Thesis On

INVESTIGATION OF THE BIODEGRADATION OF 4-CHLOROBENZOIC ACID

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08MBC016

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INTRODUCTION

A few decades ago, halogenated organic compounds have not been considered to be a chemical hazard and were not taken seriously. Fowden was once quoted to say "Present information suggests that organic compounds containing covalently bound halogens are found only infrequently in living organisms." (Fowden *et al.*, 1968). However, in the past several years more than 3800 halosubstituted organic compounds, mainly containing chlorine or bromine but a few with iodine and fluorine, have been identified. Most of these compounds are produced by living organisms such as marine and terrestrial plants bacteria, fungi, lichens, seaweeds, sponges, corals, some higher mammals and also humans; or produced during natural abiotic processes such as volcanos, forest fires, and other geothermal processes (Gribble, 1996; Gribble 2003).

Halogenated organic compounds constitute one of the largest groups of environmental pollutants. Their use as well as misuse, in industry and agriculture, is responsible for the entry of these chemicals into the environment, resulting in environmental contamination. It is therefore necessary to consider all aspects of this biologically challenging group of compounds, including their production, biodegradation, assimilation, integration and their persistence in the environment.

Microorganisms have been found to have an impact on each of these processes and thus play an important role in the global cycling of halogenated compounds (Haggblom *et al*, 2003). Because of this, a large number of microorganisms have been investigated for their ability to degrade halocompounds. This has led to the detection and isolation of various dehalogenating enzymes (Dehalogenases) that catalyze the removal of halogen atoms, following different mechanisms and under aerobic or anaerobic conditions (van Pee and Unversucht, 2003). Recent advances in microbiology and molecular techniques shed light into the diversity and functioning of these dehalogenase enzymes in several different locations. Molecular approaches towards analysis of diversity and expression especially of reductive dehalogenase-encoding genes are providing an in-depth

ACKNOWLEDGEMENTS

"The Doubter is a true man of Science; he doubts only himself and his interpretations, but he believes in Science". These great words of Claude Bernard have been an immense source of inspiration for me, since the time I decided to make a career in Science, and eventually, make Science a way of life.

It gives me great pleasure in thanking **Dr. G. Naresh Kumar**, Director, Institute of Science, Nirma University, for giving us his valuable comments and suggestions, which motivated us and helped improve our project.

The person of extra-ordinary merit is my guide, **Dr. Shalini Rajkumar**, without whom this dissertation would not have been possible. Her valuable advice, discussions about my work and constructive criticism has been of great help to me. Also, I thank her for detailed reviewing during preparation of my thesis.

I am thankful to all the faculty members – **Dr. Sri Ram Shishadhari**, **Dr. Sarika Sinha**, **Dr. Nasreen Munshi**, and **Mr. Vijay Kothari** for their constant encouragement and support.

I would also like to thank **Mr. Sachin Prajapati**, **Mr. Hasit Trivedi**, and **Mr. Bharat Anand**, for their co-operation and patience. Thank you, Sachin sir, for always willing to help fix anything that would break in the lab!

I would also like to thank all the **Ph.D. scholars** of our institute, particularly Mr. Rahul Jog, Mr. Maharshi Pandya and Mr. Mahendrapal Singh Rajput, for their guidance and help during troubleshooting.

I would like to make a special reference to **Prof. M. D. Shukla** of M.G. Science Institute, Ahmedabad, for providing us with his isolate of *Pleurotus ostreatus*. Sir, I immensely

thank you for your gift. I would extent my gratitude towards **Mr. Rushi Bhatt** of St. Xavier's College, Ahmedabad, for his generous help and support.

I would like to thank my close friend and project partner, **Rima M. Sakhpara**, for always being a constant source of support and without whom this project would not have been possible.

I thank my friends **Vinita**, **Khushboo** and **Abhishikha** for their moral support and understanding. I really enjoyed working with them and will always cherish the days spent with them in the lab.

I would like to thank my **parents** for their inspiration and encouragement in testing times, during the course of this project. Thank you, Amma and Appa, for bearing with me during all my ups and downs and for constantly telling me to never give up! No words are sufficient to express just how valuable your presence and support is for me, and I'm eternally grateful to you for everything you have given me.
And last, but certainly not the least, I would like to thank my best friend, **Aman**, for supporting me and having confidence in me and teaching me to see the brighter side of life when everything seemed lost. Thank you for always being there for me and for showing me the silver lining!

Gayatri R. Iyer

April 2010

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ABSTRACT

As part of our efforts in establishing bioremediation of a PCB metabolized dead end product, 4-Chlorobenzoic acid, we assessed the aerobic biodegradation of 4-Chlorobenzoic acid (4CBA) by three microorganisms viz. *Pseudomonas aeruginosa* MTCC 2297, *Bacillus subtilis* and *Pleurotus ostreatus*. The ability of these organisms to degrade 4CBA in liquid medium was improved by the use of continuous culture, repeated sequential subculturing and providing a combination of other carbon source. Each organism was observed to have different tolerance limit of the compound above which they could not grow. Tolerance limit in *Bacillus subtilis* was found to be 0.8 mg mL^{-1}, *Pseudomonas aeruginosa* had higher tolerance limit of 4.8 mg mL^{-1}, while it was found to be much higher, 10 mg mL^{-1}, in *Pleurotus ostreatus*. *P. aeruginosa* effectively degraded 4CBA in concentrations of 3, 3.5, 4 and 4.5 mg mL^{-1} at a rate of 0.028, 0.037, 0.049 and 0.051 mg h^{-1} respectively, while *B. subtilis* degraded 4CBA in the concentrations of 0.2, 0.4, 0.6 and 0.8 mg mL^{-1} at a rate of 0.012, 0.027, 0.041 and 0.048 mg h^{-1} respectively. The metabolite obtained from the degradation was 4-Chlorocatecol (4CC), suggesting that the pathway involved in degradation is most likely to be Oxygenolytic Dehalogenation. The fungal stain showed a clear zone of disappearance of 4CBA on Potato Dextrose Agar plates till a concentration as high as 10 mg mL^{-1}. Quantitative estimation of its degradation was not determined due to its prolonged incubation time.

knowledge on such biodegradative pathways. However, several known halogenated compounds are resistant to microbial attack and persist in the environment to become major pollutants of the biosphere and ground water and the rate of biodegradation of these compounds is largely determined by the rate of cleavage of the carbon-halogen bond (Curragh *et al*, 1994).

One of the most important classes of halogenated organic compounds is Polychlorinated Biphenyls (PCB) that contains 1 to 10 chlorine atoms linked to a biphenyl moiety. They have a low degree of reactivity, are not flammable, have high electrical resistance, good insulating properties and are very stable even when exposed to heat and pressure. They are therefore, used as coolants and insulating fluids in transformers and capacitors, plasticizers in paints and cements, pesticides, fungicides, herbicides, hydraulic fluids, lubricating oils, wood floor finishes, paints, fixatives in microscopy, surgical implants, and many others. One of major advantages, but ecologically speaking, a disadvantage, of PCB is their high thermal and chemical stability, which renders them resistant to heat or chemical degradation. Consequently, PCBs are one of the most persistent organic pollutants in the atmosphere, on a global scale. PCB have been know to cause a number of skin, respiratory, and liver disorders in humans as well as animals and have also found to be potential carcinogens. Therefore, PCBs are becoming a major cause of concern and the steps undertaken for their elimination from the environment becomes crucial. Biodegradability is related to the amount of chlorination of a specific PCB congener – higher the chlorine content, lesser is the biodegradability. The lack of degradability of PCB compounds results in bioaccumulation of PCBs in the environment.

4-Chlorobenzoic acid (4CBA) is the partial degradation product of 4-Chlorobiphenyl, an important congener of the PCB class of compounds. Moreover, 4CBA may also enter the environment directly because of its use as herbicides, fungicides, and in the manufacture of various dyes. As a result of natural production and contamination of the environment by xenobiotic compounds such as 4CBA, halogenated substances are widely distributed in the biosphere. Concern therefore arises as a result of the toxic, carcinogenic, and potential teratogenic nature of these substances (Hardman, 1991). Biodegradation studies

on 4CBA have focused mainly on the genes and enzymes that permit the degradation of 4-chlorobenzoic acid (4CBA) or 4-chlorobenzoate, by a variety of pathways, depending on the microorganism under study.

Under aerobic conditions, 4-Chlorobenzoic acid can be readily degraded to 4-Hydorxybenzoic acid (4HBA) by dehalogenation in *Psuedomonas* sp. (Adriaens *et al*., 1989), *Arthobacter* sp. (Adriaens *et al*., 1989; Schmitz *et al*., 1992) and *Alcaligenes* sp. (Layton *et al*., 1992). 4HBA is further utilized as carbon and energy source by aromatic ring-fission processes (Babbitt *et al*., 1992; Zylstra *et al*., 1989). It may also be transformed to 4-Chlorocatechol (4-CC) by benzoate dioxygenation in *Psuedomonas* spp. (Schlomann *et al*., 1990). 4-CC can be further degraded via *ortho*-cleavage or *meta*--cleavage of the aromatic ring (Layton *et al*., 1992; Schlomann *et al*., 1990). The *meta*--cleavage product of 4-CC, 5-chloro-2-hydroxymuconic semialdehyde (5C-2HMS), may be transformed to 5-chloro-2-hydroxymuconic acid (5C-2HMA) by 5C-2HMS dehyrogenase in *Psuedomonas cepacia*, and then to Chloroacetic acid, which is ultimately utilized as carbon and energy source (Arensdorf *et al*., 1995; McCullar *et al*., 1994). Hence by measuring the intermediates formed during the degradation process we can identify the pathway of metabolism of 4CBA.

Pseudomonas aeruginosa MTCC 2297 was chosen for our degradation studies since it is an isolate from a certain oil-spill and may have an implication in hydrocarbon-degradation, thus making it a potential degrader of 4CBA. Also, *Bacillus subtilis*, being a soil inhabitant, may also play an important role in the degradation of 4CBA.

Relatively little is known about the potential of fungi to degrade various types of PCBs. Degradation of PCBs by *Aspergillus niger* appeared to vary depending on the level of PCB chlorination (Dmochewitz *et al*., 1988; Murado *et al*., 1976). Also, a soil fungus, *Rhizopus japonicus*, has been reported to degrade 4-chlorobiphenyl and 4,4´-dichlorobiphenyl (DCB) (Wallnöfer *et al.*, 1973). In cases in which PCB degradation has been observed, little information concerning the degradative pathways has been provided.

The white rot fungus *Phanerochaete chrysosporium* has been examined for its ability to degrade many xenobiotic chemicals, including PCBs and investigations have shown that *P. chrysosporium* can degrade several PCB mixtures (Bumpus *et al.*, 1985; Thomas *et al.*, 1982; Zeddel *et al.*, 1993). A mechanistic interpretation of the chlorination pattern effect in *P.chrysosporium* is prevented by a lack of information regarding the enzymatic basis of PCB degradation by this organism.

Pleurotus ostreatus, another white rot fungus, has been studied extensively for its ability to efficiently degrade lignin, which is a naturally occurring aromatic polymer (Hadar *et al.*, 1993; Hataka *et al*., 1994; Karem *et al*., 1992). This capacity is assumed to result from the activities of lignin peroxidases, manganese peroxidases (MnPs), other oxidases, and laccases (Hataka *et al*., 1994; Thurston, 1994). These studies prompted the suggestion that the same fungus may have the capability to degrade ubiquitous pollutants like 4CBA.

Looking at the highly toxic and potential carcinogenic effects of 4CBA, its complete degradation is crucial to achieve detoxification. Techniques such as natural attenuation and engineered approaches can be used to check the biodegradation potential of various other microorganisms. Moreover, microorganisms that have been found to inherently play a role in the natural dechlorination cycle can be used as part of bioremediation steps. With the right microorganisms as well as monitoring tools, successful bioremediation techniques may be devised.

This project, therefore, aims to achieve the following objectives:

- To screen the microorganisms for their ability to degrade 4-Chlorobenzoic acid.
- To determine the rate and extent of degradation of 4CBA by these organisms.
- To attempt to identify key metabolites produced during the degradation.

Halogenated organic compounds are one of the largest groups of environmental pollutants because of their widespread use in herbicides, insecticides, fungicides, solvents, hydraulic and heat transfer fluids, plasticizers, intermediates for chemical syntheses etc. The distribution of these compounds in the biosphere is a major public concern because of their toxicity, bioconcentration, and persistence. An important problem is the diversity of these toxic compounds and by-products in many formulations of industrial products (Sussane *et al*., 1994). Thus mixtures of polychlorinated biphenyls (PCBs) or chlorinated terpenes, poly-chloroterphenyls, and polychloroquaterphenyls with formulations that may contain up to 105 different isomers apply to this problem.

The structures of more than 200 naturally occurring halogenated compounds have already been reported (Siuda *et al*., 1973). More than 700 naturally occurring halogenated products have been identified but the list probably is far from complete. Halogenated compounds are important in the adaptation of microorganisms in order to utilize halogenated xenobiotics.

Volatile halogenated organic compounds are released into seawater by temperate marine macroalgae. Many iodo- and bromo- substituted alkyl mono- and di-halides are found in abundance here. Thus it was concluded that the macroalgae are a major source of volatile organobromines released into the atmosphere (Gschwend *et al*., 1985).

Many widespread species of fungi have been found to ubiquitously produce large amounts of chlorinated anisyl metabolites in their natural environments (Ed-de Jong *et al*., 1984).

The biological recalcitrance of halogenated compounds is related to the number, type, and position of the halogen substituents. The carbon-halogen bond is very much regarded as intractable with increased electronegativity of the substituent. Also halogenated

substances having one or few substituents are more readily degradable than the corresponding polyhalogenated compounds. The biochemical and genetical studies of microbial dehalogenases would help us to understand and evaluate the potential for degradation of halogenated xenobiotic in microorganisms and in natural environment. Such studies might provide biotechnological solutions to tackle environmental problems by creating genetically engineered microorganisms. Such genetically engineered microorganisms might be useful for the treatment of effluents and decontamination of polluted soil or groundwater (Sussane *et al*., 1994).

Moreover, dehalogenases are also used as industrial biocatalysts to provide valuable intermediates for chemical syntheses. Novel intermediates and products can be synthesized by the biotransformation of various halogenated organic compounds by microbial or enzymatic biocatalysts, since these biocatalysts possess regiospecificities and chiral specificities that are difficult and expensive to achieve by conventional chemistry. Incomplete bacterial dechlorination can result in the accumulation of compounds that are more toxic and pose a greater health hazard than the original pollutant.

POLYCHLORINATED BIPHENYLS

Polychlorinated Biphenyls (PCB) are a class of organic compounds, containing 1 to 10 chlorine atoms linked to a biphenyl moiety. They have a low degree of reactivity, are not flammable, have high electrical resistance, good insulating properties and are very stable even when exposed to heat and pressure. They are therefore, used as coolants and insulating fluids in transformers and capacitors, plasticizers in paints and cements, pesticides, fungicides, herbicides, hydraulic fluids, lubricating oils, wood floor finishes, paints, fixatives in microscopy, surgical implants, and many others.

One of major advantage, but ecologically speaking, a disadvantage, of PCB is their high thermal and chemical stability, which renders them resistant to heat or chemical degradation. Consequently, PCBs are one of the most persistent organic pollutants in the atmosphere, on a global scale.

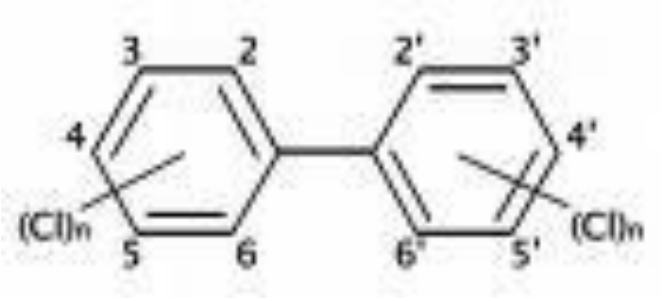

Figure 1: Polychlorinated Biphenyls (PCB)

PCB have been know to cause a number of skin, respiratory, and liver disorders in humans as well as animals and have also found to be potential carcinogens. Therefore, with the rapid industrialization across the world, PCBs are becoming a major cause of concern and the steps undertaken for their elimination from the environment becomes crucial.

The methods for the degradation of PCB have been broadly categorized as Physical, Chemical, and Biological methods. Of these, the biological method of degradation deals with the use of PCB as the sole source of carbon and energy by various microorganisms, or through the reductive dechlorination of PCB by the same. Biodegradability is related to the amount of chlorination of a specific PCB congener – higher the chlorine content, lesser is the biodegradability. The lack of degradability of PCB compounds results in bioaccumulation of PCBs in the environment.

The chemistry of halogenated organic compounds is due to the unique physicochemical properties of their halogen substituent (F, Cl, Br, or I). At the start of the series, the carbon-fluorine bond is very strong with high polarity. With increasing molecular weight of the halogen, carbon-halogen bond energies decrease markedly, i.e., F > Cl > Br > I.

Other characteristics, such as the electron-withdrawing effect of the halogen substituent impact chemical reactivity of the molecule and its heat transfer and dielectric characteristics (e.g., polychlorinated phenols; polychlorinated biphenyls, PCBs). The physical size and shape of the halogen substituent may also affect reactivity, due to steric constraints and may also hinder uptake into cells and enzymatic attack during biodegradation.

CHLOROBENZENES

Hexachlorobenzene (HCB) and other chlorobenzene congeners are also widespread pollutants with very low water solubility. Undefined cultures from a number of sources have appeared to dehalogenate HCB. Patterns of dehalogenation vary somewhat, but several generalizations are possible. HCB is most readily dehalogenated to 1,3,5-trichlorobenzene (Fathepure *et al*., 1988; Liang *et al*., 1991; Mousa *et al*., 1990). Further dehalogenation to monochlorobenzene is possible but apparently less readily catalyzed (Bosma *et al*., 1988; Liang *et al*., 1991). Monochlorobenzene appears to be stable under anaerobic conditions. Some variation in dehalogenation pathways can occur, and in some cases, as for PCB dehalogenation, variation is due to different inoculum sources. Together, these studies indicate that anaerobes are capable of the necessary steps for degradation of HCB to monochlorobenzene; however, this process has not been observed in a single system. Di- and Monochlorobenzenes can be mineralized aerobically (Schraa *et al*., 1986; De Bont *et al*., 1986; Spain *et al*., 1987; Van der Meer *et al*., 1987). Thus, as with PCBs, the proper sequence of environmental conditions may allow biological mineralization of all chlorobenzene congeners.

Chloroanilines are used in industrial syntheses and are degradation products of pesticides. Recent studies indicate that chloroanilines can be dechlorinated by organisms from aquifer material (Kuhn *et al*., 1989; Kuhn *et al*., 1990) and pond sediment (Struijs *et al*., 1989). In those studies, more highly chlorinated anilines were dechlorinated but monochloroanilines persisted. Chlororesorcinols are possible by-products of industrial

syntheses, and 4-chlororesorcinol has been shown to be reductively dechlorinated in anaerobic sewage sludge (Fathepure *et al*., 1987). The resorcinol product subsequently disappeared from enrichment cultures from the sludge, suggesting that anaerobic communities may be capable of mineralizing 4-chlororesorcinol. Chlorocatechols apparently result from the process of paper bleaching and other industrial processes and have recently been reported to be dehalogenated by enrichment cultures from marine sediments (Allard *et al*., 1991). Only certain chlorocatechol congeners were dehalogenated, and some variation in pathways was observed. In the first report of biological dehalogenation of a heterocyclic compound, Adrian and Suflita (1990) clearly demonstrated removal of bromine from the herbicide bromacil by aquifer slurries. No further evidence concerning the fate of the debrominated product was given. Chlorinated pyridines have now also been reported to dehalogenated by aquatic sediments (Liu *et al*., 1991).

Not all haloaromatic compounds are xenobiotic. The burrows of a hemichordate inhabiting a marine sediment were found to contain 2,4- dibromophenol (King *et al*., 1986). The worm apparently synthesizes the compound, which inhibits the growth of aerobic bacteria in the burrow. Subsequent investigation showed that an anaerobic microbial community from the sediment could first debrominate and then mineralize this compound (King *et al*., 1988). Red algae (Rhodophyta) also produce aryl halides. Marine sediments containing a red alga more rapidly dehalogenated various aryl halides than did sediments that apparently did not contain the alga (Paterek *et al*., 1991). This observation would be consistent with selection for a dehalogenating population during decomposition of the alga. Organochlorine compounds can also result from combustion of organic matter, as during forest fires (Ahling *et al*., 1982), and from natural transformations of humic matter in soil (Asplund *et al*., 1989). The existence of such naturally occurring compounds suggests that selective pressure for dehalogenation may have existed during the evolution of bacteria. Further investigation of the metabolism of such natural compounds will probably contribute greatly to our understanding of the metabolism of xenobiotic compounds.

4-CHLOROBENZOIC ACID

One of the major congeners of Polychlorinated Biphenyls is 4-chlorobiphenyl (4-CB). Biodegradation studies on 4-CB, a model for polychlorinated biphenyl degradation, have focused mainly on the genes and enzymes that permit the degradation of 4-CB to 4-chlorobenzoic acid (4CBA) or 4-chlorobenzoate, that may subsequently get oxidized to CO_2, depending on the microorganism under study. It may be noted here that the enzymes involved in the two-step degradation are encoded by separate sets of genes. Bacterial strains including *Arthrobacter, Alcaligenes, Pseudomonas,* and *Nocardia* sp. have been found to utilize 4CBA as a sole source of carbon and energy.

Halobenzoates themselves occur in the environment due to their use as herbicides (e.g. 2,3,6-trichlorobenzoic acid) and as partial degradation products of other xenobiotic compounds such as PCB. Many halogenated compounds are resistant to microbial attack and persist in the environment to become major pollutants of the biosphere and ground water. Moreover, the rate of biodegradation of these compounds is largely determined by the rate of cleavage of the carbon-halogen bond. (Curragh *et al*., 1994). Concern arises as a result of the toxic, carcinogenic, and potential teratogenic nature of these substances. (Hardman *et al.*, 1991).

Microbial biodegradation of a wide variety of chlorinated aliphatic compounds was shown to occur under five physiological conditions. However, any given physiological condition could only act upon a subset of the chlorinated compounds. Firstly, chlorinated compounds are used as an electron donor and carbon source under aerobic conditions. Secondly, chlorinated compounds are co-metabolized under aerobic conditions while the microorganisms are growing (or otherwise already have grown) on another primary substrate. Thirdly, chlorinated compounds are also degraded under anaerobic conditions in which they are utilized as an electron donor and carbon source. Fourthly, chlorinated compounds can serve as an electron acceptor to support respiration of anaerobic microorganisms utilizing simple electron donating substrates. Lastly chlorinated

compounds are subject to anaerobic co-metabolism becoming biotransformed while the microorganisms grow on other primary substrate or electron acceptor (Field *et al*., 2004).

The study of dehalogenating microorganisms is essential for their exploitation by humans but is also intriguing from a more fundamental scientific perspective. Studies of dehalogenation have contributed significantly to basic microbiology in the areas of ecology, physiology, and phylogeny. Dehalogenation of many compounds is known to occur only within mutualistic anaerobic microbial communities. Using undefined cultures, researchers are beginning to understand the ecology of these communities which occur in soils, sediments, intestinal tracts, and bioreactors. Studies of cell-free systems, including cell extracts, one purified enzyme, and several transition metal complexes, are beginning to lend insight into how and why microorganisms catalyze reductive dehalogenation.

DEHALOGENASES

Bacterial dehalogenases are enzymes that catalyze the cleavage of the carbon-halogen bond in halogenated compounds. There has been no evidence indicating the involvement of co-factors or metal ions in the catalytic mechanism. Dehalogenation generally makes xenobiotic compounds like 4CBA less toxic and more degradable. The majority of organisms capable of growth on halobenzoates (chlorobenzoates) metabolize the compounds via halocatechols (chlorocatechols), and dehalogenation occurs after ring cleavage, utilizing enzymes responsible for benzoate metabolism. Consequently, microorganisms capable of degrading 4CBA via 4-chlorocatechol intermediate have been reported. Another mechanism was later elucidated, in which the initial degradative step is the dehalogenation of 4CBA to yield 4-hydroxybenzoate (4HBA), which is subsequently metabolized.

The study of the biochemistry and genetics of microbial dehalogenases will therefore, help in understanding and evaluating the potential for degradation of xenobiotics in

microorganisms and in natural microcosms, in developing novel biotechnological solutions to deal with environmental problems, and in designing effective bioremediation systems. Useful bacterial strains have been constructed with more efficient catabolic activities that have been applied for the mineralization of environmental pollutants, for the treatment of industrial effluents or exhaust gases, and for the decontamination of polluted soil or groundwater. Moreover, dehalogenases have been as industrial biocatalysts to produce valuable intermediates for chemical synthesis.

DEHALOGENATION MECHANISM

The mechanism of action of dehalogenation varies from species to species, and has been categorized as: (Fetzner *et al*. 1998)

1. Reductive dehalogenation
2. Oxygenolytic dehalogenation
3. Hydrolytic halogenation
4. Thiolytic dehalogenation
5. Intramolecular substitution
6. Dehydrohalogenation
7. Hydration

Mechanistic studies on the hydrolytic dehalogenation catalyzed by dehalogenases are of importance for environmental and industrial applications. According to latest research, it is suggested that a Histidine residue (His272), polarized by Glutamate (Glu132), acts as a base, accepting a proton from the catalytic water molecule. The reaction proceeds through a metastable tetrahedral intermediate. In the formation of the products, the protonated aspartic acid (Asp108) can easily adopt conformation of the relaxed state found in the free enzyme. The overall free energy barrier of the reaction is equal to 19.5 ± 2 kcal. (Otyepka *et al*., 2008).

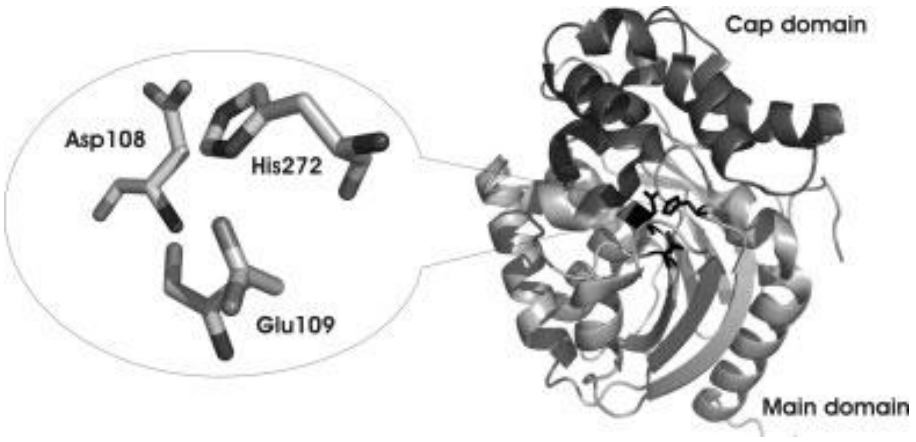

Figure 2: Structure of dehalogenase enzyme (LinB). The active site (left) consists of a nucleophile (Asp108), base (His272), and a second carboxylic acid (Glu132) is located between the cap domain (dark gray) and the main domain (light gray, right) (Otyepka *et al.*, 2008)

The mechanism of dehalogenation, as afore mentioned, largely depends on the organism under study. For example, in *Pseudomonas paucimobilis* UT26, gamma-hexachlorocyclohexane (gamma-HCH) is converted by two steps of dehydrochlorination to a chemically unstable intermediate, 1,3,4,6-tetrachloro-1,4-cyclohexadiene (1,4-TCDN), which is then metabolized to 2,5-dichloro-2,5-cyclohexadiene-1,4-diol (2,5-DDOL) by two steps of hydrolytic dehalogenation via the chemically unstable intermediate 2,4,5-trichloro-2,5-cyclohexadiene-1-ol (2,4,5-DNOL). (Nagata *et al.*, 1993).

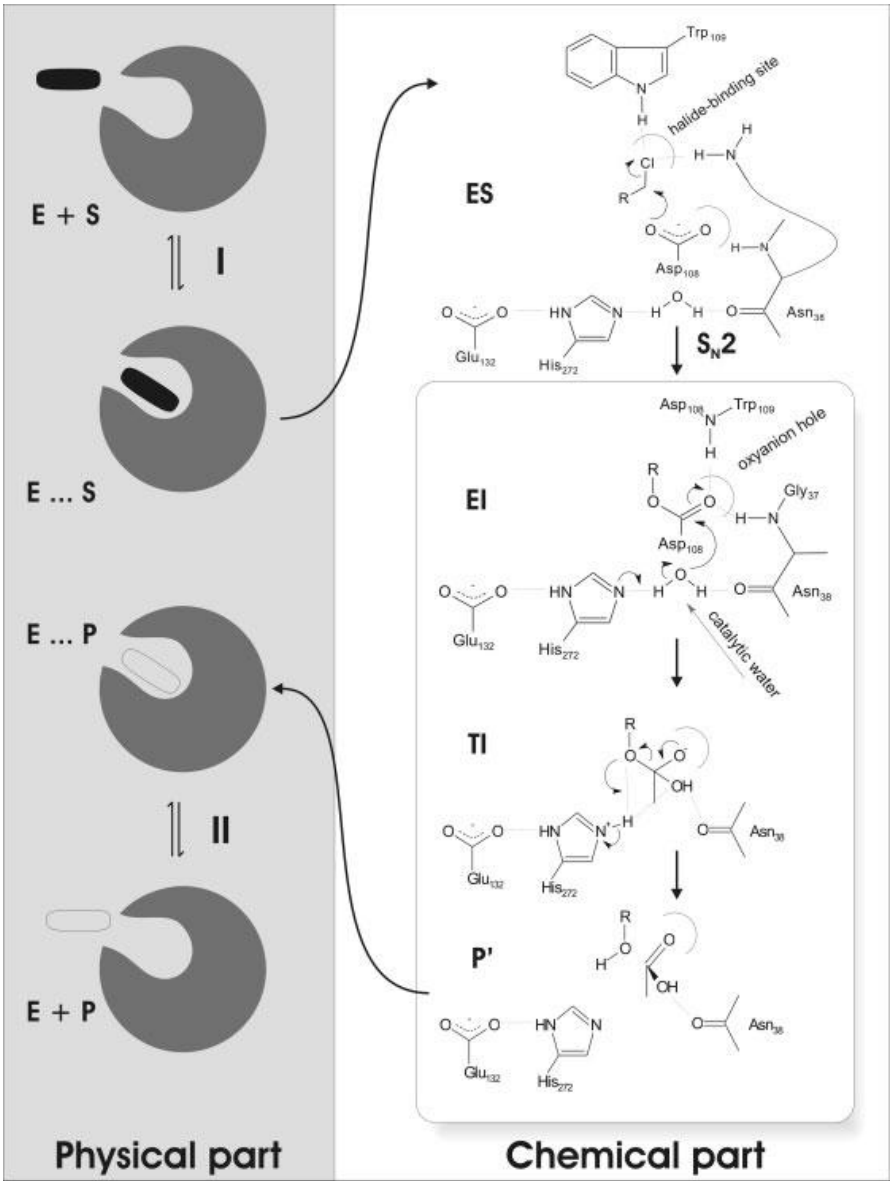

Figure 3: Hydrolytic dehalogenation catalyzed by dehalogenase. The reaction involves a ''physical step'' (left side, I) that is, enzyme-substrate complex formation (ES) followed by a two-step chemical process (right side). The first SN_2 step is followed by Ad_N hydrolysis of enzyme intermediate to give a tetrahedral intermediate decomposing to product of the reaction. This chemical reaction is followed by the second physical step, that is, product release (left side, II). Here we address in detail the Ad_N step (in rounded box). [Ester Intermediate (EI), Tetrahedral Intermediate (TI), Products (P)] (Otyepka *et al.*, 2008)

The carbon-halogen bond is cleaved either by enzymatic dehalogenation, where the carbon-halogen bond cleavage is catalyzed by specific enzymes (the dehalogenases), or by spontaneous chemical dehalogenation of unstable intermediates. In addition, there are enzymes which, because of their relaxed substrate specificity, catalyze the conversion of halogenated analogs of the corresponding unsubstituted substrate or of related compounds, which might lead to "fortuitous" dehalogenation of the substrate analog. Concerning the enzymatic cleavage of the carbon-halogen bond, seven mechanisms of dehalogenation are known so far:

a) **Reductive dehalogenation.** In the course of a reductive dehalogenation, the halogen substituent is replaced by hydrogen.
b) **Oxygenolytic dehalogenation.** Oxygenolytic dehalogenation reactions are catalyzed by monooxygenases (or dioxygenases), which incorporate one (or two) atoms of molecular oxygen into the substrate.
c) **Hydrolytic dehalogenation.** In the course of hydrolytic dehalo-genation reactions, catalyzed by halidohydrolases, the halogen substituent is replaced in a nucleophilic substitution reaction by a hydroxy group which is derived from water.
d) **"Thiolytic" dehalogenation.** In dichloromethane-utilizing bacteria, a dehalogenating glutathione S-transferase catalyzes the formation of a S-chloromethyl glutathione conjugate, with a related dechlorination taking place.
e) **Intramolecular substitution.** Intramolecular nucleophilic displacement yielding epoxides is a mechanism involved in the dehalogenation of vicinal haloalcohols.
f) **Dehydrohalogenation.** In dehydrohalogenation, HCI is eliminated from the molecule, leading to the formation of a double bond.
g) **Hydration.** A hydratase-catalyzed addition of a water molecule to an unsaturated bond can yield dehalogenation of vinylic compounds, such as 3-chloroacrylic acid, by chemical decomposition of an unstable intermediate.

4CBA is known to be readily degraded via several degradative pathways one of which is 4CBA is transformed to 4-hydroxybenzoic acid (4HBA) by dehalogenation in *Pseudomonas sp.* CBS3 (Babbitt *et al*., 1992), *Arthrobacter sp.* 4CB1 (Adriaens *et al*., 1989), *Arthrobacter sp.* SU1 (Schmitz *et al*., 1992), and *Alcaligenes sp.* A5 (Layton *et al*., 1992). 4HBA can be utilized as carbon and energy source via aromatic ring-fission processes (Zylstra *et al*., 1989). It is also transformed to 4-chlorocatechol (4CC) by benzoate dioxygenation in *Pseudomonas* strain B13 (Schlomann *et al*., 1990). 4CC can be further degraded via *ortho*-cleavage or *meta*-cleavage of the aromatic ring (McFall *et al*., 1997; Kasberg *et al*., 1995). The *meta*-cleavage product of 4CC, 5-chloro-2-hydroxymuconic semialdehyde (5C-2HMS), has been reported to be transformed to 5-chloro-2-hydroxymuconic acid (5C-2HMA) by 5C-2HMS dehydrogenase in *Pseudomonas cepacia* P166, and then to chloroacetic acid, which is ultimately utilized as carbon and energy source through TCA cycle (Arensdorf *et al*., 1994; McCullar *et al*., 1994).

These studies suggest that biodegradation might be an effective method for the removal of chlorobenzenes (4-Chlorobenzoic acid) accidentally released into the environment. Several practical concerns, however, must be addressed before biodegradation can be used in the field. First, it must be determined whether indigenous microorganisms can carry out the required transformations or whether selected strains or additional nutrients must be added. Knowledge, however, is limited concerning the indigenous microbial populations that metabolize chlorobenzenes in the environment. Stable isotope probing (SIP) coupled with metagenomics is one approach to more directly explore which organisms and genetic information may be involved in the degradation (Sul *et al.*, 2009). Second, the extent of biodegradation of the chemical must be evaluated. Mineralization must be distinguished from partial transformation. Third, the metabolic pathway should be determined to allow evaluation of whether toxic intermediates can be expected to accumulate (Nishino *et al*., 1992).

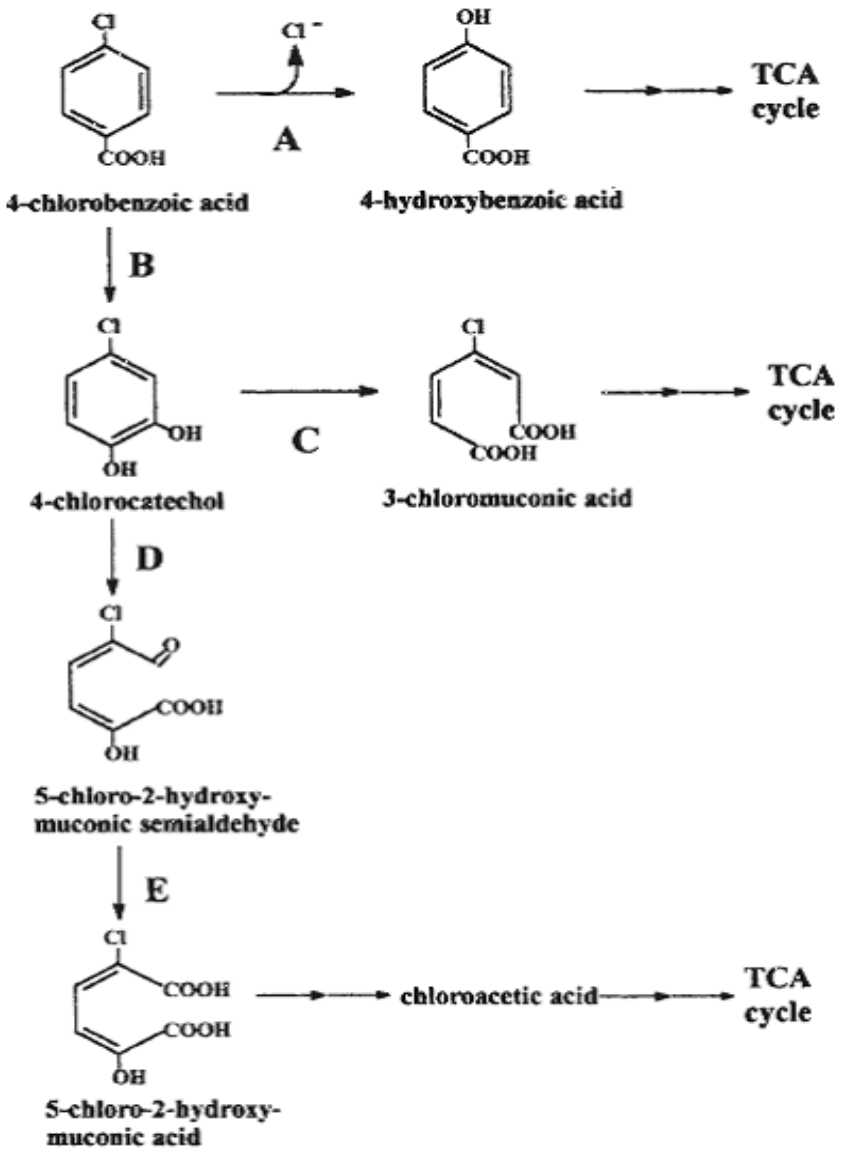

Figure 4: Pathways of degradation of 4-Chlorobenzoic acid

(Dong-In Seo *et al.*, 1997)

Mycobacterium strains isolated by their ability to grow on pyrene have often been shown to also utilize phenanthrene, fluoranthene, and high-molecular-weight alkanes as single carbon sources (Churchill *et al.*, 1999; Vila *et al.*, 2001). Recently, a *Mycobacterium* strain AP1 was isolated from an oil-polluted marine beach and was incubated with a mixture of PAHs. This strain caused a significant depletion of the three-aromatic-ring PAHs but had a limited action on the highermolecular- weight PAHs fluoranthene and

pyrene (Vila *et al*., 2009). Moreover, several new species of microorganisms capable of degradaing a variety of aromatic hydrocarbons are being isolated. *Thalassospira xianhensis* sp. nov., a marine bacterium has been isolated from an oil-polluted saline soil and shown to be an efficient degrader of PAHs (Zhao *et al.*, 2009). Similarly, *Sphingobium scionense* sp. nov., isolated from a contaminated soil has also been found to play a role in PAH-degradation (Liang *et al.*, 2010).

Pseudomonas aeruginosa

Pseudomonas aeruginosa is member of the Gamma Proteobacteria class of Bacteria, belonging to the bacterial family Pseudomonadaceae. *Pseudomonas aeruginosa* is a Gram-negative, aerobic rod measuring 0.5 to 0.8 µm by 1.5 to 3.0 µm. Almost all strains are motile by means of a single polar flagellum.
The bacterium is free-living and ubiquitous in soil and water, and on surfaces in contact with soil or water. Its metabolism is respiratory and never fermentative, but it will grow in the absence of O_2 if NO_3 is available as a respiratory electron acceptor.
The typical *Pseudomonas* bacterium in nature might be found in a biofilm, attached to some surface or substrate, or in a planktonic form, as a unicellular organism, actively swimming by means of its flagellum. *Pseudomonas* is one of the most vigorous, fast-swimming bacteria seen in hay infusions and pond water samples.
Pseudomonas aeruginosa has very simple nutritional requirements. It is often observed "growing in distilled water", which is evidence of its minimal nutritional needs. *Pseudomonas aeruginosa* has a predilection for growth in moist environments, which is probably a reflection of its natural existence in soil and water. In the laboratory, the simplest medium for growth of *Pseudomonas aeruginosa* consists of acetate as a source of carbon and ammonium sulfate as a source of nitrogen.
P. aeruginosa possesses the metabolic versatility for which pseudomonads are so renowned. Organic growth factors are not required, and it can use more than seventy-five organic compounds for growth. Its optimum temperature for growth is 37 degrees, and it is able to grow at temperatures as high as 42 degrees. It is tolerant to a wide variety of

physical conditions, including temperature. It is resistant to high concentrations of salts and dyes, weak antiseptics, and many commonly used antibiotics. These natural properties of the bacterium undoubtedly contribute to its ecological success and help explain the ubiquitous nature of the organism.

P. aeruginosa isolates normally produce three colony types. Natural isolates from soil or water typically produce a small, rough colony. A second type has a fried-egg appearance which is large, smooth, with flat edges and an elevated appearance. And a third type has a mucoid appearance, which is attributed to the production of alginate slime. The smooth and mucoid colonies are presumed to play a role in colonization and virulence.

Pseudomonas aeruginosa has been extensively worked upon for its ability to utilize and degrade various aromatic hydrocarbons and xenobiotics. *P. aeruginosa* has the capacity to degrade crude petroleum-oil (Zhang *et al*., 2005), chloromethane (Freedman *et al*., 2004), benzene, toluene, xylene (Kahraman *et al*., 2005), n-Hexadecane (Chayabutra *et al*., 2000) and various PCB congeners (Furukawa *et al*., 1986; Hoefer *et al*., 1994; Furukawa *et al*., 1995; Mulligan *et al*.., 2005). Moreover, *P. aeruginosa* MTCC 2297, being an isolate from a certain oil-spill as well as being a known biosurfactant-producer, has been shown to degrade hydrocarbons such as benzene, xylene, toluene, kerosene, petrol and hexadecane (Satpute *et al*.., 2008). Therefore, its implication in the degradation of mono-chlorinated xenobiotics, such as 4-chlorobenzoic acid, becomes very crucial.

Bacillus subtilis

Bacillus subtilis is a Gram-positive bacterium, capable of growth in the presence of oxygen, and forms a unique type of resting cell called an endospore. The organism represents a large and diverse genus of bacteria named *Bacillus*, in the Family Bacillaceae. The ubiquity and diversity of this bacteria in nature, the unusual resistance of their endospores to chemical and physical agents, the developmental cycle of endospore formation, the production of antibiotics, the toxicity of their spores and protein

crystals for many insects, and the pathogen *Bacillus anthracis*, have attracted ongoing interest in these bacteria since and Cohn and Koch's discoveries in the 1870s.

There is great diversity of physiology among these aerobic sporeformers, considering their recently-discovered phylogenetic diversity. Their collective features include degradation of most substrates derived from plant and animal sources, including cellulose, starch, pectin, proteins, agar, hydrocarbons, and others; antibiotic production; nitrification; denitrification; nitrogen fixation; facultative lithotrophy; autotrophy; acidophily; alkaliphily; psychrophily; thermophily; and parasitism. Endospore formation, universally found in the group, is thought to be a strategy for survival in the soil environment, wherein these bacteria predominate. Aerial distribution of the dormant spores probably explains the occurrence of aerobic sporeformers in most habitats examined.

Collectively, *Bacillus subtilis* are versatile chemoheterotrophs capable of respiration using a variety of simple organic compounds (sugars, amino acids, organic acids). In some cases, they also ferment carbohydrates in a mixed reaction that typically produces glycerol and butanediol. Most *Bacillus* species can be grown in defined or relatively-simple complex media. For a few bacilli (e.g. *B. subtilis*, *B. megaterium*), minimal media have been established. The majority of these genuses are mesophiles, with temperature optima between 30 and 45 degrees, but some are thermophiles with optima as high as 65 degrees. Others are true psychrophiles, able to grow and sporulate at 0 degrees. They are found growing over a range of pH from 2 to 11. In the laboratory, under optimal conditions of growth, *Bacillus* species exhibit generation times of about 25 minutes.

Like most Gram-positive bacteria the surface of the *Bacillus* is complex and is associated with their properties of adherence, resistance and tactical responses. *Bacillus subtilis* degrades pectin and polysaccharides in plant tissues, and some strains cause rots in live potato tubers. It can also grow in a minimal defined medium with no added growth factors.

Bacillus subtilis is one of the most understood prokaryotes in terms of molecular biology and cell biology. Its superb genetic amenability and relatively large size has provided tools required to investigate the bacterium form all possible aspects. It is a ubiquitous soil bacterium that can be easily isolated form soil. B. subtilis is unique in that it can choose between atleast three different genetic programs when nutrients or other resources become scarce, and/or cell density reaches a critical threshold. To adapt to adverse conditions, cells enter the stationary phase, which is characterized by the formation of single motile cells. These cells differentiate into enduring and metabolically inactive spores, or become competent and take up DNA from the environment for acquisition of new genetic material. In all three cases, different genetic programs are turned on that guide the cells through the differentiation processes.

Like *P. aeruginosa*, *B. subtilis* has also been shown to play am important role in hydrocarbon degradation. Studies carried on the biodegradation potential of *B. subtilis* show that this organism is capable of utilizing and degrading crude petroleum-oil (Das *et al.*, 2006) and several azo dyes such as p-aminoazobenzene (Zissi *et al.*, 1997), anthroquinone dyes (Itoh *et al.*, 1993) and several PCB congeners (Rouse *et al.*, 1994; Mulligan *et al.*, 2005). Such an ability of *B. subtilis* is most commonly accounted by the fact that these hydrocarbons enhance biosurfactant-production by the organism (Cooper *et al.*, 1981; Bannat *et al.*, 2000; Oliver *et al.*, 2000; Cubitto *et al.*, 2004; Mulligan *et al.*, 2005). The role of *B. subtilis* in the degradation of 4-chlorobenzoic acid can thus be suggested owing to these properties of the organism. Moreover, being a wide-spread soil-inhabiting bacterium, its role in 4CBA-degradation may be of important consequence.

Pleurotus ostreatus

Cultivation of the oyster mushroom, *Pleurotus* spp., has increased greatly throughout the world during the last few decades. Its popularity has been increasing due to its ease of cultivation, high yield potential and high nutritional value. Although commonly grown on pasteurized wheat or rice straw, it can be cultivated on a wide variety of lignocellulosic

substrates, enabling it to play an important role in managing organic wastes whose disposal is problematic.

Pleurotus species have been used by human cultures all over the world for their nutritional value, medicinal properties and other beneficial effects. They also contain a number of biologically active compounds with therapeutic activities. Oyster mushrooms modulate the immune system, inhibit tumor growth and inflammation, have hypoglycemic and antithrombotic activities, lower blood lipid concentrations, prevent high blood pressure and atherosclerosis, and have antimicrobial and other activities. Recent studies of the medicinal properties of oyster mushrooms have focused on isolated bioactive compounds. *Pleurotus* spp. cultivation is a very simple procedure in the case of log cultivation because it does not involve sophisticated equipment. However, despite its simplicity, large-scale cultivation on natural logs is not often used due to long incubation periods, low yields and environment-dependent production if conducted outdoors.

White rot fungi produce extracellular phenoloxidases and can decompose lignin efficiently. *Elfvingia applanata* has been successfully applied for the bioconversion of bisphenol A, suggesting the usefulness of white rot fungi for bioremediation. In order to attain real bioremediation, the recycling of ecomolecules in the ecosystem is important. In addition to lignin, white rot fungi are able to degrade a variety of environmentally persistent pollutants, such as chlorinated aromatic compounds, heterocyclic aromatic hydrocarbons, various dyes and synthetic high polymers. Probably, this degradability of white rot fungi is due to the strong oxidative activity and the low substrate specificity of their ligninolytic enzymes. Thus, white rot fungi and their enzymes are thought to be useful not only in some industrial processes like biopulping and biobreaching but also in bioremediation. In order to attain real bioremediation, the degradation products should be effectively mineralized and reutilized or recycled in the ecosystem.

Pleurotus are able to colonize different types of vegetable wastes, increasing their digestibility (Platt *et al*., 1984; Commanday and Macy, 1985; Rajarathnam and Bano, 1989; Villas-Boas *et al*. 2002; Zhang *et al*. 2002; Mukherjee and Nandi, 2004; Salmones

et al. 2005). Previous studies have shown the feasibility of using these kinds of wastes to produce animal feed (Calzada *et al*. 1987; Adamovic *et al*. 1998), and as substrate for mushroom production (Breene, 1990; Sermanni *et al*. 1994; Kakkar and Dañad, 1998; Yildiz *et al*. 2002).

The white rot fungus *Phanerochaete chrysosporium* has demonstrated abilities to degrade many xenobiotic chemicals. The degradation of three model polychlorinated biphenyl (PCB) congeners (4,4´-dichlorobiphenyl [DCB], 3,3´,4,4´-tetrachlorobiphenyl, and 2,2´,4,4´,5,5´-hexachlorobiphenyl) was done by *P. chrysosporium*incubation. The extent of PCB mineralization appears to decrease as the degree of PCB chlorination increases. There is also evidence indicating that PCB degradation by *P. chrysosporium* is influenced by the congeners' chlorination patterns. The effects of chlorination pattern on PCB degradation are well documented for bacteria and are believed to reflect restrictions on 2,3- or 3,4-dioxygenase attacks. A mechanistic interpretation of the chlorination pattern effect in *P. chrysosporium* is prevented by a lack of information regarding the enzymatic basis of PCB degradation by this organism.

BACTERIAL STRAINS

Two microorganisms were used in this project, to check for their ability to degrade 4 – Chlorobenzoic Acid (4CBA). *Pseudomonas aeruginosa* MTCC 2297 was obtained from Microbial Type Culture Collection, IMTECH, Chandigarh, India. *Bacillus subtilis* was revived from the stock cultures present at the Institute of Science, Nirma University, Ahmedabad, India.

FUNGAL STRAIN

Pleurotus ostreatus, a White-Rot fungus, was obtained from M.G. Science Institute, Ahmedabad, India.

MEDIA

All the media used for sub-culturing, maintenance and storage of microorganisms were purchased from Hi-media. *P. aeruginosa* and *B. subtilis* were routinely cultivated on Nutrient Broth and Nutrient Agar, while *Pleurotus ostreatus* was grown on Potato Dextrose Agar/Broth (PDA/B).

MEDIA FOR ACCLIMITIZATION

Nutrient Broth and PDB, supplemented with increasing concentrations of 4CBA, was used for acclimatizing the microorganisms to the presence of 4CBA. This was done until the maximum concentration of 4CBA in which the organisms could sustain and grow.

The organisms that grew on the highest concentration of 4CBA were then re-inoculated into Minimal Salts Medium (MSM) (Spizizien *et al.*, 1958). The composition of the medium (g L^{-1}) is as follows:

Minimal Salt Medium (g mL^{-1}):

Ammonium Sulphate	2
Monopotassium Phosphate	6
Sodium citrate	1
Magnesium Sulphate	0.2
Glucose	5
Maltose	5
Calcium Chloride	0.15
Caesin hydrolysate	5
pH	7.2
Temperature of incubation	37°C

MEDIA FOR DEGRADATION OF 4CBA

P. aeruginosa and *B. subtilis* were inoculated into MSM, containing different concentrations of 4CBA, in order to detect the tolerance limit and rate of degradation.

STOCK CULTURES

On obtaining the bacterial cultures, they were streaked onto N – Agar plates. The plates showing distinct isolated colonies were preserved as Master Plates by sealing with parafilm and refrigerating. Sub-culturing was done every 10 – 15 days.

Pleurotus ostreatus was sub-cultured every 10 – 15 days and maintained by transferring a small piece of the mycelia onto a fresh PDA plate. Once the mycelia grew to maximum length, they were refrigerated and thus preserved as master plates.

Glycerol stock cultures of both the bacterial strains were maintained in 20% glycerol in N – broth for *P. aeruginosa* and *B. subtilis*. Once the cultures grew sufficiently, glycerol was added and mixed properly. 1.5 mL of this culture was then transferred to sterile cryo-vials. These vials were sealed tightly with parafilm and stored at -20°C.

INOCULA

For inoculation into liquid medium, a single distinct colony of the bacteria was picked up and transferred into 100 mL of Nutrient broth. The flask was incubated at 37°C at 200 rpm for 24 hours.

ACCLIMATIZATION

In order to acclimatize the cells to the presence of 4CBA, 1% v/v of the prepared inoculum was inoculated into Nutrient broth, containing increasing concentrations of 4CBA. They were then transferred into MSM containing no 4CBA. Thereafter, the cultures were acclimatized by cultivation on MSM containing increasing concentrations of 4CBA, during each subsequent sub-culturing.

STANDARD CURVE FOR 4-CHLOROBENZOIC ACID

The standard curve of 4CBA was prepared in the concentration range of 5 to 50 μg mL^{-1}. 4CBA was dissolved in a water-ethanol (1:1) mixture and necessary aliquots were prepared. 4CBA was measured spectrophometrically at 234 nm (Dong-In Seo *et al.*, 1997). The standard curve was prepared by plotting Absorbance Vs Concentration of 4CBA.

GROWTH CURVE

The growth of *P. aeruginosa* and *B. subtilis* in control (MSM) and in test (MSM containing increasing concentrations of 4CBA) was monitored at regular time intervals of 1 hour by measuring the turbidity at 570 nm.

DEGRADATION OF 4-CHLOROBENZOIC ACID

The utilization of 4CBA by the microorganisms, during their growth period, was checked by estimating the reduction of 4CBA concentration in the liquid culture medium. Samples were taken at regular intervals of 1 hour for the measurement of 4CBA remaining in the medium. 1 mL sample was transferred into sterile eppendorf tubes and centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was diluted (20-folds in

case of *B. subtilis* and 100-folds in case of *P. aeruginosa*) and absorbance was taken at 234 nm. 4CBA concentration was determined using the standard curve as a reference (Dong-In Seo *et al.*, 1997).

IDENTIFICATION OF METABOLITES

Samples were taken at regular intervals of 3 hours and subjected to sonication at 500 W for 10 minutes. The cell debris was pelleted down by centrifugation at 10,000 rpm for 10 minutes at 4°C. The supernatant was diluted (as mentioned above) and a spectrum of wavelength 200 – 500 nm was generated (Dong-In Seo *et al.*, 1997).

CULTURES

Bacteria

Pseudomonas aeruginosa MTCC 2297:

P. aeruginosa MTCC 2297 was revived from the vial and maintained on Nutrient Broth, at 37°C and 200 rpm. *P. aeruginosa* appeared as small, cream-colored, slightly mucoid colonies, with characteristic green-colored pigmentation, after 24 hours of incubation, on agar plates.

Bacillus subtilis:

B. subtilis was also maintained on Nutrient Broth, at 37°C at 200 rpm. *B. subtilis* appeared as large, white-colored, serrated colonies, after 24 hours of incubation, on nutrient agar.

Fungi

The White-Rot fungus, *Pleurotus ostreatus*, was maintained on Potato Dextrose Agar (PDA) at 30°C for 7 days (Plate 1).

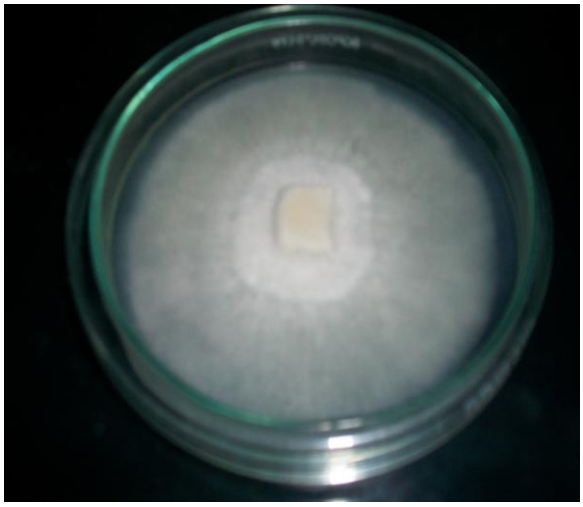

Plate 1: *P. ostreatus* grown on Potato Dextrose Agar

STANDARD CURVE FOR 4-CHLOROBENZOIC ACID

A standard curve for 4CBA was prepared by measuring the absorbance at 234 nm, which is specific for 4CBA. The detection range of the standard curve was 5 – 50 µg mL^{-1} (Figure 5).

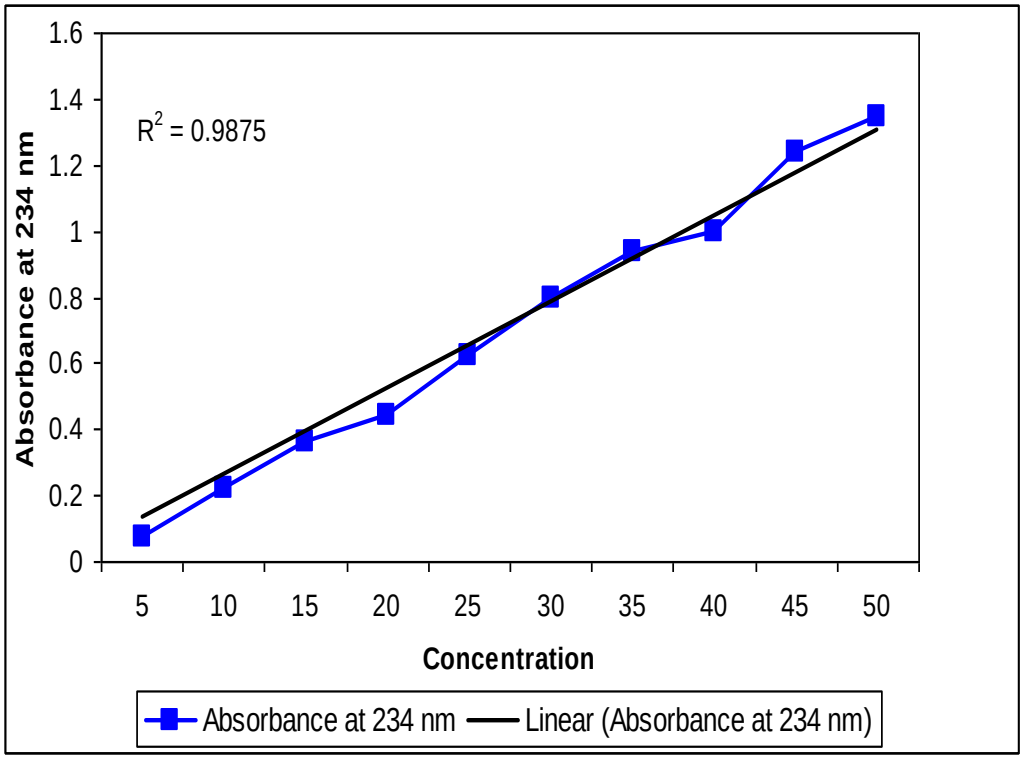

Figure 5: Standard curve of 4CBA

DEGRADATION OF 4-CHLOROBENZOIC ACID BY BACTERIA

P. aeruginosa and *B. subtilis* were tested for their ability to degrade 4CBA and both the strains were transferred into a Minimal Salts Medium, containing no 4CBA. *P. aeruginosa* and *B. subtilis* showed substantial growth at different ranges of 4CBA concentration.

Pseudomonas aeruginosa

Growth of *P. aeruginosa* on MSM, in the absence of 4CBA

P. aeruginosa was grown on MSM, lacking 4CBA, using 1% V/V of inocula. Growth of the organism was measured by measuring the absorbance at 570 nm for about 20 hours (Figure 6).

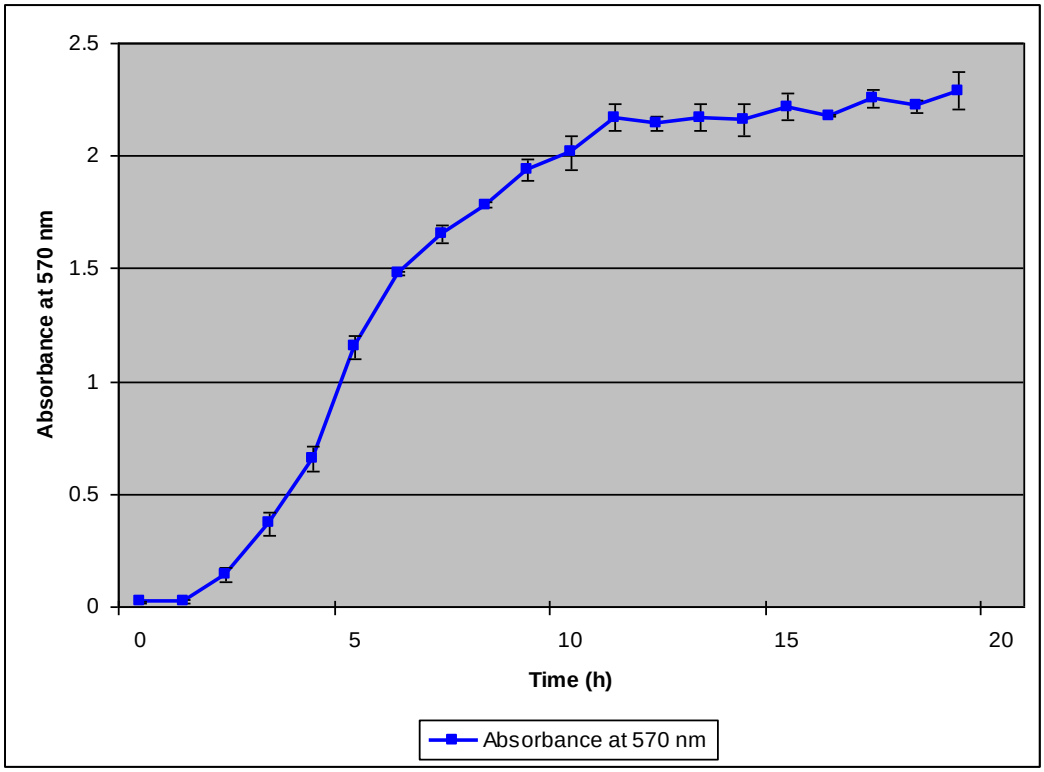

Figure 6: Growth of *P. aeruginosa* on MSM in the absence of 4CBA

*The values represent mean of triplicates ± SD

P. aeruginosa showed substantial growth on MSM with a lag phase of 2 hours and a log phase lasting 10 hours then attaining stationary phase.

Growth of *P. aeruginosa* on MSM containing various concentrations of 4CBA

Having acclimatized the organism to increasing concentrations of 4CBA, the growth pattern of *P. aeruginosa*, growing at different concentrations of 4CBA, was studied. The organism showed a tolerance limit to 4CBA of 4.8 mg mL^{-1}. Growth was measured at 570 nm (Figure 7). The range of 4CBA concentration was 3 – 5 mg mL^{-1}.

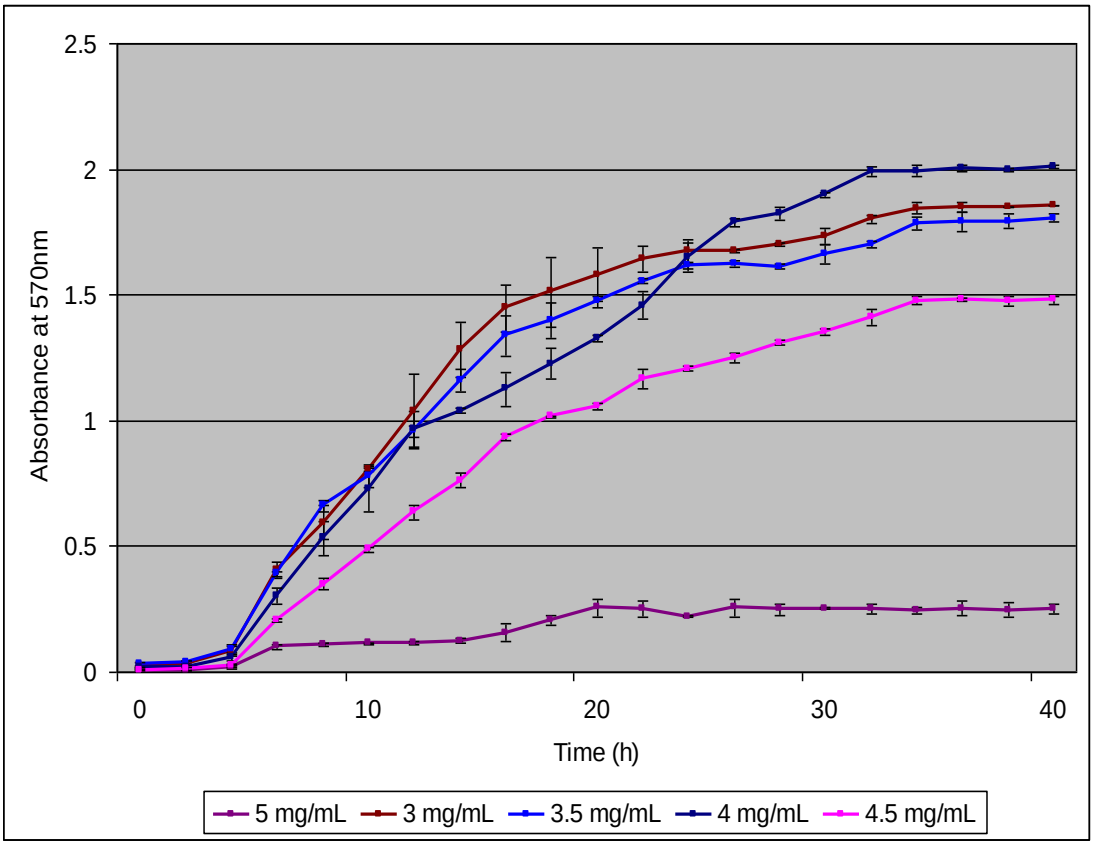

Figure 7: Growth of *P. aeruginosa* at different concentrations of 4CBA
*The values represent mean of triplicates ± SD

The growth profile of *P. aeruginosa* MTCC 2297 (acclimatized to 4CBA), grown on a double-carbon-source mineral medium, showed a normal monophasic pattern. When the cells entered the log phase, there was a marked decline in concentration of 4CBA, at all concentrations (except 5 mg mL^{-1}). Since a diauxic growth pattern was not observed, it may be possible that this strain co-metabolizes 4CBA plus glucose. The stationary phase was established typically after 10 – 12 hours. The onset of the stationary phase is slightly

later as compared to the growth pattern in control, possibly due to the presence of two carbon sources.

Earlier studies on *Pseudomonas putida* CSV86 show that when grown on naphthalene-, salicylate-, benzyl alcohol- and benzoic acid- plus glucose, biphasic growth was observed, while a monophasic growth pattern was seen using the same aromatics plus organic acids (Phale *et al*., 2006). In another study on a *Pseudomonas* sp. capable of aniline degradation, diauxic growth was not observed in the mixed substrate culture (lactate and phenol), suggesting that lactate and aniline were used simultaneously (Konopka *et al.*, 1989). Also, in *Ralstonia taiwanensis* cultures grown on medium bearing dual carbon sources (glycerol and phenol) or phenol alone, phenol was found to be firstly biodegraded for microbial growth (i.e., growth-associated degradation) with a monophasic growth pattern. Moreover, using glycerol as the nutrient source, phenol degradation seemed to be enhanced simultaneously during the consumption of glycerol for cellular growth after ca. 2 h response lag in growth (Chen *et al.*, 2007). Thus, it may be concluded that this strain utilizes glucose for cellular growth, concurrently degrading 4CBA. If 4CBA were used preferentially to glucose in the dual-carbon source medium, it would have been depleted within 10 - 12 h. However, only 35% of 4CBA had been consumed at this time. If glucose were used preferentially to 4CBA, then no 4CBA metabolism would have been observed during the time of this experiment, yet maximum 4CBA depletion occurred by 30 h.

Rate of 4-Chlorobenzoic acid degradation

P. aeruginosa grown on MSM containing 4CBA was measured at regular time intervals. The centrifuged and diluted samples were read at 234nm. This was done till a constant reading for 4CBA degradation was obtained for each concentration (Figure 8).

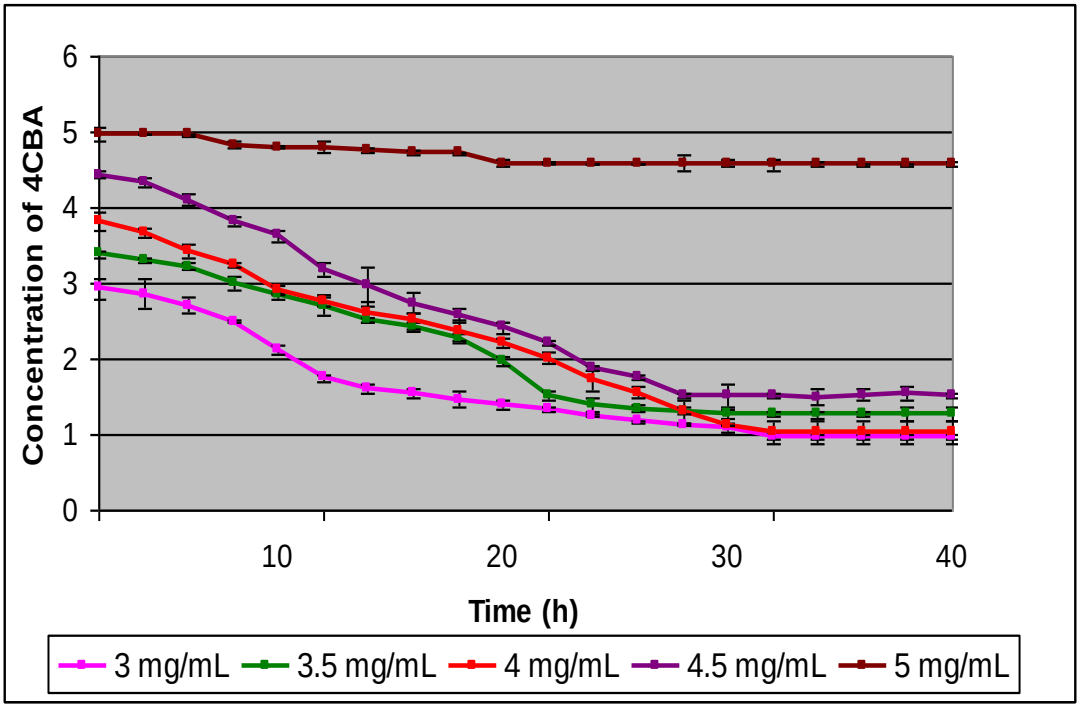

Figure 8: Degradation of 4CBA by *P. aeruginosa* grown on MSM with 4CBA
*The values represent mean of triplicates ± SD

There was an overall decline in the growth rate of cells with increasing concentration of 4CBA, with the highest growth being observed at 4 mg mL^{-1}, whereas, growth was almost completely inhibited at 5 mg mL^{-1}. The survival rates of organisms have been generally reported to decrease at higher concentrations of aromatic hydrocarbons (Ramos *et al*., 1995; Sikkema *et al*., 1994). Also, at high concentrations, aromatic hydrocarbons such as 4CBA have been shown to confer a toxic effect on cells due to destruction of the pH gradient and denaturation of some enzyme proteins in cells, leading to cell death (Warth *et al*., 1991; Sikkema *et al*., 1995). Therefore, the toxic effects of 4CBA on the survival rates of the organisms can be explained by the above mechanisms in both gram – negative and gram – positive bacterial cells.

The rate of degradation (mg h^{-1}), and the degradation potential (%) of *P. aeruginosa*, at each concentration was thus calculated (Table 1; Figure 9).

Table 1: Rate of degradation of 4CBA by *P.aeruginosa* at different concentrations of 4CBA

Initial concentration of 4CBA (mg mL^{-1})	**Final concentration of 4CBA (mg mL^{-1})**	**Rate of degradation (mg h^{-1})**	**Percent degradation (%)**
3.0	1.32 ± 0.059	0.028 ± 0.0038	56 ± 1.181
3.5	1.27 ± 0.062	0.037 ± 0.0014	64 ± 2.283
4.0	1.03 ± 0.100	0.049 ± 0.0041	74 ± 2.124
4.5	1.51 ± 0.076	0.051 ± 0.0008	67 ± 1.541
5.0	4.56 ± 0.035	0.013 ± 0.0029	10 ± 2.004

*The values represent mean of triplicates ± SD

The rate of disappearance of 4CBA at 3, 3.5, 4, and 4.5 mg mL^{-1} showed a continuous increase. The percent degradation also showed a similar trend with greater degradation at higher concentrations. Maximum degradation of 74% was observed at 4 mg mL^{-1} at the rate of 0.049 mg h^{-1}, while, a maximum rate of 0.051 mg h^{-1} was observed in the presence of 4.5 mg mL^{-1}.

The Degradation Potential of *P. aeruginosa* is presented:

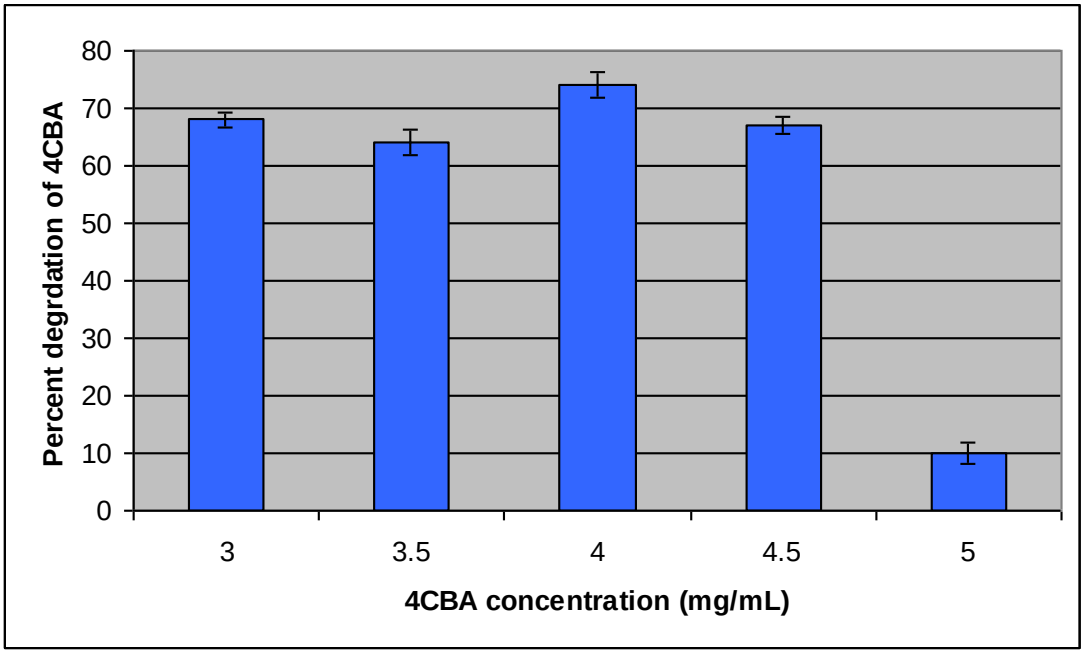

Figure 9: Degradation potential of *P. aeruginosa* at different concentrations of 4CBA
*The values represent mean of triplicates ± SD

This showed that higher concentration of 4CBA favored higher rates of degradation, although maximum degradation (%) was favored by lower concentrations of 4CBA. Increased rate of degradation with increasing concentration of 4CBA may be occurring since the cells were previously acclimatized to 4CBA, and thus the 4CBA uptake system has been previously induced (Miguez *et al.*, 1990). Higher rate of degradation with reduced degradation potential can be accounted to the possibility that the enzymes involved in the uptake and utilization of 4CBA may be getting rapidly saturated at such a high concentration.

Identification of Metabolites

During cultivation of *P. aeruginosa* in MSM containing 4.5 mg mL^{-1}, the remaining 4CBA and its metabolites were quantified by growing cell assay. As shown in the graph (Figure 10) the concentration of 4CBA decreases with time. A key metabolite of 4CBA degradation called 4- chlorocatachol (4-CC) was first detected after 6 hours of incubation, which gradually increased till 12 hours, with a simultaneous decline in 4CBA concentration. After 12 hours of incubation the samples showed a decrease in 4-CC which finally disappeared after 24 hours. The increase in 4-CC does not lead to its accumulation and any kind of affect on 4CBA degradation. A decrease in 4-CC after 12 hours also indicated that it might be getting converted to other metabolites which needs to be further investigated (Dong-In Seo *et al.*, 1997).

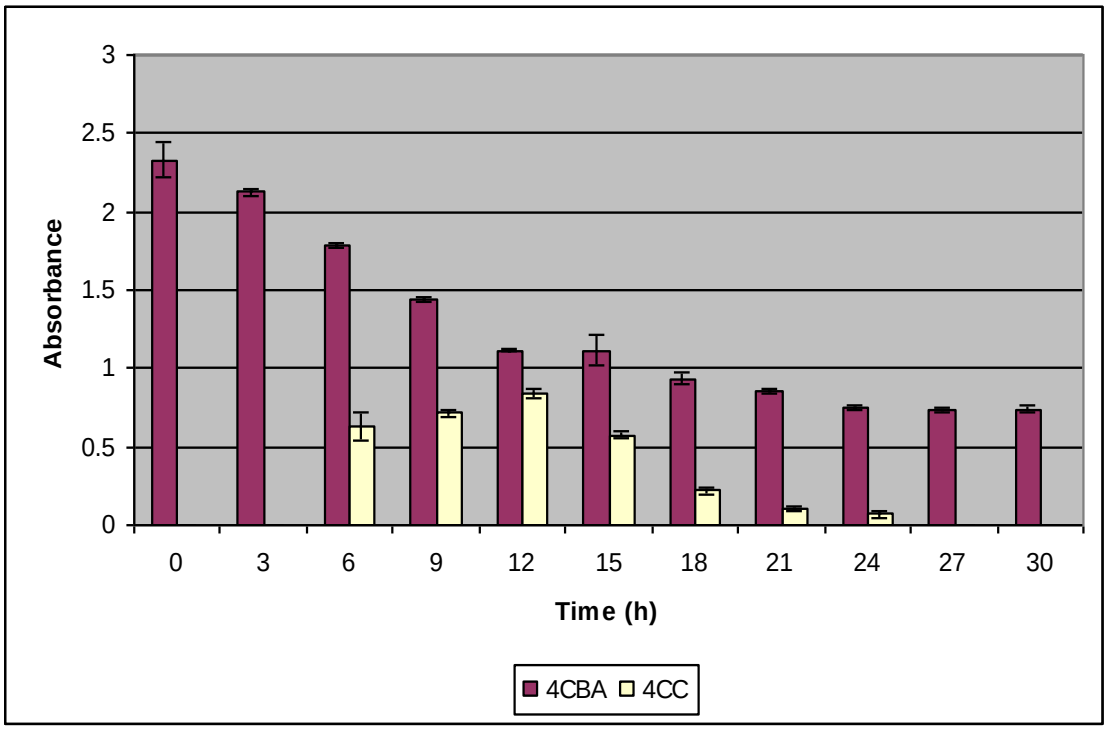

Figure 10: Detection of 4-CC in culture supertanant
*The values represent mean of triplicates ± SD

There have been reports that 4CBA is converted to 4CC in *Pseudomonas cepacia* P166 (Arensdorf *et al*., 1994) and *Pseudomonas acidovorans* M3GY (McCullar *et al*., 1994). 4CC is further metabolized to produce 5C-2HMS via *meta*--cleavage pathway. Also, 5C-2HMS was produced from 4CBA as well as 4CC by strain S-47 (Dong-In Seo *et al*., 1997) when incubated for 12 h. The enzyme involved in this degradation being dioxygenase, suggested that this is an **Oxygenolytic dehalogenation** (Dong-In Seo *et al*., 1997; Fetzner *et al*., 1998).

Bacillus subtilis

Growth of *B. subtilis* on MSM, in the absence of 4CBA

B. subtilis was grown on MSM, lacking 4CBA, using 1% V/V of inoculum. Growth of the organism was measured by measuring the absorbance at 570 nm for about 20 hours (Figure 11).

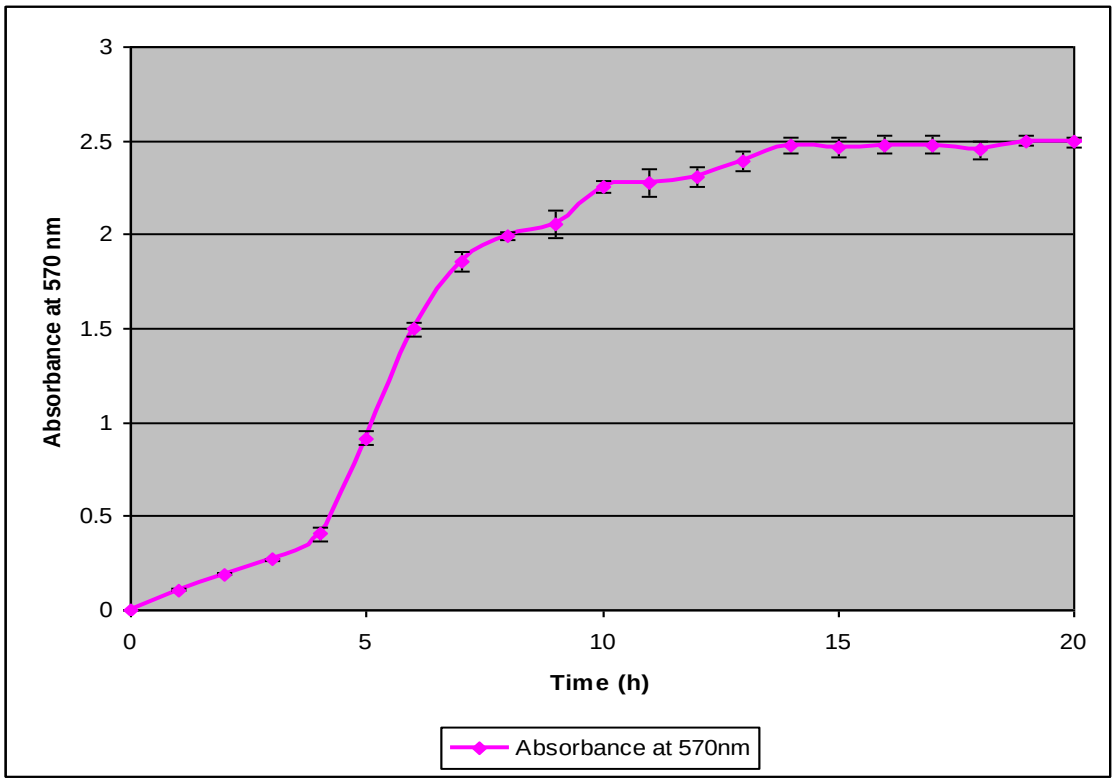

Figure 11: Growth of *B. subtilis* on MSM in the absence of 4CBA
*The values represent mean of triplicates ± SD

B. subtilis showed substantial growth on MSM with a lag phase of 4 hours and a log phase of 10 hours then attaining stationary phase.

Growth of *B. subtilis* on MSM containing increasing concentrations of 4CBA

Having acclimatized the organism to increasing concentrations of 4CBA, the growth pattern of *B. subtilis*, growing at different concentrations of 4CBA, was studied. Growth

was measured at 570 nm (Figure 12). The range of 4CBA concentration was 0.2 – 1 mg mL^{-1}. The tolerance limit of *B. subtilis* to resist the presence of 4CBA was found to be 0.8 mg mL^{-1}.

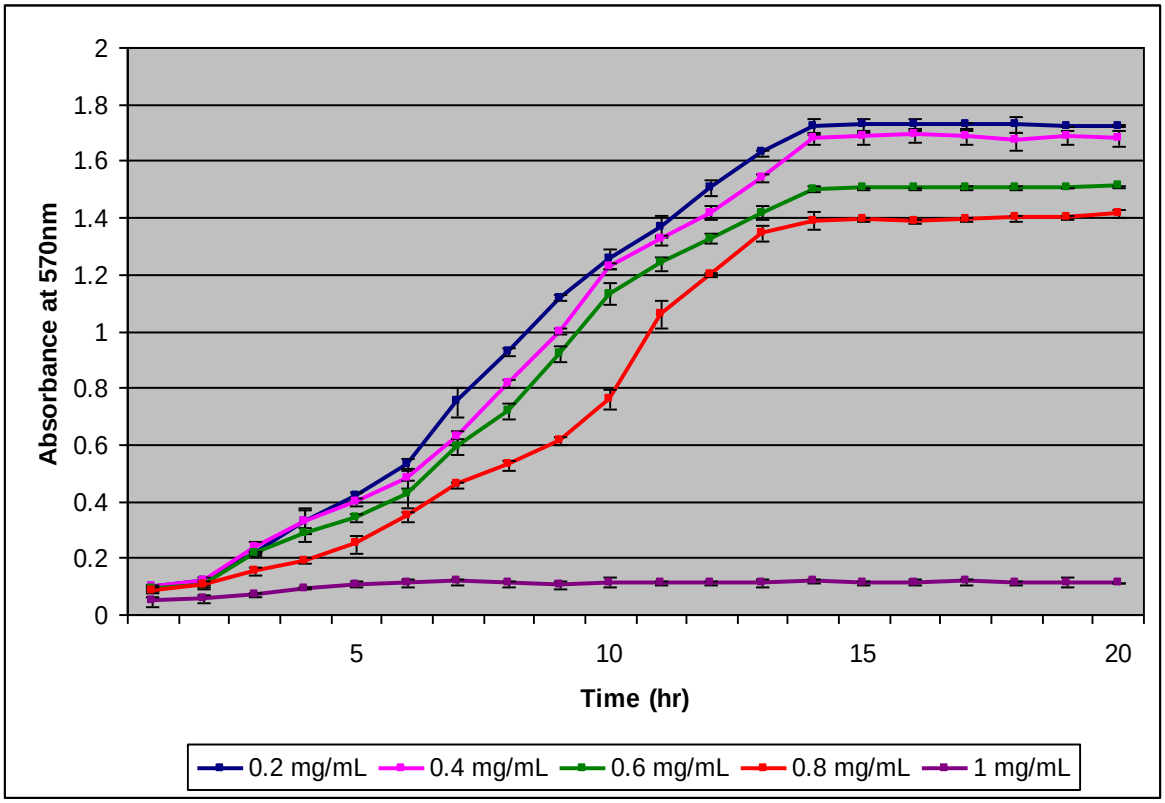

Figure 12: Growth of *B. subtilis* at different concentrations of 4CBA
*The values represent mean of triplicates ± SD

The growth profile of acclimatized *B. subtilis* cells, grown on a double-carbon-source mineral medium, showed a normal monophasic pattern, nearly identical to results in *P.aeruginosa*. It may be possible that *B. subtilis* also co-metabolizes 4CBA plus glucose. The cells had to adapt themselves to the new medium and as the cells entered the log phase, there was a marked decline in concentration of 4CBA, at all concentrations (except 1 mg mL^{-1}), with maximum growth being observed at 0.6 mg mL^{-1}. No cell-growth was observed at 1 mg mL^{-1}. This may be attributed to the fact that at relatively high concentrations, 4CBA has been found to have deleterious effects on several strains of *E. coli*, *S. aureus* and *B. subtilis*, including reduction in overall cell size and decrease

in certain membrane proteins (Park *et al.*, 2001). The rate of degradation of 4CBA increased with increasing concentration of 4CBA, up to 0.8 mg mL^{-1}. Maximum degradation was observed at 0.6 mg mL^{-1}, which is in correlation with the growth pattern. The stationary phase was established typically after 10 – 12 hours. The onset of the stationary phase was marginally delayed as compared to the growth pattern in control, possibly due to the presence of two carbon sources.

The monophasic growth pattern observed in *B. subtilis* was in correlation with earlier studies on a *Pseudomonas* sp. capable of aniline degradation, where diauxic growth was not observed in the mixed substrate culture (lactate and phenol), suggesting that lactate and aniline were used simultaneously (Konopka *et al.*, 1989). Similarly, in *Ralstonia taiwanensis* cultures grown on medium bearing dual carbon sources (glycerol and phenol) or phenol alone, phenol was found to be firstly biodegraded for microbial growth (i.e., growth-associated degradation) with a monophasic growth pattern (Chen et al., 2007). Thus, it may be concluded that this strain also utilizes glucose for cellular growth, concurrently degrading 4CBA.

Rate of 4-Chlorobenzoic acid degradation

B. subtilis grown on MSM containing 4CBA was measured at regular time intervals. The centrifuged and diluted samples were read at 234nm. This was done till maximum amount of 4CBA was degraded at each concentration (Figure 13)

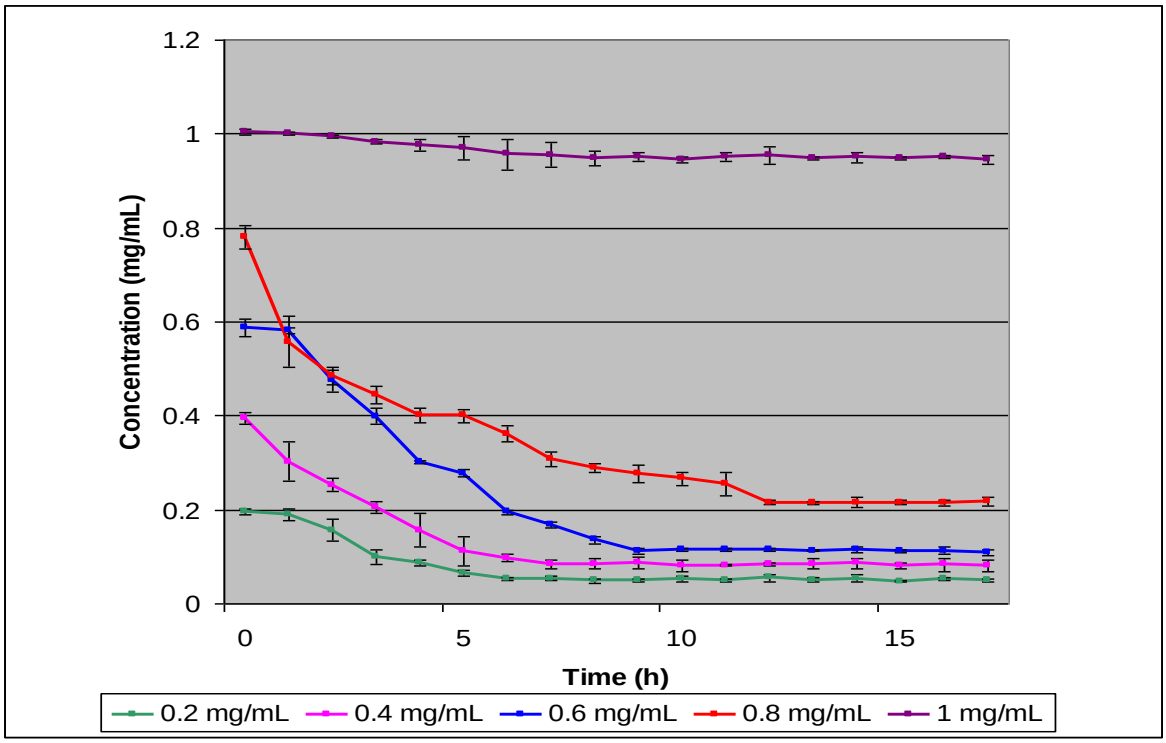

Figure 13: Degradation of 4CBA by *B. subtilis* grown on MSM with 4CBA
*The values represent mean of triplicates ± SD

There was an overall decline in the growth rate of cells with increasing concentration of 4CBA, with the highest growth being observed at 0.6 mg mL^{-1}, whereas, growth was completely inhibited at 1 mg mL^{-1}. As discussed in case of *P. aeruginosa*, reduced cell growth and survival rate at higher concentration of 4CBA in *B. subtilis* is probably due to the toxic effects of 4CBA on the cells (Warth *et al*., 1991; Sikkema *et al*., 1994; Ramos *et al*., 1995; Sikkema *et al*., 1995).

The rate of degradation (mg h^{-1}), and the degradation potential of *B. subtilis*, at each concentration was calculated.

Table 2: Rate of degradation of 4CBA by *B. subtilis* at different concentrations of 4CBA

Initial concentration of 4CBA (mg mL^{-1})	**Final concentration of 4CBA (mg mL^{-1})**	**Rate of degradation (mg h^{-1})**	**Percent degradation (%)**
0.2	0.05 ± 0.006	0.012 ± 0.0008	75 ± 0.907
0.4	0.08 ± 0.014	0.027 ± 0.0032	80 ± 3.385
0.6	0.11 ± 0.007	0.041 ± 0.0008	81 ± 0.014
0.8	0.21 ± 0.016	0.048 ± 0.0012	74 ± 0.603
1.0	0.95 ± 0.011	0.030 ± 0.0006	6 ± 0.735

*The values represent mean of triplicates ± SD

The rate of disappearance of 4CBA at 0.2, 0.4, 0.6, and 0.8 mg mL^{-1} showed a continuous increase. The percent degradation also showed a similar trend with greater degradation at higher concentrations. Maximum degradation of 81% was observed at 0.6 mg mL^{-1} at the rate of 0.041. On the other hand, the bacteria degraded 4CBA at a higher rate of 0.048 in the presence of 0.8 mg mL^{-1}. This shows that the bacterium degrades faster at higher concentration of 4CBA but relatively less amount. And the degradation potential is much higher at less concentrations of 4CBA.

The Degradation Potential of *B. subtilis* is presented:

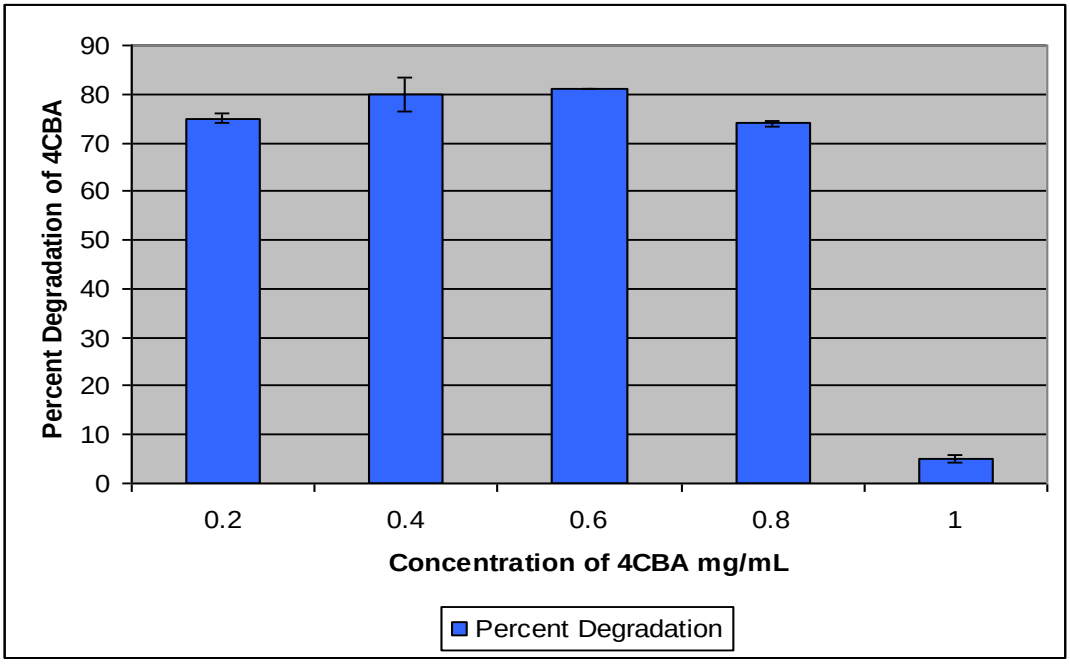

Figure 14: Degradation potential of *B. subtilis* at different concentrations of 4CBA
*The values represent mean of triplicates ± SD

Similar to *P. aeruginosa*, higher concentration of 4CBA favored higher rates of degradation, although maximum degradation (%) was favored by lower concentrations of 4CBA. Increased rate of degradation with increasing concentration of 4CBA may be occurring since the cells were previously acclimatized to 4CBA, and thus the 4CBA uptake system has been previously induced (Miguez *et al.*, 1990). Higher rate of degradation with reduced degradation potential can be accounted to the possibility that the enzymes involved in the uptake and utilization of 4CBA may be getting rapidly saturated at such a high concentration. Moreover, since 4CBA has been shown to have deleterious effects on the morphology and physiology of *B. subtilis* cells, the degradation potential was lower at 0.8 mg mL^{-1} and insignificant degradation was seen at 1 mg mL^{-1}.

Identification of Metabolites

During cultivation of *B. subtilis* in MSM containing 0.8 mg mL^{-1}, the remaining 4CBA and its metabolites were quantified by growing cell assay. As shown in the graph (Figure 15) the concentration of 4CBA decreases with time. 4-chlorocatechol (4-CC), the metabolite seen in *P. aeruginosa*, was first detected after 3 hours of incubation. 4-CC gradually increases till 9 hours with decrease in 4CBA concentration. After 9 hours of incubation the samples showed a decrease in 4-CC which finally disappeared after 24 hours. The increase in 4-CC does not lead to its accumulation and any kind of affect on 4CBA degradation. In this case also, a decrease in 4-CC after 9 hours indicated that it might be getting converted to other metabolites which needs to be further investigated (Dong-In Seo *et al*., 1997). Therefore, as observed in case of *P. aeruginosa*, the pathway of degradation is most probably **Oxygenolytic dehalogenation** (Dong-In Seo *et al.*, 1997; Fetzner *et al.*, 1998).

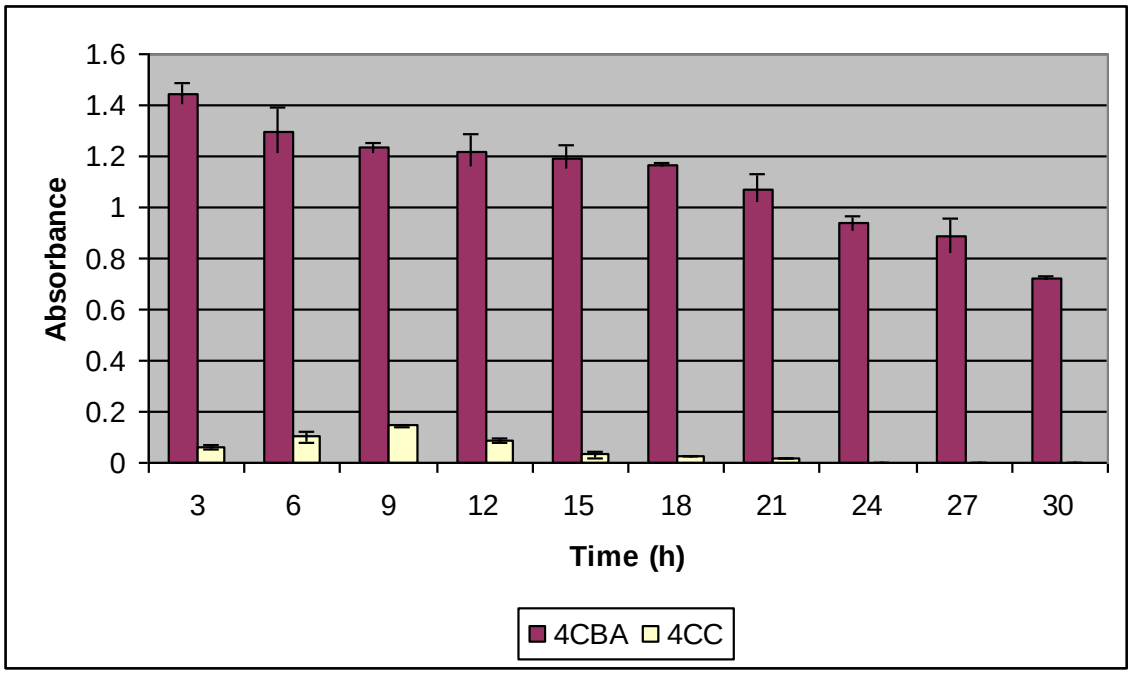

Figure 15: Detection of 4CC, key metabolite of 4CBA
*The values represent mean of triplicates ± SD

DEGRADATION OF 4-CHLOROBENZOIC ACID BY FUNGI

The White-Rot fungus, *Pleurotus ostreatus*, was also tested for its ability to degrade 4CBA. It was initially cultivated on Potato Dextrose Agar, containing no 4CBA. It was then acclimatized by cultivation on PDA, containing increasing concentrations of 4CBA, during each subsequent sub-culturing procedure.
Pleurotus ostreatus showed substantial growth up to 10 mg mL^{-1} of 4CBA present in the medium (Plates 2-5).

4CBA is insoluble in water and remains as suspended particles in an agar plate. The presence of 4CBA makes the plates appear white. *Pleurotus ostreatus* has a definite pattern of degradation on an agar plate containing 4CBA. It shows a clear zone of disappearance of 4CBA on the PDA plate after an incubation of 10-15 days.
The fungi was transferred in potato dextrose broth to obtain quantitative degradation of 4CBA. But its incubation period is much longer and is yet under experimentation.

Pleurotus ostreatus, showed extremely promising degradation capacity. When grown on Potato Dextrose Agar plates containing 4CBA (in an insoluble form), *P. ostreatus* was found to grow and utilize 4CBA upto a concentration on 10 mg mL^{-1}. Previous studies on *Pleurotus ostreatus* have shown its high efficiency in lignin degradation (Agosin *et al*., 1985; Platt *et al*., 1984). Moreover, since its lignin degradation ability is assumed to be correlated with laccase activity (Higuchi *et al*., 1990; Kerem *et al*., 1992; Youn *et al*., 1995), *P. ostreatus* has been worked upon for its ability to degrade polycyclic aromatic hydrocarbons and results show that this fungus was able to degrade catechol, phenanthrene, pyrene and anthracene almost completely (Bezalel *et al*., 1996). The results we obtained so far on the degradation of 4CBA by *P. ostreatus* are only qualitative. The quantitative determination of the extent of degradation in a particular time period is yet to be completed, since growth of this fungus in liquid medium is taking much longer than expected.

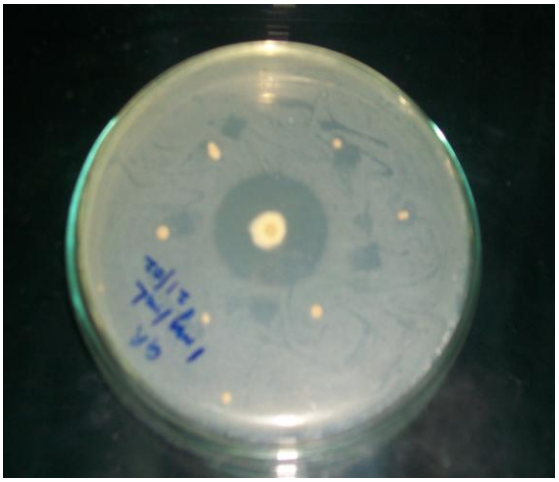

Plate 2: *P. ostreatus* growing on PDA containing 1 mg mL^{-1} 4CBA

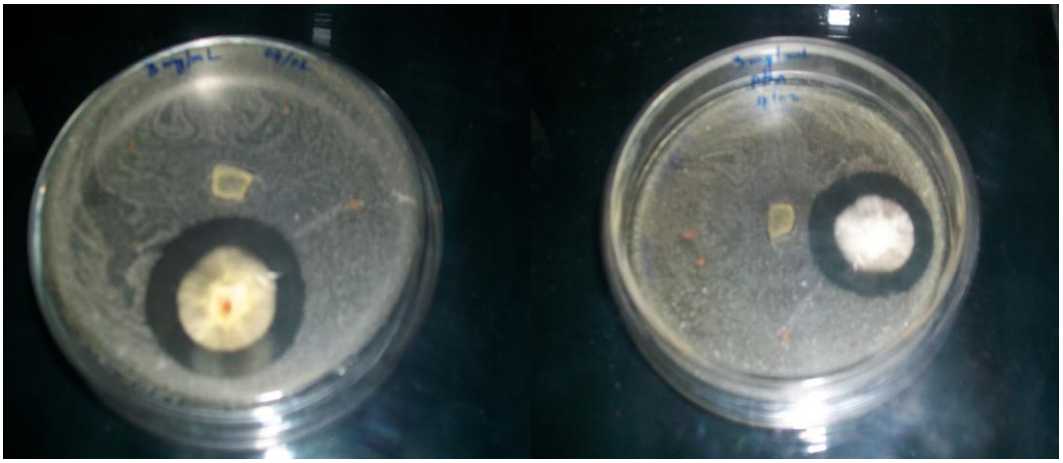

Plate 3: *P. ostreatus* growing on PDA containing 3 mg mL^{-1} 4CBA

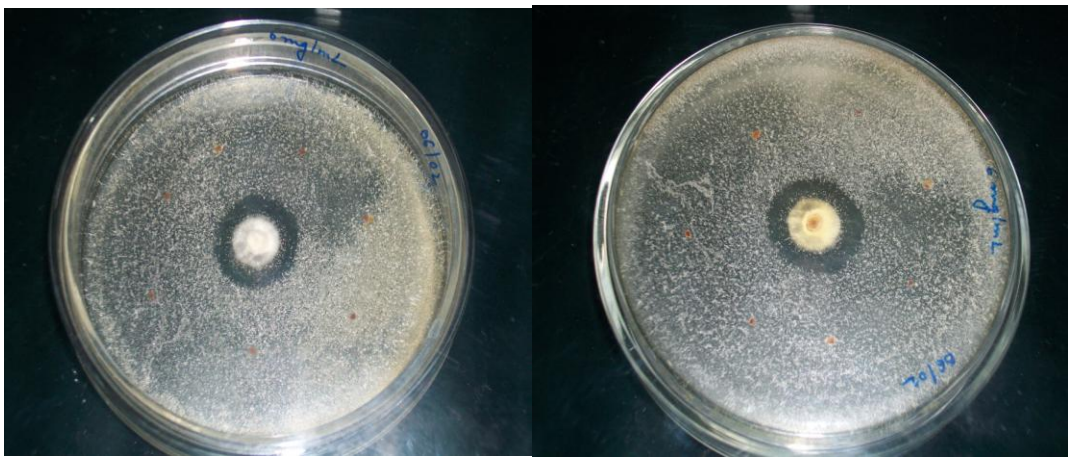

Plate 4: *P. ostreatus* growing on PDA containing 6 mg mL^{-1} 4CBA

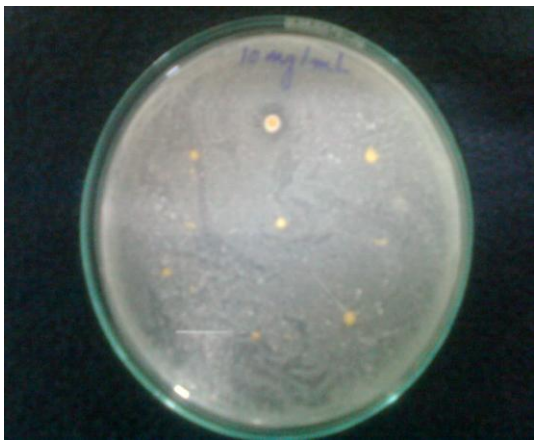

Plate 5: *P. ostreatus* grown on PDA containing 10 mg mL^{-1} 4CBA

CONCLUSION

Thus, in conclusion, *P. aeruginosa* showed the tolerance limit of 4.8 mg mL^{-1}. But its highest efficiency of biodegradation of 4CBA was found at 4 mg mL^{-1} with 74% degradation, while it showed highest rate of degradation of 0.051 mg h^{-1} at 4.5 mg mL^{-1}. The presence of 4CC as a metabolite gives clues about its pathway and the enzymes involved.

B. subtilis was able to degrade 4CBA at a much lower concentration. Its tolerance limit was found to be 0.8 mg mL^{-1}. However, *B. subtilis*, degraded 81% of 4CBA in the medium at 0.6 mg mL^{-1} concentration. Its highest rate of degradation was found to be 0.048 mg h^{-1} at 0.8 mg mL^{-1} concentration. *B. subtilis* being a wide-spread soil inhabitant may be engineered to increase its efficiency of biodegradation, thus making it an important tool in bioremediation. While, *B. circulans* has been reported to be an important hydrocarbon-degrader, especially of dichloromethane (Wu *et al.*, 2007), fewer reports on the degradation capacity of *B. subtilis* are available, and hence, this organism may be further investigated.

Although *Pleurotus ostreatus* did show excellent degradation capacity on agar plates containing 4CBA, quantitative data was not generated due to prolonged incubation time of the organism in liquid medium. Therefore, the extent of degradation and the identities of metabolic intermediates that might provide insights into the degradative pathways may be further investigated in this organism.

SUMMARY

An isolate from a certain oil spill, a very common soil inhabitant and an able fungus were selected for the degradation of a toxic soil pollutant- 4CBA. The microorganisms *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Pleurotus ostreatus* were acclimatized and checked for its tolerance limit to 4CBA. *P. aeruginosa*, *B. subtilis* and *Pleurotus ostreatus* showed a tolerance limit of 4.8, 0.8 and 10 mg mL^{-1} respectively.

Further, 4CBA degrading potential of these microorganisms was measured by inoculating them in the Minimal Salt Medium (MSM) containing various 4CBA concentrations ranging from 0.2 to 1 mg mL^{-1} in case of *B. subtilis* and 3 to 5 mg mL^{-1} in case of *P. aeruginosa*. The remaining 4CBA in the medium was measured by reading the absorbance at 234 nm. Also, the growth of the microorganisms in MSM with various 4CBA concentrations was monitored by measuring the turbidity at 570 nm.

P. aeruginosa degraded 4CBA up to 74% at the rate of 0.049 mg h^{-1} at a concentration of 4 mg mL^{-1} of 4CBA, while highest rate of degradation was found to be 0.051 mg h^{-1} in the presence on 4.5 mg mL^{-1} of 4CBA. Whereas, *B. subtilis* degraded 4CBA up to 81% at the rate of 0.041 mg h^{-1} at a concentration of 0.6 mg mL^{-1}, and its highest rate of degradation was found to be 0.048 mg h^{-1} in the presence of 0.8 mg mL-1 of 4CBA.

The degrading potential of *P. aeruginosa* when compared to *B. subtilis* is much higher. Also, the degrading potential of P. aeruginosa is higher than other strains of *Pseudomonas sp.* The metabolic efficiency of both these microorganisms can be enhanced by genetic engineering processes: by either manipulating specific catabolic pathway or manipulating the host cells. The enzymes involved in degrading 4CBA can be extensively studied to improve or engineer other strains.

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