of the sample of contamination with polysaccharides and proteins was evaluated by the ratio between O.D 260/230 nm and O.D 260/280 nm respectively. Pure samples were indicated by a value closer to 1.8.

3.5.4 DNA amplification by polymerase chain reaction (PCR)

3.5.4.1 REP-PCR based DNA fingerprinting

Reaction mixture for the REP PCR contained 1X PCR buffer (Invitrogen, USA), each deoxynucleotide triphosphate at a concentation of 200 µm, 1.5 mM $MgCl_2$, each primer at a concentration of 0.1 µm and 2.5U of Taq DNA polymerase (Invitrogen, USA) in a final volume of 100 µl. DNA amplification was performed with Genamp 2700 PCR system (Applied Biosystem, USA) by using the following program; initial denaturation at 95^{o}C for 2 min, 35 cycles at 92^{o}C for 30 sec, at 38^{o}C for 80 sec and at 60^{o}C for 200 sec,

final extension at 68^{o}C for 8 min and final soak at 4^{o}C. The REP PCR primers were REP F 5'- III ICG ICG ICA TCI GGC -3' and REP R 5'-ICG ICT TAT CIG GCC TAC-3'.

3.5.4.2 BOX- PCR amplification

BOX-PCR (based on primers targeting the highly conserved repetitive DNA sequences of the BOXA subunit of the BOX element) was conducted to obtain the genomic fingerprinting of the all efficient bacteria. 5'CTACGGCAAGGCGACGCTGACG-3' sBOXA1R: primer was used to study distinctly different species. The reaction procedure was: an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 90°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 8 min and a final extension at 72°C for 10 min. PCR products were then examined through electrophoresis in 2% agarose gel containing ethidium bromide at 40 V in 0.5XTBE buffer.

3.5.4.3 ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus PCR)

The primer pair forward (5'-GGTGATGATGCAGTGCCTCC-3') and reverse (5'-CTGCGCGCCTAATCAATAGC-3') (Versalovic et al. 1991) was used to amplify a 658-bp PCR fragment. The reaction mixture for the PCR contained 1X PCR buffer (InVitrogen Inc., USA), each dNTPs at a concentration of 200 μM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.1 μM and 2.5U of Taq DNA polymerase in a final volume of 100 μl. PCR amplifications were performed in a Gene Amp 2700, Applied Biosystem, USA) thermocycler using the following cycles: one initial denaturation at 95 °C for 3 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 67°C for 1 min and extension at 72 °C for 1 min; and a final extension at 72 °C for 6 min.

3.5.4.4 Amplification of 16S rDNA and Purification of PCR products

For amplification of 16S rRNA gene, the primers used were: Forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-ACGGGCGGTGTGTTC-3' (Weisberg et al. 1991). DNA amplification was performed with GeneAmp 2700 PCR system (Applied Biosystem, USA). Reaction mixture for the PCR contained 1X PCR buffer (InVitrogen Inc., USA), each dNTPs at a concentration of 200 μM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.1 μM and 2.5U of Taq DNA polymerase in a final volume of 100 μl. PCR conditions were as follows: Preheating at 92^{o}C for 2 min, 36 cycles at 92^{o}C for 1 min, 48^{o}C for 30 sec and 72^{o}C for 2 sec and final extension at 72^{o}C for 6 min 10 sec. Amplified DNA was verified by electrophoresis of aliquots of PCR product (5 μl) on a 1.0% agarose gel in 0.5X TBE buffer.

3.5.4.5 Gel Elution

PCR products were purified by agarose gel (0.8%) electrophoresis prior to cloning. After staining with ethidium bromide, a defined band was visualized under UV irradiation and excised. Besides removing surplus primers, nucleotides, and salts, this method possessed the advantage that incomplete (shorter) amplification fragments are also removed prior to cloning. Subsequently, the DNA was excised from the gel, using the QIAquick gel extraction kit (Qiagen Inc., USA) as per manufacturer's direction. Purified PCR products were eluted with 40 μl TE buffer (pH 8.0).

3.5.4.6 Ligation in T-vectors

The 16S rDNA amplicon was ligated into pTZ57R/T or pGEM-Teasy vector. The final reaction volume for ligation was 30μl and 10μl (Appendix I R, S) and incubated at 22^{o}C

and 4°C for pTZ57R/T and pGEM-Teasy vector respectively. The reaction mixture was kept overnight and analyzed on 0.7% agarose gel.

3.5.4.7 Transformation of DH5α cells by heat shock method

1. *E.coli* DH5a cells were taken from glycerol stock and streaked on Luria Agar (LA) plates and incubated at 37°C overnight.

2. Single colonies were isolated and inoculated in Luria Broth (LB) (Appendix I C) and incubated overnight at 37°C.

3. 100 µl of the overnight grown culture was inoculated in 50 ml LB and incubated in a shaker at 180 rpm for 5 hours and then the culture was centrifuged at 7500 rpm for 15 min at 4°C in autoclaved 30 ml tubes.

4. The supernatant was discarded and 10 ml of filtered and sterilized 0.1M $CaCl_2$ was added and the tubes were incubated in ice for 15 min.

5. The cells were again centrifuged and the supernatant was discarded and 1 ml of 0.1M $CaCl_2$ was added. The tubes were incubated in ice for 3 hours to make competent cells.

6. To 100 µl of competent cells, 5 µl of ligated product was added and mixed gently and kept in ice for 30 min for binding the plasmid to the cells.

7. Then the cells were given a heat shock treatment for exactly 2 min at 42°C in a still water bath.

8. The cells were rapidly transferred into ice and kept for 2-3 min.1 ml of LB +Ampicillin (50 µg/ml) was added to each tube and the tubes were kept at 37°C for 1 hour for expression of Ampr gene of the transformed cells.

9. 100 µl of the transformed cells were plated on LA + Amp + X-Gal + IPTG plates (Appendix I H, I, J).

10. The plates were incubated at 37°C for 16-20 hours and checked for appearance of white and blue colonies. The plates were kept in a refrigerator overnight to intensify the blue color of the colonies and differentiate between recombinants and nonrecombinants.

3.5.4.8 Blue/white screening for recombinant plasmids

After transformation of the ligated product, the *E. coli* DH5α (LacZ-) bacterial host cells were plated on Luria Agar (Appendix I C) medium containing 50 μg/ml ampicillin, for selection of transformants. X-Gal and IPTG were used to screen for colonies containing a recombinant plasmid. The cloning site in the pTZ57R/T or pGEM-Teasy vector is located in the multiple cloning site (MCS) of the plasmid's lacZαgene; if insert was present, non-functional β-galactosidase is produced, and the transformed bacterial colony is white. White colonies were picked and grown in 2 ml LB containing ampicillin (50 μg/ml) and simultaneously patch of these cultures were done on LA containing ampicillin. Plasmid was isolated (described in the proceeding section) and re-amplification of the insert was done using vector's promoter specific sequences.

3.5.4.9 Isolation and purification of plasmid DNA from bacteria by alkaline lysis method

1. Transferred a single transformed *E. coli* colony into 2 ml of LB medium containing appropriate antibiotic (ampicillin used in a final concentration of 50 μg/ml) in a capped 15-ml tube. Incubated the culture overnight at 37^{o}C with vigorous shaking.
2. Poured 1.5-2.0 ml of the above saturated culture into a microfuge tube. Centrifuged at 8000 rpm for 5 min in a microfuge.
3. Removed the medium, leaving the bacterial pellet as dry as possible.

4. Resuspended the bacterial pellet in 200 µl of ice-cold Solution I (Appendix I N) by vigorous vortexing to ensure that the bacterial pellet is completely dispersed in this solution.
5. Added 200 µl of freshly prepared Solution II (Appendix I O). Closed the tube tightly and mixed the contents by gentle inversion of the tubes five to ten times. Stored the tubes on ice.
6. Added 300 µl of ice-cold Solution III (Appendix I P). Closed the tube, mixed by inversion to disperse Solution III through the viscous bacterial lysate. Stored the tube on ice for 5-10 min.
7. Centrifuged at 12,000 rpm for 10 min at 4^{o}C in a microfuge. Transferred carefully the supernatant to a fresh tube.
8. Precipitated double stranded DNA with equal volume of isopropanol. Mixed well. Allowed the mixture to stand at room temperature for 5-10 min.
9. Centrifuged at 10,000 rpm for 10 min at 4^{o}C in a microfuge.
10. Removed the supernatant. The tube was kept in an inverted position on a paper towel to allow all of the fluid to drain away. Removed any adhering drop of fluid on the walls of the tube.
11. Air dried the pellet and dissolved in 500 µl of TE buffer (pH 8.0).
12. Added DNase free RNase solution to a final concentration of 20 µg/ml and incubate at 37^{o}C for 30 min with occasional shaking.
13. Extracted once with equal volume of phenol: chloroform.
14. Extracted once with equal volume of chloroform.

15. Transferred the upper aqueous layer to a fresh microfuge tube, and then added one-tenth volume of 3.0 M sodium acetate (pH 4.5). Added 2 volume of ethanol, mixed well and kept it at –20 ^{o}C for 1 hour.
16. Centrifuged at 10,000 rpm for 10 min at 4^{o}C in a microfuge tube. Removed the supernatant. Rinsed the pellet with 1 ml of 70% ethanol at 4^{o}C. Allowed the pellet to dry in air for 5-10 min.
17. Redissolved the pellet containing plasmid DNA in 50 µl of TE (pH 8.0). Stored the DNA at –20^{o}C for further use.

3.5.4.10 Restriction analysis of DNA samples by agarose gel electrophoresis

1. Took the DNA solution in a sterile microfuge tube and added sterile water to make up a volume of 17 µl (600 ng).
2. Added 2 µl of the appropriate 10x restriction enzyme assay buffer, mixed thoroughly by tapping the tube.
3. Added 1 µl (2-5 units) of the restriction enzyme, mixed by tapping the tube.
4. Incubated the mixture at the appropriate temperature for 1-2 hours
5. Added 4-5 µl gel-loading buffer to stop the reaction, mixed by vortexing briefly (as the DNA samples needed to be analyzed directly on agarose gel)

3.5.4.11 Sequencing

The 16S rDNA inserts were sequenced for both strands using T7 and SP6 for pGEM-T easy vector and M13 forward and reverse for pTZ57R/T vectors. The sequence was generated by chain termination method (Sanger et al., 1977) using an Applied Biosystems automatic sequencer (DNA Sequencing Facility, Department of Biochemistry, South Campus, Delhi University, New Delhi).

3.5.4.12 Analysis of sequence data

Sequences were analyzed by using CHECK-CHIMERA program of the RDP II (Maidak et al., 2001), in order to detect the presence of possible chimeric artifacts generated by PCR. Similarities were calculated for nearly complete 16S rRNA sequences using only unambiguously determined nucleotide positions. The 16S rRNA gene sequences of isolates were compared with those available in GenBank databases using BLAST program (Altschul et al., 1997) and at RDP-II (Cole et al., 2003). The sequences of closely related strains were retrieved from RDP-II and aligned using multiple alignments (Multalin) program (http://bioinfo.genotoul.fr/multalin/help.html). The evolutionary distance was calculated by Kimura 2 parameter, phylogenetic dendograms were constructed by neighbor-joining method by the use of MEGA Software (v 4.0) (Tamura et al., 2007). For analysis, 1500 bootstrap replicates were performed to assess the statistical support for the tree.

3.6 Screening of efficient bacterial stain for the degradation of chlorophenols (PCP, 2,3,4,6-TeCP, 2,4,5-TCP and 2,4,6-TCP

3.6.1 Screening

Screening was done by growing the bacterial isolates in mineral salt medium supplemented with 50 mg/l of each chlorophenol (PCP, 2,3,4,6-TeCP, 2,4,5-TCP and 2,4,6-TCP) separately. Five isolates CL2, CL3, CL5, CL7 and CL11 have showed the ability to grow on PCP supplemented media. CL2, CL5, CL7, CL9, CL10 was able to grow in 2,4,5-TCP supplemented media whereas CL4, CL5, CL7, CL9, CL11 were able to grow on 2,4,6-TCP supplemented media. All these isolates were able to grow on 2,3,4,6-TeCP amended media.

3.6.2 Inocolum preparation for degradation studies

All aerobic batch cultivations were carried out in 250 ml Erlenmeyer flasks containing 50 ml of liquid culture. The isolated strain was grown on MSM containing PCP, TeCP, 2,4,5-TCP and 2,4,6-TCP (100 mg/l) as sole carbon source for 24 hours. The cells were pelleted by centrifugation at 7,000 rpm for 10 min, cell pellets were washed twice with fresh MSM. Cell density was monitored by spectrophotometer at 600 nm (Hitachi U2900, Japan). For all experiments 10^6 cfu/ml (colony forming unit) were used and samples were incubated at 37°C and shaken at 120 rpm in dark.

3.6.3 Growth analysis

Growth of the each bacterial cells for particular chlorophenol was observed in MSM containing 100 mg/l PCP, 2,3,4,6-TeCP, 2,4,5 and 2,4,6 TCP by taking absorbance at 600 nm on Spectrophotometer from 1 to 7 days after 24 hours of interval.

3.6.4 Analysis of chloride ions

Chloride ion released in the aqueous media was determined at every 24 hour interval up to 168 hours by using 5ml of culture filtrate with an Orion ion analyzer model 940 using calibrated selective chloride ion electrode. Chloride concentration was determined using a calibration curve plotted from the log of chloride molarity for a series of standard samples ranging from 10 to 1000 mg/l.

3.6.5 High Performance Liquid Chromatography (HPLC) Analysis

Chlorophenol degradation was performed by HPLC. The cell suspension was centrifuged for 5 min at 8000 rpm and supernatant was filtered through 0.22 μm filters. HPLC was carried out with purified samples with Perkin Elmer Series (200) system. Samples (10 μl) were injected and separated on reverse phase (Licrosphere^{R} 100 RP-18 endcapped column, 250 mm x 4.6 mm i.d.) in an isocratic mode using aqueous methanol 90% (v/v) at a flow rate of 1 ml/min. The eluates were monitored at 280 nm with online diode array detector (series 200). A standard of each PCP, 2,3,4,6-TeCP, 2,4,5-TCP and 2,4,6-TCP was quantified on the basis of standard curve prepared by taking known quantities of above chlorophenol (Sigma Aldrich, USA).

3.6.6 Effect of pH and temperature on the growth and degradation

The effect of pH and temperature on the degradation of PCP, 2,3,4,6-TeCP, 2,4,5-TCP and 2,4,6-TCP was studied by growing the bacterial cells at different initial pH of 7.5, 8.5 and 9.5 in MSM supplemented with 100 mg/l of PCP, 2,3,4,6-TeCP, 2,4,5-TCP and 2,4,6-TCP as sole source of carbon separately for each positive isolate. The pH of the medium was adjusted with NaOH or HCl. The influence of temperature on PCP, 2,3,4,6-TeCP, 2,4,5-TCP and 2,4,6-TCP degradation was also determined by incubating the

samples at three different temperatures 25°C, 30°C and 37°C under shaking condition. (Lab Therm, Kuhner Shaker, Switzerland).

3.6.7 Degradation of PCP, 2,3,4,6-TeCP, 2,4,5-TCP and 2,4,6-TCP

The degradation ability of the isolated strain was analyzed in 250 ml of Erlenmeyer flask containing 50 ml of MSM supplemented with 50, 100, 200, 400 and 600 mg/l of PCP, TeCP, 2,4,5-TCP and 2,4,6-TCP separately. After that 1%, inocolumn was added to each flask and incubated on shaker. Degradation efficiency was determined and estimated by depletion of chlorophenols from culture medium.

3.7 Remediation of chlorophenol from the secondary sludge (*In-situ* remediation)

The remediation of toxic chlorophenol in the sludge was tested by inoculating the consortia of microbes into sludge. For PCP, inocolum was prepared from positive isolates (CL2, CL3, CL5, CL7 and CL11). All the cultures (CL1 to CL12) were inoculated for 2,3,4,6-TeCP. For 2,4,5-TCP inocolum was prepared from (CL2, CL5, CL7, CL9 and CL10) whereas in case of 2,4,6-TCP inocolum was prepared from (CL4, CL5, CL7, CL9 and CL11). The degradation studies were performed by inoculating 5% mixed inoculum (10^6cfu/ml) to two and half liter of conical shake flask containing one liter of secondary sludge supplemented with 100 mg/l of PCP, 2,3,4,6-TeCP, 2,4,5 and 2,4,6-TCP separately incubated at 37°C under shaking condition for two weeks in experimental and control (without inocolum) condition. Pentachlorophenol, 2,3,4,6 TeCP, 2,4,5 TCP and 2,4,6 TCP were extracted from the sludge as described by Chandra et al (2008), with little modification. The sonicated sludge sample was acidified by 1N HCl to pH 2.0; extracted three times with an equal volume of ethyl acetate by intermittent shaking for 30 min in standard separating funnel. The organic layer was dried with anhydrous sodium sulphate to absorb excess of water. Filtered samples were evaporated to dryness at 40°C,

subsequently resuspended in 1ml of methanol. Quantification of PCP, 2,3,4,6-TeCP, 2,4,5-TCP and 2,4,6-TCP was determined by HPLC.

3.8 Statistical analysis

Data were statistically analyzed by an analysis of variance (ANOVA) and when observed differences were significant, the means were compared by Tukey–Kramer Multiple Comparison Test. All the experiments in this study were performed in triplicates. Graph pad prism software was used for all analysis.

CHAPTER-4

Chapter-4

4. Isolation and characterization of chlorophenol degrading bacteria

4.1 Physiochemical characterization of secondary sludge

The characteristics of the secondary sludge generated from various processes of the pulp and paper industry depends upon the type of wood materials, process technology applied, management practices, chemical recovery efficiency and the stability of effluent treatment plant process. It had very stable chemical composition, characterized by the dominance of absorbable organic halide (AOX) and extractable organic halide (EOX). AOX was 2205±21 mg/l and EOX was about 705±55 mg/l. The pH of the secondary sludge was 7.0. The organic carbon was 28%±1.3, Nitrogen 4%±0.5, Hydrogen 3%±0.3, Sulphur 4±0.2% and Phosphorus about 1±0.1 mg/l. The surface charge or Zeta potential was about -3.1±0.5 mV. The carbohydrate and protein concentration was 73 gram per liter and 3 g/l respectively. The physiochemical characteristics of the sludge are presented in table 4.1.

4.2 Isolation of bacteria

The isolation of bacteria was done by enrichment technique. The chlorophenol degrading bacterial strains were isolated by the serial dilution method, and purified by repeated streaking on mineral salt medium (MSM) plates. Colonies appearing after incubation at 37^{o}C for 48 hours were selected for further screening. A total of 32 bacteria were isolated on the basis of morphology and were grouped into 12 CL1 to CL12, based on ARDRA (amplified ribosomal DNA restriction analysis) and Rep-PCR fingerprinting.

Table 4.1: Physiochemical characterization of secondary sludge sample

Sl.	Parameter	Concentration
1	pH	7.6 ± 0.14
2	Carbon (%)	28 ± 1.3
3	Hydrogen (%)	3 ± 0.3
4	Oxygen (%)	39 ± 2.1
5	Nitrogen (%)	4 ± 0.5
6	Sulphur (%)	4 ± 0.2
7	Phosphorus (mg/l)	1 ± 0.1
8	Carbohydrate (g/l)	73 ± 2.1
9	Proteins (mg/l)	3 ± 0.6
10	Zeta potential (mv)	-3.1 ± 0.5
11	AOX (mg/l)	2207 ± 21
12	EOX (mg/l)	706 ± 55

Values are mean ± SD (n = 3)

4.3 Morphological and biochemical characterization of bacterial isolates

The bacterial isolates showed diverse morphological and biochemical characteristics. They differ in appearance, pigmentation, colony and cell morphology, growth pattern and tolerance to various chorophenols. Pigmentation was rather discrete and the color of isolates CL1, CL2, CL4, CL6, CL8 and CL12 was yellowish, saffron, dense brown, brown, transparent and grey respectively. CL3, CL7 and CL9 were creamy and CL5,

CL10 and CL11 were whitish in color. The isolates CL7, CL8 and CL9 have shown gram negative reaction and other isolates CL1, CL2, CL3, CL5, CL6, CL10, CL11 and CL12 showed gram positive. The bacterial strain CL4 showed gram variable/positive. For capsule test, all the strains showed negative reaction. Two isolates CL3 and CL5 showed oxidase positive and other isolates showed negative while CL4, CL8, CL9 and CL12 were catalase negative. The ability to reduce nitrate was positive for isolates CL1, CL2, CL3, CL4, CL5, CL6 and CL8. For DNase test, only CL1 and CL4 showed partial positive and other isolates showed negative character. Extra cellular enzyme secretion such as urease and indole production is positive for isolates CL3, CL4, CL9, CL10, CL11, CL12 isolates. Starch hydrolysis was positive for CL3, CL5, CL7, CL9, CL10, CL11 and CL12 (Table 4.2). Total of twenty different antibiotics were tested with all isolates. Isolates CL1, CL2, CL3, CL6, CL9, CL10 and CL12 were sensitive to most of the antibiotics and resistant to only one antibiotic whereas CL4, CL5, CL7, CL8 three, four, five, six antibiotics. CL11 also showed resistance to six antibiotics (Table 4.3).

4.4 Carbohydrate fermentation test

The ability to ferment different carbohydrates by these bacterial isolates was determined. Majority of the isolates were able to ferment different carbon substrates. CL6 were able to ferment only two carbon sources whereas CL1 and CL2 fermented three carbon sources, and other isolates CL3, CL4, CL5, CL7, CL8, CL9, CL10, CL11 and CL12 were able to ferment more than five carbon sources (Table 4.4).

Table 4.2: Biochemical characterization of bacterial isolates from secondary sludge

Isolates	Gram stain	Capsule stain	Motility	Catalase test	DNase test	Oxidase test	Nitrate reduction test	Indole test	Urease test	Starch hydrolysis
CL1	+	-	+	-	±	+	+	-	-	-
CL2	+	-	+	-	-	+	+	-	-	-
CL3	+	-	+	+	-	+	+	+	+	+
CL4	Variable/ Positive	-	+	+	±	-	±	+	+	-
CL5	+	-	+	+	-	+	±	-	-	+
CL6	+	-	+	-	-	+	±	-	-	-
CL7	-	-	+	-	-	+	-	-	-	+
CL8	-	-	+	-	-	-	+	-	-	-
CL9	-	-	+	-	-	-	-	+	+	+
CL10	+	-	+	-	-	+	-	+	+	+
CL11	+	-	+	-	-	+	-	+	+	+
CL12	+	-	+	-	-	-	-	+	+	+

+ Positive; - negative and ± weak positive

Positive; - negative and ± weak positive

Table 4.3: Antibiotic sensitivity of bacteria isolated from secondary sludge of pulp and paper industry

Bacterial isolate	Antibiotic sensitivity[#]
CL1	Ceftazidime
CL2	Co-Trimoxazole
CL3	Ceftazidime
CL4	Chloramphenico; Cefuroxime; Co-Trimoxazole
CL5	Co-Trimoxazole; Cephadroxil; Cephadroxil; Penicillin
CL6	Calaritromycin
CL7	Calaritromycin; Co-Trimoxazole; Netillin; Cefaclor; Ampicillin/sublactam
CL8	Cefuroxime; Co-Trimoxazole; Cefaclor; Ampicillin/Cloxacillin; Penicillin; Ampicillin/sublactam
CL9	Ceftazidime
CL10	Ceftazidime
CL11	Roxithromycin; Co-Trimoxazole; Cefaclor; Cephadroxil; Penicillin; Ampicillin/sublactam
CL12	Cephadroxil

[#] Total 20 antibiotics were used with isolated bacteria using ICOSA, Universal -1 Kit (Hi-Media Lab., Mumbai, India).

Table 4.4: Fermentation of carbon substrate by the bacterial isolate from secondary sludge

Bacterial isolate	Carbon substrate fermentation[#]
CL1	Glucosamine, Ribose, Sodium gluconate
CL2	Dextrose, Trehalose, Maltose,
CL3	Fructose, Dextrose, Mannose, Salicin, Glucosamine, Mannitol, Ribose, Rhamnose, Cellobiose, D-Arabinose
CL4	Lactose, Glucosamine, Mannitol, Ribose, Sodium gluconate
CL5	Ribose, Glucosamine, Mannitol, Lactose, Sodium gluconate
CL6	Glucosamine, Ribose
CL7	Maltose, Dextrose, Ribose, Sodium gluconate
CL8	Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, L-Arabinose, Mannose, Sodium gluconate, Rhamnose,
CL9	Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, Raffinose, Trehalose, Mellibiose, L-Arabinose, Mannose, Inulin, Salicin, Inositol, Mannitol, Ribose, Rhamnose, Melezitose, D-Arabinose
CL10	Maltose, Fructose, Dextrose, Trehalose, Glycerol, Salicin, Mannitol, Cellobiose, D-Arabinose
CL11	Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, L-Arabinose, Mannose, Sodium gluconate, Rhamnose
CL12	Fructose, Dextrose, Mannose, Salicin, Glucosamine, Mannitol, Ribose, Rhamnose, Cellobiose, Melezitose, D-Arabinose

[#] Total of 35-carbohydrates fermentation tests were performed with isolated bacteria.

4.5 Molecular characterization

All bacterial isolates were subjected to 16S rRNA amplification and PCR results revealed a ~1.5 Kb fragment (Fig. 4.1) and the PCR products were digested with two different restriction enzymes *EcoRI, Hinf I.* Banding pattern of amplified ribosomal DNA restriction analysis (ARDRA) (Fig. 4.2 and 4.3 respectively) had shown the different banding patterns. ERIC, BOX and REP-PCR based DNA fingerprinting studies had shown the heterogenecity among the bacterial isolates (Fig. 4.4, 4.5, and 4.6 respectively). The sequences were analyzed by multiple sequence alignment (ClustalW) to check the similarities among the isolates (Table 4.5).

The 16S rRNA sequences obtained in this study were compared with NCBI and Ribosomal database project-II for homologous sequences. All the sequences were aligned using MultAlin program (http://bioinfo.genotoul.fr/multalin/help.html) and manually corrected. Phylogenetic tree was constructed by taking the sequences of present study and sequences retrieved from the GeneBank and RDP-II Database. Phylogenetic tree was constructed using MEGA software (v.4.0) (Tamura et al., 2007). Phylogenetic analysis revealed that these isolates were grouped into three major lineage of bacterial domain: *Firmicutes, Proteobacteria,* and *Actinobacteria* (Fig. 4.7). Bacterial isolates and their closest relative identity of gene sequences from NCBI database is shown in Table 4.6. The majority of isolates (CL1, CL3, CL4, CL5, CL8, CL9, CL10, CL11 and CL12) showed homology to Firmicutes and phylogenetically related to *Bacillus* sp., *B. megaterium, Planococcus* sp., *B. pumilus, Jeotgalicoccus* sp., *Exiguobacterium* sp., *Staphylococcus sciuri, B. thuringensis,* and *Bacillus* sp., respectively. In Proteobacterium only CL7 showed homology with the *Pseudomonas stutzeri.* Two isolates CL2 and CL6

were related with Actinobacteria and showed homology with *Kocuria polaris* and *Pseudonocardia* sp. The 16S rRNA gene sequences determined in this study was deposited in the GenBank of NCBI Data Library under the accession numbers EU784648 to EU784659 for CL1 to CL12 respectively (Appendix II).

The homologies among the sequences were from 66 to 94% between isolates. Minimum of 66% similarity was found in CL6 and CL7, and maximum of 94% similarity was found to CL8 and CL11 with CL10 and CL12 (Table 4.5). None of these isolates had shown 95% or more similarity among themselves (Table 4.5).

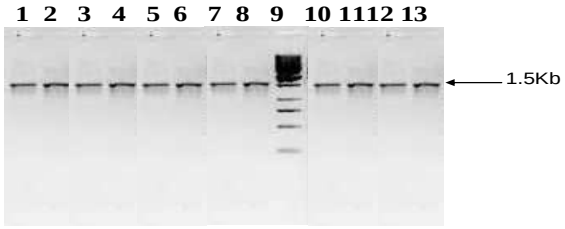


Figure 4.1: 16S rDNA amplification of bacterial isolates. Lane-1 to Lane- 8, Lane 10 to 13 and Lane 9 represent CL1 to CL8, CL9 to CL12 and 1 Kb Ladder respectively

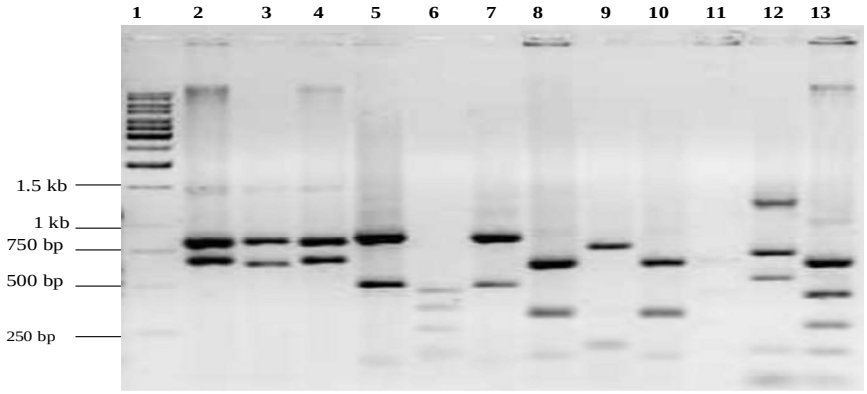


Figure 4.2: Agarose gel electrophoresis of amplified 16S rRNA digested with restriction endonuclease *EcoR I* of 12 bacterial strains representing the corresponding restriction profiles from CL1 to CL12 (Lane 2 to 13); Lane 1 represents marker of 1 kb

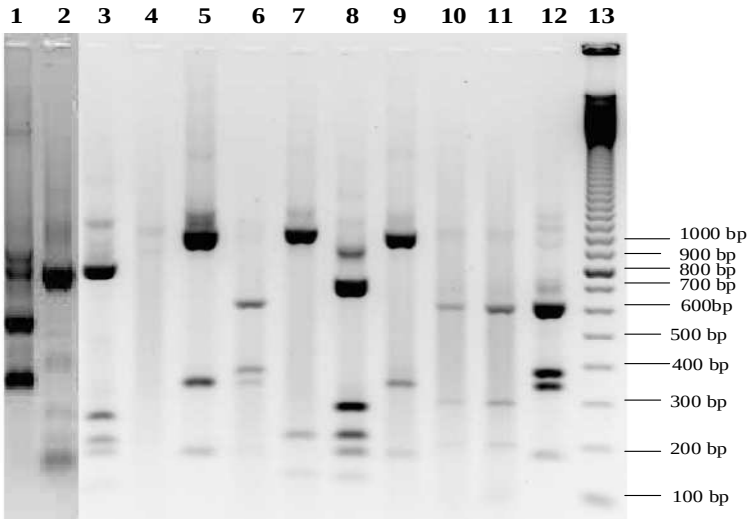


Figure 4.3: Agarose gel electrophoresis of amplified 16S rRNA digested with restriction endonuclease *Hinf I* of 12 bacterial strains representing the corresponding restriction profiles from CL1 to CL12 (Lane 1 to 12); Lanes 13 represents 100 bp marker

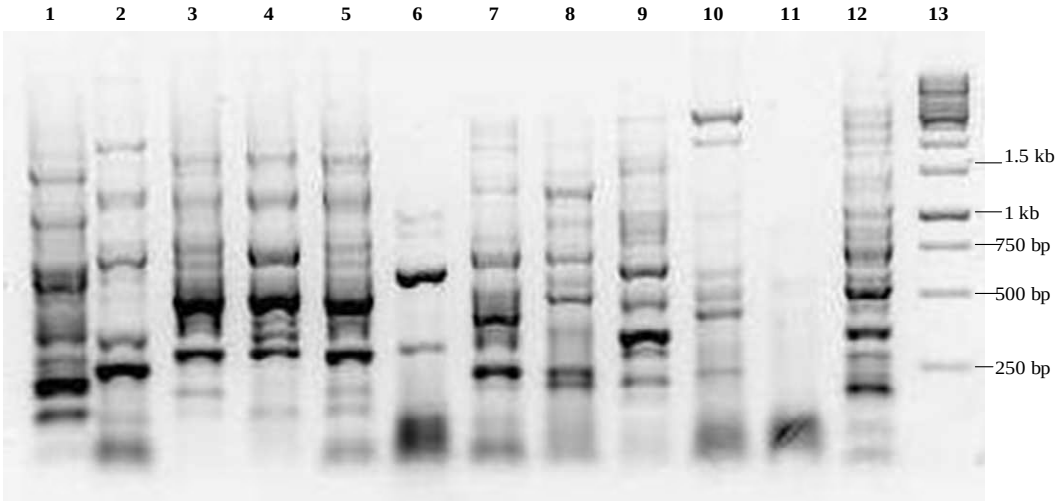


Figure 4.4: Comparison of purified genomic DNA based fingerprints generated by ERIC -PCR for different isolates, Lane 1 to 12 represent CL1 to CL12, Lane 13 represent 1 kb marker

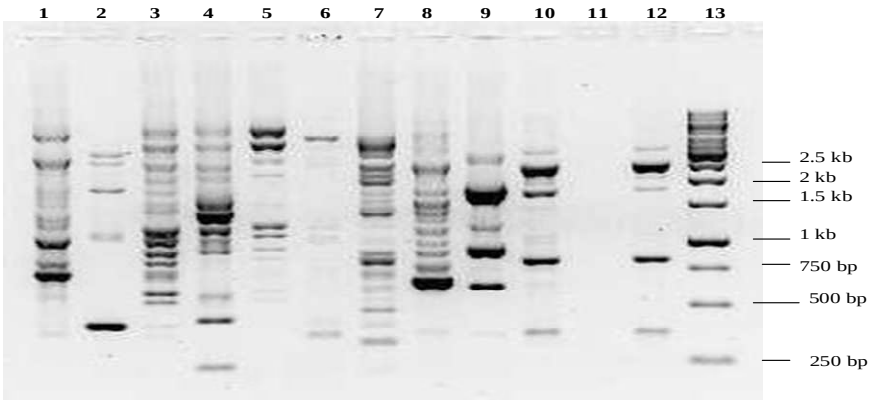


Figure 4.5: Comparison of purified genomic DNA based fingerprints generated by BOX-PCR for different isolates. Lane 1 to 12 represent CL1 to CL12 and Lane 13 represent 1 kb marker

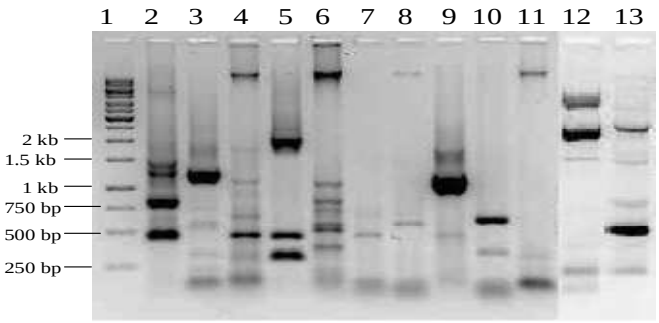


Figure 4.6: Comparison of purified genomic DNA based fingerprints generated by REP-PCR for different isolates; Lane 2 to Lane 13 represent CL1 to CL12 and Lane 1 represent 1 kb marker

Table 4.5: Percentage similarities of 16S rRNA sequences of different bacterial isolates using multiple sequence alignment (ClustalW).

SeqA	Name	Len(nt)	SeqB	Name	Len(nt)	Score
1	CL1	676	2	CL2	775	73
1	CL1	676	3	CL3	720	88
1	CL1	676	4	CL4	733	92
1	CL1	676	5	CL5	721	90
1	CL1	676	6	CL6	742	71
1	CL1	676	7	CL7	769	73
1	CL1	676	8	CL8	695	74
1	CL1	676	9	CL9	783	83
1	CL1	676	10	CL10	724	87
1	CL1	676	11	CL11	565	86
1	CL1	676	12	CL12	737	90
2	CL2	775	3	CL3	720	73
2	CL2	775	4	CL4	733	71
2	CL2	775	5	CL5	721	76
2	CL2	775	6	CL6	742	88
2	CL2	775	7	CL7	769	67
2	CL2	775	8	CL8	695	74
2	CL2	775	9	CL9	783	76
2	CL2	775	10	CL10	724	74
2	CL2	775	11	CL11	565	77

2	CL2	775	12	CL12	737	73
3	CL3	720	4	CL4	733	90
3	CL3	720	5	CL5	721	92
3	CL3	720	6	CL6	742	70
3	CL3	720	7	CL7	769	72
3	CL3	720	8	CL8	695	87
3	CL3	720	9	CL9	783	81
3	CL3	720	10	CL10	724	89
3	CL3	720	11	CL11	565	88
3	CL3	720	12	CL12	737	92
4	CL4	733	5	CL5	721	90
4	CL4	733	6	CL6	742	70
4	CL4	733	7	CL7	769	72
4	CL4	733	8	CL8	695	78
4	CL4	733	9	CL9	783	80
4	CL4	733	10	CL10	724	89
4	CL4	733	11	CL11	565	86
4	CL4	733	12	CL12	737	92
5	CL5	721	6	CL6	742	72
5	CL5	721	7	CL7	769	72
5	CL5	721	8	CL8	695	83
5	CL5	721	9	CL9	783	84
5	CL5	721	10	CL10	724	89
5	CL5	721	11	CL11	565	89
5	CL5	721	12	CL12	737	91
6	CL6	742	7	CL7	769	66
6	CL6	742	8	CL8	695	74
6	CL6	742	9	CL9	783	79
6	CL6	742	10	CL10	724	73
6	CL6	742	11	CL11	565	75
6	CL6	742	12	CL12	737	68
7	CL7	769	8	CL8	695	72
7	CL7	769	9	CL9	783	67
7	CL7	769	10	CL10	724	74
7	CL7	769	11	CL11	565	70
7	CL7	769	12	CL12	737	68
8	CL8	695	9	CL9	783	74
8	CL8	695	10	CL10	724	94
8	CL8	695	11	CL11	565	85
8	CL8	695	12	CL12	737	91
9	CL9	783	10	CL10	724	92
9	CL9	783	11	CL11	565	84
9	CL9	783	12	CL12	737	72
10	CL10	724	11	CL11	565	90
10	CL10	724	12	CL12	737	92
11	CL11	565	12	CL12	737	94

Table 4.6: Bacterial isolates and their closest relative species inferred from 16S rRNA gene sequences of NCBI database

Bacterial Isolate	Nearest match	Phylum	Accession No.	Query coverage in %	E value	% Similarity of 16S rDNA
CL1	*Bacillus* sp. (DQ416791)	Firmicutes	*EU784649*	100	0.0	99
CL2	*Kocuria polaris* (AJ278868)	Actinobacteria	*EU784648*	96	0.0	99
CL3	*Bacillus megaterium* (EU221388)	Firmicutes	*EU784650*	100	0.0	99
CL4	*Planococcus* sp. (EF471920)	Firmicutes	*EU784651*	95	0.0	99
CL5	*Bacillus pumillus* (AY030327)	Firmicutes	*EU784652*	99	0.0	99
CL6	*Pseudonocardia* sp. (AY974792)	Actinobacteria	*EU784653*	96	0.0	99
CL7	*Pseudomonas stutzeri* (FJ6133151)	Proteobacteria	*EU784654*	90	0.0	99
CL8	*Jeotgalicoccus psychrophilus* (NR025644)	Firmicutes	*EU784655*	94	0.0	99
CL9	*Exiguobacterium* sp. (AY745848)	Firmicutes	*EU784656*	97	0.0	99
CL10	*Staphylococcus sp.* (EU855191)	Firmicutes	*EU784657*	95	0.0	100
CL11	*Bacillus thuringiensis* (AM779000)	Firmicutes	*EU784658*	100	0.0	100
CL12	*Bacillus sp.* (FJ189780)	Firmicutes	*EU784659*	100	0.0	98

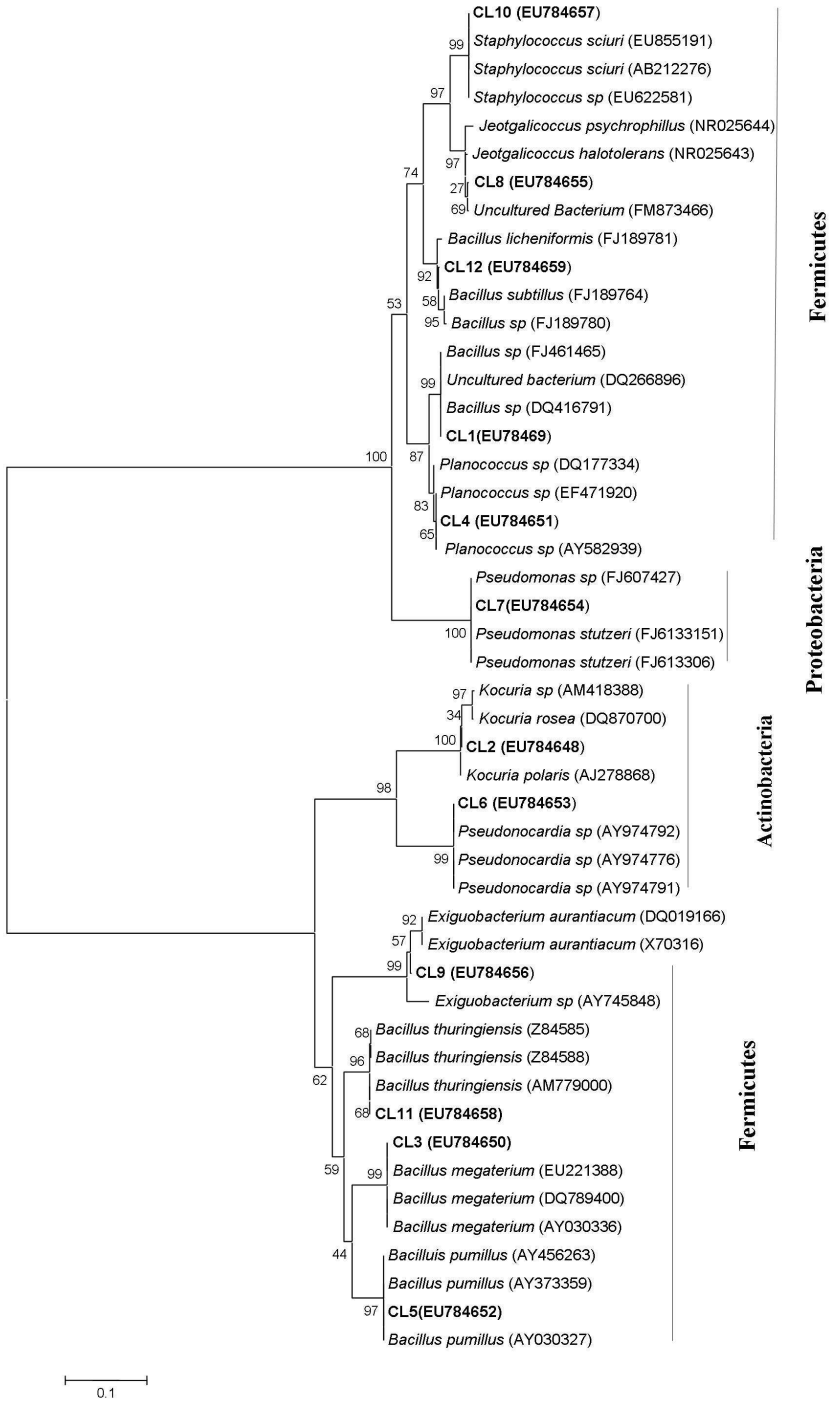


Figure 4.7: Phylogenetic relationship of 12 bacterial isolates from sludge, Neighbor-joining tree based on bacterial 16S rRNA sequence data from different isolates of current study along with sequences available in GeneBank database. Numerical value indicates bootstrap percentile from 1500 replicates. Bar indicates change per nucleotide position.

Salient findings

Twelve bacterial strains were isolated from the secondary sludge by enrichment technique. All these strains were characterized and belong tothree phylum; Fermicutes, Proteobacteria and Actinobacteria. Most of the isolates CL1, CL3, CL4, CL5, CL8, CL9 CL10, CL11 and CL12 belong to Fermicutes, two isolates CL2 and CL6 belong to Actinobacteria and only one isolate CL7 belongs to Proteobacteria. Sequence alignment of closely related bacteria and bacterial isolates from sludge showed that similarity varied from 97 to 100%, two isolates had shown 100% sequence similarity with the bacteria of the existing database CL10 and CL11. Many new strains were isolated for the degradation of chlorophenol.

CHAPTER-5

Chapter-5

5. Degradation of chlorophenols

5.1 Screening of the efficient bacteria for the chlorophenol degradation

The bacterial isolates were tested for their ability to degrade four different chlorophenolic compounds such as PCP, 2,3,4,6-TeCP, 2,4,5-TCP, 2,4,6-TCP by the enrichment technique in mineral salt medium (MSM) supplemented with 50 mg/l of each chlorophenol as sole carbon and energy source. Five isolates CL2, CL3, CL5, CL7 and CL11 have shown the ability to grow on PCP supplemented media. CL2, CL5, CL7, CL9, CL10 was able to grow in 2,4,5-TCP supplemented media whereas CL4, CL5, CL7, CL9, CL11 were able to grow on 2,4,6-TCP supplemented media. All twelve isolates were able to grow on 2,3,4,6-TeCP supplemented MSM media (Table 5.1).

Table 5.1: Screening of the efficient bacteria for their ability to grow on different chlorophenolic compounds.

Serial No.	Name	PCP	2,3,4,6-TeCP	2,4,5-TCP	2,4,6-TCP
1	CL1	-	+	-	-
2	CL2	+	+	+	-
3	CL3	+	+	-	-
4	CL4	-	+		+
5	CL5	+	+	+	+
6	CL6	-	+	-	-
7	CL7	+	+	+	+
8	CL8	-	+	-	-
9	CL9	-	+	+	+
10	CL10	-	+	+	-
11	CL11	+	+	-	+
12	CL12	-	+	-	-

+ Positive, - Negative

5.2 PCP degradation

5.2.1 Growth analysis

The bacterial strains isolated from the pulp and paper industry were tested for the utilization of PCP by each bacterial strain for their ability to utilize PCP in liquid batch culture. The bacterial growth and utilization of PCP was estimated. The growth was assessed by taking absorbance at 600 nm of the bacterial suspension observed for CL2, CL3, CL5, CL7 and CL11 in MSM containing PCP (100 mg/l). All five isolates were able to grow and utilize PCP as an energy source. The growth of CL2 and CL3 increased significantly up to fifth days and declined later, whereas growth of CL5 increased up to sixth day and reached a stationary phase. For CL7 growth was high from the first day compared to other isolates, but maximum growth achieved on third day. The growth of CL11 increased significantly up to fourth day and decreased thereafter. Initially for all the isolates initially the growth was slower but the growth increased significantly in the medium after first day and the maximum growth achieved at third and fourth days of the incubation period (Table 5.2 and Figure 5.1.).

Table 5.2: Growth of bacterial isolates in mineral salt medium (MSM) supplemented with 100 mg/l (0.37 mM) of PCP.

Time (Day)	CL2	CL3	CL5	CL7	CL11
0	0.025±0.01dA	0.029±0.01aA	0.029±0.01cA	0.030±0.01bA	0.033±0.01eA
1	0.074±0.01cdAB	0.062±0.01aB	0.059±0.01cB	0.116±0.01bA	0.062±0.01dB
2	0.111±0.01cB	0.089±0.01aB	0.089±0.01cB	0.300±0.01bA	0.090±0.01dB
3	0.315±0.03bB	0.121±0.01abB	0.142±0.01cB	1.067±0.14aA	0.120±0.01cB
4	0.350±0.02abB	0.153±0.01abC	0.283±0.02bBC	0.852±0.07aA	0.228±0.01aBC
5	0.396±0.01aB	0.155±0.02abcC	0.323±0.04bBC	0.880±0.10aA	0.223±0.02abBC
6	0.403±0.01aBC	0.140±0.01bcD	0.637±0.07aB	0.843±0.08aA	0.219±0.02abCD
7	0.392±0.01aB	0.127±0.03cC	0.612±0.04aB	0.835±0.08aA	0.201±0.02bC

Values sharing a common lowercase letter within column and uppercase letter within the row are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3).

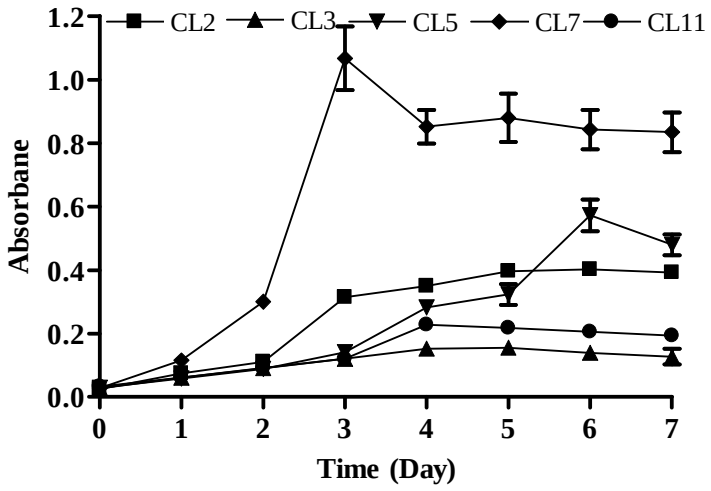


Figure 5.1: Growth profiles of CL2, CL3, CL5, CL7 and CL11 in mineral salt media containing 100 mg/l (0.37 mM) of PCP as a sole carbon and energy source

5.2.2 Chloride release assay

During the course of bacterial treatment PCP was mineralized and the concentration of inorganic chloride ion in culture medium increased. The two are interrelated i.e., concentration of chloride ion is directly linked with the depletion of PCP. During PCP utilization CL2 released 1.6 mM chloride ion; CL3 and CL11 released 1.7 mM whereas CL5 and CL7 released chloride up to 1.8 mM (Table 5.3 and Fig. 5.2).

Table 5.3: Chloride ion released in MSM supplemented with 100 mg/l (0 .37 mM) of PCP by the bacterial isolates during course of incubation. The initial chloride ions present in the medium (control) were normalized to zero (Values are given in mM).

Time (Day)	CL2	CL3	CL5	CL7	CL11
0	0.00±0.00eE	0.00±0.00eE	0.00±0.00eE	0.00±0.00eE	0.00±0.00eE
1	0.82±0.14dC	0.51±0.02dBC	0.42±0.51dB	0.82±0.03dAB	0.42±0.51dA
2	0.93±0.35cA	0.85±0.07cBC	0.45±0.55dD	1.10±0.14cdCD	0.54±0.65cAB
3	1.35±0.21bcA	1.24±0.06bA	1.08±0.01cB	1.28±0.30cB	1.10±0.14bA
4	1.60±0.20aAB	1.63±0.19aA	1.35±0.21bC	1.55±0.07bBC	1.40±0.28aA
5	1.55±0.71aA	1.68±0.26aA	1.63±0.19abA	1.70±0.14aAB	1.40±0.27aA
6	1.56±0.40 a A	1.70±0.14 a A	1.75±0.17 ab A	1.81±0.01aA	1.57±0.10aA
7	1.55±0.20a A	1.70±0.14a A	1.80±0.12aA	1.80±0.02aA	1.77±0.10aA

Values sharing a common lowercase letter within column and uppercase letter within the row are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3).

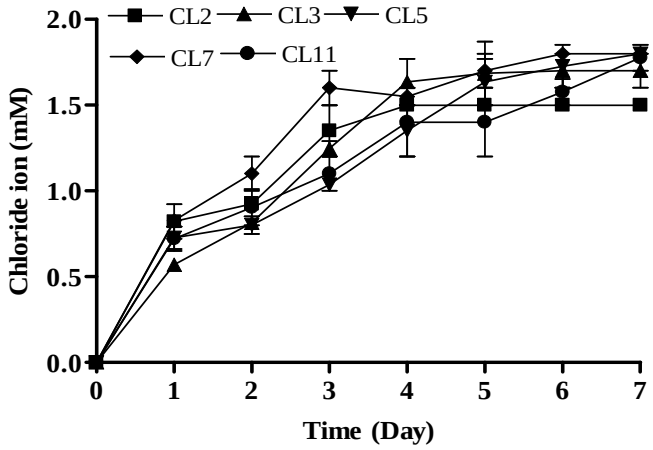


Figure 5.2: Release of chloride ion in the mineral salt medium containing 100 mg/l (0.37 mM) of PCP by CL2, CL3, CL5, CL7 and CL11 at different time interval during incubation period; error bars represent the standard deviation

5.2.3 Effect of pH on PCP degradation

The effect of pH on the degradation of PCP was studied by growing the bacterial cells at three pH condition; 7.5, 8.5, and 9.5 in MSM supplemented with 100 mg/l or 0.37 mM of PCP as a sole source of carbon and energy. The maximum PCP removal was observed at pH range of 7.5 to 8.5 by all five isolates. Significant difference was observed among the isolates; CL2 and CL7 were able to remove 97% whereas CL3, CL5 and CL11 were able to remove 93% of PCP at pH 7.5. At pH of 8.5 the degradation capacity also increased for all five PCP degrading bacterial isolates. The optimum pH for the degradation was within the pH range of 7.5 to 8.5, shown in Table 5.4 and Fig. 5.3. At pH 9.5 two isolates CL2 and CL5 have same degradation efficiency as at pH 7.5 but with CL3, CL7 and CL11 degradation potential decreased up to 82%. Result showed that increase in pH of the medium decreased the growth of bacterial isolates (Table 5.4).

Table 5.4: Effect of pH on the growth and degradation of PCP by five bacterial strains

	Degradation (%)			Absorbance (600 nm)		
Isolate	pH 7.5	pH 8.5	pH 9.5	pH 7.5	pH 8.5	pH 9.5
CL2	98±1aA	99±1aA	99±1aA	0.206±0.01bA	0.203±0.01bA	0.200±0.01bA
CL3	93±1bA	94± bA	87±1bB	0.113±0.01cA	0.091±0.01cA	0.073±0.01cB
CL5	94±1bB	99±1aA	99±1aA	0.158±0.01 c A	0.145±0.01bB	0.141±0.01bB
CL7	98±1a A	99±1aA	98±14aA	0.470±0.02 a A	0.414±0.01aB	0.413±0.01aB
CL11	95±1bA	90±1cA	82±1bB	0.479±0.01 a A	0.426±0.01aB	0.418±0.01aB

Values sharing a common lowercase letter within column and uppercase letter within the row are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3).

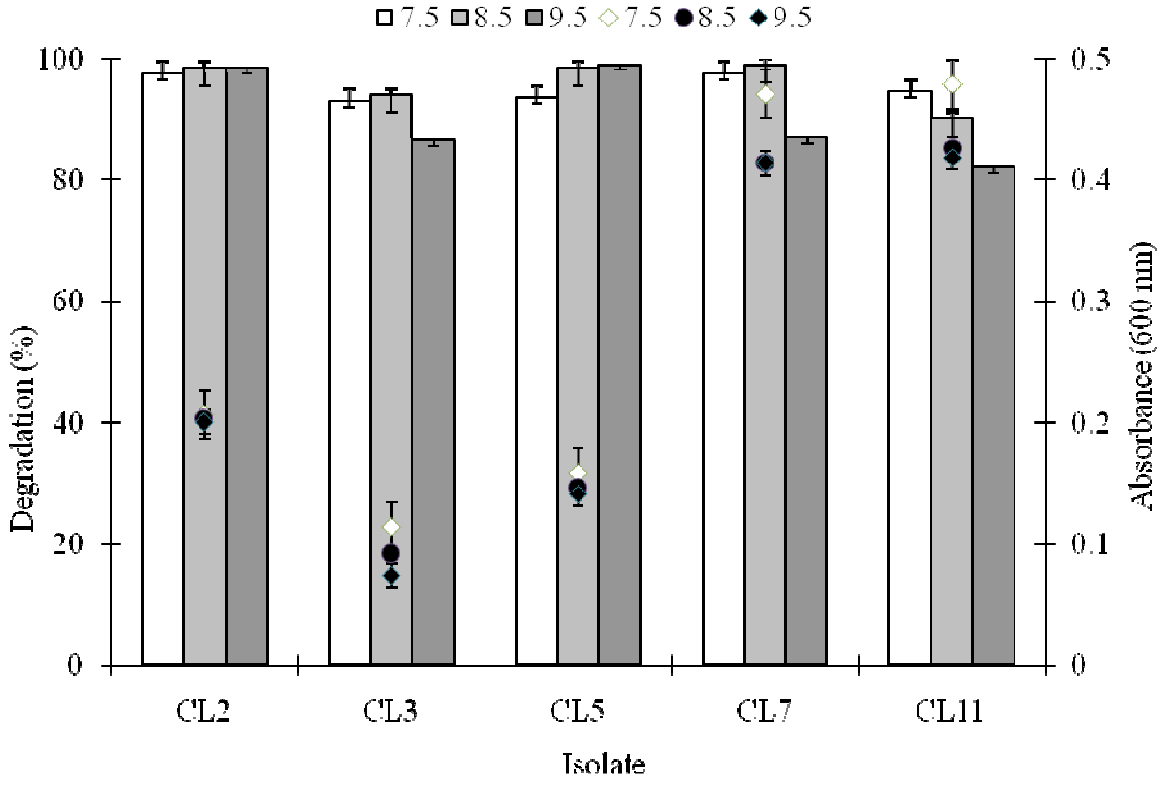


Figure 5.3: Effect of pH on the growth and degradation of PCP by CL2, CL3, CL5, CL7 and CL11 isolates; error bars represent the standard deviation

5.2.4 Effect of temperature on PCP degradation

Results showed that at temperature 25 and 30°C microbial growth and degradation of PCP are both lower as compared to those at 37°C. Degradation gradually increased with increase in temperature from 25 to 37°C. At temperature 25°C, CL2 and CL7 were able to degrade 85%, CL3 74%, whereas CL5 and CL11 66% and 63% PCP respectively. At temperature 30°C all the five isolates were able to degrade about 90% of PCP except CL11 which degraded 82%. At 37°C all five isolates CL2, CL3, CL5, CL7 and CL11 removed PCP up to 95% (Table 5.5 and Figure 5.4).

The influence of temperature on PCP degradation was studied by growing the bacterial isolates at 25, 30 and 37°C under the shaking condition. All five isolates CL2, CL3, CL5, CL7 and CL11 showed lower growth at 25, 30 as compared to 37°C. The growth gradually increased with increase of temperature, so the result revealed that, temperature of 30 to 37°C is more suitable for the growth of the bacterial isolates (Table 5.5 and Figure 5.4).

Table 5.5: Effect of temperature on the growth and degradation of PCP by five bacterial stains

	Degradation (%)			Absorbance (600 nm)		
Isolate	25°C	30°C	37°C	25°C	30°C	37°C
CL2	85±4aB	94±2aA	96±1aA	0.112±0.01bC	0.164±0.01cB	0.206±0.01bA
CL3	74±3bB	91±2aA	95±2aA	0.091±0.02bcB	0.105±0.01cdA	0.113±0.01cA
CL5	66±1cB	94±1aA	95±1aA	0.124±0.01bC	0.136±0.01cB	0.158±0.01cA
CL7	85±4aB	90±1aA	94±1aA	0.321±0.01aB	0.448±0.02aA	0.470±0.02aA
CL11	63±1cC	82±1bB	92±1abA	0.300±0.02aC	0.351±0.04bB	0.479±0.01aA

Values sharing a common lowercase letter within column and uppercase letter within the row are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3).

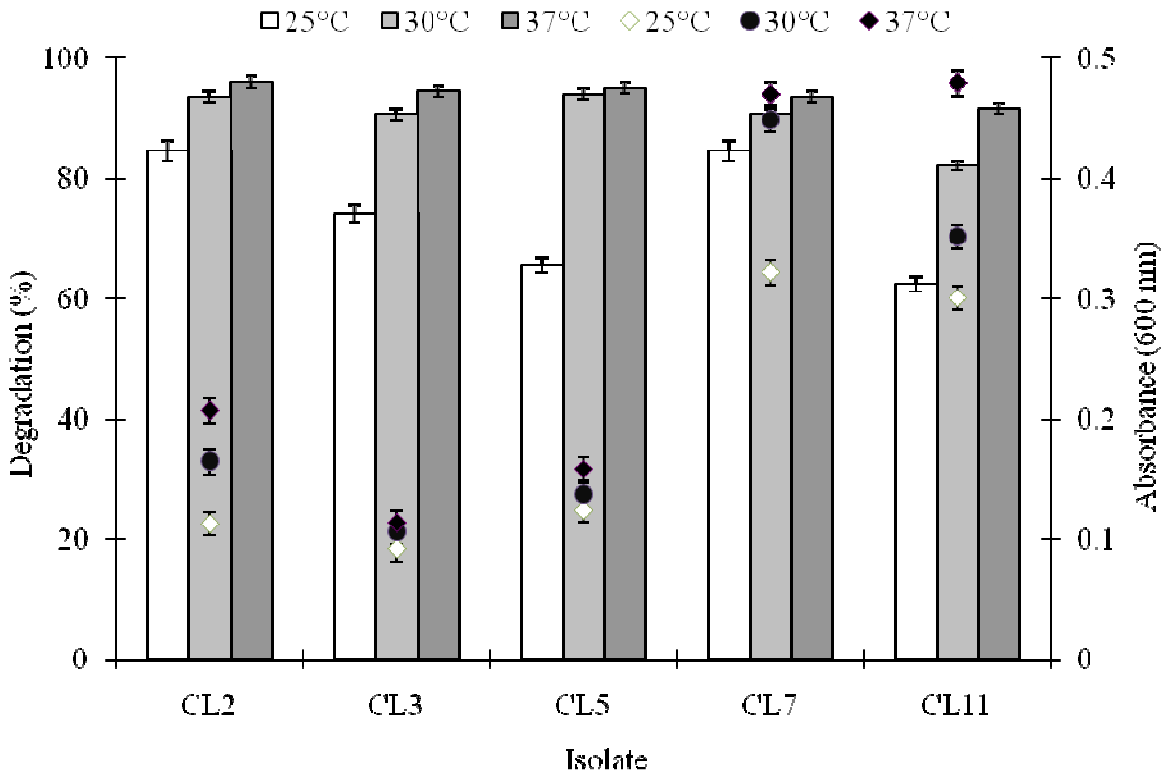


Figure 5.4: Effect of temperature on the growth and degradation of PCP by CL2, CL3, CL5, CL7 and CL11 isolates; error bars represent the standard deviation

5.2.5 Degradation of PCP

Degradation of PCP by individual strain were investigated in terms of growth for seven days in different concentrations and utilization of PCP as substrate. The isolate CL2 showed 90% degradation of PCP up to 400 mg/l and there was slight decrease in the degradation at 600 mg/l which was upto 87%. The same results were also observed with CL3 when degradation at 600 mg/l was 83% and below this concentration degradation was more than 90%. For CL5 and CL7 degradation was 90% upto 600 mg/l. For CL11, degradation of more than 90% was observed upto 400 mg/l but at 600 mg/l the degradation decreased to 88% (Table 5.6a and Figure 5.5a&b). All the isolates from the present study have shown effective degradation potential. CL2, CL3, CL11 have shown

90% degradation at 400 mg/l but at 600 mg/l, there was slight decrease in degradation potential which was up to 85%. CL5 and CL7 were able to degrade 90% of PCP at 600 mg/l. Final absorbance was also taken at different concentration of PCP. All the bacterial strains were able to grow at 600 mg/l PCP. With increase in concentration of PCP there was a decrease in growth of bacterial isolates. CL2, CL5 and CL11 show uniform growth in MSM supplemented with PCP. Maximum growth was observed with CL7 whereas CL3 has shown least growth (Table 5.6b).

Table 5.6a: Degradation of PCP (in %) by bacterial isolates at different concentrations

Concentration (mg/l)	CL2	CL3	CL5	CL7	CL11
50	97±2aA	94±3aA	97±2aA	98±4aA	98±3aA
100	96±1aA	94±1aA	96±1aA	97±5aA	97±4aA
200	95±1aA	95±1aA	95±1abA	97±4aA	96±6aA
400	90±1abA	92±1aA	91±2abA	92±6aA	94±3aA
600	87±3bB	83±4bB	90±1bA	90±6aA	88±9aA

Values sharing a common lowercase letter within column and uppercase letter within the row are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

Table 5.6b: Effect of concentration of PCP on the growth of bacterial isolates

Concentration (mg/l)	CL2	CL3	CL5	CL7	CL11
50	0.367±0.01aC	0.107±0.01aE	0.450±0.02aB	0.864±0.01aA	0.181±0.01aD
100	0.360±0.01aB	0.107±0.01aC	0.459±0.01aB	0.838±0.08aA	0.156±0.01aC
200	0.317±0.01abB	0.105±0.01aC	0.279±0.01bB	0.666±0.06abA	0.123±0.01bC
400	0.279±0.01bB	0.112±0.01aC	0.233±0.01cB	0.657±0.03abA	0.095±0.01bcC
600	0.214±0.01cB	0.108±0.01aC	0.220±0.01cB	0.549±0.03bA	0.104±0.01cC

Values sharing a common lowercase letter within column and uppercase letter within the row are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3).

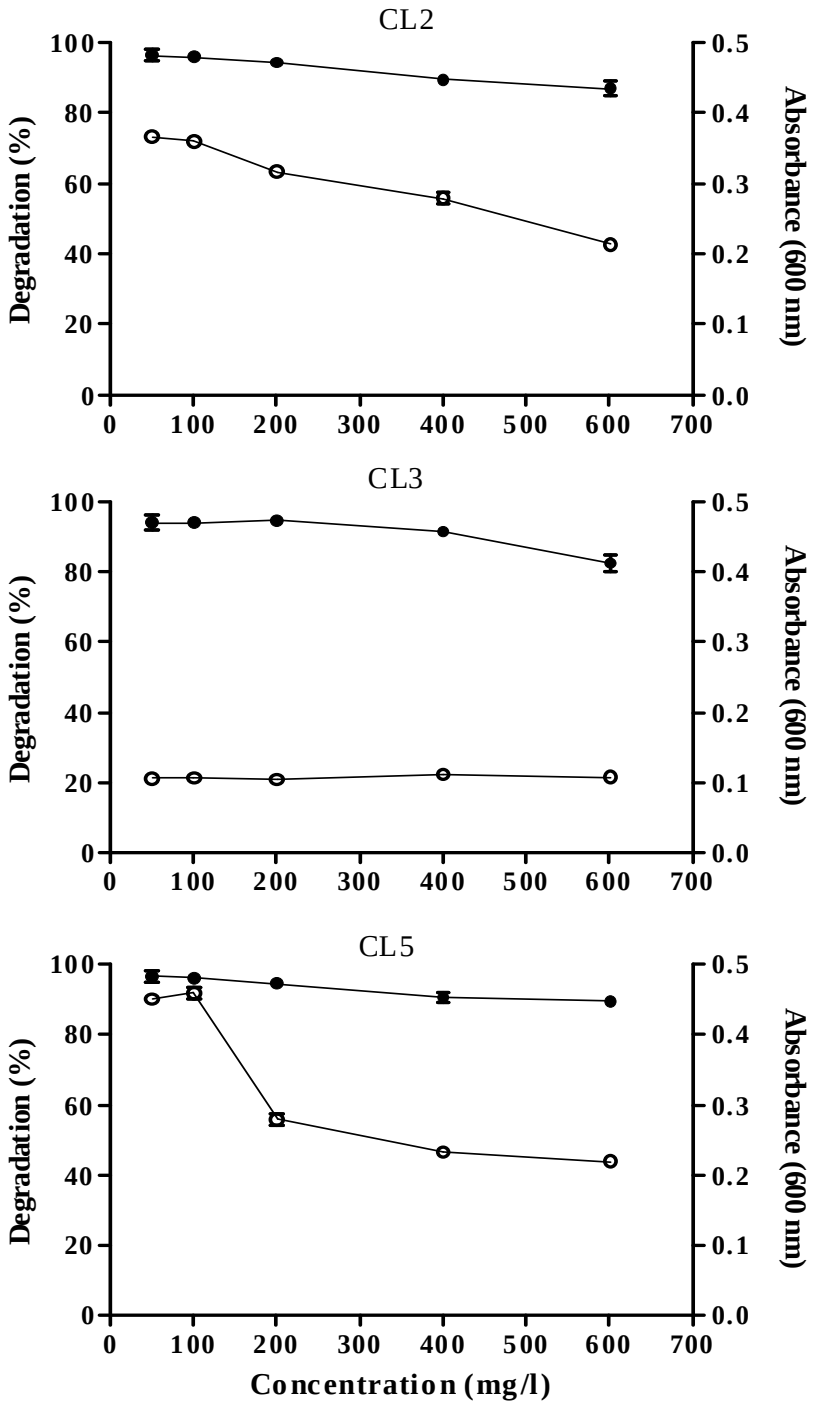


Figure 5.5a: Degradation of PCP (50 to 600 mg/l) and growth of bacterial isolates by CL2, CL3 and CL5; open circle represent the absorbance and filled circle degradation. Error bars represent the standard deviation

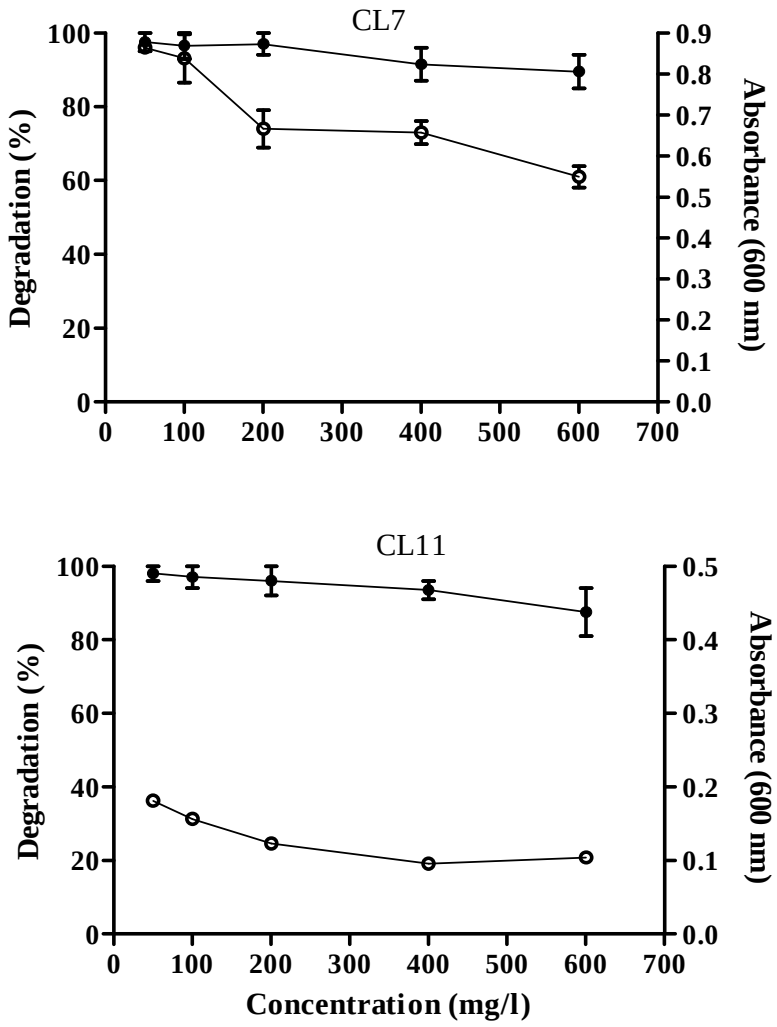


Figure 5.5b: Degradation of PCP (50 to 600 mg/l) and growth of bacterial isolates CL7 and CL11; open circle represent the absorbance and filled circle degradation. Error bars represent the standard deviation

Salient findings

Five PCP degrading bacterial strains were screened from the secondary sludge of pulp and paper mill. These five isolates were morphologically, biochemically and molecularly characterized and identified as one *Kocuria* sp. (CL2), three *Bacillus* sp.(CL3, CL5, CL11) and one *Pseudomonas* sp.(CL7). These isolates were identified as PCP degrader.

Kocuria sp. (CL2), *Bacillus* sp. (*Bacillus megaterium*, (CL3), *B. pumulis* (CL5) and *B. thuringenins* (CL11) and *Pseudomonas stutzeri* (CL7) were five isolates which used PCP as sole source of carbon and energy, and were capable of degrading this compound with release of chloride ion. HPLC analysis revealed all these strains were able to degrade PCP upto a concentration of 600 mg/l. This is the first report on the PCP degradation by the *Kocuria* species.

All five isolates were able to remove PCP effectively at PH 7.5. The degradation by *Bacillus* sp. is higher at pH 8.5 but at pH 9.5 degradation was lower for all the bacterial isolates, and optimum temperature for the degradation was 37°C.

As compared to the physiological characteristics and PCP degradation potential of the PCP degrading strains reported by earlier researchers, these isolates have higher potential for PCP degradation with more flexible growth conditions.

5.3 2,3,4,6-Tetrachlorophenol degradation

5.3.1 Growth studies

Utilization of 2,3,4,6-TeCP by all twelve bacterial strains (CL1, CL2, CL3, CL4, CL5, CL6, CL7, CL8, CL9, CL10, CL11 and CL12) in MSM containing 100 mg/l (0.43 mM) was observed. All isolates used 2,3,4,6-TeCP as carbon and energy source. The growth pattern of these isolates was presented in Figure 5.6 (a&b). Four bacterial strains CL1, CL2, CL4 and CL12 have shown similar growth pattern and maximum absorbance value observed was about 0.5 whereas CL3, CL7, CL8 and CL10 have shown comparatively less growth in this medium than other isolates. Growth of CL5, CL6 and CL12 showed highest growth with this chlorophenol than other isolates on sixth day, absorbance was recorded to be about 0.6. Whereas growth of CL9 and CL11 followed similar pattern and maximum absorbance recorded was 0.2, the growth was higher in case of CL3, CL7, CL8 and CL10. Maximum growth was recorded in fourth and fifth day of incubation period (Table 5.7 and Fig. 5.6a&b).

Table 5.7: Growth of bacterial isolates in mineral salt medium (MSM) supplemented with 100 mg/l (0.43 mM) of 2,3,4,6-TeCP

Time (Days)	CL1	CL2	CL3	CL4	CL5	CL6	CL7	CL8	CL9	CL10	CL11	CL12
0	0.030±0.01dA	0.025±0.01bA	0.033±0.01bA	0.033±0.01cA	0.026±0.01fA	0.024±0.01fA	0.028±0.01dA	0.026±0.01bA	0.028±0.01bA	0.026±0.01bA	0.029±0.01dA	0.026±0.01eA
1	0.090±0.01dA	0.102±0.02bA	0.083±0.02abA	0.097±0.01cA	0.087±0.01efA	0.068±0.00efA	0.065±0.01cdA	0.066±0.01abA	0.105±0.01abA	0.087±0.01abA	0.066±0.01cdA	0.089±0.01dA
2	0.126±0.01dB	0.334±0.01aA	0.100±0.01abB	0.206±0.02bB	0.102±0.01deB	0.090±0.01eB	0.091±0.01bcB	0.093±0.02abB	0.116±0.02abB	0.101±0.01abB	0.106±0.02bcdB	0.103±0.01dB
3	0.336±0.02bA	0.425±0.10aA	0.147±0.04aB	0.500±0.03aA	0.157±0.01dB	0.158±0.02dB	0.118±0.01abB	0.144±0.04abB	0.144±0.04ab B	0.145±0.04aB	0.149±0.04abcB	0.134±0.01dB
4	0.474±0.02aA	0.490±0.01aA	0.158±0.02aDE	0.529±0.01aA	0.312±0.01cB	0.317±0.01cB	0.137±0.01aE	0.161±0.03aB	0.249±0.03aBC	0.162±0.02aDE	0.224±0.01aCD	0.313±0.01cB
5	0.519±0.01aAB	0.484±0.00aB	0.153±0.01aE	0.578±0.01aA	0.370±0.06cC	0.477±0.01bB	0.154±0.01aE	0.167±0.03aDE	0.248±0.05aD	0.163±0.02aDE	0.211±0.01aDE	0.282±0.01bC
6	0.518±0.01aBC	0.425±0.02aC	0.130±0.01aD	0.546±0.03aAB	0.604±0.02aAB	0.633±0.01aA	0.131±0.01abD	0.145±0.03abD	0.234±0.04aD	0.145±0.03aDE	0.203±0.01aDE	0.528±0.01aABC
7	0.490±0.01aAB	0.413±0.01aB	0.124±0.02abC	0.491±0.04aAB	0.495±0.02bAB	0.588±0.01aA	0.116±0.01abC	0.135±0.03abC	0.208±0.06aC	0.134±0.02aC	0.187±0.01abC	0.487±0.03aAB

Values sharing a common lowercase letter within column and uppercase letter within the row are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

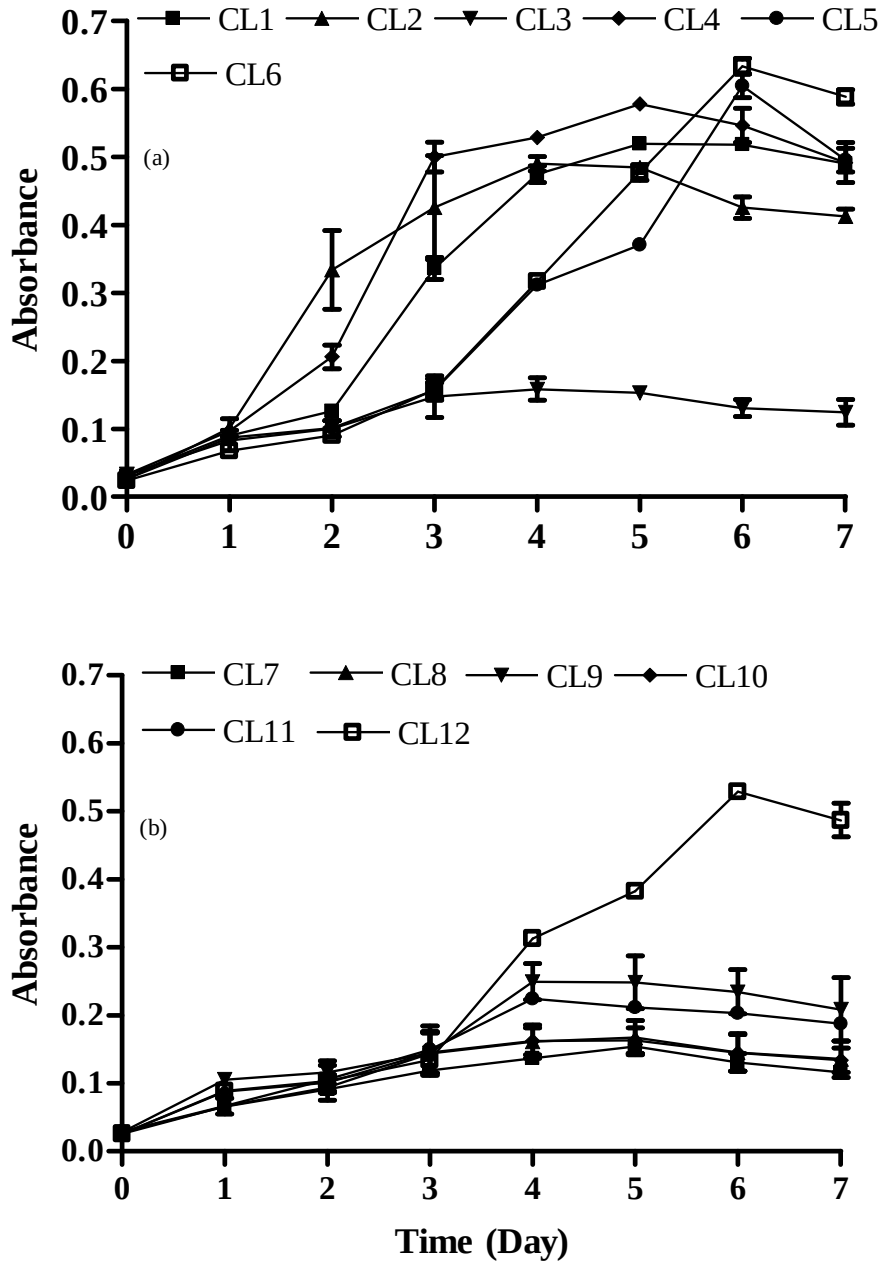


Figure 5.6 a&b: Growth pattern of CL1 to CL12 bacterial isolates in mineral salt medium containing 100 mg/l (0.43 mM) of 2,3,4,6-Tetrachlorophenol as sole carbon and energy source; error bars represent the standard deviation.

5.3.2 Chloride release assay

Bacterial isolates showed an increase in the concentration of inorganic chloride. For 2,3,4,6-TeCP all these isolates released chloride ion up to 1.7 mM except CL10 that released the same upto 1.6 mM. CL1, CL4 and CL11 released chloride ion upto 1.7 mM on fourth day of incubation whereas CL2, CL3, CL5, CL6, CL7, CL8, CL9 and CL12 released the same by 1.7 mM on fifth day. Chloride ion concentration gradually increased in the medium with the passage of time. Maximum chloride ion release was observed on third and fourth day of incubation. Table 5.8 and Fig. 5.7 (a&b) show the pattern of inorganic chloride ion release by the isolates.

Table 5.8: Chloride ion released in MSM supplemented with 100 mg/l (0.43 mM) of 2,3,4,6-TeCP by the bacterial isolates incubation.

Time (Days)	CL1	CL2	CL3	CL4	CL5	CL6	CL7	CL8	CL9	CL10	CL11	CL12
1	0.44±0.1dEF	0.64±0.4dBCD	0.56±0.4dCDEF	0.52±0.1dDEF	0.77±0.3dAB	0.62±0.1eBCDE	0.42±0.1eF	0.74±0.4dABC	0.42±0.2dF	0.91±0.1eA	0.50±0.1dDEF	0.87±0.3dA
2	0.89±0.1cDEF	1.15±0.2cABC	0.72±0.5dF	0.83±0.1cEF	1.0±0.2cCD	0.95±0.3dCDE	0.74±0.3dF	1.27±0.3cAB	0.91±0.1cDEF	1.29±0.1dA	0.87±0.3cEF	1.33±0.1cA
3	1.30±0.1bDE	1.56±0.3bAB	1.19±0.3cE	1.54±0.1bAB	1.4±0.1bBCD	1.47±0.3cBC	1.29±0.1cDE	1.33±0.1bCDE	1.49±0.1bBC	1.47±0.3cBC	1.29±0.1bDE	1.68±0.3bA
4	1.71±0.1aABC	1.56±0.2bA	1.76±0.3bABC	1.70±0.3aABC	1.66±0.3bABC	1.72±0.3bBC	1.56±0.4bC	1.70±0.3aABC	1.56±0.3bC	1.54±0.1bcBC	1.70±0.3aABC	1.60±0.2bAB
5	1.70±0.1aABCD	1.72±0.2aABCDE	1.70±0.1abCDE	1.70±0.4aABC	1.70±0.2aAB	1.70±0.1abEF	1.70±0.1aF	1.70±0.4aDEF	1.70±0.1aEF	1.52±0.1bBCDE	1.70±0.1aEF	1.70±0.1aA
6	1.70±0.6aAB	1.70±0.2aA	1.70±0.1aBCD	1.70±0.1aABC	1.70±0.1aABC	1.70±0.2aCD	1.70±0.1aD	1.70±0.2aCD	1.70±0.4aBCD	1.60±0.2abBCD	1.70±0.2aCD	1.70±0.1aABC
7	1.70±0.2aAB	1.70±0.1aA	1.70±0.4aCD	1.70±0.1aABC	1.70±0.1aBC	1.70±0.2aD	1.70±0.2aCD	1.70±0.1aCD	1.70±0.1aCD	1.62±0.1aCD	1.70±0.1aD	1.70±0.1aBC

Value sharing a common lowercase letter within column and uppercase letter within the row are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

The initial chloride ions present in the medium (control) were normalized to zero

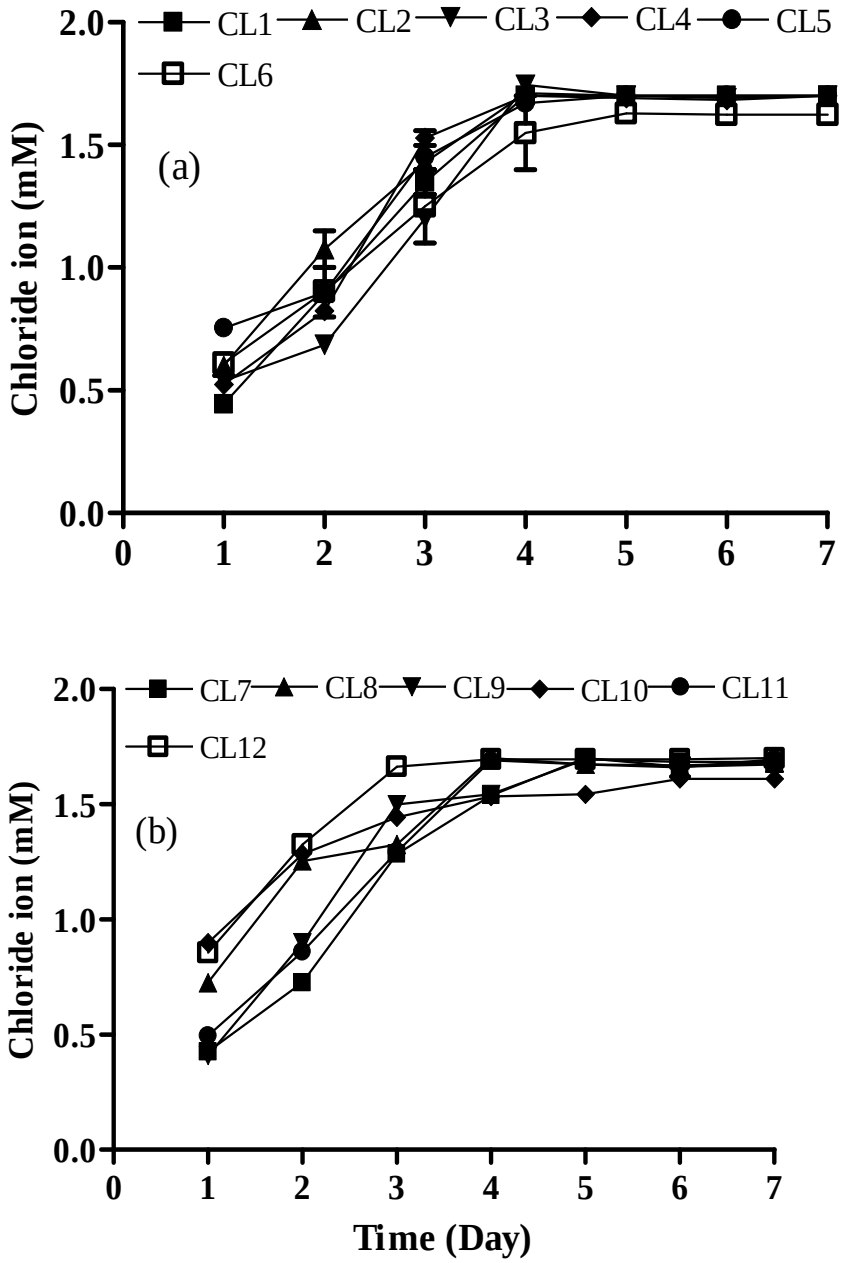


Figure 5.7: Release of chloride ion in the mineral salt medium containing 100 mg/l (0.43 mM) 2,3,4,6-TeCP by CL1 to CL12 isolates at different time interval during incubation; error bars represent the standard deviation

5.3.3 Effect of pH on 2,3,4,6-TeCP degradation

All the isolates were able to degrade 2,3,4,6-TeCP effectively, those are able to remove 90% and above of 2,3,4,6-TeCP at pH 7.5 to 8.5. CL3, CL8, and CL9 removed maximum at pH 7.5, which was upto 93%, 85% and 93% respectively. Bacterial isolates CL1, CL2, CL4, CL5, CL6, CL7, CL10, CL11 and CL12 removed higher percentage of 2,3,4,6-TeCP at pH 8.5 upto 90% as compared to those at pH 7.5. At pH 9.5 there was a decrease in the efficiency of degradation by all these bacterial isolates (Table 5.9 and Fig. 5.8).

The growth of bacterial isolates at pH 7.5 to 8.5 was approximately similar for these isolates but at pH 9.5 the absorbance relatively decreased. Degradation also decreased at higher pH (Table 5.9 and Fig. 5.8).

5.3.4 Effect of temperature on 2,3,4,6-TeCP degradation

Temperature directly affected both the growth and degradation of all the bacterial isolates used in this study. At 25 and 30°C, the degradation was lower by all the twelve bacterial isolates. Degradation increased gradually with increase in temperature. At 25 and 30°C, the degradation was less by 10 to 20%. Degradation was observed to be maximum at 37°C for all those isolates as compared to those at 25 and 30°C (Table 5.10 and Fig.5.9). Absorbance was also higher at 37°C, this temperature might be more favorable for the optimum growth of the bacterial isolates (Table 5.10).

Table 5.9: Effect of pH on the growth and degradation of 2,3,4,6-TeCP by the twelve bacterial isolates

	Degradation (%)			Absorbance (600 nm)		
Isolate	pH 7.5	pH 8.5	pH 9.5	pH 7.5	pH 8.5	pH 9.5
CL1	76±5bcB	90±1aA	86±1aA	0.413±0.01bA	0.341±0.03cB	0.242±0.03abC
CL2	94±1aA	96±1aA	85±4aB	0.213±0.01cA	0.213±0.02dA	0.182±0.06bcB
CL3	93±1aA	91±2aA	85±2aB	0.106±0.01dA	0.104±0.07eB	0.084±0.09dC
CL4	92±4aA	95±1aA	88±1aB	0.226±0.01cA	0.205±0.08dB	0.133±0.04cC
CL5	83±2bB	92±1aA	86±1aB	0.427±0.01bA	0.426±0.05bA	0.221±0.02bB
CL6	82±0bB	94±4aA	87±4aA	0.487±0.02aA	0.494±0.07aA	0.289±0.01aB
CL7	86±1bA	74±3cB	70±2cB	0.105±0.06dA	0.105±0.05eA	0.077±0.02dB
CL8	85±2bA	83±1bA	73±1cB	0.105±0.04dA	0.100±0.01efA	0.072±0.07dB
CL9	93±1aA	90±2aA	79±4abB	0.153±0.04cdA	0.143±0.02eA	0.119±0.04cB
CL10	91±2aA	94±1aA	75±0cB	0.106±0.02dA	0.098±0.09efA	0.091±0.09dB
CL11	84±3bB	92±1aA	74±3cC	0.163±0.02cdA	0.150±0.07eA	0.132±0.01cB
CL12	82±1bB	93±2aA	71±0cC	0.425±0.01bA	0.417±0.08bA	0.207±0.07bB

Value sharing a common lowercase letter within column and uppercase letter within the row are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3).

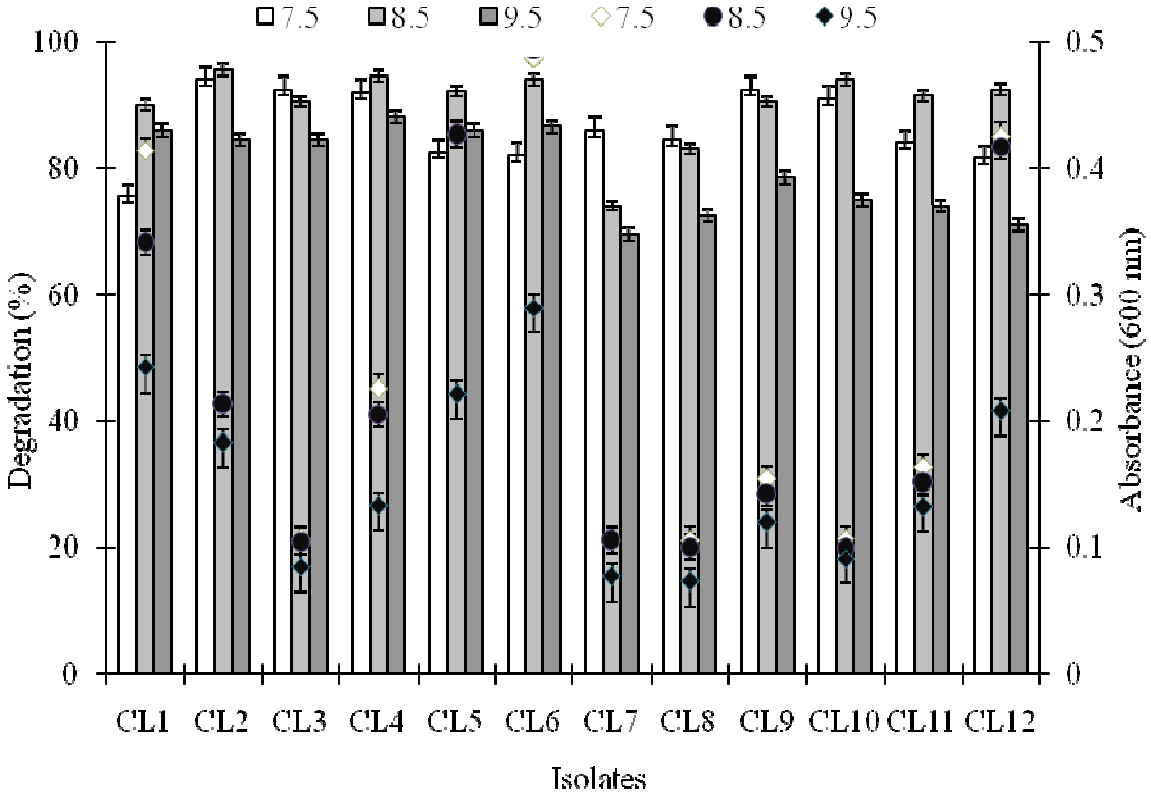


Figure 5.8: Effect of pH on growth and degradation of 2,3,4,6-TeCP by CL1 to CL12 error bars represent the standard deviation

Table 5.10: Effect of temperature on the growth and degradation of 2,3,4,6-TeCP by twelve bacterial isolates

	Degradation (%)			Absorbance (600 nm)		
Isolate	25°C	30°C	37°C	25°C	30°C	37°C
CL1	70 ±2bC	85±4bB	96±1aA	0.182±0.09bB	0.195±0.02bA	0.213±0.02bA
CL2	69±2cB	75±0bB	91±2aA	0.085±0.05eB	0.103±0.02cA	0.106±0.01cA
CL3	71±3bC	81±2bB	95±1aA	0.171±0.02cB	0.213±0.02bA	0.226±0.01bA
CL4	77±1bC	88±0abB	92±1aA	0.304±0.01aB	0.416±0.01aA	0.427±0.02aA
CL5	71±2bB	91±1aA	94±4aA	0.300±0.01aC	0.425±0.05aB	0.487±0.06aA
CL6	88±1aA	86±1abA	74±3bB	0.094±0.01eA	0.104±0.07cA	0.105±0.04cA
CL7	70±2bB	72±1bB	83±1bA	0.081±0.01eB	0.107±0.01cA	0.105±0.04cA
CL8	74±1bB	75±3bB	91±1aA	0.106±0.02dB	0.141±0.02 bcA	0.153±0.02bcA
CL9	66±4cC	78±0bB	94±2aA	0.104±0.06dA	0.106±0.01cA	0.106±0.02cA
CL10	77±0bB	78±2bB	92±1aA	0.121±0.01dC	0.149±0.01bcB	0.163±0.01bcA
CL11	72±1bB	79±1bB	93±1aA	0.210±0.01bC	0.310±0.08aB	0.425±0.02aA
CL12	72±1bB	92±1aA	96±1aA	0.182±0.01bC	0.195±0.01bB	0.213±0.01bA

Value sharing a common lowercase letter within column and uppercase letter within the row are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3).

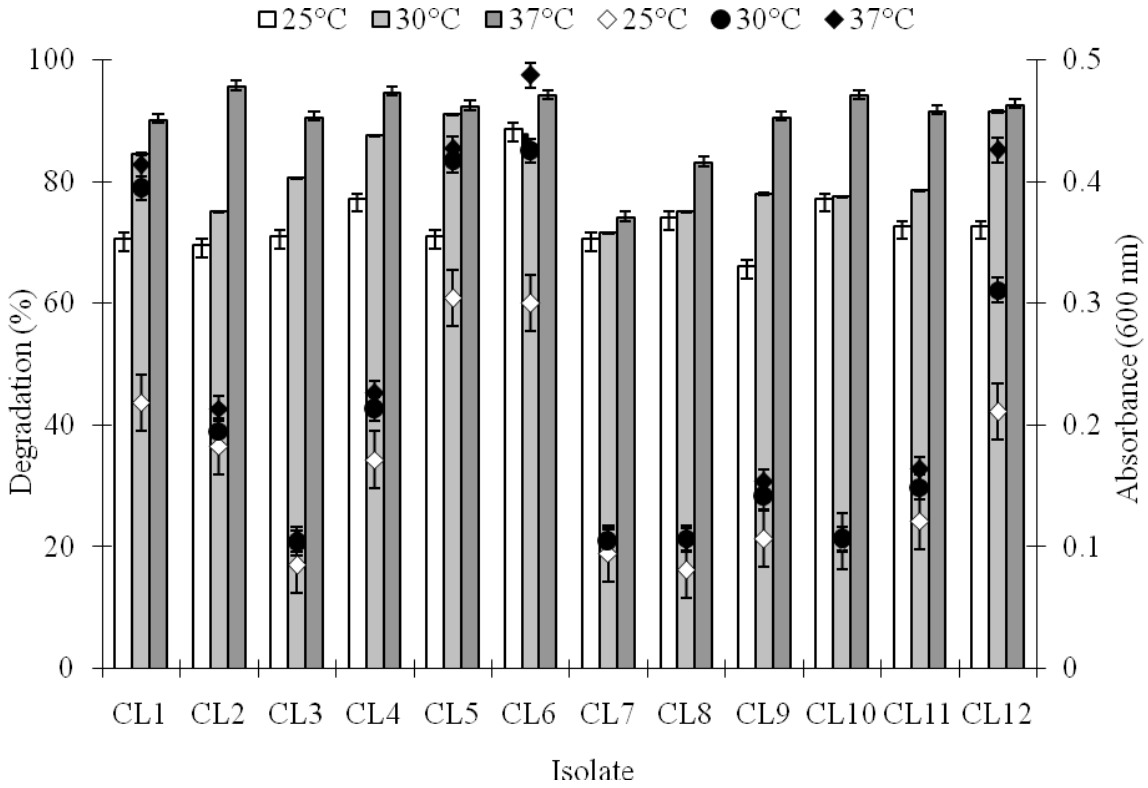


Figure 5.9: Effect of temperature on the growth and degradation of 2,3,4,6-TeCP by CL1 to CL12; error bars represent the standard deviation

5.3.5 Degradation of 2,3,4,6-TeCP

The degradation studies of 2,3,4,6-TeCP was analyzed by high pressure liquid chromatography. CL1, CL11 and CL12 were able to remove 95% of 2,3,4,6-TeCP at 200 mg/l and the degradation decreased with increase in concentration (Fig. 5.10). CL2, CL3, CL4 and CL5 were able to remove 90% at 200 mg/l but at 400 and 600 mg/l the degradation decreased up to 83%. Isolates CL6, CL9 and CL10 were able to remove 90% of 2,3,4,6-TeCP at 600 mg/l, two isolates CL7 and CL8 have relatively lower degradation efficiency upto 85% at 200 mg/l. With increased concentration the degradation efficiency decreased upto 73%. Simultaneously release of chloride ion into the medium and disappearance of chlorophenol from the medium took place showing the degradation (Table 5.11a and Fig 5.10a&b).

Final absorbance at seven days was also taken to check the growth of bacterial isolates present in the MSM supplemented with 2,3,4,6-TeCP. All these isolates were able to grow on this chlorophenol. An increase in the concentration reduces the growth of the bacterial isolates. CL1, CL6 and CL12 grew more as compared with those of other isolates used for the degradation of this chlorophenol. Growth pattern of all the isolates at different concentration is given in (Table 5.11b and Figure 5.10a&b).

Table 5.11:

a) Degradation (%) of 2,3,4,6-TeCP at different concentration by the bacterial isolates

Concentration (mg/l)	CL1	CL2	CL3	CL4	CL5	CL6	CL7	CL8	CL9	CL10	CL11	CL12
50	99±1aA	94±1aABC	94±1aABC	93±1a BC	93±1aBC	97±1aAB	86±2aD	91±1aCD	98±1aAB	98±1aAB	96±3aAB	98±1aAB
100	98±1aA	93±1abC	93±1aBC	93±1a C	93±1aC	98±1aA	83±1aD	87±1aD	98±1aA	97±1aAB	97±1aABC	97±2aABC
200	96±1aAB	91±1abBC	92±1aBC	90±0ab CD	92±1aBC	98±1abA	82±3aDE	86±1aE	96±1aAB	98±1aA	96±1aAB	96±1aAB
400	92±1bAB	88±3abBC	89±1aAB	86±1abBC	89±2aAB	94±2bcA	82±1aBC	86±1aC	91±1aAB	94±2abA	91±1aAB	90±1aAB
600	90±2bA	86±1bAB	88±4aAB	82±5b ABC	88±4aAB	91±1cA	74±3bC	80±2aBC	91±1aA	91±1b A	90±4aAB	87±3bAB

Values sharing a common lowercase letter within column and uppercase letter within the row are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

b) Effect of concentration of 2,3,4,6-TeCP on the growth of different isolates

Concentration (mg/l)	CL1	CL2	CL3	CL4	CL5	CL6	CL7	CL8	CL9	CL10	CL11	CL12
50	0.487±0.03aA	0.404±0.01aC	0.105±0.01 aF	0.463±0.02 aB	0.476±0.02 aA	0.476±0.02 aA	0.107±0.02 aF	0.106±0.04 aF	0.163±0.02 aE	0.114±0.02 aF	0.183±0.02 aD	0.462±0.01 aB
100	0.413±0.02bC	0.213±0.02bE	0.106±0.01 aG	0.226±0.01 bD	0.427±0.02 bB	0.487±0.06 aA	0.105±0.04 aG	0.105±0.04 aG	0.153±0.02 aF	0.106±0.02 aG	0.164±0.02 bF	0.425±0.01 aB
200	0.413±0.02bA	0.126±0.01cC	0.107±0.06 a D	0.113±0.02 cCD	0.293±0.02 cB	0.283±0.01 bB	0.108±0.04 aD	0.108±0.05 aD	0.123±0.02 bC	0.109±0.04 aD	0.113±0.02 cCD	0.296±0.01 1bB
400	0.213±0.02cB	0.103±0.02dCD	0.103±0.02 a CD	0.123±0.02 dC	0.235±0.01 dA	0.223±0.02 bAB	0.104±0.02 aCD	0.105±0.07 aCD	0.094±0.07 cD	0.103±0.02 aCD	0.089±0.01 dD	0.224±0.01 bAB
600	0.213±0.02cB	0.103±0.02dCD	0.104±0.02 a CD	0.103±0.02 eCD	0.230±0.02 dA	0.217±0.06 bA	0.104±0.04 aCD	0.105±0.04 aCD	0.089±0.01 cD	0.110±0.04 aD	0.099±0.01 eD	0.233±0.02 bA

Value sharing a common lowercase letter within column and uppercase letter within the row are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

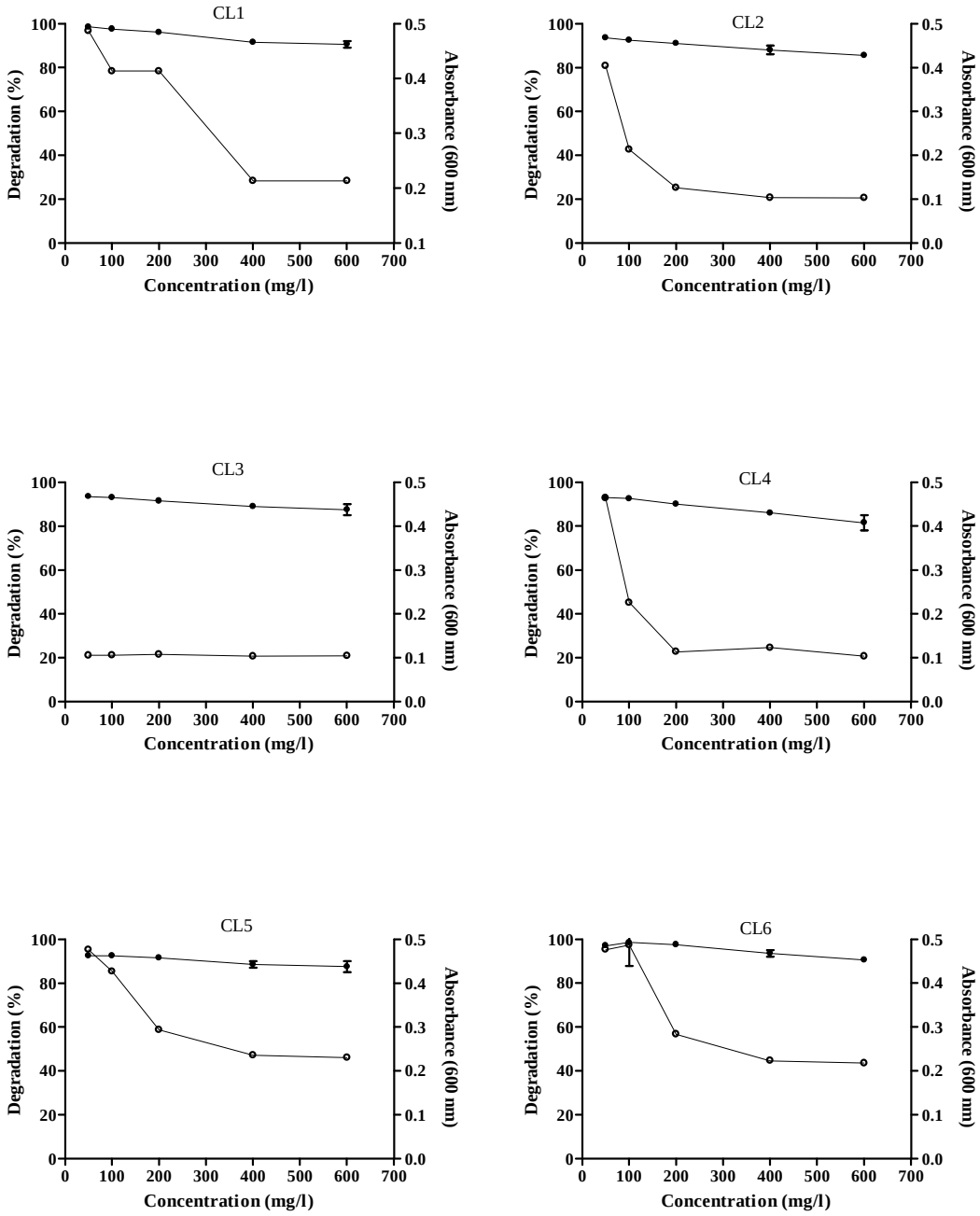


Figure 5.10a: Degradation of 2,3,4,6-TeCP at different concentration by bacterial isolates CL1 to CL6. Open circle represents the absorbance and filled circle degradation. Error bars represent the standard deviation

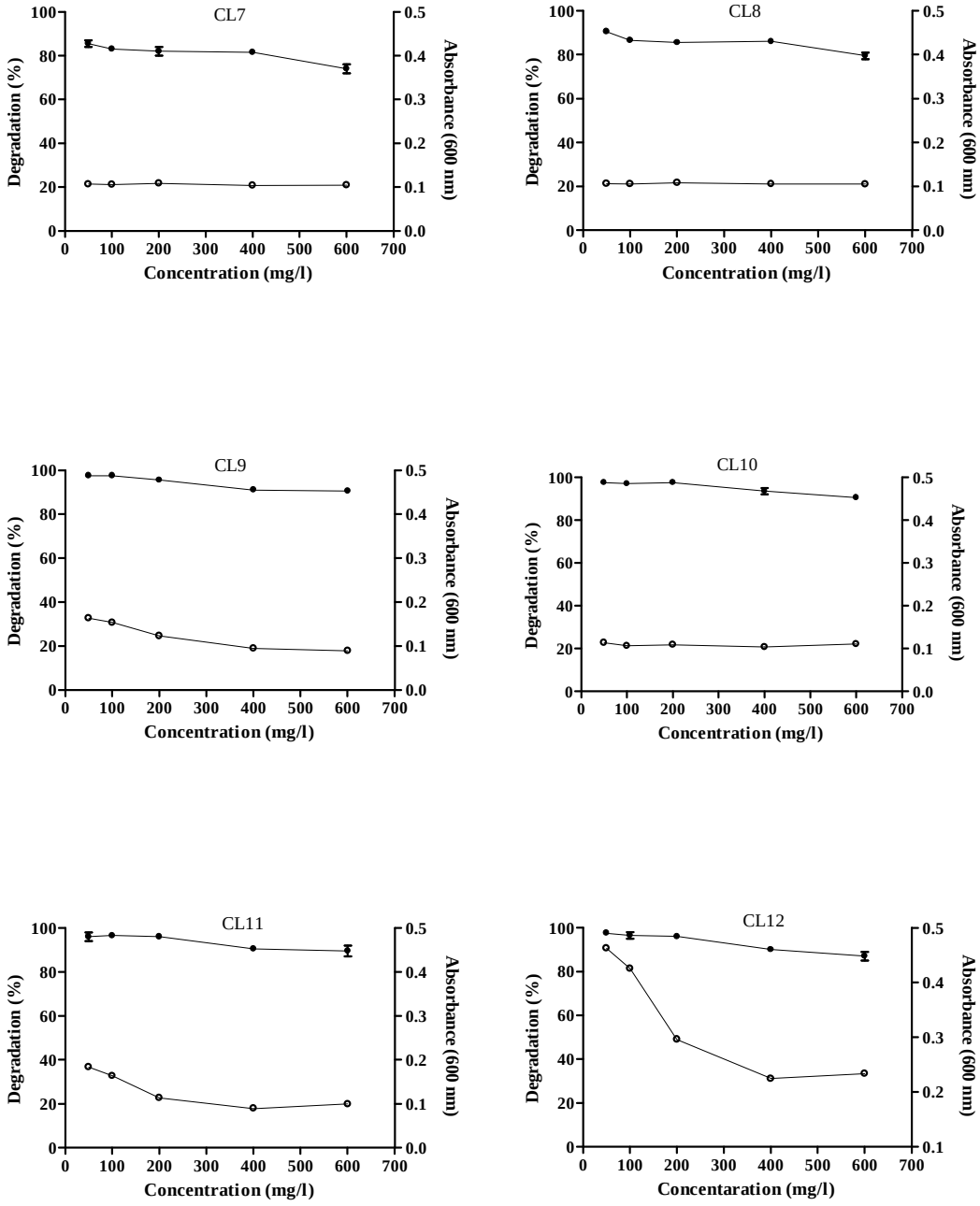


Figure 5.10b: Degradation of 2,3,4,6-TeCP at different concentrations by bacterial isolates CL7 to CL12. Open circle represents the absorbance and filled circle degradation. Error bars represent the standard deviation

Salient findings

All twelve bacterial isolates (CL1, CL2, CL3, CL4, CL5, CL6, CL7, CL8, CL9, CL10, CL11, CL12) were able to degrade 2,3,4,6-TeCP effectively.

The degradation ability of all the isolates (CL1 to CL12) was approximately 90% at 600 mg/l except CL7 and CL8 which can degrade upto 75 to 80% of 2,3,4,6-TeCP.

Maximum removal of 2,3,4,6-TeCP was observed at pH range of 7.5 to 8.5 but increased pH affects the degradation process and reduced the degradation drastically. 37°C is the optimum for the removal of 2,3,4,6-TeCP. At temperature 25 and 30°C degradation efficiency decreased.

5.4 2,4,5-TCP Degradation

5.4.1 Growth studies

The bacterial isolates; CL2, CL5, CL7, CL9 and CL10 showed utilization of 2,4,5-TCP as a carbon and energy source in MSM supplemented with 100 mg/l (0.50 mM) of 2,4,5-TCP. The growth of CL2 significantly increased upto fifth day and decreased thereafter. For CL7 the growth maintained uniformly and achieved maximum absorbance in third day which was approximately 0.5. The growth of CL5 reached high on sixth day and declined thereafter. Growth of CL5 was the highest among all other isolates. CL9 and CL10 showed uniform growth pattern on this chlorophenol, maximum absorbance was recorded in fourth day of incubation (Table 5.12 and Fig. 5.11).

Table 5.12: Growth of bacterial strain in mineral salt medium (MSM) supplemented with 100 mg/l (0.50 mM) of 2,4,5-TCP

Time (Day)	CL2	CL5	CL7	CL9	CL10
0	0.026±0.04eA	0.030±0.01eA	0.029±0.02dA	0.027±0.03cA	0.027±0.06dA
1	0.123±0.01dA	0.080±0.01deA	0.152±0.05cdA	0.084±0.07bA	0.069±0.01cdA
2	0.388±0.02cA	0.103±0.01dC	0.241±0.04bcB	0.099±0.01bC	0.094±0.07bcC
3	0.507±0.07abA	0.144±0.02dC	0.430±0.02aB	0.130±0.02bC	0.118±0.01abcC
4	0.526±0.01abA	0.303±0.02cC	0.412±0.01aB	0.229±0.08aC	0.153±0.01aC
5	0.552±0.01aA	0.371±0.07cC	0.390±0.04abB	0.217±0.01aD	0.153±0.01aE
6	0.517±0.01dB	0.627±0.08bA	0.375±0.05abC	0.207±0.08aD	0.130±0.01abD
7	0.487±0.03eA	0.490±0.04aA	0.369±0.06abA	0.197±0.02aB	0.120±0.02dB

Values sharing a common lowercase letter within column and uppercase letter within the row are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

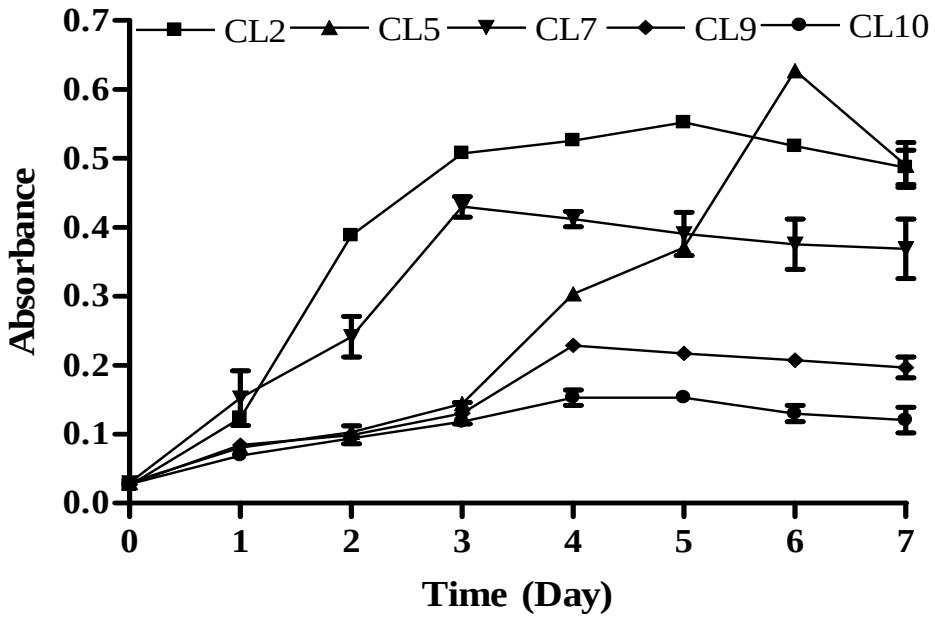


Figure 5.11: Growth curve of CL2, CL5, CL7, CL9 and CL10 in mineral salt medium containing 100 mg/l (0.50 mM) of 2, 4, 5-Trichlorophenol as sole carbon and energy source; error bars represent the standard deviation

5.4.2 Chloride release assay

Chloride release was assayed by these five isolates, which were able to degrade 2,4,5-TCP and released inorganic chloride ion into the MSM. Chloride ion concentration increased with increase of time. Initially isolate like CL2 released 0.28 mM of inorganic chloride on first day of incubation. As incubation period increased chloride ion concentration also increased upto 1.2 mM. Whereas CL5 and CL9 showed similar chloride ion release on the first day about 0.35 mM and reached upto 1.09 mM. CL9 and CL10 also followed the same pattern. On first day chloride ion release was 0.35 mM, during course of incubation chloride ion concentration increased to 1.12 mm. Chloride release into MSM was linked with the utilization of 2,4,5-TCP as carbon and energy source by the bacterial isolates (Table 5.13 and Fig. 5.12).

Table 5.13: Chloride ion release in MSM supplemented with 100 mg/l (0.50 mM) of 2,4,5-TCP by bacterial isolates

Time (Day)	CL2	CL5	CL7	CL9	CL10
1	0.28±0.2cC	0.36±0.3cBC	0.51±0.3cA	0.39±0.1dABC	0.47±0.1dAB
2	0.71±0.7bAB	0.59±0.4bcB	0.85±0.1bA	0.63±0.2cAB	0.86±0.2cA
3	0.89±0.5abA	0.89±0.4bcA	1.0±0.1abA	0.85±0.1bA	0.97±0.1bA
4	0.95±0.3abA	1.04±0.6aAB	1.18±0.4aA	1.07±0.1aAB	0.97±0.1bAB
5	1.20±0.4aA	1.07±0.6aA	1.20±0.5aA	1.09±0.3aA	1.12±0.1aA
6	1.20±0.4aA	1.07±0.8aA	1.18±0.3aA	1.09±0.3aA	1.12±0.1aA
7	1.18±0.4aA	1.09±7aA	1.12±2aA	1.07±1aA	1.07±1aA

Values sharing a common lowercase letter within column and uppercase letter within the row are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3).

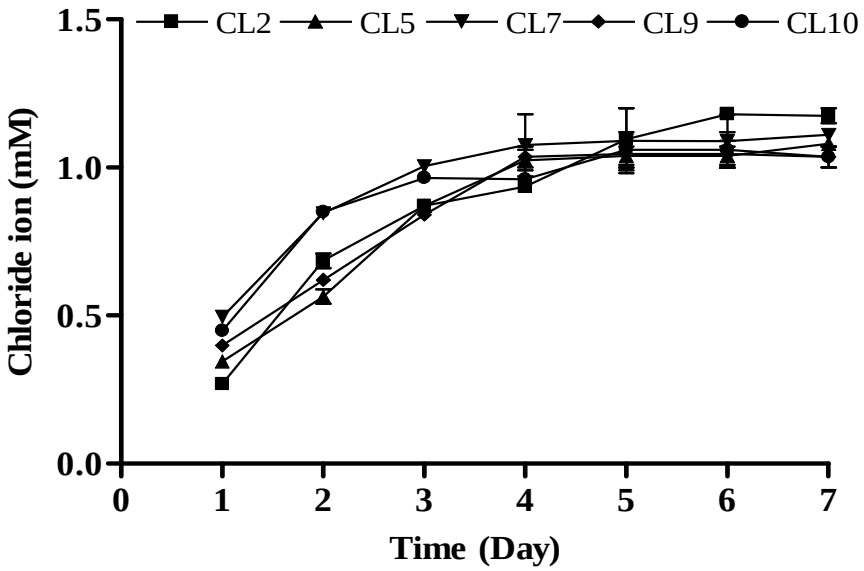


Figure 5.12: Release of chloride ion in the mineral salt medium containing 100 mg/l (0.5 mM) 2,4,5-TCP by CL2, CL5, CL7, CL9 and CL10 at different time interval during incubation error bars represents the standard deviation

The initial chloride ions present in the medium (control) were normalized to zero

5.5 2,4,6-TCP degradation

5.5.1 Growth studies

The bacterial isolates; CL4, CL5, CL7 CL9 and CL11 were able to utilize 2,4,6-TCP in MSM supplemented with 100 mg/l (0.50 mM) as source of carbon and energy. The growth gradually increased in the medium with increase in time but at fifth day the growth was stable for all the isolates. The maximum growth was obtained for CL4 followed by CL5 when compared to other isolates. In case of CL7, CL9 and CL11, the growth significantly increased upto fourth day and decreased there after (Table 5.17 and Fig. 5.16).

Table 5.17: Growth of bacterial strains in mineral salt medium (MSM) supplemented with 100 mg/l (0.50 mM) of 2,4,6-TCP as sole carbon and energy source

Time (Day)	CL4	CL5	CL7	CL9	CL11
0	0.027±0.06cA	0.026±0.04dA	0.027±0.06cA	0.030±0.02dA	0.030±0.0dA
1	0.099±0.01cA	0.095±0.05dAB	0.070±0.01bcBC	0.090±0.04cdABC	0.066±0.06cC
2	0.439±0.06bA	0.114±0.02dB	0.093±0.06abcB	0.107±0.02cB	0.096±0.01bcB
3	0.499±0.04abA	0.150±0.05cdB	0.120±0.04abB	0.139±0.03bcB	0.128±0.05bB
4	0.528±0.01abA	0.314±0.01bcB	0.152±0.01aD	0.234±0.01aC	0.240±0.02aC
5	0.587±0.02aA	0.445±0.11abA	0.158±0.01aB	0.221±0.01aB	0.223±0.01aB
6	0.532±0.03abA	0.610±0.01aA	0.145±0.03aB	0.211±0.01abB	0.207±0.07aB
7	0.483±0.03abA	0.525±0.05aA	0.133±0.03abC	0.200±0.02abB	0.204±0.01aB

Values sharing a common lowercase letter within column and uppercase letter within the row are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

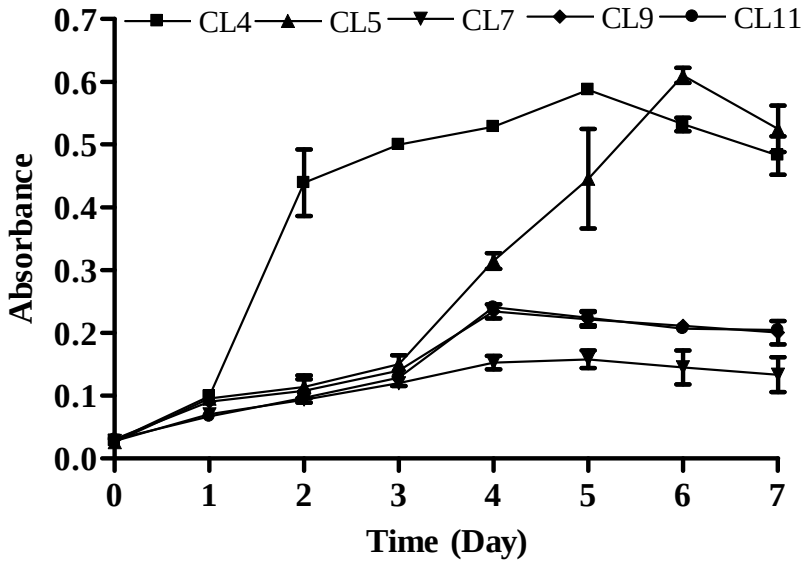


Figure 5.16: Growth curve of CL4, CL5, CL7, CL9 and CL11 in mineral salt medium containing 100 mg/l (0.50 mM) of 2, 4, 6-TCP as sole carbon and energy source; error bars represent the standard deviation

5.5.2 Chloride release assay

Concentration of chloride ion increased in medium during the course of incubation. Initially CL4 released 0.47 mM of chloride. CL5 released slightly less amount of chloride (0.33 mM) but during the course of incubation significantly increased to 1.15 mM. CL7 released 0.47 mM initially and 1.15 mM on fifth day of incubation. CL9 and CL11 released inorganic chloride ion from 0.56 mM to 1.16 mM. Initially CL11 released maximum chloride ion (0.60 mM). Overall all these isolates were able to release up to1.15 mM of chloride ion (Table 5.18. and Fig. 5.17).

Table 5.18: Chloride ion released in MSM supplemented with 100 mg/l (0.50 mM) of 2,4,6-TCP by the bacterial isolates during course of incubation

Time (Days)	CL4	CL5	CL7	CL9	CL11
1	0.47±0.1cB	0.33±0.2dC	0.47±0.1dB	0.56±0.2cAB	0.62±0.1cA
2	0.65±0.4cB	0.69±0.1cAB	0.85±0.1cA	0.72±0.4bAB	0.79±0.1cAB
3	0.85±0.1cBC	0.94±0.3bAB	1.00±0.1bA	0.86±0.2bBC	0.83±0.1bC
4	1.09±0.1bAB	1.03±0.4abAB	1.15±0.2aA	1.00±0.3aAB	0.97±0.1aB
5	1.12±0.6aA	1.03±0.1aA	1.09±0.3abA	1.16±0.3aA	1.00±0.1aA
6	1.09±0.4aA	1.15±0.1aA	1.09±0.1abA	1.16±0.1aA	1.10±0.3aA
7	1.09±0.1aA	1.07±0.2aA	1.09±0.1abA	1.12±0.1aA	1.12±0.3aA

Values sharing a common lowercase letter within column and uppercase letter within the row are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3).

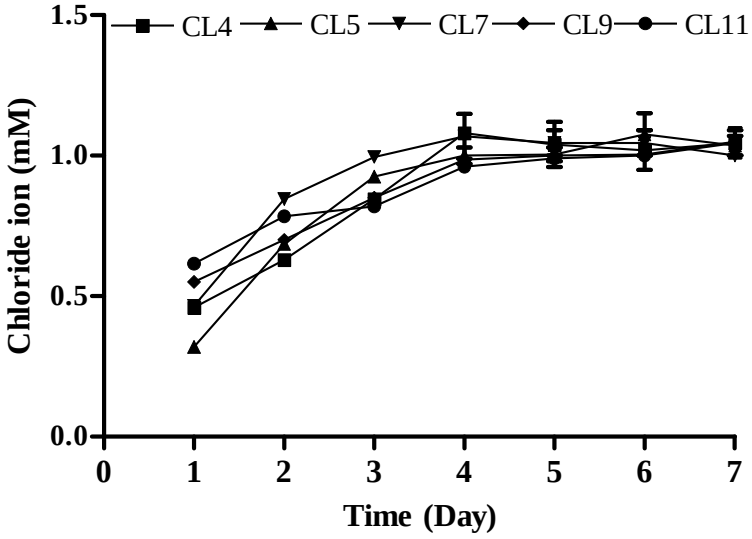


Figure 5.17: Release of chloride ion in the mineral salt medium containing 100 mg/l (0.50 mM) of 2, 4, 6-TCP by CL4, CL5, CL7, CL9 and CL11 at different time interval during incubation, error bars represents the standard deviation. The initial chloride ion concentration present in the medium (control) was normalized to zero

5.5.3 Effect of pH on 2,4,6-TCP degradation

The effect of pH on 2,4,6-TCP degradation was tested for CL4, CL5, CL7, CL9, and CL11. These isolates effectively removed 2,4,6-TCP at pH 7.5; with increase in pH the degradation efficiency decreased for all the isolates. For CL4, degradation decreased form 93 to 88%. For CL5, degradation drastically decreased to 65%. Degradation decreased for CL7 from 90 to 70%. For CL9 and CL11, degradation decreased to 85%. The growth of these isolates was also affected with increase and decrease in pH. Maximum growth was observed at pH 7.5 (Table 5.19 and Figure 5.18).

Table 5.19: Effect of pH on the growth and degradation of 2,4,6-TCP by the bacterial isolates

	Degradation (%)			Absorbance (600 nm)		
Isolate	pH 7.5	pH 8.5	pH 9.5	pH 7.5	pH 8.5	pH 9.5
CL4	93±1aA	90±4aA	88±4aB	0.223±0.02bA	0.218±0.07bA	0.165±0.01bB
CL5	97±1aA	67±3bB	65±4bB	0.424±0.01aA	0.409±0.03aB	0.308±0.01aC
CL7	89±10bA	72±4bB	69±2bB	0.107±0.02dA	0.102±0.02dA	0.083±0.01cB
CL9	94±4aA	87±6aB	86±4aB	0.143±0.02cA	0.140±0.01cA	0.112±0.01bcB
CL11	91±2abA	85±5aA	88±1aA	0.153±0.02cA	0.127±0.07cB	0.099±0.01cC

Values sharing a common lowercase letter within column and uppercase letter within the row are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3).

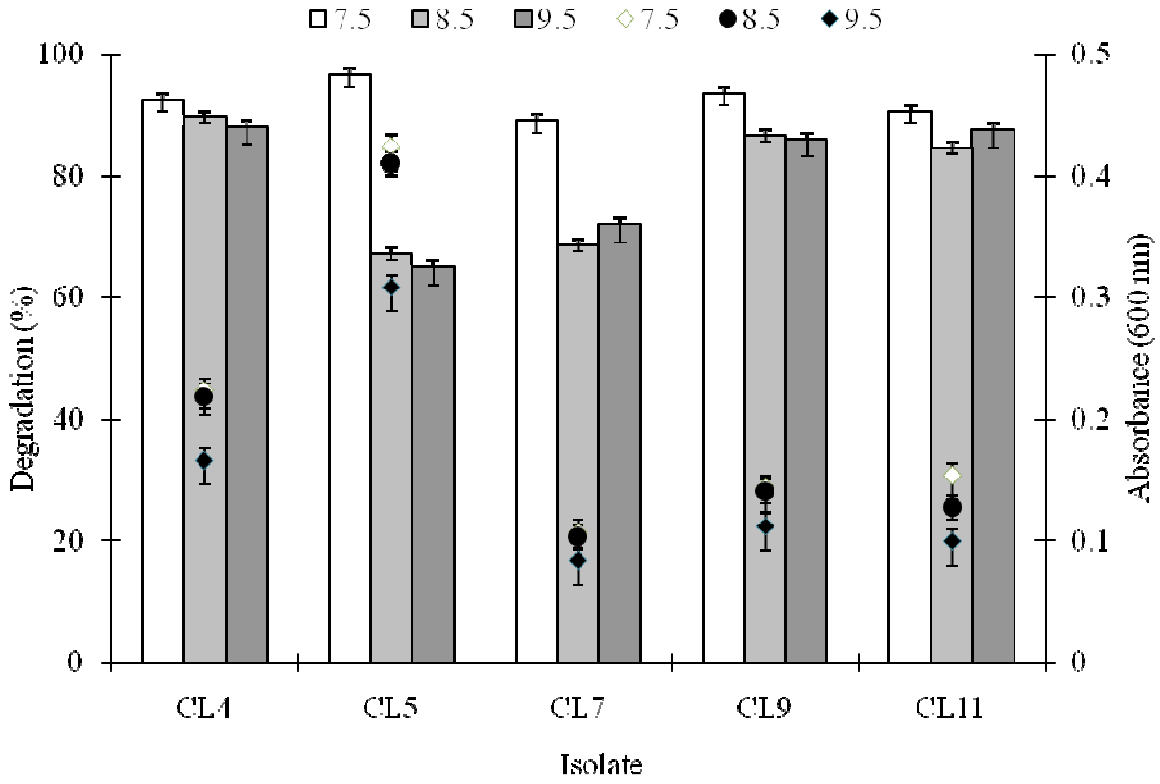


Figure 5.18: Effect of pH (7.5, 8.5 and 9.5) on the growth and degradation of 2,4,6-TCP by CL4, CL5, CL7, CL9 and CL11; error bars represent the standard deviation

5.5.4 Effect of temperature on 2,4,6-TCP degradation

The effect of three different temperature condition on the degradation of 2,4,6-TCP was studied. The removal efficiency of 2,4,6-TCP was lower at 25°C for all the five isolates. Whereas degradation was high at 37°C and 30°C, degradation was lower at 30°C (about 75%). Simultaneously the growth of these isolates was also affected at 25°C. But with increase in temperature from 30 to 37°C growth gradually increased. Except CL9 and CL11 isolates showed same growth pattern at 30 and 37°C (Table 5.20 and Figure 5.19).

Table 5.20: Effect of temperature on the growth and degradation of 2,4,6-TCP by the bacterial isolates

	Degradation (%)			Absorbance (600 nm)		
Isolates	25°C	30°C	37°C	25°C	30°C	37°C
CL4	85±4aB	92±1aA	93±1aA	0.163±0.03bC	0.209±0.01bB	0.223±0.02bA
CL5	67±2bB	74±2bA	93±1aA	0.365±0.01aB	0.415±0.01aA	0.424±0.01aA
CL7	74±1bB	89±10aA	94±2aA	0.071±0.01dB	0.106±0.01dA	0.107±0.02dA
CL9	86±4aB	91±1aA	92±0aA	0.108±0.04cC	0.132±0.07cB	0.143±0.02cA
CL11	87±1aB	94±3aA	93±1aA	0.087±0.07dC	0.120±0.02cB	0.153±0.02cA

Values sharing a common lowercase letter within column and uppercase letter within the row are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

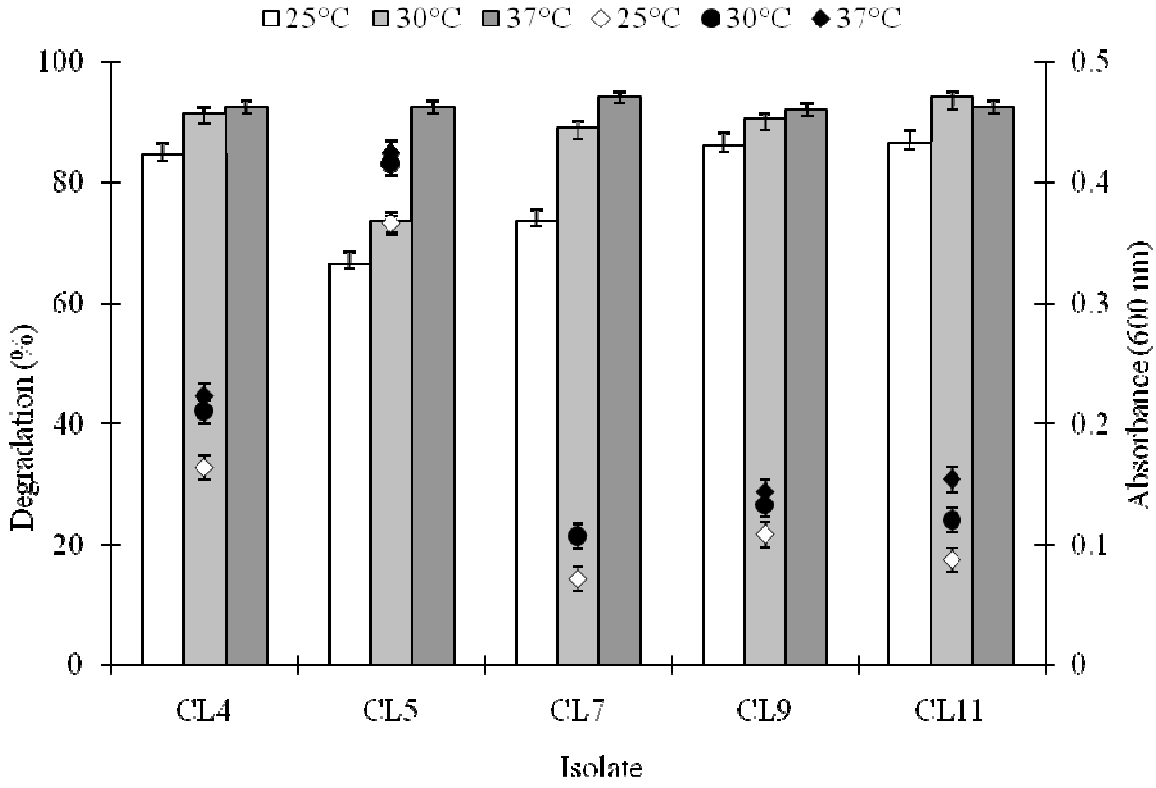


Figure 5.19: Effect of temperature (25, 30 and 37°C) on the growth and degradation of 2,4,6-TCP by CL4, CL5, CL7, CL9 and CL11 isolates; error bars represent the standard deviation

5.5.5 Degradation studies

HPLC analysis revealed that CL4, CL5, CL9, CL11 isolates showed high potential to utilize 2,4,6-TCP and remove 95% at all the concentrations tested in this study. Whereas slight decrease in degradation was noticed at higher concentration upto 90%, CL7 showed slightly less removal capacity up to 86% at concentration of 200 mg/l. At 400 and 600 mg/l concentration degradation was observed to be 80% (Table 5.21a and Fig. 5.20a&b).

All the isolates were able to grow and utilize 2,4,6-TCP as carbon energy source. The maximum growth was noticed for CL4 followed by CL5 as compared to CL7, CL9 and CL11 isolates. Increase in the concentration of 2,4,6-TCP decreased the growth of these isolates. CL7, CL9 and CL11 showed similar growth pattern (Table 5.21b and Fig. 5.20).

Table 5.21:

a) Degradation of 2,4,6-TCP at different concentration by bacterial isolates

Concentration (mg/l)	CL4	CL5	CL7	CL9	CL11
50	94±2aA	95±1aA	86±1aB	94±1aA	93±1aAB
100	94±1aA	94±1abA	86±1abB	94±1aA	92±1aA
200	94±2aA	93±1abA	82±1abA	94±1aA	92±1aA
400	94±2aA	93±1abA	82±1abB	92±1aA	92±1aA
600	92±1aA	92±1bA	82±1bB	91±2aA	92±1aA

Values sharing a common lowercase letter within column and uppercase letter within the row are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

b.) Growth (absorbance at 600 nm) of bacterial isolates in different concentration of 2,4,6-TCP

Concentration (mg/l)	CL4	CL5	CL7	CL9	CL11
50	0.453±0.02aB	0.486±0.02aA	0.105±0.01aE	0.183±0.02aD	0.196±0.04aC
100	0.223±0.02bB	0.424±0.01bA	0.107±0.02aE	0.143±0.02bD	0.153±0.02bC
200	0.125±0.01cB	0.301±0.05cA	0.100±0.02aC	0.133±0.02bB	0.121±0.01cB
400	0.113±0.01dB	0.235±0.01dA	0.103±0.02aB	0.090±0.01cB	0.107±0.02dB
600	0.106±0.01dB	0.236±0.01dB	0.106±0.01aB	0.103±0.02cB	0.106±0.01dB

Values sharing a common lowercase letter within column and uppercase letter within the row are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

Salient findings

CL4, CL5, CL7, CL9 and CL11 isolates were screened for the degradation of 2,4,6-TCP as sole carbon and energy sources. All these five isolates were able to remove 2,4,6-TCP by more than 90% at 600 mg/l except CL7 which removed upto 85%. The most favorable pH for the removal of 2,4,6-TCP was 7.5 by these isolates. There was a decrease in degradation about 10 to 15% at pH 8.5 and 9.5 as compared to pH 7.5. All these isolates removed 2,4,6-TCP maximum at 37°C.

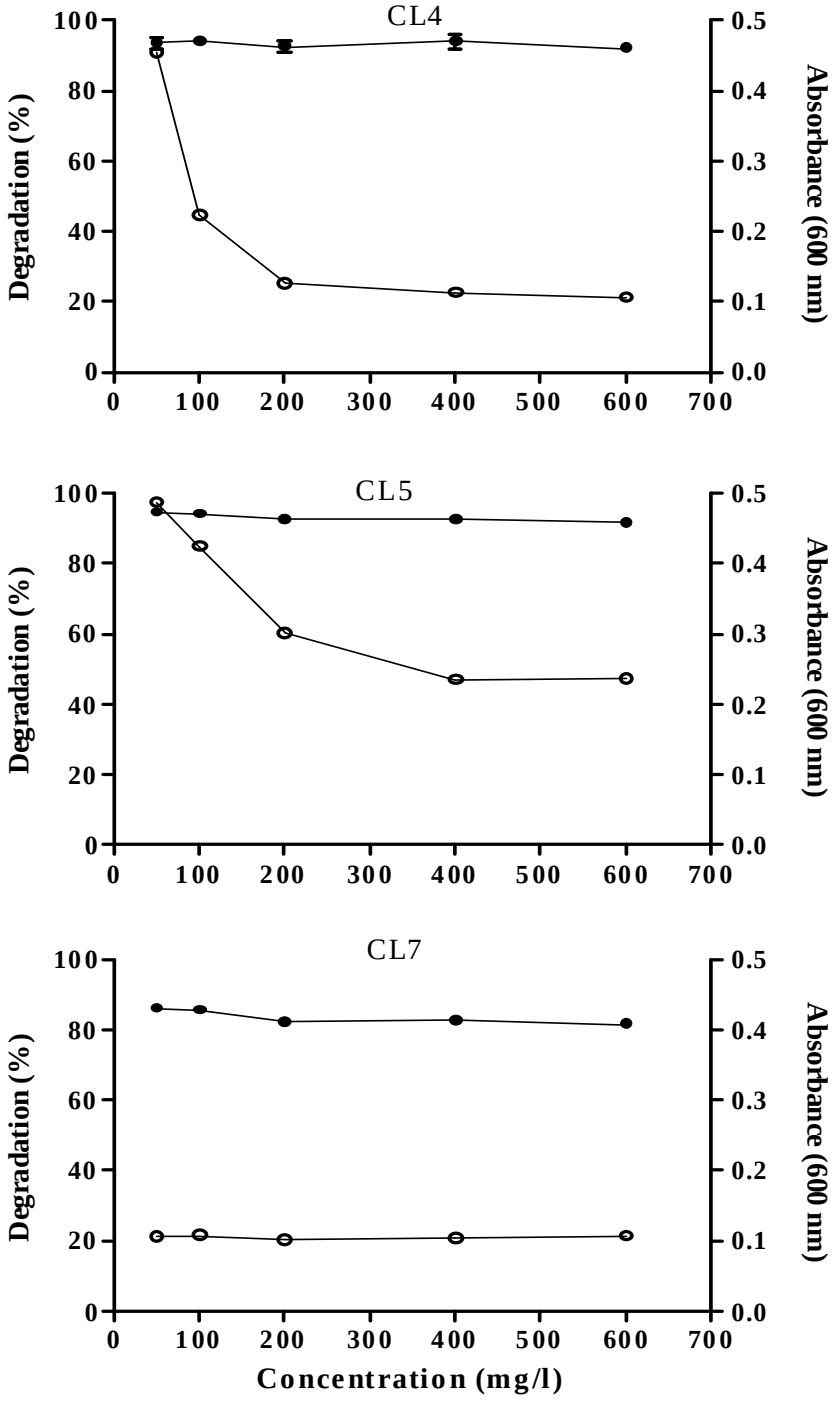


Figure 5.20a: Degradation of 2,4,6-TCP at different concentration by CL4, CL5 and CL7 isolates. Open circle represents the absorbance and filled circle degradation. Error bars represent the standard deviation

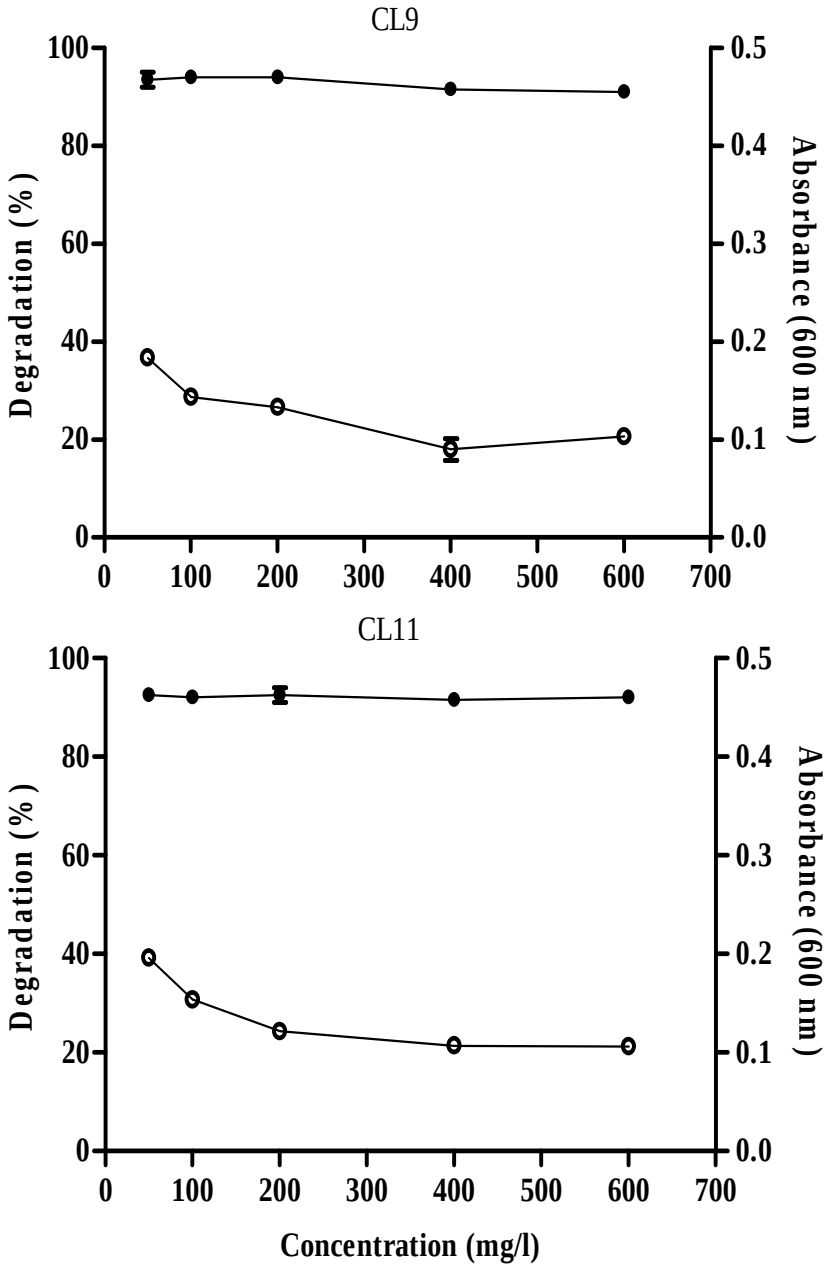


Figure 5.20b: Degradation of 2,4,6-TCP at different concentration by CL9 and CL11 isolates. Open circle represents the absorbance and filled circle degradation. Error bars represent the standard deviation

5.6 Growth of bacterial isolates in MSM containing sodium glutamate

To check the growth pattern of all these isolates into the medium other than chlorophenol as a sole carbon and energy source, MSM supplemented with sodium glutamate was used. This is easily available carbon source into the medium. These isolates showed quite high growth in the glutamate-containing medium as compared to chlorophenol (3 to 5 chlorine content) as a sole source of carbon and energy. The maximum growth in terms of absorbance in chlorophenol containing medium was 1.0, whereas in glutamate it was 2.5 (maximum) and 0.6 (minimum) for CL10, which is very high due to easily available carbon source.

CL1, CL2, CL6 and CL11 showed similar growth pattern in this medium. Growth gradually increased and absorbance reached maximum up to 2.0 at fifth and sixth day of incubation. CL3 showed maximum growth on third day of incubation. Whereas CL4 and CL5 showed moderate growth, maximum absorbance reached in fifth and sixth day which was upto 1.0. CL7 and CL8 have also showed good growth upto sixth day and maximum absorbance was 1.5 and 2.0 respectively. CL9 and CL12 have similar growth pattern, maximum growth was achieved within third and fourth day of incubation and maximum absorbance was 1.0. CL10 showed comparatively less growth into this medium; which was fast upto three days and maximum absorbance was upto 0.6 in third day. After that it remained stable (Table 5.22 and Fig. 5.21a&b).

Table 5.22: Absorbance at 600 nm of bacterial strain in mineral salt medium (MSM) supplemented with 4 g/l of sodium glutamate as sole carbon and energy source

Time (Day)	CL1	CL2	CL3	CL4	CL5	CL6	CL7	CL8	CL9	CL10	CL11	CL12
0	0.027±0.01eA	0.029±0.03dA	0.025±0.04eA	0.027±0.02cA	0.028±0.03cA	0.029±0.02dA	0.028±0.07dA	0.027±0.02fA	0.026±0.01dA	0.024±0.03cA	0.025±0.02dA	0.025±0.07dA
1	0.128±0.05eC	0.049±0.03cA	0.471±0.04dA	0.118±0.08bcC	0.126±0.02cC	0.398±0.01cAB	0.510±0.01cA	0.346±0.06eAB	0.159±0.08dC	0.235±0.04bBC	0.162±0.04dC	0.507±0.13cA
2	0.276±0.01deFG	1.382±0.22bA	0.818±0.04bBCD	0.184±0.03bcG	0.704±0.07bCDE	0.448±0.02cEFG	1.066±0.05bAB	0.920±0.01dBC	0.510±0.04cDEF	0.633±0.01aCDE	0.876±0.05cBC	0.898±0.11bBC
3	0.648±0.08cdE	1.824±0.12abA	1.123±0.03aD	0.473±0.02bE	1.157±0.06aCD	1.202±0.01bCD	1.281±0.05abCD	1.578±0.09cB	1.251±0.05abCD	0.647±0.07aE	1.366±0.04bBC	1.311±0.01aCD
4	0.842±0.04cE	1.935±0.16aA	0.841±0.02bE	0.454±0.05bF	1.132±0.11aD	1.629±0.04aBC	1.647±0.02aB	1.700±0.11bcAB	1.251±0.06abD	0.660±0.08aEF	1.456±0.12abBC	1.377±0.03aCD
5	1.316±0.05bDE	1.988±0.18aAB	0.719±0.01cFG	0.870±0.08aFG	1.067±0.10aEF	1.718±0.07aABC	1.527±0.19aCD	2.066±0.08aA	1.278±0.07aDE	0.664±0.06aG	1.623±0.01aBCD	1.367±0.06aCDE
6	2.043±0.01aA	2.049±0.03aA	0.678±0.01cE	1.222±0.01aC	0.947±0.06abD	1.627±0.09aB	1.421±0.14abBC	1.937±0.10abA	1.121±0.01abC	0.668±0.03aE	1.507±0.07abB	1.367±0.06aBC
7	1.647±0.31abAB	1.934±0.04aA	0.649±0.01cE	1.046±0.07aCDE	0.945±0.07abDE	1.506±0.17aABC	1.322±0.12bBCD	1.835±0.10abcA	1.003±0.15bCDE	0.610±0.01aE	1.490±0.05abABC	1.250±0.03aBCD

Values sharing a common lower case letter within column and upper case letter within the row are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3).

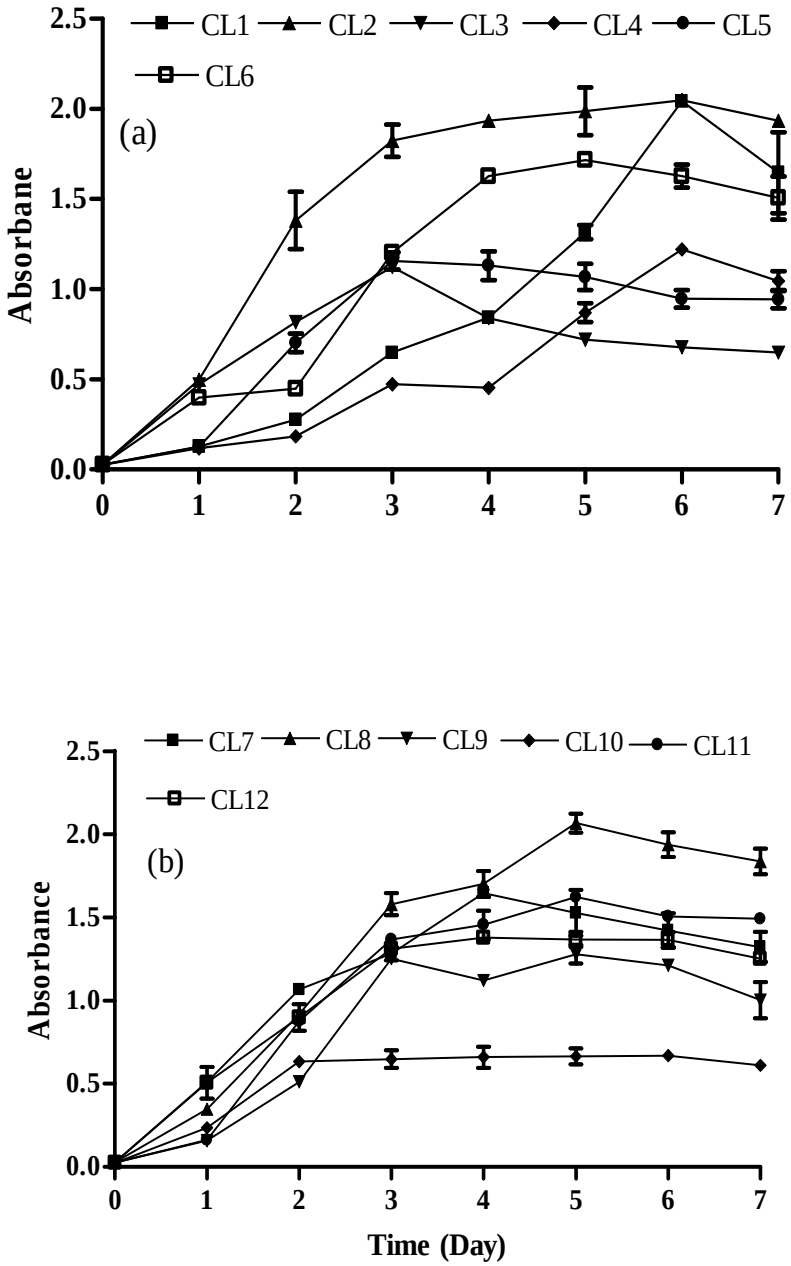


Figure 5.21a&b: Growth profile of CL1 to CL12 in mineral salt media containing 4 g/l of sodium glutamate as carbon and energy source; error bars represent the standard deviation

CHAPTER-6

Chapter-6

6. *In situ* remediation

6.1 *In situ* remediation

The remediation of chlorophenols in the secondary sludge was assessed by adding the mixed inocolum or consortium into the sludge. Initially PCP, 2,3,4,6 TeCP, 2,4,5 and 2,4,6 TCP were extracted from the sludge and analyzed. Concentration of PCP, 2,3,4,6 TeCP, 2,4,5 and 2,4,6 TCP was found to be 0.029, 0.038, 0.012 and 0.283 mg/l respectively. Therefore 100 mg/l of each chlorophenol was spiked to the sludge separately.

Removal of PCP in the sludge was tested by inoculating the five isolates (CL2, CL3, CL5, CL7 and CL11) together into the sludge. An HPLC chromatogram of control and experimental samples shown in Fig 6.1 revealed that the consortium was able to remove 100% of PCP from the sludge within a two weeks period.

To observe removal of 2,3,4,6-TeCP from sludge all twelve isolates (CL1 to CL12) inoculated into the sludge in the form of consortia. HPLC analysis clearly showed that consortium effectively removed 100% of 2,3,4,6-TeCP from sludge (Fig. 6.2)

For 2,4,5-TCP removal a consortium of CL2, CL5, CL7, CL9 and CL10 was added to the sludge. All these five isolates were able to completely remove 2,4,5-TCP from the sludge (Fig. 6.3). 2,4,6-TCP was removed by the consortium (CL4, CL5, CL7, CL9 and CL11) from the sludge. This consortium removed completely 2,4,6-TCP from the sludge (Fig. 6.4).

The survival of the bacterial isolates was monitored by determining the growth in the form of colony forming units (CFU). The growth of the bacterial strains increased with time both in sludge supplemented with PCP and other chlorophenols and sludge alone.

The growth of consortia was higher in sludge alone compared to sludge amended with PCP, 2,3,4,6-TeCP, 2,4,5-TCP and 2,4,6-TCP (Table 6.1) due to HPLC analysis revealed that all four consortia are capable of mineralizing chlorophenol from the sludge. It is able to remove up to 100% chlorophenol from the sludge. These results suggested that the isolated bacterial consortia have potential to remove PCP, 2,3,4,6-TeCP, 2,4,5-TCP, 2,4,6-TeCP in the sludge.

Table 6.1: Growth of bacterial isolates in terms of colony forming unit (CFU) in the sludge alone and sludge supplemented with chlorophenol after two weeks.

Chlorophenol	No. of cells (after two weeks) sludge alone	No. of cells (after two weeks) supplemented with chlorophenol
PCP	9.9×10^6	9.78×10^6
2,3,4,6-TeCP	7×10^6	7.0×10^6
2,4,5-TCP	9.5×10^6	9.2×10^6
2,4,6-TCP	9.5×10^6	9.0×10^6

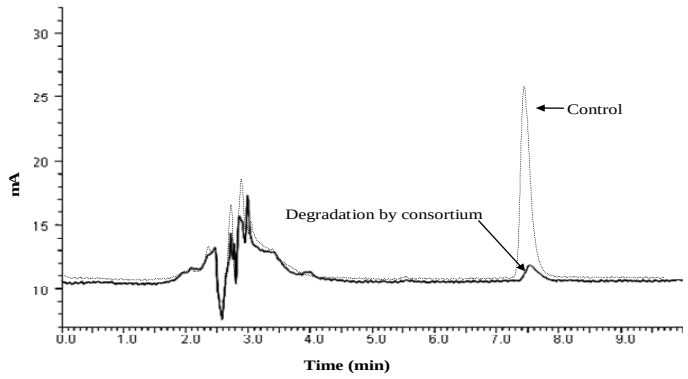


Figure 6.1: HPLC chromatogram of PCP degradation by consortium in sludge compared with control after two week incubation period; PCP was separated at retention time of 7.65 minutes.

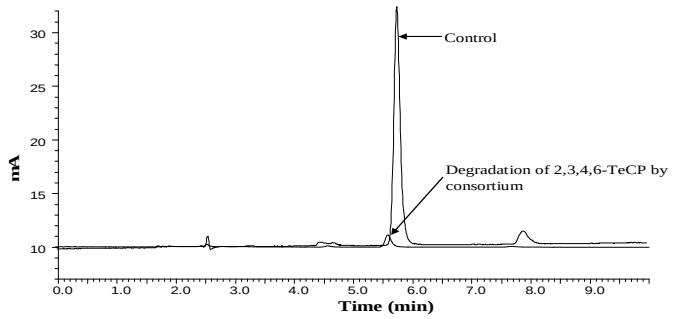


Figure 6.2: HPLC chromatogram of 2,3,4,6-TeCP degradation by consortium in sludge compared with control after two weeks incubation period; 2,3,4,6-TeCP was separated at retention time of 5.85 minutes

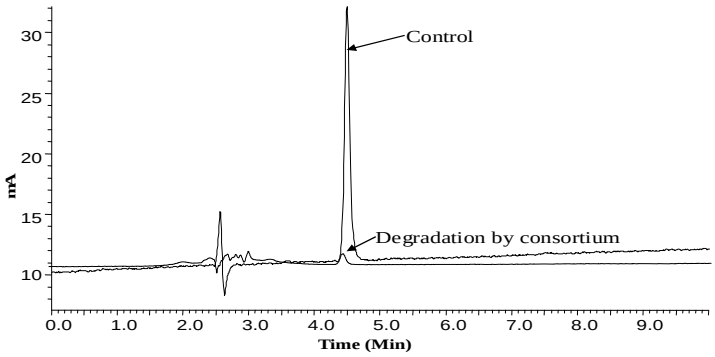


Figure 6.3: HPLC chromatogram of 2,4,5-trichlorophenol degradation by consortium in sludge compared with control after two weeks incubation period; 2,4,5-trichlorophenol was separated at retention time of 4.15 minutes.

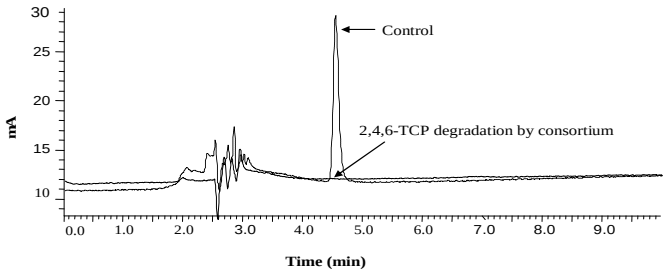


Figure 6.4: HPLC chromatogram of 2,4,6-trichlorophenol degradation by consortium in sludge compared with control after two weeks incubation period; 2,4,6-trichlorophenol was separated at retention time of 4.65 minutes.

6.2 Physiochemical characterization of secondary sludge

Secondary sludge sample were characterized before and after treatment with the consortia of bacterial isolates of PCP, 2,3,4,6-TeCP, 2,4,5-TCP, 2,4,6-TCP separately. Results showed that the sludge enriched with selected strains to degrade PCP can reduce AOX and EOX by 47% and 48% respectively in addition to complete elimination of PCP (Table 6.2). Other parameters remained almost same. These results indicate the efficiency of the microbial consortia for the bioremediation purpose at pilot scale.

For 2,3,4,6-TeCP all the twelve isolates were used for the *in situ* remediation of secondary sludge. This consortia was able to remove 2,3,4,6-TeCP from the sludge completely within two week of incubation period. Apart from 2,3,4,6-TeCP, consortia were able to remove of AOX by 67% and EOX by 70% from the sludge. So the consortia may be helpful for the remediation purposes. There was no notable change in the other parameters in the sludge samples (Table 6.2).

In case of 2,4,5-TCP the consortia removed chlorophenol completely from the sludge and th consortia removed AOX and EOX by 61% and 63% from the sludge respectively.

For 2,4,6-TCP consortia removed completely the chlorophenol from the sludge. It also removed AOX and EOX by 63% and 70% respectively from the sludge.

Table 6.2: Characterization of secondary sludge before and after treatment by consortia of PCP, TeCP, 2,4,5-TCP, 2,4,6-TCP degrading isolates

Parameter	Secondary sludge (as such)	Secondary sludge (with PCP)	Secondary sludge (with TeCP)	Secondary sludge (with 2,4,5-TCP)	Secondary sludge (with 2,4,6-TCP)
pH	7.5±0.14	6.9±0.35	7.0±0.42	7.1±0.64	7.2±0.49
Carbon (%)	29±1.2	26±1.4	23±2.3	27±1.3	23±1.4
Hydrogen (%)	2.9±0.3	2±0.2	2±0.0	2±0.1	1.7±0.2
Oxygen (%)	39±2.2	35±1.4	33±1.4	32±2.1	31±0.8
Nitrogen (%)	5±0.3	4±0.6	4±0.9	5±0.2	3±2.5
Sulphar (%)	4±0.4	3±0.5	3±0.9	3±0.4	3±0.4
Phosphorus (mg/l)	1±0.0	1±0.0	1±0.2	1±0.0	1±0.0
Carbohydrate (g/l)	73±1.9	77±1.4	76±1.4	74±1.4	73±1.4
Proteins (mg/l)	2.7±0.7	3±0.6	3±0.2	3±0.2	3±0.1
Zeta potential (mv)	-3.1±0.5	-2.5±0.2	-3±0.7	-2.7±0.1	-2.4±0.1
AOX (mg/l)	2400±27	1278±78	802±70	929±38	891±21
EOX (mg/l)	884±51	464±83	265±9	323±26	267±15

Values are mean ± SD (n =3)

Salient findings

All twelve bacterial isolates were efficient for the degradation of chlorophenol in shake flask level as well as at pilot scale. Mixed culture of efficient isolates for each chlorophenol were able to remove 100% of chlorophenol from the secondary sludge. Apart from all four chlorophenols PCP, 2,3,4,6-TeCP, 2,4,5-TCP, 2,4,6-TCP these isolates removed AOX and EOX in significant amount potentially from the sludge without any addition of extra carbon and energy source.

CHAPTER-6

Chapter-6

6. *In situ* remediation

6.1 *In situ* remediation

The remediation of chlorophenols in the secondary sludge was assessed by adding the mixed inocolum or consortium into the sludge. Initially PCP, 2,3,4,6 TeCP, 2,4,5 and 2,4,6 TCP were extracted from the sludge and analyzed. Concentration of PCP, 2,3,4,6 TeCP, 2,4,5 and 2,4,6 TCP was found to be 0.029, 0.038, 0.012 and 0.283 mg/l respectively. Therefore 100 mg/l of each chlorophenol was spiked to the sludge separately.

Removal of PCP in the sludge was tested by inoculating the five isolates (CL2, CL3, CL5, CL7 and CL11) together into the sludge. An HPLC chromatogram of control and experimental samples shown in Fig 6.1 revealed that the consortium was able to remove 100% of PCP from the sludge within a two weeks period.

To observe removal of 2,3,4,6-TeCP from sludge all twelve isolates (CL1 to CL12) inoculated into the sludge in the form of consortia. HPLC analysis clearly showed that consortium effectively removed 100% of 2,3,4,6-TeCP from sludge (Fig. 6.2)

For 2,4,5-TCP removal a consortium of CL2, CL5, CL7, CL9 and CL10 was added to the sludge. All these five isolates were able to completely remove 2,4,5-TCP from the sludge (Fig. 6.3). 2,4,6-TCP was removed by the consortium (CL4, CL5, CL7, CL9 and CL11) from the sludge. This consortium removed completely 2,4,6-TCP from the sludge (Fig. 6.4).

The survival of the bacterial isolates was monitored by determining the growth in the form of colony forming units (CFU). The growth of the bacterial strains increased with time both in sludge supplemented with PCP and other chlorophenols and sludge alone.

The growth of consortia was higher in sludge alone compared to sludge amended with PCP, 2,3,4,6-TeCP, 2,4,5-TCP and 2,4,6-TCP (Table 6.1) due to HPLC analysis revealed that all four consortia are capable of mineralizing chlorophenol from the sludge. It is able to remove up to 100% chlorophenol from the sludge. These results suggested that the isolated bacterial consortia have potential to remove PCP, 2,3,4,6-TeCP, 2,4,5-TCP, 2,4,6-TeCP in the sludge.

Table 6.1: Growth of bacterial isolates in terms of colony forming unit (CFU) in the sludge alone and sludge supplemented with chlorophenol after two weeks.

Chlorophenol	No. of cells (after two weeks) sludge alone	No. of cells (after two weeks) supplemented with chlorophenol
PCP	9.9×10^6	9.78×10^6
2,3,4,6-TeCP	7×10^6	7.0×10^6
2,4,5-TCP	9.5×10^6	9.2×10^6
2,4,6-TCP	9.5×10^6	9.0×10^6

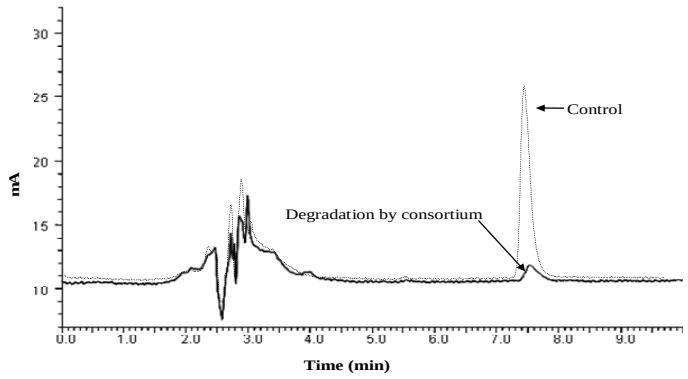


Figure 6.1: HPLC chromatogram of PCP degradation by consortium in sludge compared with control after two week incubation period; PCP was separated at retention time of 7.65 minutes.

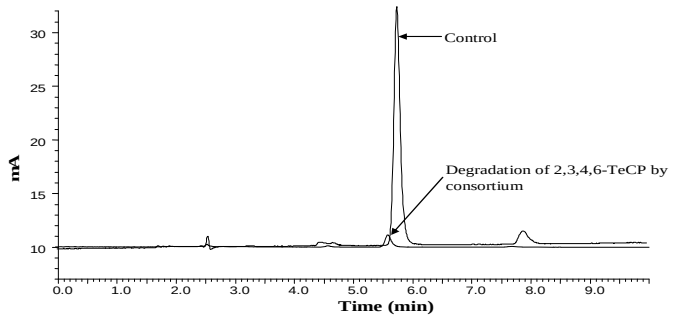


Figure 6.2: HPLC chromatogram of 2,3,4,6-TeCP degradation by consortium in sludge compared with control after two weeks incubation period; 2,3,4,6-TeCP was separated at retention time of 5.85 minutes

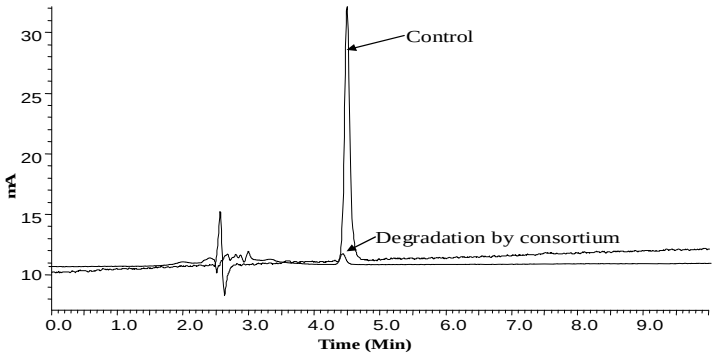


Figure 6.3: HPLC chromatogram of 2,4,5-trichlorophenol degradation by consortium in sludge compared with control after two weeks incubation period; 2,4,5-trichlorophenol was separated at retention time of 4.15 minutes.

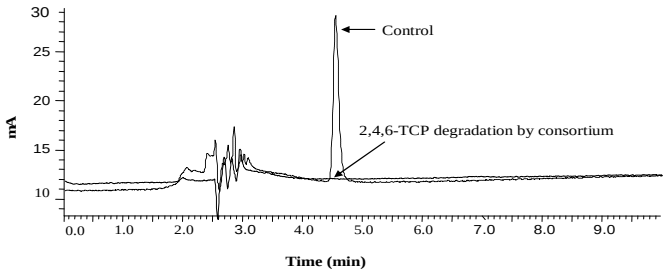


Figure 6.4: HPLC chromatogram of 2,4,6-trichlorophenol degradation by consortium in sludge compared with control after two weeks incubation period; 2,4,6-trichlorophenol was separated at retention time of 4.65 minutes.

6.2 Physiochemical characterization of secondary sludge

Secondary sludge sample were characterized before and after treatment with the consortia of bacterial isolates of PCP, 2,3,4,6-TeCP, 2,4,5-TCP, 2,4,6-TCP separately. Results showed that the sludge enriched with selected strains to degrade PCP can reduce AOX and EOX by 47% and 48% respectively in addition to complete elimination of PCP (Table 6.2). Other parameters remained almost same. These results indicate the efficiency of the microbial consortia for the bioremediation purpose at pilot scale.

For 2,3,4,6-TeCP all the twelve isolates were used for the *in situ* remediation of secondary sludge. This consortia was able to remove 2,3,4,6-TeCP from the sludge completely within two week of incubation period. Apart from 2,3,4,6-TeCP, consortia were able to remove of AOX by 67% and EOX by 70% from the sludge. So the consortia may be helpful for the remediation purposes. There was no notable change in the other parameters in the sludge samples (Table 6.2).

In case of 2,4,5-TCP the consortia removed chlorophenol completely from the sludge and th consortia removed AOX and EOX by 61% and 63% from the sludge respectively.

For 2,4,6-TCP consortia removed completely the chlorophenol from the sludge. It also removed AOX and EOX by 63% and 70% respectively from the sludge.

Table 6.2: Characterization of secondary sludge before and after treatment by consortia of PCP, TeCP, 2,4,5-TCP, 2,4,6-TCP degrading isolates

Parameter	Secondary sludge (as such)	Secondary sludge (with PCP)	Secondary sludge (with TeCP)	Secondary sludge (with 2,4,5-TCP)	Secondary sludge (with 2,4,6-TCP)
pH	7.5±0.14	6.9±0.35	7.0±0.42	7.1±0.64	7.2±0.49
Carbon (%)	29±1.2	26±1.4	23±2.3	27±1.3	23±1.4
Hydrogen (%)	2.9±0.3	2±0.2	2±0.0	2±0.1	1.7±0.2
Oxygen (%)	39±2.2	35±1.4	33±1.4	32±2.1	31±0.8
Nitrogen (%)	5±0.3	4±0.6	4±0.9	5±0.2	3±2.5
Sulphar (%)	4±0.4	3±0.5	3±0.9	3±0.4	3±0.4
Phosphorus (mg/l)	1±0.0	1±0.0	1±0.2	1±0.0	1±0.0
Carbohydrate (g/l)	73±1.9	77±1.4	76±1.4	74±1.4	73±1.4
Proteins (mg/l)	2.7±0.7	3±0.6	3±0.2	3±0.2	3±0.1
Zeta potential (mv)	-3.1±0.5	-2.5±0.2	-3±0.7	-2.7±0.1	-2.4±0.1
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Salient findings

All twelve bacterial isolates were efficient for the degradation of chlorophenol in shake flask level as well as at pilot scale. Mixed culture of efficient isolates for each chlorophenol were able to remove 100% of chlorophenol from the secondary sludge. Apart from all four chlorophenols PCP, 2,3,4,6-TeCP, 2,4,5-TCP, 2,4,6-TCP these isolates removed AOX and EOX in significant amount potentially from the sludge without any addition of extra carbon and energy source.

CHAPTER-7

Chapter-7

7. Discussion

7.1 Isolation and characterization of chlorophenol degrading bacteria from the secondary sludge

Microbial diversity offers an immense field of environment friendly options for mineralization of contaminants or their transformation into less harmful non-hazardous compounds. There is a general interest in studying the diversity of indigenous microorganisms capable of degrading different pollutants because of their varied effects on the environment. Efforts have been made to characterize bacterial communities and their responses to pollutants, to isolate potential degraders and to identify the genes involved in degradation processes (Watanabe 2001).

Present study results showed widespread chlorophenol degradation among bacteria from secondary sludge. A total of 32 bacterial isolates were isolated from secondary sludge of a typical pulp and paper industry, 12 isolates were screened for the degradation of chlorophenols. From which five isolates degraded PCP, five degraded 2,4,5-TCP and 2,4,6-TCP and all twelve were able to degrade 2,3,4,6-TeCP. Morphological, biochemical and partial 16S rRNA gene sequences of the isolates revealed that the polychlorophenol-degrading bacteria present in secondary sludge formed a diverse group. Phylogenetic analysis placed the isolates in three major lineages of the bacterial domain that is Femicutes, Proteobacteria and Actinobacteria. The Fermicutes consist of phylum Bacillus, Proteobacteria consist phylum Pseudomonas and Actinobacteria consist of phylum, Actinomycetes. Present study showed that many phylogenetic branches have the ability to degrade polychlorophenols up to 600 mg/l. The Fermicutes consisted of nine

isolates. Five of them were close phylogenetic relatives of the genera *Bacillus* sp. (CL1, CL3, CL5, CL11, and CL12). Earlier Chandra et al. (2008) reported the most potent and novel PCP degrading microorganisms designated as *Bacillus cereus* ITRC S6 and *Serratia marcescens* ITRC S9. The bacterial consortia *Bacillus subtilis*, *Bacillus lentimorbus*, *Bacillus mycoides* and *Bacillus subbilus* effectively degraded 2-chlorophenol, 3-chlorophenol and 2, 4-dichlorophenol at degradation rates of between 1.7 and 6.7 μmole /l/hour (Herrera et al, 2008). Present study showed that all these isolates *Bacillus* sp. (CL1), *Bacillus megaterium* (CL3), *Bacillus pumilus* (CL5), *Bacillus thuringiensis* (CL11), and CL12 (*Bacillus* sp.) effectively removed chlorophenols. It also indicates that *Planococcus* sp. (CL4), a fermicute is likely to represent a new chlorophenol-degrading bacterium based on 16S rRNA gene sequences.

Two actinobacteria, *Kocuria polaris* (CL2) and *Exiguobacterium* sp. (CL6) were also not reported earlier for any chlorophenol degradation. Therefore, these isolates represent new chlorophenol-degrading taxa. The degradation ability of this group display different characteristics than reported earlier.

Earlier the gram-positive isolates that degraded 2,4,6-TCP, 2,3,4,6-TeCP and PCP were nocardioform and actinomycetes. *Nocardioides simplex* strain 3E has been reported to degrade 2,4,5- trichlorophenoxyacetic acid via 2,4,5-TCP (Golovleva et al, 1992). Results indicated the presence of a chlorophenol-degrading cluster within the Proteobacteria isolates CL7 grouped most closely with *Pseudomonas stutzeri.* Chlorophenol degradation by these species has not been reported earlier. From previous studies this group includes several genera that are known to contain xenobiotic-degrading species (Busse et al, 1992).

7.2 Degradation of chlorophenols (PCP, 2,3,4,6-TeCP, 2,4,5-TCP, 2,4,6-TCP)

A large variety of bacteria are known which can utilize chlorophenols as a carbon and energy source under aerobic conditions. The earliest reports of bacterial utilization of chlorophenols include those of Chu and Kirsch (1972) and Tyler and Finn (1974). Chu and Kirsch (1972) described a bacterial strain (KC 3) capable of mineralizing [14C]-PCP to $^{14}CO2$ when supplied as a sole carbon source. PCP mineralization was shown to be linked to cell growth. Basically two main strategies are used to degrade chlorophenols by aerobic bacteria utilizing these compounds as a carbon and energy source (Solyanikova and Golovleva 2004). Lower chlorinated phenols (1 to 2 chlorine substitution) are initially attacked by monooxygenases yielding chlorocatechols as the first intermediate (chlorocatechol pathway), which are subject to ring cleavage prior to dechlorination. On the other hand, polychlorinated phenols (3 to 5 chlorines) are converted to chlorohydroquinones as the initial intermediates (hydroquinone pathway). Subsequent reactions progressively remove chlorines from the ring prior to ring cleavage.

Several unique pathways of chlorophenol degradation by aerobic bacteria have come to light. 4-CP degradation by *Arthrobacter ureafaciens* strain CPR706 and *Arthrobacter chlorophenolicus* strain A6 is initiated by a dechlorination, yielding 1, 4-hydroquinone as the intermediate (Bae et al, 1997; Nordin et al, 2005). The proposed pathway involves the conversion of 4-CP to hydroquinone to hydroxyquinol to maleylacetate (Nordin et al, 2005). A ring cleaving hydroxyquinol dioxygenase is a key enzyme in the pathway. When a gene coding for the dioxygenase is disrupted, *A. chlorophenolicus* has negligible growth on 4-CP (Nordin et al, 2005). The monooxygenase from *Ralstonia eutropha* strain JMP134 (pJP4) catalyzes successive dechlorination reactions of 2,4,6-TCP to 2,6-

dichlorohydroquinone and then to 6-chlorohydroxyquinol prior to ring cleavage by a hydroxyquinol dioxygenase (Matus et al, 2003). A unique property of the monooxygenase is that this dechlorination reaction does not require O_2, instead the dechlorination of 2,6-dichlorohydroquinone is catalyzed by a hydrolytic activity associated with the monooxygenase (Xun and Webster 2004). Several aerobic bacteria implicated in the rapid degradation of higher chlorinated phenols are known to convert chlorophenols to methylated biotransformation products. *Mycobacterium* strains were shown to ortho-methylate a variety of chlorophenols (Haggblom et al, 1988b). The preferred substrates of the methylation were hydroxyl groups doubly flanked by chlorine groups. Chlorinated hydroquinones were readily ortho-methylated (Haggblom et al, 1988a), accounting for the occurrence of methylated chlorohydroquinone intermediates during PCP biodegradation (Suzuki 1983). Direct methylation of PCP resulted in the formation of pentachloroanisole in soil (D'Angelo and Reddy 2000).

Lee et al. (1998) reported that *Pseudomonas* sp. (Bu34) was able to grow up to 4000 mg/l of PCP. Radehaus and Schimdt (1992) reported complete mineralization of PCP (160 mg/l) in a week by *Pseudomonas* sp. strain RA2. Shah and Thakur (2002) observed 72% PCP removal by *Pseudomonas fluorescence* (TE3*)* in 96 hours when grown with 100 mg/l PCP. Sharma and Thakur (2008) observed that *Pseudomonas aeruginosa* (PCP2) utilized 60% of PCP within 96 hours of incubation. Premlatha and Rajkumar (1994) recorded the complete degradation of PCP (800 mg/l) by *Pseudomonas aeruginosa* in 6 days with glucose as a co-substrate. Singh et al. (2007) reported that the bacterial isolate *Serratia marcescens* isolated from pulp and paper mill waste was able to degrade 90.3% of PCP within 168 hours when grown with 300 mg/l of PCP. Chandra et al. (2006)

recorded 62.7% PCP degradation by *Bacillus cereus* when grown with 300 mg/l of PCP. Yang et al. (2006) showed that *Sphingomonas chlorophenolica* could not degrade PCP when the concentration was above 600 mg/l. Current isolates (CL2, CL3, CL5, CL7 and CL11) were able to utilize PCP as sole carbon source and degrade more than 90% of PCP up to 600 mg/l of PCP in the culture media. Chloride ions released by these isolates while degrading PCP were assayed. The results clearly showed that the chloride ion concentration increased significantly with increase of PCP degradation. Other studies have also shown the release of chloride ions while PCP is degraded in the medium (Kennes et al. 1996). Wolski et al. (2006) reported the degradability of PCP at pH values from 6.3 to 8.0, with maximum rate of PCP degradation at pH 6.3 by *Pseudomonas* species. *Sphingomonas chlrophenolia* removed 90% of PCP when the initial pH was 9.2 and could not remove the PCP when the initial pH value was below 6.0 (Yang et al. 2006). Present isolates were able to remove the PCP up to 90% when the initial pH was 7.5 but at pH 8.5 to 9.5 the degradation efficiency of two isolates CL2 and CL5 was not affected whereas degradation efficiency of other isolates CL3, CL7 and CL11 decreased with increase in pH upto 82%. Crawford and Mohn (1985) observed the degradation of PCP between 24 and 35^{o}C by *Flavobacterium* species. Present isolates were able to degrade PCP up to 90% at a temperature of 37^{o}C.

Degradation of 2,3,4,6-TeCP by the *Sphingomonas* isolates K74 and MT1 and the *Nocardioides* sp. K44 was associated with 73–79% inorganic chloride release. This together with the absence of methylation products gave evidence of mineralization. Highest affinity of 2,3,4,6-TeCP mineralization was observed for the *Nocardioides* sp.K44. This strain was also sensitive to 2,3,4,6-TeCP showing partial inhibition for

TeCP above 3 mg/l of 2,3,4,6-TeCP. The groundwater *Sphingomonas* sp. K74 showed the highest specific degradation rate of 2,3,4,6-TeCP. Study of the long-term contamination of the aquifer has resulted in a diverse community of 2,3,4,6-TeCP degrading bacteria as indicated by analysis of 17 isolates. (Männistö et al.1999, 2001). Present research shows that all the twelve isolates were able to remove 2,3,4,6-TeCP. CL1, CL11 and CL12 were able to remove 2,3,4,6-TeCP up to 200 mg/l but at concentration 400 and 600 mg/l, degradation was decreased to 95% and 87% respectively. CL2, CL3, CL4 and CL5 were able to remove 90% of 2,3,4,6-TeCP at 200 mg/l but at 400 and 600 mg/l concentration the degradation decreased upto 83%. These isolates have the degradation potential at all the concentrations tested in this study. CL6, CL9 and CL10 were able to remove 90% of 2,3,4,6-TeCP at 600 mg/l, whereas, two isolates CL7 and CL8 have relatively less degradation capacity upto 85% at 200 mg/l but at 400 and 600 mg/l the degradation efficiency decreased upto 73%. Release of chloride ion and HPLC analysis on chlorophenol concentration proved the degradation of 2,3,4,6-TeCP.

CL1, CL5 and CL11 were able to remove 76, 83 and 84% of 2,3,4,6-TeCP respectively at pH 7.5. Removal efficiency of these isolates increased at pH 8.5 upto 90, 92 and 91% respectively. At pH 9.5 removal efficiency again decreased to 86% for CL1 and CL5 and 74% for CL11. For CL7 degradation efficiency was maximum at pH 7.5 upto 69%. Other isolates were able to remove 90% and above of 2,3,4,6-TeCP at pH 7.5 to 8.5. There is a decrease in the efficiency of degradation at higher pH of 9.5.

Very little work has been reported on 2,4,5-TCP biodegradation relative to other chlorophenols. It seems that TCP is remarkably more resistant to biodegradation than

other trichlorophenols (Golovleva et al. 1990). There are a few reports on bacteria that degrade 2,4,5-TCP. *Pseudomonas cepacea* AC1100 (Kilbane et al, 1982) and *Nocardioides simplex* 3E (Golovleva et al, 1990) were able to grow on 2,4,5-TCP. The present study reported some new species for 2,4,5-TCP degradation which includes *Kocuria polaris* (CL2), *Bacilllus pumillus* (CL5), *Exiguobacterium* sp. (CL9), *Pseudomonas stutzeri* (CL7) and *Bacillus thuringensis* (CL11) have been reported for the degradation of 2,4,5-TCP. These isolates were effective for the mineralization of 2,4,5-TCP by forming biomass and release of chloride ion into the medium. This would suggest that 2,4,5-TCP was immediately dechlorinated as soon as primary degradation or uptake had occurred without significant accumulation of chlorinated intermediates. It was observed that CL2, CL5, and CL9 had higher potency to utilize 2,4,5-TCP and removed up to 90% at 600 mg/l. Whereas CL7 and CL10 are able to utilize 90% at 400 mg/l, the degradation potential decreased to 82% at 600 mg/l at seven days of incubation period for both the two cases.

The growth of CL5 and CL7 was significantly higher at all three pH (7.5, 8.5 and 9.5) compared to CL2, CL9 and CL10. There was slight decrease in the growth when initial pH of the medium was 8.5 and 9.5. All these isolates removed approximately 90% of 2,4,5-TCP at pH 7.5. 2,4,5-TCP removal efficiency significantly decreased when the initial pH of the medium was kept at 8.5. CL5, CL7 and CL10 were able to remove 81%, whereas CL9 removed 86%. Only for CL2 removal efficiency was not affected at pH 8.5, whereas at pH 9.5 the removal efficiency decreased for all isolates up to 75%. In case of temperature, removal efficiency was less at 25 and 30^{o}C. The growth of all the isolates increased as the temperature increased. The maximum growth was obtained at 37^{o}C. The

2,4,5-TCP degradation efficiency also increased with increase in the temperature. The optimum temperature for the removal of 2,4,5-TCP was observed at 37^{o}C in this study. Earlier Maltseva and Oriel (1997) isolated an alkaliphilic bacterial strain, *Nocardiodes* sp. strain M6, that could degrade up to 100 mg/l of 2,4,5-TCP. Golovleva et al. (1990) found that a *Nocardiodes simplex* strain 3E, isolated from soils contaminated with 2,4,5-T, could aerobically degrade 2,4,5-TCP at concentration up to 40 μM, but was toxic at a concentration of 80 μM. Similar results by Madsen and Aamand (1992) showed that 2,4,5-TCP could be dechlorinated at concentration less than or equal to 40 μM. Utkin et al. (1995) also showed that *Desulfitobacterium dehalogenans* could readily dehalogenate 2,4,5-TCP.

The biological degradation of 2,4,6-TCP by heterotrophic microorganisms is a useful strategy to eliminate the compound and thus detoxify polluted environments. Earlier reports have shown that 2,4,6-TCP can be degraded in aerobic conditions by mixed cultures (Puhakka et al. 1995; Maltseva and Oriel 1997) or pure cultures (Apajalahti and Salkinoja-Salonen 1986; Li et al. 1991; Kiyohara et al. 1992; Clément et al. 1995; Tomasi et al. 1995; Bae et al. 1997) isolated from a variety of sources using conventional or immobilized-cell systems (Puhakka and Järvinen 1992; Langwaldt et al. 1998). Present study dealt with the selection of an aerobic microbial isolates that efficiently metabolizes 2,4,6-TCP as the sole source of carbon and energy.

Through the present study some new species such as *Planococcus* sp. (CL4) *Bacilllus pumillus* (CL5), *Pseudomonas stutzeri* (CL7), *Exiguobacterium* sp. (CL9) and *Bacillus thuringensis* (CL11) for the degradation of 2,4,6-TCP have been found. The isolates are quite comparable for the mineralization of 2,4,6-TCP by forming biomass and release of

chloride ion into the MSM. This would suggest that 2,4,6-TCP was immediately dechlorinated as primary degradation or uptake had occurred without significant accumulation of chlorinated intermediates. Similar results have been reported earlier for 2,4-dichlorophenol (DCP) and 2,4,6-TCP by Beltrame et al. (1982), Kharoune et al. (2002) and Aranda et al. (2003). HPLC analysis also confirmed the loss of 2,4,6-TCP from the medium. At pH 7.5 all these isolates removed more than 90% 2,4,6-TCP but at pH 8.5 and 9.5 removal efficiency for CL4, CL9 and CL11 was 85% and for CL5 and CL7 was up to 65%. Degradation was maximum at 37°C. These bacterial isolates from the sludge of pulp and paper mill have the ability to degrade the 2,4,6-TCP at higher concentration (upto 600 mg/l). Lora et al. (2000) found that 2,4,6-TCP was degradable at concentrations into the milimolar range. Utkin et al. (1995) showed that *Desulfitobacterium dehalogenans* JW/IU-DC1 could readily dehalogenate 2,3,4 - 2,3,6- and 2,4,6-TCP.

7.3 *In situ* remediation

For i*n situ* remediation to be successful, the role of appropriate microbes and the suitable environment are crucial for degradation to occur. The right microbes are bacteria or fungi, which have the physiological and metabolic capabilities to degrade the pollutants. Bioremediation offers several advantages over conventional techniques such as landfilling or incineration. Bioremediation can be done on site, is often less expensive and site disruption is minimal, it eliminates waste permanently, eliminates long-term liability, and has greater public acceptance, with regulatory encouragement, and it can be coupled with other physical or chemical treatment methods.

Microorganisms present in the microcosms form (mixed culture) can easily remove the toxic effect of both the parent and intermediate compounds. Mixed cultures with their multiple interactions are more prone to completely mineralize the chlorinated organics to CO_2, unlike pure cultures, which can only degrade the target compounds (Zeikus and Johnson 1991). Such studies would be more meaningful and practical. They have many advantages over pure cultures, such as increased overall growth rates, better utilization of substrates, potential for bringing about multistep transformations, reduced potential for phage infections, and ability to recover quickly when growth conditions are disturbed (Zahn and Braunbeck 1993).

Numerous studies have evaluated the biodegradability of chlorophenol in soil and compost to clean up the chlorinated phenols. The degradation was achieved by the addition of chlorphenol degrading microorganisms or enzyme to enhance the bioremediation process of contaminated site. Lamar et al. (1993) observed that *Pseudomonas sordida* removed 89% of PCP from a soil contaminated with 672 mg PCP/kg soil. According to Laine and Jorgensen (1996) PCP degrading enrichment culture degraded 99% of PCP in a sandy loam soil contaminated with 450 mg PCP/kg in 130 days. PCP-remediated soil enriched with PCP-degrading microorganisms (10^2 cells/g of dry soil) rapidly mineralized 6 mg [14C]-PCP/ kg soil by 56% in 4 weeks. Addition of *Sphingomonas chlorophenolica* RA2 (10^8 cells/g dry soil) greatly accelerated the mineralization of 30 mg [14C]-PCP per kg added to soil (Miethling and Karlson 1996). The PCP was mineralized by 80% within one month whereas non-inoculated controls required 7 months. Over 90% of the chlorophenols were removed from municipal solid

waste and soil during the, composting period of 8 weeks with highly contaminated soils of 850 mg PCP/kg soil (Laine and Jorgensen 1997).

Murialdo et al, 2003 isolated a *Pseudomonas* sp. strain from a consortium that degrades PCP. It was shown that glucose and glutamate have positive effects on its population density. This microorganism could be used very effectively for *in situ* bioremediation in an environment which is highly contaminated with PCP and other chlorinated phenols.

Though i*n situ* remediation of PCP was reported previously deals mainly with compost, manure and soil no reports are available with removal of PCP from the sludge.

In the present study, the consortium or microcosms has shown potential to mineralize 100 % PCP within two weeks when the concentration of PCP is more than 100 mg/l.

In situ remediation of 2,3,4,6-TeCP released into the environment may undergo transformation by natural chemical or biological processes. 2,3,4,6-TeCP is stable to hydrolysis and oxidation, but the compound can be metabolized by microorganisms. Treatments of 2,3,4,6-TeCP contaminated wastewater have been performed successfully in pilot-scale at room temperature, mixtures of PCP, 2,3,4,6-TeCP and 2,4,6-TCP were mineralized by 99.9% at loading rates of 1 kg chlorophenol/m^3 reactor/d when these compound were used as the sole source of carbon and energy (Jarvinen et al, 1994; Puhakka et al, 1995). The consortia of the bacterial isolates from the present study were able to degrade 2,3,4,6-TeCP completely from the sludge sample when the concentration was 100 mg/l. The recovery of inorganic chloride was in agreement with the degradation of the chlorophenols (Jarvinen et al, 1994).

Observation of Gonzalez et al (1996) on the study of the mineralization of 2,4,5-TCP in soil not previously exposed to chloroorganics revealed that soil mineralized up to 45% of

2,4,5-TCP after 60 days of incubation when it was exposed to 100 ppm of this compound. Minimal half-lives of 35-170 days were estimated from rates of $^{14}CO_2$ evolution in soil incubated with 1-100 ppm of 2,4,5-TCP. Soil exposed to 500 ppm or in incubations with sterile soil with 10 or 100 ppm of 2,4,5-TCP was mineralized by less than 2%.

Valeria et al. (1996) reported microorganisms that mineralize 2,4,5-TCP (100 mg/l of 2,4,5-TCP, 30 days of incubation) in plain soils. Aerobic degradation of 2,4,5-TCP is also observed in surface water samples by (Larsson and Lemkemeier 1989).

Many chlorinated phenols 2-, 3-, and 4-CP; 2,4-, 2,6- and 3,4-DCP; 2,4,6-, and 2,4,5-TCP; and PCP incubated under aerobic conditions with a Canadian clay loam grassland soil were biodegraded (Baker and Mayfield 1980). Four chlorinated phenolic compounds (2-CP, 2,4-DCP, 2,4,6-TCP and PCP) were degraded by two subsurface soil samples collected from two different states in the USA (Smith and Novak 1987). High concentrations of 2,4,6-TCP up to 5000 mg/kg were readily degraded by pristine forest soil which was not previously exposed to chlorophenols (Sanchez et al, 2004). Very few reports are available with removal of 2,4,5-TCP and 2,4,6-TCP from the sludge. The consortia of the bacterial isolates from the present study were able to degrade 2,4,6-TCP completely from the sludge sample when the concentration was 100 mg/l. It is believed that these isolates have high potential to remove the 2,4,6-TCP and can be applied to remediate 2,4,5-TCP and 2,4,6-TCP contaminated sites.

There are numerous examples in which soil, compost and manure have been utilized to degrade various chlorophenols under aerobic condition but only a few reports are available regarding the treatment of sludge system. In the present study, the each

consortium has shown potential to completely mineralize PCP, 2,3,4,6-TeCP, 2,4,5-TCP, 2,4,5-TCP (100 mg/l) within two weeks.

On the basis of the above results, it could be concluded that environmental contaminations may be viewed as an ecosystem malaise, while bioremediation can be regarded as a kind of environmental medicine. It is possible that the existing bacterial isolates can be applied for pilot and scale-up process for reduction of pollutant in pulp and paper mill effluent and contaminated site of chlorophenols.

These isolates also have ability to significantly reduce the amount of COD, AOX and EOX from the secondary sludge. An earlier study Knudsen et al. (1994) also provides evidences of reduction of pollutant by the bacterial isolates from the activated sludge process. Shere and Daly (1982) claimed that thermo-mechanical pulping (TMP) wastewater was readily degraded by the activated sludge process. Hansen et al. (1999) suggested upgrading the activated sludge plant by the addition of floating biological bed in series that increased COD and BOD removal from 51 to 90% and 70 to 93%, respectively. Chandra (2001) reported efficient removal of color, BOD, COD, phenolics, and sulfide by microorganisms such as *Pseudomonas putida, Citrobacter* sp., and *Enterobacter* sp. in the activated sludge process. Mohamed et al. (1989) reported removal of chlorinated phenols, 1,1-dichlorodimethyl sulfone (DDS), and chlorinated acetic acids in an oxygen activated sludge effluent treatment plant. Demirbas et al. (1999) reported AOX removal by the activated sludge process. Bryant et al. (1992) reported AOX removal of 46% on average from two activated sludge systems studied. High removals of BOD, COD, AOX, and chlorinated phenolics have been achieved in the activated sludge process (Saunamaki, 1997; Schnell et al., 2000). Kennedy et al. (2000) reported that the

activated sludge was successful in removing nearly all detectable toxicity from bleached kraft pulp mills at low level.

SUMMARY

Summary

Microbial diversity, the richness of species in environmental sites, provides a reservoir of resources which can be exploited for the environment. However, little is known about the true diversity of bacterial life. Despite the acknowledged value of microorganisms, the level of understanding of the diversity and the key roles in sustaining global life support systems is still very scarce. Exploring the range of microbial biodiversity is the key to developing effective and environment friendly 'green' technologies. Bioremediation is one such process that exploits the catabolic abilities of microorganisms to degrade harmful and toxic xenobiotics. To maximize the potential benefits of microbial community in combating pollution, it is vital to develop fundamental understanding or degradative potential of microbes under various conditions, its biochemical systems and molecular biology. Chlorophenolic compounds are mostly toxic and recalcitrant and hence their discharge into the environment must be regulated. Biodegradation of chlorophenolic compounds under aerobic as well anaerobic natural environment has been observed. Aerobic degradation takes place via formation of catechlol. The recalcitrance of chlorophenols increase with increase of number of chlorine substituent in phenol ring. Position of chlorine substitution also affects the biodegradadibility of such substances. Present study deals with the isolation and characterization of polychlorophenol degrading bacteria from the secondary sludge, and degradation studies of PCP, 2,3,4,6-TeCP, 2,4,5-TCP and 2,4,6-TCP at laboratory level in mineral salt medium and at pilot scale in secondary sludge itself.

Bacterial isolation was done by enrichment method in mineral salt medium supplemented with 50 mg/l of PCP, 2,3,4,6-TeCP, 2,4,5-TCP, 2,4,6-TCP as the sole carbon source for

energy. Five potential isolates CL2, CL3, CL5, CL7 and CL11 were selected for the degradation of PCP. All twelve isolates were capable to degrade 2,3,4,6-TeCP. CL2, CL5, CL7, CL9 and CL10 were selected for 2,4,5-TCP degradation and for 2,4,6-TCP five isolates CL2, CL5, CL7, CL9 and CL11 were screened. Further, these bacterial isolates were morphologically, biochemically, and molecularly (16S rDNA sequence analysis) characterized. Sequence data of the isolates was analyzed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree was constructed based on 16S rDNA sequences by neighbor-joining method using MEGA software (v 4.0). The majority of isolates (CL1, CL3, CL4, CL5, CL8, CL9, CL10, CL11 and CL12) are phylogenetically related to *Bacillus* sp. (CL1), *B. megaterium (CL3), Planococcus* sp. (CL4), *B. pumillus* (CL5), *Jeotgalicoccus* sp.(CL8), *Exiguobacterium* sp.(CL9), *Staphylococcus sciuri* (CL10), *B. thuringensis* (CL11), and *Bacillus* sp. (CL12) and showed homology to Firmicutes. In Proteobacterium only CL7 showed homology with the *Pseudomonas stutzeri.* Two isolates CL2 and CL6 were related with Actinobacteria and showed homology with *Kocuria polaris* (CL2) and *Pseudonocardia* sp .(CL6). The 16S rRNA gene sequences determined in this study was deposited in the GenBank of NCBI data library under accession numbers EU784648 to EU784659 for CL1 to CL12 respectively. Most of the identified bacteria isolated from sludge were new which have been not reported earlier for the degradation of any particular chlorophenol.

Five isolates (CL2, CL3, CL5, CL7 and CL11) have demonstrated the ability to grow on PCP amended media and degrade more than 90% PCP up to 600 mg/l. Biomass formation and the release of chloride ion were monitored and it was observed that significant increase in chloride ion concentration takes place with increase of incubation

time. The maximum degradation was achieved by these isolates at the pH range of 7.5 to 8.5 and temperature 37°C.

For 2,3,4,6-TeCP each of CL1 to CL12 was able to remove 2,3,4,6-TeCP up to 95% at a concentration of 600 mg/l. Two isolates CL7 and CL8 have relatively lower degradation capacity (about 75%). Simultaneous increase in chloride ion and disappearance of 2,3,4,6-TeCP from the medium analyzed by HPLC showed the degradation abilities of these isolates. Degradation was maximum at 7.5 pH. Increase in pH of the medium does not support the growth and degradation. At lower temperature 25 and 30°C the degradation was lower, maximum degradation was achieved at 37°C.

CL2, CL5 and CL9 were able to remove up to 95% of 2,4,5-TCP at all the concentrations in the present study; CL7 and CL10 have slightly less degradation capability. At higher concentration of 400 and 600 mg/l this was up to 82% of 2,4,5-TCP. pH 7.5 and temperature 37°C were the most favoured for the degradation of 2,4,5-TCP.

CL4, CL5, CL9 and CL11 removed up to 95% of 2,4,6-TCP, whereas CL7 degraded slightly lower which was up to 80%. Decrease in degradation at higher concentration by these isolates was observed; which are upto 90%. Release of chloride ion showed the dechlorination of the compound. Maximum degradation was achieved at pH 7.5. 30 and 37°C were suitable for the degradation by these isolates except CL5 which has lower degradation capability at 30°C.

In situ remediation of PCP, 2,3,4,6-TeCP, 2,4,5-TCP and 2,4,6-TCP by these isolates was proved very effective in microcosm form in separate batch in sludge spiked with 100 mg/l chlorophenols which were completely removed from the industrial sludge.

The most significant inference of this study is that degradation of four different chlorophenols (PCP, 2,3,4,6-TeCP, 2,4,5-TCP and 2,4,6-TCP) was possible with the efficient bacterial isolates. These isolates are also efficient to degrade the chlorophenol in the secondary sludge. These results indicated that the isolated bacterial isolates have higher potential to remove chlorophenolic compounds in the sludge and can be used for bioremediation of chlorophenolics contamination in pulp and paper mill waste or sites contaminated with polychlorophenols.

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APPENDIX

Appendix I

(A) Nutrient Agar

Ingredient	Quantity (g/l)
Agar	12.0
Peptic digest of animal tissue	5.0
Beef extract	1.50
Yeast extract	1.50
Sodium chloride	5.0

Boiled to dissolve the medium completely, sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min, pH 7.4 ± 0.2

(B) Mineral Salt Medium (MSM)

Ingredient	Quantity (mg/l)
KH_2PO_4	800
Na_2HPO_4	800
$MgSO_4.7H_2O$	200
$CaCl_2.2H_2O$	10
NH_4Cl	500

And 1 ml of trace metal solution which includes following ingredients

$FeSO_4.7H_2O$	5
$ZnSO_4.7H_2O$	4
$MnSO_4.4H_2O$	0.2
$NiCl_2.6H_2O$	0.1
H_3BO_3	0.15
$CoCl_2.6H_2O$	0.5
$ZnCl_2$	0.25
EDTA	2.5

pH adjusted to 7.0 with 5 N NaOH and HCl, sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min

(C) Luria-Bertani (LB) Medium

Ingredient	Quantity (g/l)
NaCl	10.0
Beef extract	5.0
Tryptone	10.0
Agar	10.0

pH adjusted to 7.0 with 5 N NaOH, sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min, added filtered ampicillin 50 µg/ml to prepare LB - Ampicillin plates

(D) Nitrate agar

Ingredient	Quantity (g/l)
Agar	12.0
Beef extract	3.0
Peptic digest of animal tissue	5.0
Potassium nitrate	1.0

Suspended 21 g of nitrate agar in 1000 ml distilled water, boiled to dissolve the medium completely, sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min

(E) Starch Agar

Ingredient	Quantity (g/l)
Peptic digest of animal tissue	5.0
Meat extract	3.0
Starch (soluble)	2.0
Agar	15.0

Final pH was adjusted to 7.2± 0.1

(F) DNase Test Agar

Ingredient	Quantity (g/l)
Casein enzyme hydrolysate	15.0
Papic digest of soyabean meal	5.0
Deoxyribonucleic acid (DNA)	2.0
Sodium chloride	5.0
Agar	15.0

Final pH was adjusted to 7.3± 0.2

(G) Modified Universal Buffer (5X)

Tris (hydroxyl methyl) amino methane	3.02g
Maleic acid	2.90 g
Citric acid	3.50 g
Boric acid	1.57 g
NaOH (1N)	122 ml
Water	up to 250 ml

Final pH was adjusted to 5.5± 0.2

(H) LB/amp+ agar plates

Prepared LB broth as above, added agar (15 g/l), autoclaved, and cooled to 50°C, added ampicillin 50 µg/ml, Poured plates and stored at 4°C

(I) IPTG stock solution (0.1M)

1.2 g IPTG
Added water to 50 ml final volume, filtered and stored at 4°C

(J) X-Gal (2ml)

100 mg 5-bromo, 4 chloro, 3-indolyl, D galactoside dissolved in 2ml N,N-dimethylformamide, covered with aluminum foil and stored at 20°C

(K) LB plates with Ampicillin/IPTG/X-Gal

Prepared LB plates with ampicillin as above; 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-Gal spread over the surface of LB ampicillin plate and allowed to absorb for 30 minutes at 37°C prior to use

(L) TBE Buffer (10x)

Tris-HCl	0.09 M (pH 8)
Boric acid	0.9 M
EDTA	0.02 M (pH 8)

(M) Molybdate-tartarate solution

i) Dissolved 12 g of ammonium molybdate in about 250 ml distilled water
ii) Dissolved 0.291 g of antimony potassium tartarate in 100 ml of distilled water
Added the two solutions (i) & (ii) to 1L of 5N H_2SO_4, mixed thoroughly and made the volume to 2L with distilled water

(N) Plasmid Extraction Solution I (10X)

Tris-HCl	25 mM (pH 8.0)
Glucose	50 mM
Na_2EDTA	10 mM

(O) Plasmid Extraction Solution II

NaOH	5M
SDS	10%

(P) Plasmid Extraction Solution III

5.0 M K-acetate (pH 4.5)

(Q) Agarose Gel Loading Dye (6X)

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol in water	30.0%

(R) Ligation reaction of amplicon in pTZ57R/T

Plasmid pTZ57R/T (50ng/µl)	3µl
Amplicon (75ng/µl)	4µl
Buffer (10X)	3µl
T4 Ligase	1µl
H_2O	19µl

(S) Ligation reaction of amplicon in pGEM-Teasy

Plasmid pGEM-Teasy (50 ng/µl)	1µl
Amplicon (75 ng/µl)	1µl
Buffer (2X)	5µl
T4 Ligase	1µl
H_2O	2µl

Primers

M13 forward primer	5''-GTAAAACGACGGCCAGT-3’
M13 reverse primer	5’-CAGGAAACAGCTATGAC-3’
T7 primer	5’-TAATACGACTCACTATAGGG-3’
SP6 primer	5’-ATTTAGGTGACACTATAG-3’
Rep forward primer	5’-IIIICGICGICATCIGGC -3’
Rep reverse primer	5’-ICGICTTATCIGGCCTAC - 3’
BoxA1R PCR	5’CTACGGCAAGGCGACGCTGACG-3’
ERIC PCR forward primer	5’-GGTGATGATGCAGTGCCTCC-3’
ERIC PCR reverse primer	5’-CTGCGCGCCTAATCAATAGC-3’
16S rDNA forward primer	5’-AGAGTTTGATCCTGGCTCAG-3’
16S rDNA reverse primer	5’-ACGGGCGGTGTGTTC-3’

Appendix II

Bacillus sp. CL1 16S ribosomal RNA gene, partial sequence

```
LOCUS       EU784649               1515 bp    DNA     linear   BCT
28-JUN-2008
DEFINITION  Bacillus sp. CL1 16S ribosomal RNA gene, partial
sequence.
ACCESSION   EU784649
VERSION     EU784649.1  GI:192383708
KEYWORDS    .
SOURCE      Bacillus sp. CL1
  ORGANISM  Bacillus sp. CL1
            Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1515)
  AUTHORS   Karn,S.K., Chakarbarti,S.K. and Reddy,M.S.
  TITLE     Diversity of chlorophenolics degdrading bacteria in
secondary
            sludge of pulp and paper industry
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1515)
  AUTHORS   Karn,S.K., Chakarbarti,S.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2008) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road,
Patiala,
            Punjab 147004, India
FEATURES             Location/Qualifiers
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                     /mol_type="genomic DNA"
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     rRNA            <1..>1515
                     /product="16S ribosomal RNA"
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 121 caacctgccc tgtagattgg gataactccg ggaaaccggg gctaataccg ataatccac
 181 cagaccacat ggtccggtgt tgaaaggcgg cttcggctgt cactacagga gggcccgcg
 241 gcgcattagc tagttggtgg ggtaacggcc caccaaggcg acgatgcgta ccgacctga
 301 gagggtgatc ggccacactg ggactgagac acggcccaga ctcctacggg ggcagcagt
 361 agggaatctt ccacaatgga cgaaagtctg atggagcaac gccgcgtgag gaagaaggt
 421 tctcggatcg taaaactctg ttgtggggga agaacaagta ccggagtaac gccggtacc
 481 ttgacggtac ctcattagaa agccacggct aactacgtgc cagcagccgc gtaatacgt
 541 aggtggcaag cgttgtccgg aatcattggg cgtaaagcgc gcgcaggcgg cccttaagt
 601 ctgatgtgaa agcccacggc tcaaccgtgg agggtcattg gaaactgggg acttgagtg
 661 cagaagagga aagcggaatt ccaagtgtag cggtgaaatg cgtagagatt ggaggaaca
 721 ccagtggcga aggcggcttt ctggtctgta actgacgctg aggcgcgaaa cgtggggag
 781 caaacaggat tagataccct ggtagtccac gccgtaaacg atgagtgcta gtgttaggg
 841 ggtttccgcc ccttagtgct gcagctaacg cattaagcac tccgcctggg agtacggtc
 901 gcaagactga aactcaaagg aattgacggg ggcccgcaca agcggtggag atgtggttt
 961 aattcgaagc aacgcgaaga accttaccag gtcttgacat cccgccgacc cctaggaga
1021 ctaggccttc ccttcgggga cggcggtgac aggtggtgca tggttgtcgt agctcgtgt
1081 cgtgagatgt tgggttaagt cccgcaacga gcgcaaccct tgatcttagt gccagcatt
1141 cagttgggca ctctaaggtg actgccggtg acaaaccgga ggaaggtggg atgacgtca
1201 aatcatcatg ccccttatga cctgggctac acacgtgcta caatggatgg acaaagggc
1261 tgcgaacccg cgaggggcag ccaatcccat aaaaccattc tcagttcgga tgtaggctg
1321 caactcgcct acatgaagct ggaatcgcta gtaatcgtgg atcagcatgc acggtgaat
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1501 cgtaacaagg taacc
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Kocuria sp. CL2 16S ribosomal RNA gene, partial sequence

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LOCUS       EU784648                743 bp    DNA     linear   BCT
28-JUN-2008
DEFINITION  Kocuria sp. CL2 16S ribosomal RNA gene, partial sequence.
ACCESSION   EU784648
VERSION     EU784648.1  GI:192383707
KEYWORDS    .
SOURCE      Kocuria sp. CL2
  ORGANISM  Kocuria sp. CL2
            Bacteria; Actinobacteria; Actinobacteridae;
Actinomycetales;
            Micrococcineae; Micrococcaceae; Kocuria.
REFERENCE   1  (bases 1 to 743)
  AUTHORS   Karn,S.K., Chakarbarti,S.K. and Reddy,M.S.
  TITLE     Diversity of chlorophenolics degdrading bacteria in
secondary
            sludge of pulp and paper industry
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 743)
  AUTHORS   Karn,S.K., Chakarbarti,S.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2008) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road,
Patiala,
            Punjab 147004, India
FEATURES             Location/Qualifiers
     source          1..743
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                     /mol_type="genomic DNA"
                     /strain="CL2"
                     /db_xref="taxon:543426"
     rRNA            <1..>743
                     /product="16S ribosomal RNA"
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 61 atgatgccca gcttgctggg cggattagtg gcgaacgggt gagtaatacg tgagtaacct
121 gcccttgact ctgggataag cctgggaaac tgggtctaat actggatact accgtccacc
181 gcatggtggg tggtggaaag ggttttactg gttttggatg ggctcacggc ctatcagctt
241 gttggtgggg taatggctca ccaaggcgac gacgggtagc cggcctgaga gggtgaccgg
301 ccacactggg actgagacac ggcccagact cctacgggag gcagcagtgg ggaatattgc
361 acaatgggcg gaagcctgat gcagcgacgc cgcgtgaggg atgacggcct tcgggttgta
421 aacctctttc agtagggaag aagcgagagt gacggtacct gcagaagaag cgccggctaa
481 ctacgtgcca gcagccgcgg taatacgtag ggcgcaagcg ttgtccggaa ttattgggcg
541 taaagagctc gtaggcggtt tgtcgcgtct gctgtgaaag cccggggctc aaccccgggt
601 ctgcagtggg tacgggcaga ctagagtgca gtaggggaga ctggaattcc tggtgtagcg
661 gtgaaatgcg cagatatcac gaggaacacc gatggcgaag gcaggtctct ggggctgtta
721 ctgacgctga ggagcgaaag cat
//
```

Bacillus sp. CL3 16S ribosomal RNA gene, partial sequence

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LOCUS       EU784650                 687 bp    DNA     linear   BCT
28-JUN-2008
DEFINITION  Bacillus sp. CL3 16S ribosomal RNA gene, partial
sequence.
ACCESSION   EU784650
VERSION     EU784650.1  GI:192383709
KEYWORDS    .
SOURCE      Bacillus sp. CL3
  ORGANISM  Bacillus sp. CL3
            Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 687)
  AUTHORS   Karn,S.K., Chakarbarti,S.K. and Reddy,M.S.
  TITLE     Diversity of chlorophenolics degdrading bacteria in
secondary
            sludge of pulp and paper industry
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 687)
  AUTHORS   Karn,S.K., Chakarbarti,S.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2008) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road,
Patiala,
            Punjab 147004, India
FEATURES             Location/Qualifiers
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                     /mol_type="genomic DNA"
                     /strain="CL3"
                     /db_xref="taxon:543422"
     rRNA            <1..>687
                     /product="16S ribosomal RNA"
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121 gtaacacgtg ggcaacctgc ctgtaagact gggataactt cgggaaaccg aagctaatac
181 cggataggat cttctccttc atgggagatg attgaaagat ggtttcggct atcacttaca
241 gatgggcccg cggtgcatta gctagttggt gaggtaacgg ctcaccaagg caacgatgca
301 tagccgacct gagagggtga tcggccacac tgggactgag acacggccca gactcctacg
361 ggaggcagca gtagggaatc ttccgcaatg gacgaaagtc tgacggagca acgccgcgtg
421 agtgatgaag gctttcgggt cgtaaaactc tgttgttagg gaagaacaag tacaagagta
481 actgcttgta ccttgacggt acctaaccag aaagccacgg ctaactacgt gccagcagcc
541 gcggtaatac gtaggtggca agcgttatcc ggaattattg ggcgtaaagc gcgcgcaggc
601 ggtttcttaa gtctgatgtg aaagcccacg gctcaaccgt ggagggtcat tggaaactgg
661 ggaacttgag tgcagaagag aaaagcg
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Planococcus sp. CL4 16S ribosomal RNA gene, partial sequence

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LOCUS       EU784651                621 bp    DNA     linear   BCT
28-JUN-2008
DEFINITION  Planococcus sp. CL4 16S ribosomal RNA gene, partial
sequence.
ACCESSION   EU784651
VERSION     EU784651.1  GI:192383710
KEYWORDS    .
SOURCE      Planococcus sp. CL4
  ORGANISM  Planococcus sp. CL4
            Bacteria; Firmicutes; Bacillales; Planococcaceae;
Planococcus.
REFERENCE   1  (bases 1 to 621)
  AUTHORS   Karn,S.K., Chakarbarti,S.K. and Reddy,M.S.
  TITLE     Diversity of chlorophenolics degdrading bacteria in
secondary
            sludge of pulp and paper industry
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 621)
  AUTHORS   Karn,S.K., Chakarbarti,S.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2008) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road,
Patiala,
            Punjab 147004, India
FEATURES             Location/Qualifiers
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                     /product="16S ribosomal RNA"
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181 ctctcatgag gctgcacgga aagacggttt cggctgtcac tgcaggatgg gcccgcggcg
241 cattagctag ttggtgaggt aacggctcac caaggccacg atgcgtagcc gacctgagag
301 ggtgatcggc cacactggga ctgagacacg gcccagactc ctacgggagg cagcagtagg
361 gaatcttccg caatggacga aagtctgacg gagcaacgcc gcgtgagtga agaaggtttt
421 cggatcgtaa aactctgttg tgagggaaga acaagtacca actaactact ggtaccttga
481 cggtacctca ccagaaagcc acggctaact acgtgccagc agccgcggta atacgtaggt
541 ggcaagcgtt gtccggaatt attgggcgta aagcgcgcgc aggcggtcct ttaagtctga
601 tgtgaaagcc cacggctcaa c
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Bacillus sp. CL5 16S ribosomal RNA gene, partial sequence

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LOCUS       EU784652                675 bp    DNA     linear   BCT
28-JUN-2008
DEFINITION  Bacillus sp. CL5 16S ribosomal RNA gene, partial
sequence.
ACCESSION   EU784652
VERSION     EU784652.1  GI:192383711
KEYWORDS    .
SOURCE      Bacillus sp. CL5
  ORGANISM  Bacillus sp. CL5
            Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 675)
  AUTHORS   Karn,S.K., Chakarbarti,S.K. and Reddy,M.S.
  TITLE     Diversity of chlorophenolics degdrading bacteria in
secondary
            sludge of pulp and paper industry
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 675)
  AUTHORS   Karn,S.K., Chakarbarti,S.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2008) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road,
Patiala,
            Punjab 147004, India
FEATURES             Location/Qualifiers
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                     /mol_type="genomic DNA"
                     /strain="CL5"
                     /db_xref="taxon:543423"
     rRNA            <1..>675
                     /product="16S ribosomal RNA"
ORIGIN
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121 ctgcctgtaa gactgggata actccgggaa accggagcta ataccggata gttccttgaa
181 ccgcatggtt caaggatgaa agacggtttc ggctgtcact tacagatgga cccgcggcgc
241 attagctagt tggtggggta atggctcacc aaggcgacga tgcgtagccg acctgagagg
301 gtgatcggcc acactgggac tgagacacgg cccagactcc tacgggaggc agcagtaggg
361 aatcttccgc aatggacgaa agtctgacgg agcaacgccg cgtgagtgat gaaggttttc
421 ggatcgtaaa gctctgttgt tagggaagaa caagtgcgag agtaactgct cgcaccttga
481 cggtacctaa ccagaaagcc acggctaact acgtgccagc agccgcggta atacgtaggt
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661 agaggagagt ggaaa
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Pseudonocardia sp. CL6 16S ribosomal RNA gene, partial sequence

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LOCUS       EU784653                717 bp    DNA     linear   BCT
28-JUN-2008
DEFINITION  Pseudonocardia sp. CL6 16S ribosomal RNA gene, partial
sequence.
ACCESSION   EU784653
VERSION     EU784653.1  GI:192383712
KEYWORDS    .
SOURCE      Pseudonocardia sp. CL6
  ORGANISM  Pseudonocardia sp. CL6
            Bacteria; Actinobacteria; Actinobacteridae;
Actinomycetales;
            Pseudonocardineae; Pseudonocardiaceae; Pseudonocardia.
REFERENCE   1  (bases 1 to 717)
  AUTHORS   Karn,S.K., Chakarbarti,S.K. and Reddy,M.S.
  TITLE     Diversity of chlorophenolics degdrading bacteria in
secondary
            sludge of pulp and paper industry
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 717)
  AUTHORS   Karn,S.K., Chakarbarti,S.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2008) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road,
Patiala,
            Punjab 147004, India
FEATURES             Location/Qualifiers
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     rRNA            <1..>717
                     /product="16S ribosomal RNA"
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301 actgggactg agacacggcc cagactccta cgggaggcag cagtggggaa tattgcgcaa
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481 gtgccagcag ccgcggtaac acgtagggtg cgagcgttgt ccggaattat tgggcgtaaa
541 gagctcgtag gcggtgtgtc gcgtcggccg tgaaaacttg gggcttaact ctgagcgtgc
601 ggtcgatacg ggcatcactt gagttcggca ggggagactg gaattcctgg tgtagcggtg
661 aaatgcgcag ataccaggag gaacaccggt ggcgaaggcg ggtctctggg ccgatac
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Pseudomonas sp. CL7 16S ribosomal RNA gene, partial sequence

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LOCUS       EU784654                697 bp    DNA     linear   BCT
28-JUN-2008
DEFINITION  Pseudomonas sp. CL7 16S ribosomal RNA gene, partial
sequence.
ACCESSION   EU784654
VERSION     EU784654.1  GI:192383713
KEYWORDS    .
SOURCE      Pseudomonas sp. CL7
  ORGANISM  Pseudomonas sp. CL7
            Bacteria; Proteobacteria; Gammaproteobacteria;
Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1  (bases 1 to 697)
  AUTHORS   Karn,S.K., Chakarbarti,S.K. and Reddy,M.S.
  TITLE     Diversity of chlorophenolics degdrading bacteria in
secondary
            sludge of pulp and paper industry
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 697)
  AUTHORS   Karn,S.K., Chakarbarti,S.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2008) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road,
Patiala,
            Punjab 147004, India
FEATURES             Location/Qualifiers
     source          1..697
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                     /strain="CL7"
                     /db_xref="taxon:543428"
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241 ggtgaggtaa aggctcacca aggcgacgat ccgtaactgg tctgagagga tgatcagtca
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421 cactttaagt tgggaggaag ggcagtaagt taataccttg ctgttttgac gttaccaaca
481 gaataagcac cggctaactt cgtgccagca gccgcggtaa tacgaagggt gcaagcgtta
541 atcggaatta ctgggcgtaa agcgcgcgta ggtggttcgt taagttggat gtgaaagtcc
601 cgggctcaac ctgggaactg catccaaaac tggcgagcta gagtatggca gagggtggtg
661 gaatttcctg tgtagcggtg aaatgcgtag atatagg
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Jeotgalicoccus sp. CL8 16S ribosomal RNA gene, partial sequence

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LOCUS       EU784655                656 bp    DNA     linear   BCT
28-JUN-2008
DEFINITION  Jeotgalicoccus sp. CL8 16S ribosomal RNA gene, partial
sequence.
ACCESSION   EU784655
VERSION     EU784655.1  GI:192383714
KEYWORDS    .
SOURCE      Jeotgalicoccus sp. CL8
  ORGANISM  Jeotgalicoccus sp. CL8
            Bacteria; Firmicutes; Bacillales; Jeotgalicoccus.
REFERENCE   1  (bases 1 to 656)
  AUTHORS   Karn,S.K., Chakarbarti,S.K. and Reddy,M.S.
  TITLE     Diversity of chlorophenolics degdrading bacteria in
secondary
            sludge of pulp and paper industry
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 656)
  AUTHORS   Karn,S.K., Chakarbarti,S.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2008) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road,
Patiala,
            Punjab 147004, India
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181 cggggcagag tgacaggtgg tgcatggttg tcgtcagctc gtgtcgtgag atgttgggtt
241 aagtcccgca acgagcgcaa cccttaagtt tagttgccat cattaagttg ggcactctag
301 actgactgcc ggtgacaaac cggaggaagg tggggacgac gtcaaatcat catgcccctt
361 atgatttggg ctacacacgt gctacaatgg acaggttaca aagggcagcg acgccgcgag
421 gccaagcgaa tcccataaaa ctgttctcag ttcggattgg agtctgcaac tcgactccat
481 gaagctggaa tcgctagtaa tcgtagatca gcatgctacg gtgaatacgt tcccgggtct
541 tgtacacacc gcccgtcaca ccacgaaagt cgataacacc tgaagccggt gggccaacct
601 ttatggaggc agccgtcgaa ggtgggattg atgattgggg tgaagtcgta acaagg
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Exiguobacterium sp. CL9 16S ribosomal RNA gene, partial sequence

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LOCUS       EU784656                712 bp    DNA     linear   BCT
28-JUN-2008
DEFINITION  Exiguobacterium sp. CL9 16S ribosomal RNA gene, partial
sequence.
ACCESSION   EU784656
VERSION     EU784656.1  GI:192383715
KEYWORDS    .
SOURCE      Exiguobacterium sp. CL9
  ORGANISM  Exiguobacterium sp. CL9
            Bacteria; Firmicutes; Bacillales; Bacillales Family XII.
Incertae
            Sedis; Exiguobacterium.
REFERENCE   1  (bases 1 to 712)
  AUTHORS   Karn,S.K., Chakarbarti,S.K. and Reddy,M.S.
  TITLE     Diversity of chlorophenolics degdrading bacteria in
secondary
            sludge of pulp and paper industry
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 712)
  AUTHORS   Karn,S.K., Chakarbarti,S.K. and Reddy,M.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2008) Department of Biotechnology and
            Environmental Sciences, Thapar University, Bhadson Road,
Patiala,
            Punjab 147004, India
FEATURES             Location/Qualifiers
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                     /strain="CL9"
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  JOURNAL   Unpublished
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  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2008) Department of Biotechnology and
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REFERENCE   2  (bases 1 to 533)
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  TITLE     Direct Submission
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Pentachlorophenol degradation by *Pseudomonas stutzeri* CL7 in the secondary sludge of pulp and paper mill

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Abstract

A pentachlorophenol (PCP) mineralizing bacterium was isolated from the secondary sludge of pulp and paper mill and identified as *Pseudomonas stutzeri* strain CL7. This isolate used PCP as its sole source of carbon and energy and was capable of degrading this compound as indicated by stoichiometric release of chloride and biomass formation. *P. stutzeri* (CL7) was able to mineralize a high concentration of PCP (600 mg/L) than any previously reported *Pseudomonad* with PCP as sole carbon source. As the concentration of PCP increased from 50 to 600 mg/L, the reduction in the cell growth was observed and the PCP degradation was more than 90% in all studied concentrations. This isolate was able to remove 66.8% of PCP from the secondary sludge of pulp and paper mill when supplemented with 100 mg/L of PCP and grown for two weeks. This study showed that the removal efficiency of PCP by CL7 was found to be very effective and can be used in PCP remediation of pulp paper mill waste in the environment.

Key words: pentachlorophenol; 16S rRNA; secondary sludge; bioremediation; pulp and paper mill

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Introduction

The manufacturing process of pulp and paper utilizes huge amount of lignocellulosic components of plants and chemicals and generates effluents consisting complicated mixtures of several hundred types of compounds. Chlorinated phenols are major environmental pollutants discharged from pulp and paper mill. Chlorophenols from the pulp and bleaching process are found both in free (hexane extractable) and bound (extractable with strong alkali) forms in dissolved organic matter and particles (Palm et al., 1995). Among the chlorophenols, pentachlorophenol (PCP) is expected to be recalcitrant to aerobic biodegradation due to its high chlorinated ring structure; generally aromatic compounds with higher amounts of chlorine are more recalcitrant to biodegradation (Anandrajah et al., 2000). The United States Environmental Protection Agency has registered PCP in the list of priority of pollutants and the safe permissible limits of PCP in water is 0.30 μg/L (US EPA, 1999). However pulp and paper mill effluent contains far above the permissible limit of PCP even after the treatment at industrial scale (Raj et al., 2005). In addition, this compound is very harmful to microorganisms because it destroys membrane function due to its ability to uncouple oxidative phosphorylation (Copley, 2000; Ito and Ohnishi, 1982). The accumulation of PCP through the food chain has been established and it is considered to be mutagenic or at least co-mutagenic to human, thus the PCP exposure in environment poses significant health hazards (Chandra et al., 2008). In spite of these properties, several microorganisms have been isolated which have the ability to degrade PCP.

A large variety of bacteria are known which can utilize chlorophenols as a carbon and energy sources under aerobic conditions. Lee et al. (1998) reported that *Pseudomonas* sp. (Bu34) was able to grow up to 4000 mg/L of PCP. Radehaus and Schimdt (1992) reported complete mineralization of PCP (160 mg/L) in a week by *Pseudomonas* sp. strain RA2. Shah and Thakur (2002) observed 72% PCP removal by *Pseudomonas fluorescence* (TE3) in 96 hours when grown with 100 mg/L PCP. Sharma and Thakur (2008) observed that *Pseudomonas aeruginosa* (PCP2) utilized 60% of PCP within 96 hours of incubation. Premlatha and Rajkumar (1994) recorded the complete degradation of PCP (800 mg/L) by *Pseudomonas aeruginosa* in 6 days with glucose as a co-substrate. Singh et al. (2007) reported that the bacterial isolate *Serratia marcescens* from pulp and paper mill waste was able to degrade 90.3% of PCP within 168 hr when grown with 300 mg/L of PCP. There are still many unknown bacteria that have tremendous degradation capacity for PCP present in nature and it is important to asses the potential of bacterial strain indigenous to sites contaminated with PCP. In the present study, PCP degrading bacterium *Pseudomonas stutzeri* was isolated from the secondary sludge of pulp and

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paper mill and characterized. The PCP degradation ability of this isolate with different concentrations of PCP and the removal of PCP from the secondary sludge of pulp and paper mill were also determined.

1 Materials and methods

1.1 Sample collection and isolation of PCP degrading bacterium

Secondary sludge samples of pulp and paper mill were collected from M/s Shree Gopal Unit (BILT) Yamunanagar, Haryana (India). The samples were taken in pre-sterilized 250 mL conical flasks and immediately preserved at 4°C. Bacteria were isolated by the serial dilution technique, and purified by repeated streaking on nutrient agar plates. Colonies appearing after incubation at 37°C for 48 hr were selected for further screening. PCP tolerant bacterial strains were isolated by the nutrient enrichment technique in mineral salt medium (MSM) (Sharma et al., 2009) supplemented with 50 mg/L pentachlorophenol as the sole carbon source for energy. The medium contained the following components (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 (mg/L): $FeSO_4{\cdot}7H_2O$, 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; EDTA, 2.5 and agar 1.0%. The pH was adjusted to 7.3 ± 0.2. PCP was added to the medium after autoclaving. The potent isolates were screened for the degradation studies and one of the efficient isolates was selected and designated as CL7 for further studies.

1.2 Morphological and biochemical characterization

The identification of PCP degrading bacterium (CL7) was conducted according to Bergey's Manual of Systematic Bacteriology (Holt et al., 1994). The CL7 isolate was subjected to microscopic examination for the shape and size. Gram stain, catalase, oxidase, citrate utilization, lysine, ornithine TDA, nitrate reduction, urease and starch hydrolysis tests were performed by standard methods. Antibiotic profiling were performed by using ICOSA universal -1 kit (Hi-Media Laboratories, Mumbai, India) having twenty different antibiotics of various concentrations according to the manufacturer's instructions. A total of 35 carbohydrate fermentation tests were performed according to the manufacturer's direction (Himedia Lab., Mumbai, India).

1.3 16S rRNA gene amplification and sequence analysis

Genomic DNA extraction, amplification of 16S rRNA gene and sequence analysis were performed as described in Krishna et al. (2008). The 16S rRNA gene sequence determined in this study was deposited in the GenBank of NCBI data library under the accession number EU784654.

1.4 Degradation of PCP by the isolated strain

The degradation studies were performed by inoculating 1% inoculum (10^6 cfu/mL) of CL7 in 250 mL Erlenmeyer flasks containing 50 mL of MSM supplemented with 100 mg/L PCP. The flasks were incubated at 37°C under shaking conditions (120 r/min) up to 168 hr. The growth of the bacterial cells was determined by measuring the optical density at 600 nm. The degradation of PCP in the culture filtrate was determined by high-performance liquid chromatography (HPLC) as described by Yang et al. (2006). Briefly, the cell suspension was centrifuged to separate the biomass at 8000 r/min for 5 min and the supernatant was passed through 0.22 μm filters. HPLC was performed with a PerkinElmer System (USA) equipped with a Merck Lichrospher 100 RP-18 (USA) end capped (5 μm) column at flow rate of 1 mL/min. The solvent system was methanol, water and glacial acetic acid in the volume ratio of 90:10:0.02. The UV detector absorbance wavelength was fixed at 280 nm. A standard PCP was run under the same conditions. The percent utilization was estimated by measuring the peak area of the PCP.

Chloride ion released in the aqueous media was determined at every 24 hr of interval up to 168 hr using 5 mL of culture filtrate. The level of chloride ion was measured with an Orion ion analyzer model 940 (NyCo Systems, USA) using calibrated selective chloride ion electrode. Chloride concentration was determined using a calibration curve plotted from the log of chloride molarity for a series of standard samples ranging from 10 to 1000 mg/L.

The effect of pH on the degradation of PCP was studied by growing the bacterial cells at different initial pH of 7.5, 8.5 and 9.5 in MSM supplemented with 100 mg/L of PCP as sole source of carbon. The pH of the medium was adjusted with NaOH or HCl. The influence of temperature on PCP degradation was also determined by incubating the samples at three different temperatures 25, 30 and 37°C under shaking condition.

The effect of different concentrations of PCP on the growth of CL7 and degradation ability of this strain was also studied. The bacterial strain CL7 was inoculated (10^6 cfu/mL) to 250 mL Erlenmeyer flasks containing 50 mL of MSM supplemented with different concentrations (50, 100, 200, 400, 600 mg/L) of PCP. The flasks were incubated at 37°C under shaking conditions for 168 hr. The growth of the bacterial cells was determined by measuring the optical density at 600 nm and the degradation of PCP in the culture filtrate by HPLC.

1.5 Degradation of PCP in the secondary sludge

The PCP degradation ability of CL7 was analyzed in the secondary sludge by inoculating 5% of inoculum in 2.5 L conical flasks containing one liter of sludge supplemented with 100 mg/L of PCP. The flasks were incubated at 37°C under shaking condition for two weeks. PCP was extracted from the sludge by the method described by Chandra et al. (2008) with a little modification. The sonicated sludge sample was acidified by 1 mol/L HCl to pH 2.0; extracted three times with an equal volume of

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ethyl acetate by intermittent shaking for 30 min in standard separating funnel. The organic layer was dried with anhydrous sodium sulphate to absorb excess of water. Filtered samples were evaporated to dryness at 40°C, subsequently resuspended in 1 mL of methanol. Quantification of PCP present in the bacterial degraded sludge was determined by HPLC.

The data were analyzed by analysis of variance, and the means were compared with Tukey's test at $p < 0.05$. Three replicates were maintained for each treatment

2 Results and discussion

2.1 Characterization of CL7 isolate

The biochemical characterization of CL7 isolate is presented in Table 1. CL7 is Gram negative, motile, aerobic, oxidase- and urease positive and able to hydrolyze starch. Susceptible to different antibiotics such as calithromycin, co-trimoxazole, netilin, cefaclor and ampicillin. Acid is produced from fructose, sodium gluconate, glycerol, salicin, ONPG, esculin, citrate, and malonate. The bacterium was further identified by 16S rRNA sequence analysis. The nucleotide BLAST and RDP-II analyses showed that CL7 belong to the phylum proteobacteria and family *Pseudomonadaceae*. Phylogenetic analysis revealed that CL7 have very close similarity with *Pseudomonas stutzeri* and hence identified as *P. stutzeri* (Fig. 1).

2.2 Degradation of PCP by CL7

P. stutzen CL7 was able to grow and utilize PCP as an energy source. The growth of bacterial strain was significantly increased up to 72 hr and decreased thereafter and attained stationary phase up to 168 hr. It was observed that CL7 utilized more than 60% of PCP within 24 hr and above 90% at 72 hr. The PCP was completely mineralized after 120 hr of incubation (Fig. 2). The bacterial growth curve with correspondence to liberation of chloride ion as shown in Fig. 3, revealed that the strain CL7 achieved good growth with simultaneous liberation of chloride ion. The initial concentration of chloride ion was 200 mg/L but during the course of bacterial treatment the liberation of inorganic chloride ion in culture medium increased up to 478 mg/L at the 168 hr treatment. The growth of CL7 and degradation of PCP were observed under culture conditions such as different temperatures and initial pH values. The results revealed that CL7 was able to grow well at 37°C and degrade 93.5% of PCP at this temperature compared to 25 and 30°C (Fig. 4a). The effects of initial pH value on growth and degradation of PCP are shown in Fig. 4b. These results indicated a higher PCP degradation (96.5% and 94.5%) between pH 7.5 and 8.5. The growth

Table 1 Cultural and biochemical characteristics of *P. stutzeri* (CL7) isolated from the sludge of pulp and paper mill

Characteristics	Observation
Form	Rods
Gram stain	–
Motility	+
Catalase test	–
Oxidase test	+
Nitrate reduction test	–
Urease test	+
Starch hydrolysis	+
Antibiotic sensitivity	Calaritromycin; co-trimoxazole; netillin; cefaclor; ampicillin/sublactam
Fructose, sodium gluconate glycerol, salicin, ONPG, esculin, citrate, malonate	+

+: positive; – : negative.

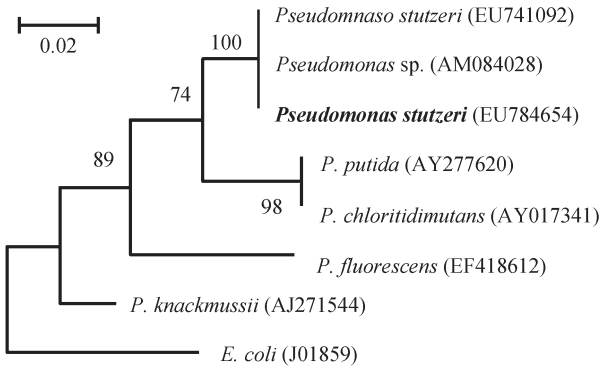

Fig. 1 Neighbor-joining tree based on 16S rRNA sequence CL7 of current study along with sequences available in GenBank database. Numerical values indicate bootstrap percentile from 1500 replicates.

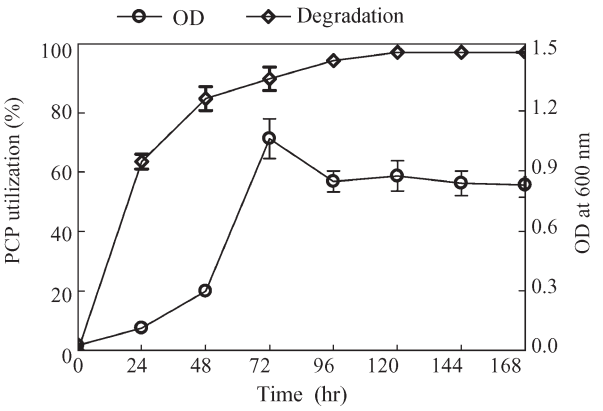

Fig. 2 Growth curve of *P. stutzeri* CL7 in mineral salt media containing 100 mg/mL of PCP as a sole carbon or energy source.

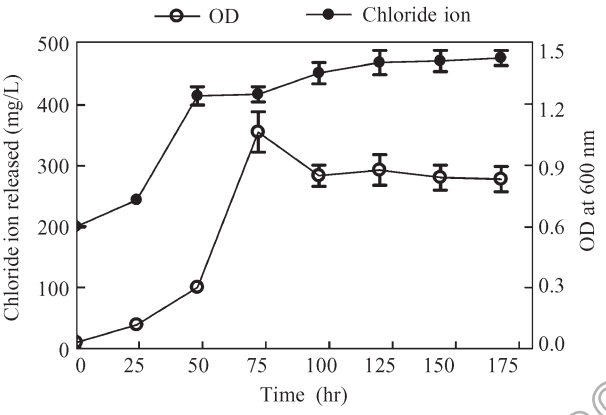

Fig. 3 Release of chloride ion in the medium at different time intervals due to the degradation of PCP by *P. stutzeri* CL7.

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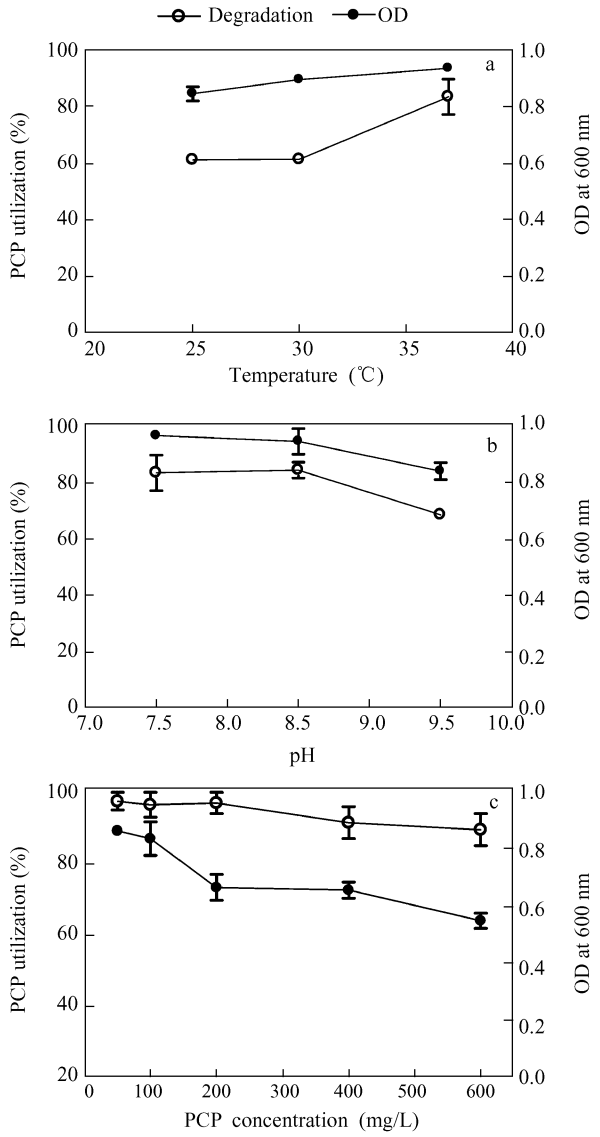

Fig. 4 Effect of temperature (a) and pH (b) and different PCP concentrations (c) on the growth and degradation of PCP by *P. stutzeri* CL7.

of the bacterial strain and the degradation of PCP were reduced at pH 9.5. The growth of CL7 was significantly reduced as the concentration of PCP in the mineral salt medium increased. It was able to degrade 89% PCP at all concentrations. More than 95% degradation of PCP was recorded up to 200 mg/L of PCP and decreased at 400 and 600 mg/L (Fig. 4c).

The bacterial strain CL7 was capable of completely mineralizing PCP when grown in 100 mg/L of PCP utilizing it as sole source of carbon. Some previous reports also showed the degradation of PCP by other *Pseudomonas* species (Radehaus and Schimdt, 1992; Shah and Thakur, 2002; Sharma and Thakur, 2008). The release of chloride ion in the medium increased with increase in time. The liberation of chloride ion from the medium can be considered as the result of mineralization of chlorinated compounds by the bacterium thereby release of chloride in the medium. Available data of earlier studies indicated that chlorinated phenols were mineralized to chlorine free end products (Homada et al., 1987; Kennes et al., 1996; Radehaus and Schimdt, 1992; Mohn and Kennedy, 1992). The maximum PCP degradation was observed at pH between 7.5 and 8.5 in this study. Wolski et al. (2005) reported the degradability of PCP at pH values from 6.3 to 8.0, with maximum rate of PCP degradation at pH 6.3 by *Pseudomonas* species. The optimum temperature for the growth and degradation of PCP was observed at 37°C in this study. Sharma et al. (2009) reported the temperature range of 25–35°C for PCP degradation by *Acinetobacter* species. The growth and degradation of PCP decreased in the medium as the concentration of PCP increased from 50–600 mg/L. The possible explanation for reduction in degradation of PCP by the bacterial strain CL7 might be due to decreased activity of the degrading enzymes at lower pH, as the pH of the medium decreased significantly at higher concentration (data not shown). The present study results showed that CL7 was able to utilize PCP as a sole source of carbon.

2.3 Degradation of PCP in sludge

The bacterial strain has been shown to degrade PCP in mineral salt medium at higher concentrations in this study. Therefore, this strain was used for the treatment of pup and paper mill sludge. The initial PCP concentration of the sludge was about 0.029 mg/L, and therefore, 100 mg/L of PCP was amended to the sludge to study the efficacy of this strain in degradation of PCP. The survival of this strain was monitored by determining the growth in the form of colony forming units (cfu). The growth of the bacterial strain increased from the initial cfu of 2.38×10^4 to 10.78×10^6 after two weeks. HPLC analysis shown in Fig. 5 revealed that this strain is capable of mineralizing PCP from the sludge. It is able to remove up to 66.8% PCP from the sludge.

Many biological treatment methods are utilized to clean up soils contaminated with chlorinated phenols. One approach towards *in-situ* remediation is the addition of microbial cells or enzyme to enhance the bioremediation process of contaminated site. Although *in-situ* remediation of PCP studies was reported previously, they mainly deal with compost, manure and soil (Miethling and Karlson, 1996; Laine and Jorgensen, 1997). Very few reports are

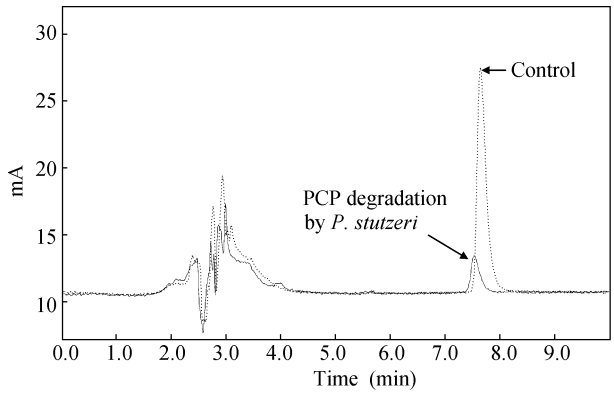

Fig. 5 HPLC chromatogram of PCP degradation in the sludge by *P. stutzeri* CL7 compared with control.

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available with removal of PCP from the sludge. In the present study, CL7 mineralized 66.8% PCP from the sludge where the PCP concentration was more than 100 mg/L. Chandra et al. (2008) also reported the removal of PCP from the effluent of pulp and paper by 85% and 90% by *Bacillus cereus* and *Serratia marcescens* respectively where the PCP concentration was 50.3 mg/L. These results suggested that CL7 is more efficient in degradation of PCP from pulp and paper mill sludge.

3 Conclusions

In conclusion, the results obtained by this study indicated that the bacterial strain CL7 is able to mineralize high concentrations of PCP. This strain also degraded 66.8% of PCP from the sludge within two weeks of treatment. These results highlight the potential of this bacterium to be used in bioremediation of high strength PCP contaminated pulp and paper mill sludge.

Acknowledgments

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ORIGINAL PAPER

Degradation of pentachlorophenol by *Kocuria* sp. CL2 isolated from secondary sludge of pulp and paper mill

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Abstract A pentachlorophenol (PCP) degrading bacterium was isolated and characterized from sludge of pulp and paper mill. This isolate used PCP as its sole source of carbon and energy and was capable of degrading this compound, as indicated by stoichiometric release of chloride and biomass formation. Based on morphology, biochemical tests, and 16S rRNA gene sequence analysis this strain was identified as *Kocuria* sp. CL2. High Performance Liquid Chromatography (HPLC) analysis revealed that this strain was able to degrade PCP up to a concentration of 600 mg/l. This is first time we are reporting the degradation of PCP by the *Kocuria* species. This isolate was also able to remove 58.64% of PCP from the sludge within two weeks. This study showed that the removal efficiency of PCP by CL2 was found to be very effective and can be used in degradation of PCP containing pulp paper mill waste in the environment.

Keywords *Kocuria* sp. CL2 · Sludge · Biodegradation · Characterization · Chloride ion · Pentachlorophenol

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Introduction

The chlorophenolic compounds are common environmental contaminants originating mainly from the use of the compounds as wide spectrum biocides in industry, agriculture, and their formation during pulp bleaching and some direct industrial waste discharge (Vallecillo et al. 1999). These are 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). Among chlorinated phenols, pentachlorophenol (PCP) has widely been used as wood and leather preservative, owing to their toxic effect on bacteria, mould, fungi and algae (Kaoa et al. 2005). PCP is toxic to all life forms as it inhibits the oxidative phosphorylation (Yang et al. 2006) and extensive exposure to PCP could cause cancer, acute pancreatitis, immunodeficiency and neurological disorders (Sai et al. 2001).

The US Environmental Protection Agency (USEPA) has listed PCP as a priority contaminant (Bock et al. 1996). 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). The biodegradation of PCP has been studied in both aerobic and anaerobic systems. Aerobic degradation of PCP has been studied extensively and several bacterial isolates such as *Mycobacterium chlorophenolicum* (Wittmann

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et al. 1998), *Rhodococcus chlorophenolicum* (Apajalahti et al. 1986), *Flavobacterium* (Gonzalez and Hu 1991), *Novosphingobium lentum* (Dams et al. 2007) *Pseudomonas* species (Kaoa et al. 2005) and *Sphingomonas chlorophenolica* (Yang et al. 2006) were found to utilize PCP as a sole source of carbon and energy. There are still many unknown bacteria that have tremendous degradation capacity for PCP present in nature and it is important to asses the potential of bacterial isolates indigenous to sites contaminated with PCP. In this study, a PCP degrading bacterium was isolated and characterized from sludge of pulp and paper industry and tested for its ability to degrade PCP under in vitro and in situ conditions.

Material and methods

Isolation and enrichment of PCP degrading bacterium

Sludge sample was collected from the effluent treatment plant site of a pulp and paper mill at M/s Shree Gopal Unit-Yamunanagar, Haryana, India. The samples were taken into sterilized conical flask and preserved at 4°C. Bacteria were isolated by the serial dilution technique, and purified by repeated streaking on nutrient agar plates. Colonies appearing after incubation at 37°C for 48 h were selected for further screening. Screening of PCP tolerant bacterial strains were done by the nutrient enrichment technique in mineral salt medium (MSM) (Dams et al. 2007) supplemented with 50 mg/l (0.185 mM) of pentachlorophenol as a sole source of carbon for energy. MSM was used in enrichment culture and degradation studies. The medium contained the following components at the specified concentrations (in 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. The pH of the medium was adjusted to 7.5. PCP was added to the medium after autoclaving. All reagents used were of analytical grade. Synthetic PCP was purchased from Sigma Aldrich chemicals (USA). All solutions were prepared in sterile Milli-Q water (Millipore direct Q3, Bangalore, India). Pentachlorophenol was dissolved in small amounts of methanol and Mili1-Q water was added to make the required volume where the methanol concentration did not exceed more than 0.2%.

One bacterial isolate with highest PCP degradation efficiency was selected and designated as CL2 for further study. This isolate was deposited at Microbial Culture Collection Centre (MTCC), Institute of Microbial Technology, Chandigarh, India.

Biochemical and phenotypic characteristics

The identification of PCP degrading bacterium was carried out according to Bergey's Manual on Systematic Bacteriology (Holt et al. 1994). Catalase activity was determined by detective bubble formation with 3% H_2O_2 solution. Oxidase was determined by using paper disc with tetramethyl-*p*-phenylenediamine. Nitrate reduction by using nitrate agar plate; DNAase test by DNAase agar medium with methyl green as an indicator. Starch hydrolysis was determined on starch hydrolyzing agar by detecting cleared zones formed around the colonies. Motility, Indole and Urease activities were determined by using MIU-media kit (Hi-Media Laboratories, India) and antibiotic profiling was also done by using ICOSA universal-1 kit (Hi-Media Laboratories, India) having twenty different antibiotics of various concentrations according to the manufacturer's instructions. The antibiotics and their concentrations are: Norfloxacin (10 μg), Gentamicin (10 μg), Chloramphenicol (30 μg), Cefuroxime (30 μg), Ciprofloxacin (5 μg), Cefaperazone (75 μg), Ceftazidime (30 μg), Roxithromycin (30 μg), Calaritromycin (15 μg), Co-Trimoxazole (25 μg), Netillin (30 μg), Cefaclor (30 μg), Cephotaxime (30 μg), Cephadroxil (30 μg), Azithromycin (15 μg), Ampicillin/Cloxacillin (10/10 μg), Penicillin (10 units), Amikacin (30 μg), Sparfloxacin (5 μg) and Ampicillin/sublactam (10/10 μg).

16S rRNA sequence and phylogenetic analysis

Genomic DNA was extracted from overnight grown cultures by a simple lysis protocol, as described in Kapley et al. (2001). One μl of purified genomic DNA served as template. 16S rRNA gene was amplified using GeneAmp 2700 PCR systems (Applied Biosystems, USA) with the following set of primers: forward primer: 5′-AGAGTTTGATCCTGGCTCAG-3′ and

reverse primer: 5′-ACGGGCGGTGTGTTC-3′ as described in Weisburg et al. (1991). Reaction mixture for the PCR contained 1× PCR buffer; 200 µM of dNTPs; 1.5 mM $MgCl_2$, 0.1 µM of each primer and 2.5 units of Taq DNA polymerase (Invitrogen, USA) in a final volume of 100 µl sterile MQ water. The PCR was performed with an initial denaturation at 92°C for 2 min followed by 36 cycles of 92°C for 1 min, 48°C for 30 s and 72°C for 2 min and a final extension of 72°C for 6 min. The amplification product was gel purified using QIAgel extraction kit (Qiagen, USA), and ligated into the pGEM-T easy vector as per manufacturer's instructions (Promega Inc., USA). Ligated plasmid was transformed into *E. coli* DH5α cells by calcium chloride treatment and heat shock method. After screening of the positive clones, the partial sequence was generated by chain termination method using an Applied Biosystems automated DNA sequencer (DNA sequencing facility, Delhi University, India). The sequence was compared against the available DNA sequences from type strains in GenBank (http://www.ncbi.nlm.nih.gov/) using BLASTN sequence match tool. The sequences were aligned using MultAlin (http://bioinfo.genotoul.fr/multalin/multalin.html) program and the alignment was manually corrected and phylogenetic tree was constructed using the MEGA 4.0 (Tamura et al. 2007) software. The sequence was deposited in GenBank database under the accession number EU784649.

Degradation of PCP by isolated strain

The degradation studies were performed by inoculating 1% inoculum (10^6 CFU/ml) of the bacterial strain CL2 in 250 ml Erlenmeyer flasks containing 50 ml of MSM supplemented with 100 mg/l (0.37 mM) of PCP. The flasks were incubated at 37°C under shaking conditions (120 rpm) up to 168 h. Growth of the bacterial cells was determined by measuring the optical density at 600 nm and the PCP degradation was analysed by HPLC. The cell suspension was centrifuged (5 min, 8000 rpm) and supernatant was filtered through 0.22 µm filters. HPLC was carried out with purified samples on Perkin Elmer Series (200) system. Samples (10 µl) were injected and separated on reverse phase (Licrosphere® 100 RP-18 endcapped column, 250 mm × 4.6 mm i.d.) in an isocratic mode using aqueous methanol 90% (v/v) at a flow rate of 1 ml/min. The eluates were monitored at 280 nm with online diode array detector (series 200). PCP was quantified on the basis of standard curve prepared by taking known quantities of PCP (Sigma–Aldrich, USA). Chloride ion released in the aqueous medium was determined at every 24 h of interval up to 168 h using 5 ml of culture filtrate. The level of chloride ion was then measured with an Orion ion analyzer model 940 using calibrated selective chloride ion electrode. The electrode was calibrated by adding known concentrations of sodium chloride standards to the MSM used in the experiments.

The effect of different concentrations of PCP on the growth of CL2 and degradation ability of this strain was also studied. The bacterial strain CL2 was inoculated to 250 ml Erlenmeyer flasks containing 50 ml of MSM supplemented with different concentrations (50, 100, 200, 400, 600 mg/l or 0.185, 0.37, 0.75, 1.5 and 2.2 mM) of PCP. The flasks were incubated at 37°C under shaking conditions for 168 h. The growth and the degradation of PCP in the culture filtrate were determined as mentioned previously.

Degradation studies of PCP in sludge

The degradation ability of the isolated strain CL2 was analyzed in the sludge by inoculating 5% of inoculum in 2.5 l conical flasks containing 1 l of sludge supplemented with 100 mg/l (0.37 mM) of PCP. The flasks were incubated at 37°C under shaking condition for 2 weeks. PCP was extracted from the sludge by the method described in Chandra et al. (2006). The sludge sample was acidified by 1 N HCl to pH 2.0; extracted thrice with an equal volume of ethyl acetate by intermittent shaking for 30 min in standard separating funnel. The organic layer was dried with anhydrous sodium sulphate to absorb excess of water. Filtered samples were evaporated under vacuum at 40°C, subsequently resuspended in 1 ml of methanol. Quantification of PCP present in the bacterial degraded sludge sample was determined by HPLC. The growth of CL2 was determined in both PCP amended and unamended sludge.

Statistical analysis

Data were statistically analysed by analysis of variance (ANOVA) and the mean differences were compared by Tukey–Kramer Multiple Comparison

Test at $p < 0.05$. Three replicates were maintained for each treatment. All the analyses were performed using GraphPad Prism (v 4.03) software.

Results

Isolation and identification of PCP degrading isolate

Totally 36 bacteria were isolated from the sludge and screened them on PCP containing media. The bacterial colonies grown on this media were further screened and the bacterial strain CL2 was selected based on the growth and utilization of PCP as sole source of carbon and energy. The colonies were morphologically vermilion in color, non-spreading, smooth, wet, and non transparent. The results of the biochemical characterization of the CL2 strain are presented in Table 1. The shape of the bacteria are cocci, motile and positive for Gram reaction, oxidase and nitrate reduction tests and negative for capsule, catalase, DNAase, indole, urease and starch hydrolysis. This isolate was sensitive to Calaritromycin; Cotrimoxazole; Netillin; Cefaclor; Ampicillin/sublactam. Partial sequence data of the isolate was analysed by BLAST search. The BLAST analysis of this sequence showed 99% of sequence similarity with *Kocuria polaris*. A phylogenetic tree was constructed based on 16S rRNA gene sequence by maximum parsimony using MEGA software (v 4.0). The phylogenetic analysis revealed that CL2 was grouped with *Kocuria polaris* and hence designated it as *Kocuria* sp. CL2 (Fig. 1).

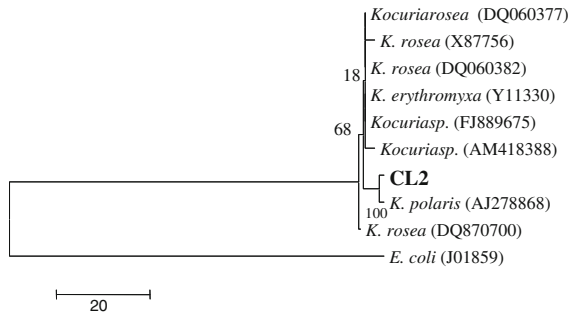

Fig. 1 Maximum parsimony tree based on 16S rRNA sequence of current study along with sequences available in GenBank database. Numerical values indicate bootstrap percentile from 1000 replicates

Degradation studies

The bacterial strain CL2 was able to grow and utilize PCP as an energy source. The growth of bacterial strain was increased up to 120 h and then attained stationary phase. The CL2 utilized 55 % of PCP within 24 h and 95% after 96 h when 100 mg/l (0.37 mM) of PCP was used (Fig. 2). During the course of bacterial treatment PCP was mineralized and the liberation of inorganic chloride ion in culture medium was observed (Fig. 3). The chloride ion concentration increased with increase in PCP degradation. The amount of PCP utilized and the release of chloride ion (mentioned in parenthesis) for different

Table 1 Biochemical characteristic features of the aerobic bacterial isolate CL2 of the sludge and comparison with other *Kocuria* species

Test	CL2	*K. rosea*[a]	*K. varians*[a]
Form	Coccus	Coccus	Coccus
Colony color (Nutrient Agar)	Vermilion	Orange/red	Yellow
Cell grouping	Individuals	Individuals	Individuals
Gram stain	+	+	+
Capsule stain	−	NA	NA
Motility	+	+	NA
Catalase test	−	+	NA
DNase test	−	NA	NA
Oxidase test	+	−	−
Nitrate reduction test	+	+	+
Indole test	−	−	NA
Urease test	−	−	+
Starch hydrolysis	−	+	−

+, Positive; −, Negative and NA, no data available

[a] Data obtained from Reddy et al. (2003)

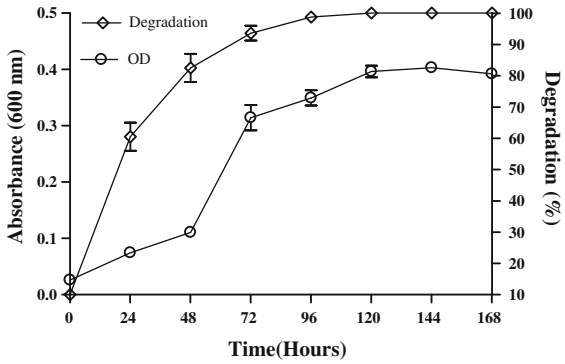

Fig. 2 Growth curve and degradation of *Kocuria* sp. CL2 in mineral salt media containing 100 mg/l (0.37 mM) of PCP as a sole carbon or energy source

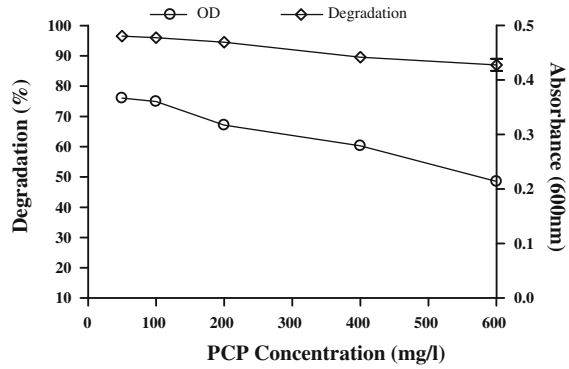

Fig. 4 Effect of different concentrations of PCP on the growth and utilization of PCP by *Kocuria* sp. CL2 grown for 168 h

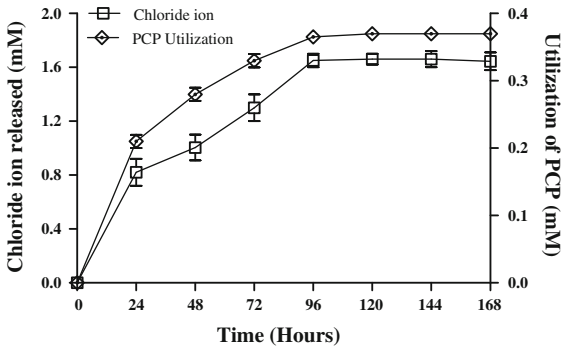

Fig. 3 Release of chloride ion in the medium at different time intervals due to degradation of PCP (100 mg/l or 0.37 mM) by *Kocuria* sp. CL2

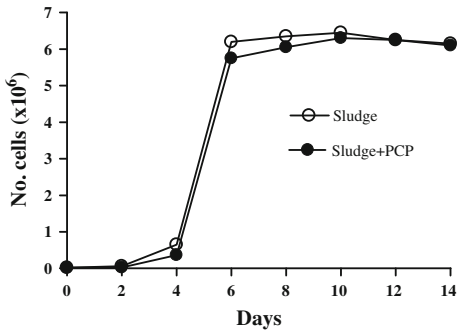

Fig. 5 Growth pattern of CL2 (CFU/ml) grown in the sludge supplemented with or without PCP

time intervals; 24, 48, 72, 96, 120, 144 and 168 h were 0.20 (0.82), 0.29 (1.10), 0.34 (1.30), 0.37 (1.6), 0.37 (1.6), 0.37 (1.6), and 0.37 (1.6) mM respectively. The liberation of chloride ion in the culture filtrate can be considered as the result of mineralization of PCP. The growth of CL2 was significantly reduced as the concentration of PCP in the mineral salt medium increased. Up to 200 mg/l, 95% removal of PCP was observed, and slightly decreased at 400 and 600 mg/l (Fig. 4).

Degradation of PCP in sludge

The removal of PCP from the sludge was tested by inoculating the bacterial strain CL2 into sludge. The initial PCP concentration in the sludge used for this study was about 0.029 mg/l, and therefore 100 mg/l of PCP was added to the sludge to study the efficacy of this strain in degradation of PCP. The survival of this strain was monitored by determining the growth in the form of colony forming units (CFU). The growth of the bacterial strain increased with time both in sludge supplemented with PCP and sludge alone. The growth of CL2 was higher in sludge alone compared to sludge amended with PCP (Fig. 5). HPLC analysis shown in Fig. 6 revealed that this strain is capable of mineralizing PCP from the sludge. It is able to remove up to 58.64% PCP from the sludge. These results suggested that the isolated bacterial strain CL2 have potential to remove PCP in the sludge.

Discussion

There is extensive evidence that chlorophenols are mineralized by bacteria that utilize chlorophenolic compounds as a carbon and energy source. The evidence is based on the stoichiometric release of

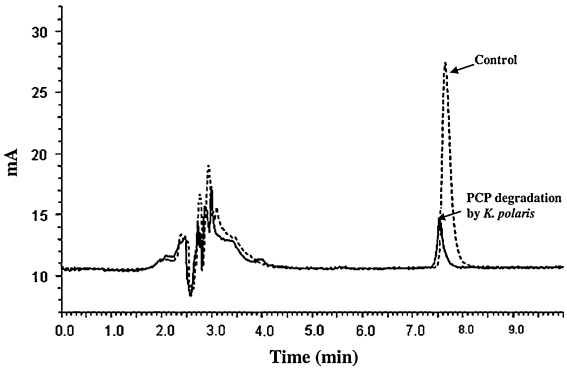

Fig. 6 HPLC chromatogram showing the PCP degradation in sludge by *Kocuria* sp. CL2

inorganic chloride and the concomitant production of biomass linked to chlorophenol utilization (Radehaus and Schmidt 1992). We are reporting for the first time that *Kocuria* sp. CL2 able to metabolize pentachlorophenol as carbon and energy source. This strain is capable of tolerating up to 600 mg/l of PCP and also degraded 90% of PCP in the culture medium.

The lower chlorinated phenols are attacked by monooxigenase yielding chlorocatechol as the first intermediates (Chlorocatechol pathway), which are subjected to ring cleavage prior to dechlorination. On the other hand polychlorinated phenols (3 to 5 chlorine) are converted to chlorohydroxyquinones as the initial intermediate (hydroxyquinone pathway). Subsequent reaction progressively removes chlorine from the ring prior to ring cleavage (Solyanikova and Golovleva 2004). The results of this study clearly show that CL2 strain is able to utilize PCP as chloride ion concentration increased significantly with increase of PCP degradation. The liberation of chloride ion from the medium can be considered as the result of mineralization of chlorinated compounds by the bacterium thereby release of chloride in the medium. Available data of earlier studies indicated that chlorinated phenols are mineralized to chlorine free end products (Homada et al. 1987; Radehaus and Schmidt 1992; Mohn and Kennedy 1992).

In the present study the bacterial strain CL2 was able to grow and remove the PCP more than 87% in 600 mg/l indicating the efficiency *Kocuria* sp. CL2 to remove PCP at higher concentrations. Apart from PCP, this strain also capable of degrading 2,4,5-Trichlorophenol and 2,3,4,6-Tetrachlorophenol (unpublished data). Numerous studies have evaluated the biodegradability of PCP in soil and compost to clean up the chlorinated phenols. Though in situ remediation of PCP studies was reported previously, they mainly deal with compost, manure and soil (Miethling and Karlson 1996; Laine and Jorgensen 1997). Very few reports are available with removal of PCP from the sludge. In the present study, CL2 mineralized 58.64% PCP from the sludge where the PCP concentration was more than 100 mg/l.

In conclusion, the results obtained by this study indicated that the bacterial strain CL2 is able to mineralize high concentrations of PCP. This strain also degraded 58.64% of PCP from the sludge within 2 weeks of treatment. These results highlight the potential of this bacterium to be used in bioremediation of high strength PCP contaminated pulp and paper mill sludge.

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Characterization of pentachlorophenol degrading *Bacillus* strains from secondary pulp-and-paper-industry sludge

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ABSTRACT

Pentachlorophenol (PCP) degrading *Bacillus* strains CL3, CL5, and CL11 were isolated from the secondary sludge of a pulp paper mill and characterized. These isolates were identified as *Bacillus megaterium*, *Bacillus pumilus*, and *Bacillus thuringensis* based on their 16S rRNA sequence analysis. These isolates were able to grow and utilize PCP as a carbon and energy source. HPLC analysis and stoichiometric release of chloride in the medium confirmed the degradation ability of these isolates. The removal efficiency of PCP by these bacterial isolates was highly significant and they were able to degrade more than 90% of PCP when grown at high concentration of PCP (600 mg l^{-1}). Consortia of these isolates removed 77% of PCP from the sludge. The removal efficiency of PCP by the bacterial isolate used in this study was found to be more efficient than what has been reported with other *Bacillus* species. These results suggest that the bacterial isolates are very effective PCP degraders and can be used in remediation of PCP-contaminated sites.

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1. Introduction

Chlorophenolic compounds are common environmental contaminants, mainly due to their use as wide-spectrum biocides in industry and agriculture, their formation during pulp bleaching, and direct industrial waste discharge (Vallecillo et al. 1999). The most common sources of chlorinated phenols in the environment include production of chlorine from the bleaching of pulp, combustion of organic matter, partial transformation of phenoxy pesticides such as 2,4-dichlorophenoxyacetic acid and 2,4,6-trichlorophenoxyacetic acid, treatment of wood against fungi and insects, and preservation of rawhides in leather tanning industries (Shukla et al. 2001). The toxicity of these compounds tends to increase with their degree of chlorination (Fetzner and Lingens 1994). Among chlorinated phenols, pentachlorophenol (PCP) has been widely used as a wood and leather preservative owing to its toxicity toward bacteria, mould, algae, and fungi (Kaoa et al. 2004). Pentachlorophenol is toxic to all forms of life since it is an inhibitor of oxidative phosphorylation (Shen et al. 2005). Moreover, it is recalcitrant to degradation because of its stable aromatic ring and high chloride content, thus persisting in the environment (Copley 2000). This compound is listed among the priority pollutants of the U.S. Environmental Protection Agency (EPA 1987).

Chemical and physical-based clean-up methods have some disadvantages, including their high costs and the possibility of causing secondary pollution. The biodegradation of PCP has been studied in both aerobic and anaerobic systems. Several bacterial strains capable of degrading PCP under aerobic conditions have been reported, principally members of the genus *Pseudomonas* sp., *Sphingomonas chlorophenolica*, *Rhodococcus chlorophenolicum*, *Flavobacterium* sp., *Arthrobacter* sp., and *Corynebacterium* sp. (Field and Sierra-Alvarez 2008, and references therein). Very few PCP-degrading *Bacillus* have been reported (Chandra et al. 2006). In this investigation, we have isolated and characterized three *Bacillus* strains that are capable of degrading high concentrations of PCP.

2. Material and methods

2.1. Isolation and enrichment

Pulp and paper mill secondary sludge samples were collected from the M/s Shree Gopal Unit (BILT) Yamunanagar, Haryana, India. The samples were collected into pre-sterilized 250-ml conical flasks and immediately transferred at 4 °C. The potential PCP-degrading bacterial strains were isolated by the serial dilution technique, and purified by repeated streaking on nutrient agar

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plates. Colonies appearing after incubation at 37 °C for 48 h were selected for further screening. Selection of PCP-tolerant bacterial strains was done in mineral salt medium (MSM; Dams et al. 2007) supplemented with 50 mg l^{-1} (0.19 mM) PCP as the sole carbon source. The composition of MSM (in mg l^{-1}) was KH_2PO_4, 800; Na_2HPO_4, 800; $MgSO_4 \cdot 7H_2O$, 200; $CaCl_2 \cdot 2H_2O$, 10; NH_4Cl, 500; and 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; $CoCl2 \cdot 6H_2O$, 0.5; $ZnCl_2$, 0.25; and EDTA, 2.5. PCP was added to the medium after autoclaving. The pH was adjusted to 7.3 ± 0.2 prior to autoclaving. Three potent isolates were selected for the degradation studies. These strains were designated as CL3, CL5, and CL11.

2.2. Morphological and biochemical characterization

The biochemical characterization of the three isolates was done in accordance with *Bergey's Manual of Systematic Bacteriology* (Holt et al. 1994). Each pure culture was subjected to microscopic examination for morphological analysis. Capsule stain was determined with 20% copper sulfate. Catalase activity was determined based on formation of bubbles in the presence of 3% H_2O_2 solution. Oxidase was performed on paper discs using tetramethyl-*p*-phenylenediamine. Nitrate reductase was detected on nitrate agar plates and DNase on DNase agar medium with methyl green as an indicator. Starch hydrolysis was demonstrated from clearing zones formed around the colonies grown on starch containing agar. Motility, indole formation, and urease activities were determined by using an MIU-media kit (Hi-Media Laboratories, India). Antibiotics profiling was performed with an ICOSA universal-1 kit (Hi-Media Laboratories, India).

2.3. Amplification of 16S rRNA genes and sequence analyses

Genomic DNA was extracted according to Kapley et al. (2001) from overnight grown cultures. 16S rRNA genes were amplified from 1 μl purified genomic DNA using a GeneAMP 2700 PCR (Applied Biosystems, USA) with the following set of primers: forward: 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse: 5′-ACGGGCG GTGTGTTC-3′ as described by Weisburg et al. (1991). Reaction mixture for the PCR contained 1X PCR buffer, 200 μM of dNTPs, 1.5 mM $MgCl_2$, 0.1 μM of each primer, and 2.5 units of Taq DNA polymerase (Invitrogen, USA) in a final volume of 100 μl. The PCR was performed with an initial denaturation at 92 °C for 2 min followed by 36 temperature cycles as follows: 92 °C for 1 min, 48 °C for 30 s, 72 °C for 2 min, and a final extension at 72 °C for 6 min. The amplicons were purified with the QIA gel extraction kit (Qiagen, USA), and ligated into the pGEM-T easy vector as per the manufacturer's instructions (Promega Inc., USA). Ligated plasmids were transformed into *Escherichia coli* DH5α cells. Recombinant plasmids were sequenced using an Applied Biosystems automated DNA sequencer (DNA Sequencing facility, Delhi University, India). The sequences were compared against the available DNA sequences in GeneBank (http://www.ncbi.nlm.nih.gov/) using the BLASTN tool. The sequences were aligned using the MultAlin (http://bioinfo.genotoul.fr/multalin/multalin.html) program and the alignment was manually corrected and a phylogenetic tree was constructed using the MEGA 4.0 (Tamura et al. 2007) software.

2.4. Degradation studies

The degradation studies were performed by inoculating strains CL3, CL5, and CL11 at 1% inoculum (10^6 CFU ml^{-1}) in 250-ml Erlenmeyer flasks containing 50 ml of MSM supplemented with 100 mg l^{-1} (0.37 mM) of PCP. The flasks were incubated at 37 °C at 120 rpm for up to 168 h. Growth was determined by measuring the OD_{600nm}, and PCP depletion was quantified by HPLC by sampling 1 ml of culture at 24-h intervals. Chloride ion concentrations in cultures were measured at 24-h intervals up to 168 h using a chloride ion-specific electrode (Orion ion analyzer model 940) that was standardized against a wide range of known chloride standards in MSM.

HPLC was carried out on a Perkin Elmer Series 200 system. The cell suspension was centrifuged (8000 rpm, 5 min) and the supernatant was filtered through a 0.22-μm filter. Ten μl samples were injected on a reverse phase Lichrosphere^R 100 RP-18 endcapped column (250 mm × 4.6 mm i.d.). The column was eluted in an isocratic mode using aqueous methanol (90%, v/v) at a flow rate of 1 ml min^{-1}. PCP was monitored at 280 nm with an online diode array detector (series 200). PCP was quantified from standard curve prepared by injecting known quantities of PCP (Sigma Aldrich, USA) on the same column.

The effect of PCP concentration on the growth of strains CL3, CL5, and CL11 and on their PCP-degrading ability were also examined. The isolates were inoculated to 250-ml Erlenmeyer flasks containing 50 ml of MSM supplemented with different concentrations (50, 100, 200, 400, and 600 mg l^{-1}, corresponding to 0.19 mM, 0.37 mM, 0.75 mM, 1.5 mM, and 2.2 mM) of PCP separately. The flasks were incubated at 37 °C with shaking for 168 h. The growth of the bacterial cells was measured at OD_{600nm} and the degradation of PCP in the culture by HPLC as described above.

The effect of pH on the degradation of PCP was studied by growing each strain at an initial pH of 7.5, 8.5, or 9.5 in MSM supplemented with 100 mg l^{-1} of PCP as the sole carbon source. The pH of the medium was adjusted with NaOH or HCl. The influence of temperature on PCP degradation was also determined by incubating the samples at 25 °C, 30 °C, or 37 °C under shaking.

2.5. Degradation studies of PCP in sludge

The degradation ability of a consortium comprised of strains CL3, CL5, and CL11 was analyzed in 2.5-l conical flasks containing 1 liter of sludge supplemented with 100 mg l^{-1} of PCP and inoculated with 5% of inoculum. The flasks were incubated at 37 °C under shaking for two weeks. PCP was extracted from the sludge according to Chandra et al. (2006). The sludge was sonicated and acidified to pH 2.0 with 1 N HCl. It was then extracted three times with an equal volume of ethyl acetate by intermittent shaking for 30 min in separating funnels. The organic layer was dried over anhydrous sodium sulphate. Filtered samples were evaporated under vacuum at 40 °C, subsequently resuspended in 1 ml of methanol, and injected on an HPLC to quantify the remaining PCP.

2.6. Statistical analysis

Data were subjected to analysis of variance using ANOVA software and the averages were compared by the Tukey–Kramer Multiple Comparison Test at $p < 0.05$. Three replicates were prepared for each treatment. All the analyses were performed using GraphPad Prism (v 4.03) software.

3. Results

3.1. Isolation, characterization, and identification

Three bacterial isolates, CL3, CL5, and CL11, were selected based on their ability to grow on PCP-amended medium. The biochemical characterization results are presented in Table 1.

The 16S rRNA gene sequences determined in this study have been deposited in GenBank of NCBI database under the accession numbers EU784650, EU784652, and EU784658 for CL3, CL5, and CL11, respectively. A BLAST search revealed that CL3 is closely

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Table 1
Biochemical characterization of pentachlorophenol-degrading *Bacillus* strains isolated from the secondary sludge of pulp and paper industry.

Test	CL 3	CL 5	CL 11
Gram stain	+	+	+
Capsule stain	−	−	−
Motility	+	+	+
Catalase test	+	+	−
DNase test	−	−	−
Oxidase test	+	+	+
Nitrate Reduction test	+	±	−
Indole test	+	−	+
Urease test	+	−	+
Starch Hydrolysis	+	+	+
Antibiotic Sensitivity	Ceftazidime	Co-Trimoxazole, Cephadroxil, Cephadroxil, Penicillin	Roxithromycin, Co-Trimoxazole, Cefaclor, Cephadroxil, Penicillin, Ampicillin/sublactam

+, Positive; −, negative and ±, weak positive.

related to *Bacillus megaterium*, CL5 to *Bacilluspumilus*, and CL11 to *Bacillus thuringensis.*

3.2. Degradation studies

All three isolates were able to grow and utilize PCP as an energy source. The growth of CL3 increased significantly up to 120 h and declined later, whereas growth of CL5 increased up to 144 h and reached a stationary phase. The growth of CL11 increased significantly up to 96 h and decreased thereafter (Fig. 1A, B, C). Degradation of PCP increased with time for all the isolates. All the isolates were able to completely remove PCP from the medium within 168 h (Fig. 1A, B, C). The fact that chloride is released in the medium during growth is an evidence for PCP mineralization (Fig. 2).

Strain CL5 reached higher OD_{600nm} values than did other isolates. The growth decreased as the concentration of the PCP increased in the medium. All these isolates were able to degrade more than 90% of PCP at a concentration of 400 mg l^{-1}. Strains CL3 and CL11 were able to remove slightly less PCP (up to 80%) when grown at 600 mg l^{-1} compared to strain CL5, which was able to remove 91% of PCP at the same concentration (Fig. 3A, B, C). These results show that all the three *Bacillus* isolates have the ability to degrade the PCP at high concentrations (600 mg l^{-1}).

The removal of PCP by these isolates was tested at pH values of 7.5, 8.5, and 9.5 and at an initial concentration of 100 mg l^{-1} PCP. The growth of strain CL5 was significantly higher at all the pH levels tested compared to strains CL3 and CL11. All isolates removed 90% of PCP between pH 7.5 to 8.5, and the PCP removal efficiency was significantly decreased when the initial pH of the medium was 9.5 with CL3 and CL11. The PCP degradation efficiency of strain CL5 was higher at pH 8.5 and 9.5 compared to 7.5 (Fig. 4A). Removal of PCP was less efficient at 25 °C than at 30 °C or 37 °C. The growth of the CL5 isolate increased as the temperature increased, and the maximum growth was recorded at 37 °C. The PCP degradation was greater at 30 °C and 37 °C than at 25 °C but the removal was similar at both 30 °C and 37 °C (Fig. 4B).

3.3. PCP removal in sludge

Removal of PCP in the sludge was tested by inoculating the three isolates together into a sludge. The initial PCP concentration in the sludge was 0.029 mg l^{-1} and an additional 100 mg l^{-1} of PCP was amended to the sludge before examining the degradation potential

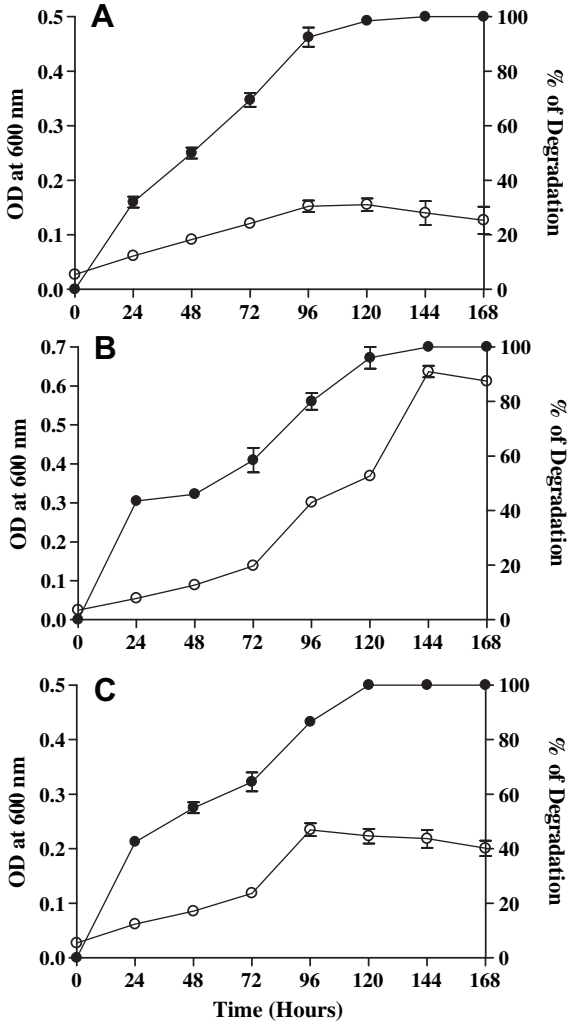

Fig. 1. Growth and PCP degradation of (A) *Bacillus megaterium* CL3, (B) *B. pumilus* CL5, and (C) *B. thuringensis* CL11 isolates grown in the presence of 100 mg l^{-1} (0.37 mM) of PCP. No growth or PCP removal was found in uninoculated control samples. Open circle represents the growth and filled circles PCP degradation. Error bars represent the standard deviation.

of the consortium in sludge. An HPLC chromatogram of control and experimental samples shown in Fig. 5 reveals that the consortium was able to remove 77% of PCP from the sludge within a two-week period.

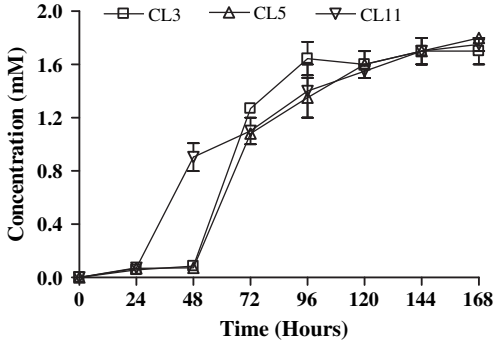

Fig. 2. Release of chloride ion in the medium during degradation of PCP (100 mg l^{-1} (0.37 mM) by *B. megaterium* CL3, *B. pumilus* CL5, and *B. thuringensis* CL11 at different time intervals. The initial chloride ions present in the medium (control) were normalized to zero. Error bars represent the standard deviation.

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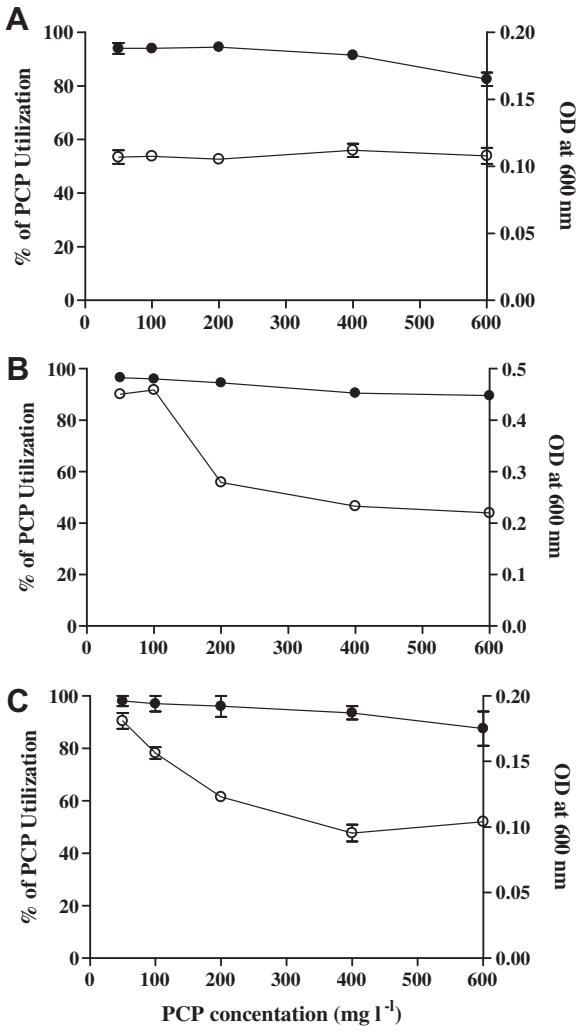

Fig. 3. Effect of different concentrations of PCP on the growth and utilization of PCP by (A) *B. megaterium* CL3, (B) *B. pumilus* CL5, and (C) *B. thuringensis* CL11. The OD_{600nm} values were recorded at 168 h. Open circle represents the growth and filled circles the PCP degradation. Error bars represent the standard deviation.

4. Discussion

A large variety of bacteria are known that can utilize chlorophenols as a carbon and energy source under aerobic conditions. Two main strategies are used to degrade chlorophenols by aerobic bacteria, utilizing these compounds as a carbon and energy source (Solyanikova and Golovleva 2004). Lower chlorinated phenols (1 to 2 chlorine substituents) are initially attacked by monooxygenases, yielding chlorocatechols as the first intermediates (chlorocatechol pathway), which are subject to ring cleavage prior to dechlorination. On the other hand, polychlorinated phenols (3 to 5 chlorines) are converted to chlorohydroquinones as the initial intermediates (hydroquinone pathway). Subsequent reactions progressively remove chlorines from the ring prior to ring cleavage.

The batch culture experiments performed revealed that PCP was biodegraded with stoichiometric release of chloride anions by the CL3, CL5, and CL11. The simultaneous release of chloride ions also corroborated the previous findings of dechlorination during PCP degradation as described by Mohn and Kennedy (1992). Chandra et al. (2006) reported that *Bacillus cereus* $ITRCS_6$ degraded 67% of 300 mg l^{-1} PCP after 168 h. The aerobic bacterial strain *Serratia marcescens* could utilize up to 300 mg l^{-1} PCP within 168 h (Singh

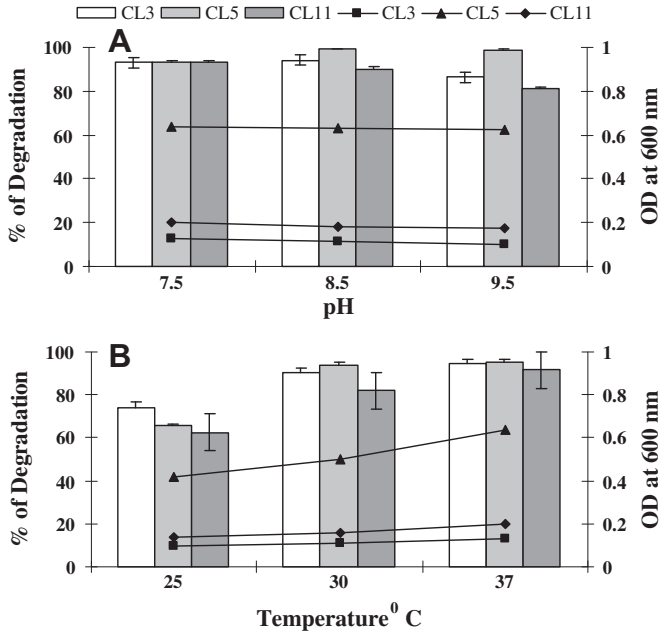

Fig. 4. Effect of different (A) pH, and (B) temperature on the growth and degradation efficiency of the *Bacillus* isolates. Bars represent the degradation and lines represent the growth of the isolates. Error bars represent the standard deviation.

et al., 2007). Shah and Thakur (2002) have observed that *Pseudomonas fluorescens* could degrade PCP up to 100 mg l^{-1}. The *Bacillus* isolates from the present study tolerated 600 mg l^{-1} of PCP and also degraded more than 90% of it. Therefore, the removal efficiency of PCP by the *Bacillus* isolates used in this study is more efficient than what has so far been reported in the literature.

Bacterial degradation of PCP is affected differently by the pH. Yang et al. (2006) reported that *S. chlorophenolica* removed 90% of PCP when the initial pH was 9.2 and that it could not remove PCP when the pH value was below 6.0. Barbeau et al. (1997) found that bacterial activity was apparently reduced when the pH was less than 6.0. Edgehill (1994) also found that the growth rate for *Arthrobacter* species at pH 7.4 was higher than under acidic conditions in the presence of PCP. Our results also indicated that PCP degradation was high for pH values ranging between 7.5 and 8.5, but strain CL5 showed higher degradation ability between pH 8.5 and 9.5 than at pH 7.5.

Temperature is another important environmental factor that may influence the rates at which pollutants are degraded (Trevors 1982) through altering both microbial activity and the physical and

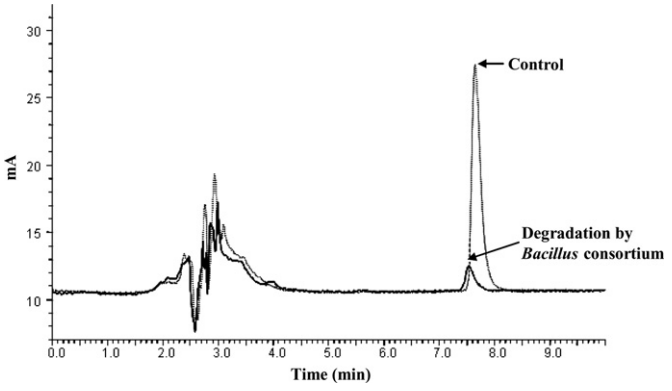

Fig. 5. HPLC chromatogram showing PCP degradation in sludge by the consortium of *Bacillus* species.

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chemical properties of pollutants (Providenti et al. 1993; Miller et al. 2004). Crawford and Mohn (1985) showed significant removal of PCP between 24 and 35 °C by *Flavobacterium* sp., but removal was ineffective below 12 °C or above 40 °C. Our results also show that at lower temperature the removal was less efficient than at 37 °C.

In-situ remediation of PCP released into the environment may undergo transformation by natural chemical or biological processes. Biodegradation is a significant process under aerobic conditions. PCP is stable to hydrolysis and oxidation, but the compound can be metabolized by microorganisms. Treatment of PCP-contaminated wastewater has been performed successfully in pilot-scale and field studies by aerobic organisms such as *Flavobacterium* species and *Rhodococcus* species (Salkinoja-Salonen et al. 1983; Apajalahti and Sakinoja-Salonen1986). Aerobic PCP biodegradation products include both oxidized residues and less chlorinated derivatives (Davis et al. 1994). Some reports are also available with compost, manure, and soil (Laine and Jorgensen 1997). Reports related to the removal of PCP from sludge are scarce. In the present study, the consortium has shown potential to mineralize 77% PCP within two weeks for a PCP concentration slightly above 100 mg l^{-1}.

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