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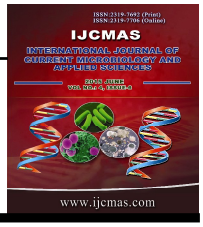

Review Article

Xenobiotic Degradation by Bacterial Enzymes

S.K. Jha, Paras Jain* and H.P. Sharma

Laboratory of Plant Physiology and Biotechnology, University Department of Botany, Ranchi University, Ranchi-834008, Jharkhand, India

**Corresponding author*

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A B S T R A C T

In agriculture and public health wide spread use of pesticide causes severe environmental problems and potential health hazards namely reproductive abnormalities, skin disease, headache, nausea, vomiting, fatigue, gastro-intestinal problems, cancer and neurological symptoms etc. Synthetic chemicals, i.e. pesticides have been prevalent in protection for eatables, building materials and clothing. Usually they are used against different harmful pests affecting different families of crops. Even Integrated Pest Management (IPM) which is a philosophy of pest management rather than a specific, defined strategy, combining physical, cultural, biological, chemical control and the use of resistant varieties does not put a complete tab on pesticides. A considerable investment of time and effort is required to determine the optimum strategy, for long term environmental and economic benefits. There are large group of pesticides which are used to increase crop production, but in present chapter two groups are discussed namely pyrethroids, organohalogen. Pesticides like β-cyfluthrin [α-cyano-4-fluoro-3-phenoxybenzyl-3(2,2,dichlorovinyl)-2,2-dimethycyclo-propanecarboxylate],dichloroehane,1,2-dichloroethane have entered in environmental factors like soil water & eatables through different routes and causing widespread environmental contamination. Simple culinary process was tested for decontamination of β- cyfluthrin was found quite efficient for 0 and day 1 sample in reducing the pesticide residues from fruit. The lime water soaking was found better for decontamination of eggplant fruits than simple washing with tap water. Though different pesticides are not systemic, but they penetrate the skin of different fruits. Therefore there is an urgent need for the detoxification of these compounds. The study was carried out to screen microorganisms and isolate through serial dilution and enrichment culture isolate. It is an efficient and prudent way to determining their ability to degrade β-cyfluthrin. There are only few reports available to biodegrade β-cyfluthrin and cypermethrin through different-strains of fungi. For study purpose known microorganisms viz., *Bacillus subtilis, Bacillus polymyxa, Klebsiella planticola* and *Proteus vulgaris; Burkholderia cepacia* have shown maximum degradation of these compounds up to 37.0%. Two degradation product were isolated by the preparative TLC technique and were identified as α-cyano-4-fluoro-3-phenoxybenzyl alcohol, and 3(2,2-dichlorovinyl)-2,2 dimethyl cyclopropanoic acid. Through enrichment culture method from a soil having history of pesticide, a strain S1 was isolated and through 16S ribosomal DNA sequence had 100% identity to the sequence of *P. stutzeri (Saikia et al., 2004)*. The product formed during degradation of β- cyfluthrin were identified as cyano-4-fluorobenzylalchol-3(2,2 dichlorovinyl)-2–dimethyl cyclopropane carboxylate (M.W. 341); 4-fluoro-3- phenoxy-α–cyanobenzylalcohol (M.W. 243) and 3, (2,2 dichlorovinyl) 2,2 dimethyl cyclopropanoic acid (M.W. 208) To located the biodegradation gene(s) of *Pseudonones stutzari* strain S1, capable of degrading β-cyfluthrin; molecular studies was further done. A mutant was formed *in vitro* having insertional Tn5 was deficient in biodegradation of β- cyflurthrin. The mutant of the same strain transfer the Tn5 plasmid in another strain of *E. coli* DH5 and this transfer was confirmed by Kanamycin resistant and PCR analysis of *E. coli* DH5. The plasmid of strain S1 was eliminated through EtBr along with heat shock treatment and showed that there was no biodegradation activity for β cyfluthrin. Thus it was confirmed that biodegradation genes are present on the plasmid which was identified as DNA circular molecule of 18.0 kb size. Through literature it is confirmed that the gene (s) code for enzymes found on the plasmid and not on the main bacterial chromosome. Based on that we are aware with xylenes, Napthalenes, octanes and camphores and the gene present on four types of plasmid XYL, NAH, OCT and CAM.

Introduction

A xenobiotic purely a synthetic compound is a man made compound which is not found in nature. Most xenobiotic compounds, however, are recalcitrant and some of them are biomagnified to dangerous/toxic levels. A xenobiotic compound is a man-made compound either not found in nature e.g DDT (dichlorodiphenyl tetrachloroethane), BHC (benzene hexachloride), organo-phosphates, etc. or found in a far lower concentration than liberated by man e.g. phenols, aromatic hydrocarbons, metals, etc. Biomagnification is the phenomenon of progressive increase in the concentration of a xenobiotic compound as it passes through the food-chain for example; DDT is absorbed by plants and microorganisms, which may be eaten by some animals including fish. The fish are food for birds. The DDT not only passes on in the food chain, but goes on accumulating mainly because it is the recalcitrant to biodegradation and it accumulates in body fats. Consequently certain sea-birds accumulated DDT in their livers to a toxic level so that they laid fragile (thin-shelled) eggs. In addition, recurrent exposure to low levels of such chemicals would also lead to their accumulation in human and other biological entities.

Most of the xenobiotic compounds for example, Pesticides however, are recalcitrant (the compounds that resist biodegradation and thereby persist in environment) and some of them are biomagnified to dangerous/ toxic level Xenobiotic waste/residues may be biodegradable, poorly biodegradable or recalcitrant and non biodegradable. Application of pesticides is increasing for the protection from pests and insects. Pesticides is inevitable and its use and spared is continued from ancient times. In past commonly used pesticides like S, Cu, Hg, Zn, CaO (lime), Boredaux mixture (40 gm$CaSo_4$+ 40gm $Ca(OH)_2$) in 5 liter water, Burgundy mixture ($CuSo_4$, washing soda crystal) into 50 gallons of water are used regularly to increase the crop yield to feed the uncontrollable growing population and to keep them in good health. Biomagnification/bioconcentration/biological amplification is the condition of progressive increase in the concentration of a xenobiotic compound as it passes through the food chain. Although there is integrated pest management and growing awareness and attempts are being made to reduce their use, certain chemicals are very persistent in nature and they remain in the environment for example aldrine (5yr.), chlordane (12yr), DDT (10yr) HCH (11yr) and zineb (75yr). Many of these compounds are toxic for different life forms. Persistent means, the pesticide molecule retain its molecular integrity and hence physical, chemical & functional characteristics in environment, may remain transported and distributed for considerable period of time. Therefore it is necessary to detoxify the residues of pesticides from food commodities.

Hazards from xenobiotic

The xenobiotic present a number of potential hazards to man and the environment, which are briefly listed below:-

1. Toxicity – Many xenobiotics like halogenate and aromatic hydrocarbons are toxic to bacteria, lower eukaryoutes and even humans. At low concentrations, they may cause various skin problems and reduce reproductive potential.

2. Carcinogenicity – Certain halogenated hydrocarbons have been shown to be arcinogenic.

3. Progressive Build up in the Environment. Many xenobiotics are recalcitrant and persist in the environment so that there is a buildup in their concentration with time.

4. Bioaccumulation. Many xenobiotics, including DDT and PCB's are recalcitrant and lipophilic; as a consequence they show bioaccumulation or biomagnifications often by a factor of 10'4 -10'6. Biomagnifications occurs mainly because of the following two reasons (i) these compounds are continuously taken up from the environment and accumulated in the lipid deposits of body, e.g. a 100- fold accumulation of DDT by plankton from water.

Types of recalcitrant xenobiotic compounds

Halocarbons

These compounds contain different numbers of halogen (e.g.,Cl,Br,F,I) atoms in the place of H atoms. $CHCl_3$, Ferons, CCl_3F, CCl_2F_2, $CClF_3$, CF_4, insecticides like DDT, BHC, lindane.

Polychlorinated Biphenyls (PCB)These compounds have two covalently linked benzene rings having halogens substituting for H.PCB are used as plasticisers, insulator coolants in transformers and as heat exchange fluids. They are both biologically and chemically inert to various degrees, which increases with the number of chlorine atoms present in the molecule.

Synthetic polymers: These compounds are produced as plastics e.g. polyethylene, polystyrene, polyvinyl chloride, etc., and nylons which are used in garments, wrapping materials etc. They are recalcitrant mainly due to their insolubility in water and molecular size.

Alylbenzyl sulphonates: These are surface active detergents superior to soaps. The sulphonate group present at one end resists microbial degradation while the other end (non polar alkyl end) becomes recalcitrant if it is branched (resistance increases with the degree of branching). At present alkylbenzyl sulphonates having nonbranched alkyl ends are used. These are biodegraded by β-oxidation from their alkyl ends.

Oil mixtures: Oil is a natural products has many components and is biodegradable the different components being degraded at different rates. Biodegration is able to handle small oil seepages. But when large spills occurs the problem of pollution become acute while is recalcitrant mainly because of its insolubility in water and due to their toxicity of some of its component.

Other xenobiotic compounds: A number of pesticides are based on aliphatic, cyclening structures containing substitution of nitro, sulphonate, methoxy, amino and carbomyl groups in addition they also contain halogens. These substitution made them recalcitrant.

Polychlorinated Biphenyls (PCB,s),synthetic polymers, Alkylbenzyl Sulphonates, Oil Mixture., Members of pesticides. In recent past large number of novel, manmade synthetic chemicals was came in to application to control the spread of disease.

Classifications of pesticides- on the basis of chemical nature are

(i) Organochlorines: These are chlorinated hydrocarbons. Heence, they are called organochlorines. e.g. DDT, BHC, Lindane, Dieldrin

(ii) Organophosphates: These consists of phosphorus bonded to carbon atoms of

organic radicals. e.g. Parathion and Melathion.

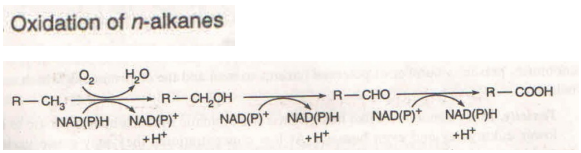
(iii) Carbamates: These are N-methyl and N,N-dimethyl carbamic esters of phenols and heterocyclic enols. e.g. Carbaryl and pirimicarb

(iv) Pyrethroids: Pyrethrum when produced synthetically, it provide new group of insecticides.

(v) **Trizines:** These pesticides have their own limitations because they fail to control different kind of disease caused by various causal organisms besides that sometimes the pathogenic organism undergoes in to mutations therefore become resistant to different pesticides. Soon its demerits was realized because these chemicals were highly toxic, persistent in nature and it not only kill the harmful pest but many friendly pest which are useful in many activities of food chain, biodiversity and pollen dissemination.

General feature of biodegradation of xenobiotics

Since xenobiotics consist of a wide range of compounds, their degradation occurs via a large number of metabolic pathways. Degaradation of alkenes and aromatic hydrocarbons generally occurs as follows: (i) an oxygenase first introduces a hydroxyl group to make the compound reactive, (2) the hydroxyl group is then oxidized to a carboxyl group, (3) the ring structure is opened up (in case of cyclic compounds), (4) the linear molecule is degraded by B-oxidation to yield acetyl coA which is, metabolized in usual manner.



Similarly, an alicyclic hydrocarbon, e.g., cyclohexane, is oxidized as follows: (1) first an oxygenase adds an -OH group in the ring, (2) then another oxygenase forms an ester in the form of a lactone, (3) which is then hydrolysed to open the ring structure to yield a linear molecule. In both these oxidation the mono-oxygenase are involved which add oxygen to asingle position in the molecule. In contrast, oxidation of benzene ring may involve a di-oxygenase which adds oxygen at two positions in the molecule in a single step.

Both mono and di-oxygenase are of a variety of types: some react best with short chain alkanes, while others act as cyclic alkanes. But these enzymes are not very specific and each enzyme oxidizes a limited range of compounds.

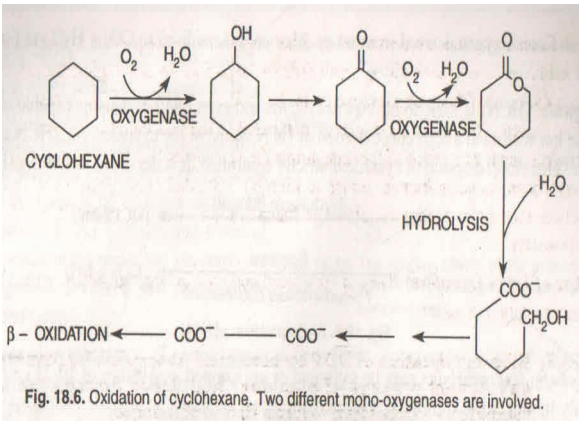


Fig. 18.6. Oxidation of cyclohexane. Two different mono-oxygenases are involved.

Thus Xenobiotics are degraded by awide variety of micro-organisms, each of which degrades a small range of compounds. Frequently oxidation of Xenobiotics involves cytochrome P450 or rubredoxin. In addition the halogen or other substituent groups are either modified or removed as one of the initial reactions or sometimes it is achieved later in the process.

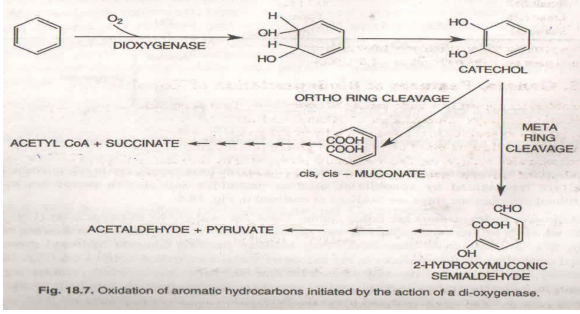


Fig. 18.7. Oxidation of aromatic hydrocarbons initiated by the action of a di-oxygenase.

Hydrocarbon degradation

The degradation of hydrocarbon is briefly outlined below:

1. Halomethanes are transferred into methanol by enzyme methane mono oxygenase which uses them as substrate. This enzyme occurs in a number of methylotrophs. Alternatively a glutothione dependent hydrolase catalyses oxidative dechlorination of halomethanes into methanol. This reaction is anaerobic and uses oxygen derived from water. Methanol is oxidized to CO_2 +H_2O via formaldehyde and formic acid.

2. Cyanide (HCN): is toxic to biological system and even microorganism capable of degrading cyanide cannot withstand a high concentration of HCN. Some of the cyanides e.g. HCN and CH_3CN are volatile. Therefore disposal of strictly controlled. It is degraded by fungal hydratase into $HCONH_2$ and by pseudomonas fluorescence into CO_2 and NH_2.

3. Aliphatic Hydrocarbon may be saturated or unsaturated. N-alkanes of 10-24 carbons are most deadly biodegradable. Similarly aliphatics are easier to degrade than unsaturated ones and branched chain show decreased bio-degradation. Biodegradation of n-alkanes is catalysed by oxygenases to produce carboxylic acids, which is then degraded by B-oxidation. Oxidation may involve the methane group at one end of n-alkane molecule, or it may occur as beta methylene group. Sometimes, both terminal methyl grops are oxidized to yield a di-carboxylic acid.

4. Alicyclic Hydrocarbons are present naturally in waxes from plants, crude oil, microbial lipids and are represented by Xenobiotics used as pesticides and also in petroleum products.

5. Aromatic hydrocarbons are rather stable. These are oxidized by di-oxygenases to catechol which is further metabolized by two separate pathways. (i) IN case of ortho-linkage cleavage pathway, a 1,2-dioxygenase cleaves the ring between the two adjacent hydroxyl groups and sequential catabolism of the product cis, cis-muconate yields Succinate+acetyl CoA. (ii)alternatively the enzyme 2,3, di-oxygenase cleaves the ring between the carbon atoms having an OH group and adjacent carbon lacking an OH group (meta cleavage). The product at the end of the reaction is acetaldehyde and pyruvate. Both ortho and meta are involved in degradation of aromatic hydrocarbon. Benzene is degraded by meta pathway.

6. Polycyclic hydrocarbon contains two or more rings. Generally one of the terminal rings is attacked by di-oxygenase. Degradation of complex molecule containing aliphatic, aromatic, alicyclic

or heterocyclic components is difficult to generalize but the following features are observed,(i) amide, ester or other bonds are first attacked and further degradation of the product so generated takes place (ii) if these bonds are absent or inaccessible aliphatic chains are degraded. (iii) if aliphatic chains are branched the aromatic component of the complex molecule may be attacked. (iv) the site and mode of attack depends on the molecular structure, the microorganism involved and the environmental conditions in general recalcitrance of various benzene derivatives increases with substituent groups (at meta position): COOH=OH<-NH_2< -O-CH_3< -SO_3 <-NO2. Further the greater the number of substituent groups on the benzene ring, higher the rate of recalcitrance. As the position of substituent group also affects recalcitrance as meta>ortho>para.

Methods of degradation

There are several ways which are unambiguously applied to different pesticide (because it has different quality and composition of waste and residues. Detoxification methods may be classified as:

(i) Physical
(ii) Chemical
(iii) Biological

The physical methods comprising of washing, cooking, peeling, sun drying, brushing, solvent washing/ removal, washing with water, soaps; adjuvant or surfactant, burial and disposal. The chemical alterations include oxidation, reduction, nucleophilic displacement, hydrolysis and high energy decomposition. The Biological methods include are through biocatalysis; enzymatic oxidation and microbial degradation either by using them as a carbon source in place of other substrate as a carbon or by co-metabolism.

Mechanism of biodegradation-

Mechanism involved in chemical bond cleavage. Xenobiotic compounds may be recalcitrant due to one or more of the following reasons:

I. Presence of halogens in the place of hydrogen in the molecule the carbon-halogen bond is highly stable and its cleavage requires considerable energy.
II. Substitution of H by other group like nitro, sulphonate, methoxy, amino and carbonyl groups.
III. Cyclic structures, aromatic compounds, cyclo alkanes and hetrocyclic compounds are more recalcitrant than linear chain or aliphatic compounds,
IV. Branched linear chains resist biodegradation.

Biodegradation involves oxidation by cyctochrome P-450, the most common monooxygenase reaction are those employing heme protein P-450. Like mitochondrial cytochromeoxidase, cytochrome P-450 can react with O_2 and bind CO. (CO complex of its reduced form absorb 450nm, thus name P-450). Cytochrome P-450 catalyzes (hydroxylation) OH in which organic substrate, RH is hydroxylated to R-OH; incorporating one oxygen atom of O_2.

Detoxification of xenobiotics may involve one of the following mechanisms:

- Hydrolysis- breaking of ester bond (C-O-C) in malathion; activation of herbicide (eg. Dichlorofop methyl)
- Hydroxylation- replacing H with OH in 2,4-D.
- Dehalogenation- reductive (replacement with H), Hydrolytic (replacement with OH)for DDT into DDE.
- Dealkylation e.g- demethylation of chloroneb
- Methyaltion eg. Pentachlorophenol conversion of 2,4 dinitrophenol to 2-amino-4 and 4- amino-2-nitrophenol
- Nitro- reduction
- Deamination
- Ether cleavage eg. 2,4-D loss of phytotoxicity
- Nitrile to amide conversion
- Conjugation eg. Pyrene + glucose= glucoside conjugtate microorganisms involved in biodegradation:

Microorganisms involved in biodegradation

Apart from bacteria and fungi, algae are also used in pesticide decontamination although to lesser extent. Several green and blue-green algae (Mukherjee *et al.,* 2004), isolated from soil or water, have been found to degrade organophosphorus insecticide chlorpyriphos, monocrotophos and quinalphos. *Actinomycets* has shown the degradation of herbicide pendimethalin (Gopal *et al.,* 2005).

Pseudomonas sp. or *aspergillus niger* could hydroxylate 2,4-D present in a factory waste. Degradation of three benzonitrile herbicide,bromoxynil(3,5-dibromohydroxy-benzonitrile),ioxynil(3,5diiodo-4-hydroxy-benzonitrile), dichlobenil (2,6-dichloro-benzonitrile), and their mixture by the soil microorganism *Agrobacterium radiobacter* was studied in batch culture. The mixture of bacterial culture, enhancing the degradation of carbofuran residues to 96% in 10 days. (Mohapatra and Awasthi, 1997). The bacteria feed on chicken and low manure, old newspapers, straw and wood chips can be used to clean up dangerous organochlorin such as DDT. Bacteria that grow on rotting waste can break down DDT (Anonymous, 2000). Chemists at Oxford University, Southern England "tweaked" a gene from *pseudomonas putida,* to make an enzyme that attack soil pollutants like dichlorobenzene etc. The ability of plasmid transfer can be exploited to create microorganisms with novel characteristics. For example, *Alcaligenes sp*. degrade 4-chlorophenol to 5-chlo-2-hydroxymuconic semialdehyde (by meta cleavage of the ring) which is toxic. *Pseudomonas strain* B13 has a plasmid-borne gene which encode the enzyme 1,2-di-oxygenase, this enzyme cleaves 4-chlorophenol by ortho pathway.

White rot fungi, which can degrade lignin present in wood, has also shown ability to degrade wide varieties of different chemicals, even those, which did not have any structural relationship with liginn. Among the various species, *Phaenerochaete chrysosporium* was reported to be the most important as it could degrade several environmentally persistent organic pollutants like DDT, lindane, benzopyrene, azo-dyes and dioxin. Degradation of lindane by white rot fungi has also been reported. Two different species of white rot fungi namely *Cyathus bulleri* and *Phanerochaete sordida* were studied for their ability to degrade the compound. Degradation of various organophosphorus compounds by different fungi has also been documented. Highest degradation has been observed in case of *Aspergillus sydouri* followed by *A. flavus* and *Fusarium oxysporum*. *Aspergillus*

niger has demonstrated the ability degrade-endosulfan up to 40% by 1st day, followed by fast dissipation to 98.6% by day 15. *A. niger* could also degrade chlorpyrifos. Up to 95.7% degradation of chlorpyrifos has been obtained by using the fungus *Trichoderma viride* within 14 days. Degradation β-cyfluthrin by as strain of *T. viride* and identification of the products formed are also documented. Metolachlor, an herbicide could be degraded by a mixed fungal culture isolated from a metolachlor acclimated field soil. Processes like hydrolytic dechlorination. N-dealkylation and amide bond cleavage appeared to be the dominant ways of transformation of metolachlor.

An aerobic bacterium capable of degrading β-cygluthrin was isolated from soil, with known history of its disposal by enrichment culture using the following procedure:

- Soil was collected from fields having history of different types of pesticides application
- 9ml of saline water was inoculated with 1g soil sample,
- 1ml supernatant was taken from it and was transferred to fresh 9ml solution. This procedure was repeated 8 time or serially diluted and plated on mineral salts agar medium.
- It was incubated at 30˚C for 3 days.
- Different types of bacterial colonies appearing on the medium were purified by repeated streaking on mineral salts glucose agar medium.
- Pure cultures of different types of bacteria were obtained on mineral salt glucose agar (MSGA) slants.

Origin and distribution of dehalogenase gene sequences

Analysis of the genetic organization of biodegradation pathway provides insight into the genetic processes that led to their evolution. It appears that catabolic genes for xenobiotic compounds are often associated with transposable elements and insertion sequences. They are also frequently located on transmissible plasmids. One striking example of a mobile element that has assisted catabolic genes in their dissemination is IS 1071. This insertion element flanks the haloacetate dehalogenase gene dehH2 on plasmid pUO1 in *Moraxella sp*. Strain B (Kawasaki *et al.,* 1992), the haloalkane dehalogenase gene *dhaA* on the chromosome in *P. pavonaceae* 170 (Poelarends *et al.,* 2000a), the atrazine degradative genes *atzA, atzB* and *atzC* on plasmid pADP-1 in *Pseudomonas sp.*ADP (Wackett, 2004), the aniline degradative genes on plasmid pTDN1 in *Pseudomonas putida* UCC22 (Fukumori and Saint, 1997), and presumably also the p-sulfobenzoate degradative genes on plasmids pTSA and pPSB in *Comamonas testosteronei* strains T-2 and PSB-4 respectively (Junker and Cook, 1997). These observations clearly indicate that gene mobilization between and within replicons is an important process during genetic adaptation. It also suggests that genes that are involved in biodegradation of xenobiotics were recruited from a 'pre-industrial' gene pool by integration, transposition, homologous recombination and mobilization.

Although insight in the sequence of events that led to the current genetic make-up of biodegradation pathways is lacking, the general nature of some of the processes involved is understood. Less is known about the origin of the structural genes that encode critical enzymes, such as dehalogenases, and the degree of divergence that occurred during evolution of the current sequences. The possibility to rapidly evolve new enzyme selectivities by laboratory evolution is known since the 1970s, most notably

through the work of P.H. Clarke and coworkers who showed that the substrate specificity of *Pseudomonas aeruginosa* amidase can be changed can be changed by mutagenesis and selection on plates (Betz *et al.,* 1974; Paterson and Clarke, 1979). This approach was called experimental enzyme evolution and is conceptually similar to directed evolution.

Theoretically, it is possible that a current gene for a specific critical (dehalogenase) reaction was already present in the pre-industrial gene pool. Alternatively, there could be a short evolutionary pathway that led from an unknown pre-existing gene to the gene as we currently find it in a biodegradation pathway. It has even been suggested that a new sequences for enzyme acting on a synthetic compound could evolve through the activation of an unused alternative open reading frame of a preexisting internal repetitious coding sequence (Ohno, 1984). Here it should be noted that the similarity of a dehalogenase to members of an enzyme superfamily that catalyse other reactions generally does not provide intermation about the process of adaptation to xenobiotic compounds.

No such primitive dehalogenase has yet been detected, with the notable exception of TriA, the enzyme that dehalogenates the herbicide atrazine (vide infra).

One way to obtain information about the evolutionary origin of dehalogenase genes is to compare the dehalogenase sequences that have been detected in different bacterial cultures, If closely related sequences are present, this would make it possible identify sequence differences and to determine the effect of the mutations on substrate selectivity. Another approach is to search for sequences that are closely related to dehalogenases in databases of sequenced genomes.

Analysis of sequence databases such as Pfam and of the literature revealed that in several cases the same dehalogenase sequence has repeatedly been detected in organisms that have been isolated from different geographical locations. Thus, identical or almost identical haloalkane dehalogenases, dichloromethane dehalogenases and atrazine chlorohydrolases have been detected in isolates from different areas (Table 2).

The current version of the NCBI databases lists more than 250 proteome sequences of bacteria and 31 archaeal proteomes. When these were searched for 15 different dehalogenase sequences that have been detected in organisms isolated on halogenated compounds as carbon source, it appeared that no single closely related counterpart of dehalogenase genes was present in these sequenced bacterial genomes (Table 2).

Table 2 Number of positive hits obtained with protein sequences of dehalogenases and alkane hydroxylases as queries in Blast-P searches against protein sequence databases.

At the same time, it appears that more distantly related dehalogenase sequences are quite common. For example, large numbers of putative haloalkane dehalogenase and haloacid dehalogenase sequence are present in the whole-genome database and in the Sargasso Sea database (Table 2).

Haloalkane dehalogenase from *Xanthobacter autotrophicus* (DhlA)

Haloalkane dehalogenase (DhlA) was originally discovered in *X. autorophicus* GJ10, a nitrogen-fixing hydrogen bacterium that was enriched with 1,2-dichloroethane as the sole carbon source. Subsequently, identical DhlA-encoding genes have been

discovered in several other strains of *X. autotrophicus*, isolated in the Netherlands and Germany and in isolates of *Ancylobacter aquaticus* obtained with 1,2-dichloroethane or chloroethylvinyl ether as the growth substrate (van den Wijngaard *et al.*, 1992). Recently a strain of *Xanthobacter flavus* was isolated in South Korea and this organism also possessed and identical dehalogenase (Song *et al.*, 2004). In fact, DhlA is still the only known hydrolytic haloalkane dehalogenase that operates in 1,2-dichloroethane degrading bacteria; no variants have been described and at least 12 identical copies of this gene have been obtained from different environmental isolates.

Rhodococcus haloalkae dehalogenase (DhaA)

Another abundant type of haloalkane dehalogenase is the one form *Rhodococcus erythropolis* (DhaA). Mutually identical copies of the dhaA genes have been detected in different organisms, of which the taxonomy has been rather confusing due to errors in classification. The first dhaA sequence was determined by Kulakova and colleagues (1997), using a strain of *Rhodococcus* (*Rhodococcus rhodochrous* NCIMB 13064) that was isolated on 1-chlorobutane. Later sequencing of several other haloalkane dehalogenase gens from 1-chlorobutane, 1-chlorohexane and 1,6 dichlorohexane-degrading gram positive organisms, some of which had been isolated before strain NCIMB 13064, showed that these possessed identical *dhaA* sequences. Poelarands and colleagues (2000b) have reclassified several organisms and using 16s rRNA gene sequencing they showed that *R. erythropolis* Y2 (England), *R. rhodochrous* NCIMB 13064 (N. Ireland), *Corynebacterium* sp. Strain m15 (Japan), *Arthrobacter* strain HA1 (Switzerland), strain GJ70 (the Netherland, originally called *Acinetobacter*) and strain TB2 (USA) should all be classified as *R. erythropolis*.

The N-terminal part of the cap domain of wild-type DhlA harbours two short tandem sequence repeats (shown as arrows), which might be signs of recent genetic adaptation to 1, 2-dichloroethane. This idea is supported by the following observations. First, as shown in panel A, changes are observed in this part of the cap domain (shown in boxes, three tandem duplications, a large deletion and two substitutions) when DhlA is forced to evolve dehalogenase activity toward 1-chlorohexane, a substrate not used by the wild-type enzyme (experimental enzyme evolution; Pries *et al.*, 1994).

Second, in a pairwise sequence alignment the closest homologue of DhlA in the database (a putative protein from *Erythrobacter litoralis* TCC2594) shows a gap precisely in this region of the cap domain (panel B). Third, from the current 1,2-dichloroethane dehalogenase sequence (DhlA) a pre-industrial sequence (primitive) was proposed and constructed. The encoded protein is inactive with 1,2-dichloroethane (DCE), but does convert 1,2-dibromoethane (DBE) (panel C).

When the primitive dehalogenase is subjected to ITCHY mutagenesis, a technique that allows the introduction of random repeats and deletions in the N-terminal part of the cap domain, some mutants carrying repeats (D2, lC12 and 3B2) had evolved 1,2-dichloroethane dehalogenase activity (Pikkemaat and Janssen, 2002) (panel D).

On the basis of these findings, we propose that a primitive dehalogenase with a shorter stretch of sequence in the N-terminal region

of the cap (as observed in the *E. litoralis* putative dehalogenase) was recruited from the pre-industrial environmental gene pool, and evolved into the current DhlA by a short volutionary pathway that included generation of short duplications and substitutions.

Atrazine chlorohydrolase (AtzA)

The bacterial degradation of the herbicide atrazine by *Pseudomonas* ADP starts with a hydrolytic dechlorination catalysed by an enzyme called atrazine chlorohydrolase (AtzA). Identical atrazine chlorohydrolase gene have been obtained from different source including an *Arthrobacter* from china (Cai *et al.*, 2003), strains from France (Rousseaux *et al.*, 2001), an undescribed β-protobacterium strainCDB21 from Japan of which the sequence was deposited by lwaski and collegues (EMBL Accession number AB19409700), and four other isolates from different location including an *Alcaligenes,a Ralstonia* and an *Agrobacterium* sp.(de Souza *et al.*, 1998).

Table.1 Screening of bacteria for degradation of pesticides

Bacteria	% of Degradation of Pesticide				
	Atrazine	Chloropyrifos	Metribuzine	Imidacloprid	Fenvalerate
Bacillus Subtilis	60	60	25.41	32.13	ND
Proteus vulgaris	ND	85	18.89	19.72	80
Pseudomonas striata	ND	30	88.73	62.95	12
Kelbsiella plenticola	ND	12	19.48	36.68	ND
Bacillus Polymyxa	7	Nil	74.48	Nil	82

Table.2 Number of positive hits obtained with protein sequences of dehalogenases and alkane hydroxylases as queries in Blast-P searches against protein sequence databases

Query protein	GenBank GI number	Super family	Filtered by conserved pattern or residues	No of identical genes found in isolates	No. of homologues in microbial genomes	No. of homologues in Archae genomes	No. of homologues in Sargasso Sea
DcmA	482502	GST	126H-W*	11 (> 96%)	2 (33–22%)	0	4 (30–25%)
DhlA	729681	α/β-HF	124D-W-G, 289H*	12 (100%)	15 (50–26%)	1 (30%)	35 (41–21%)
DhaA	3114657	α/β-HF	106D-W-G, 272H*	7 (> 98%)	28 (53–23%)	1 (26%)	44 (49–22%)
LinB	9789853	α/β-HF	108D-W-G, 273F*	3 (> 99%)	10 (69–25%)	0	43 (65–21%)
DhlB	3122178	HAD	8D, 39R, 147K#	7 (49–39%)	39 (57–25%)	3 (26–24%)	119 (52–23%)
HAD-Ps	3122176	HAD	10D, 41R, 151K#	12 (77–39%)	35 (55–23%)	4 (27–23%)	115 (50–24%)
HheA	15213645	SDR	135S-X(7,17)-Y-X(3)-R*	2 (99%), 3 (49–48%)	6 (30–21%)	0	4 (31–21%)
HheB	15209119	SDR	126S-X(7,17)-Y-X(3)-R*	1 (98%)	16 (45–19%)	1 (27%)	10 (53–21%)
HheC	15213643	SDR	132S-X(7,17)-Y-X(3)-R*	2 (100–92%), 2 (49%)	6 (30–20%)	0	5 (31–21%)
CaaD	10637969	4-OT	9R-X-X-R*	0	0	0	6 (35–29%)
LinA	51859616	DH	25D, 73H#	3 (> 99%)	4 (30–27%)	0	3 (31–25%)
CbzA	2392484	ECH	90H, 145D#	6 (86–51%)	21 (41–26%)	1 (28%)	48 (37–27%)
AtzA	32455822	DA	60H-X-H, 243H, 327D*	4 (> 98%)	134 (36–20%)	12 (31–27%)	20 (32–19%)
TriA	42558845	DA	76H-X-H, 251H, 287H*	2 (> 99%)	156 (36–21%)	21 (26–20%)	32 (34–21%)
PcpC	22417110	GST	12S-X-C*	1 (96%)	2 (37–23%)	0	49 (41–19%)
AlkB	113639	AH	269 N-Y-X-E-H-Y-G*	9 (100–77%)	21 (50–32%)	0	2 (82%), 49 (50–25%)
AlkM	2623971	AH	281 N-Y-X-E-H-Y-G*	12 (90–62%)	1 (100%), 20 (50–32%)	0	51 (56–28%)

Protein sequences (column 2) were compared by BlastP (Altschul *et al.*, 1990; 1997) to the NCBI whole microbial database (295 proteomes, April 2005) and to the NCBI environmental Sargasso sea proteins (Venter *et al.*, 2004). Hits with an E-score < 0.01 were considered homologues. Output lists were filtered by looking for conserved residues in a multiple alignment (#, column 4) or by using PHI-BlastP with a conserved pattern (* column 4). If uncertain, sequences in the low-similarity region were used as Blast queries against the Uniprot database to confirm their identity. Results in columns 5–8 give the number of homologues followed by the percentage range of amino acid identities. In some cases very high scores are mentioned separately. AlkB, AlkM: only hits containing motif C (see text) were counted. AlkM scores 100% in one case in column 6 because the whole genome of the original host has been sequenced.
GST, glutathione transferase; α/β-HF, α/β-hydrolase fold family; HAD, haloacid dehalogenase; SDR, short chain dehydrogenase reductase; 4-OT, 4-oxalocrotonate tautomerase; DH, dehydratase; ECH, enoyl CoA hydratase; DA, deaminase; AH, alkane hydroxylase. The abbreviations for the proteins and their functions are given in the text.

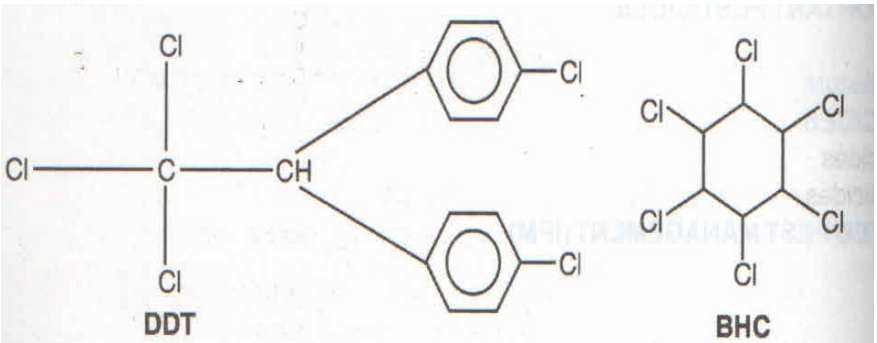


Fig 1 A-B. Common organochlorine insecticides.

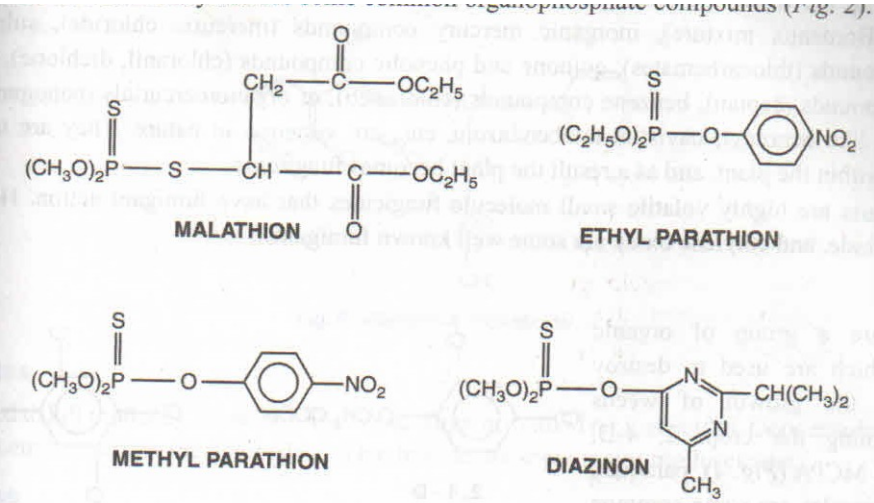


Fig. 2. Common organophosphate insecticides.

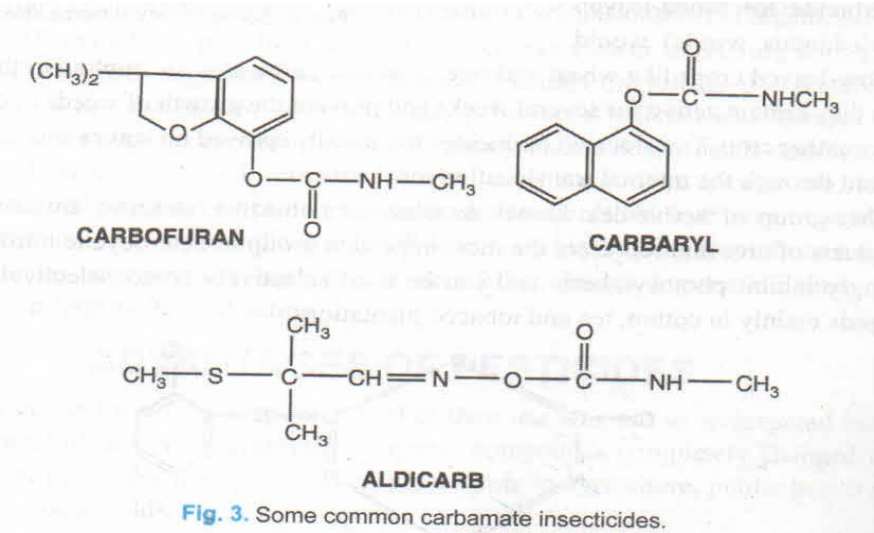


Fig. 3. Some common carbamate insecticides.

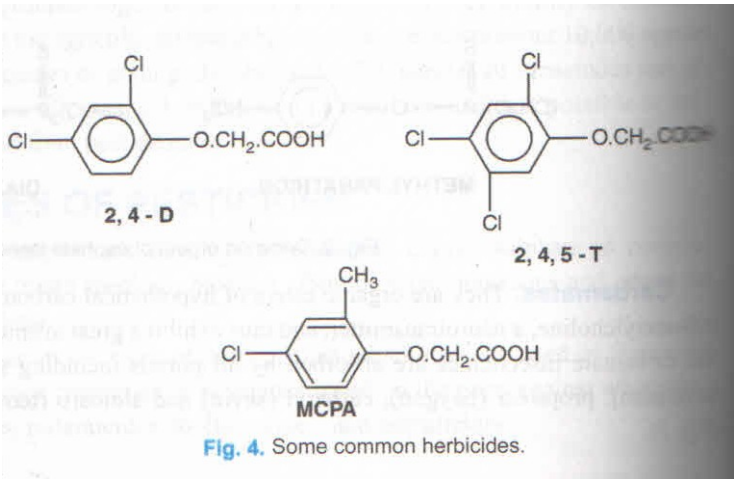


Fig. 4. Some common herbicides.

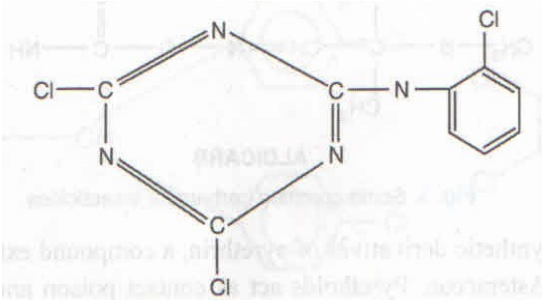


Fig. 5. Anilazine-a triazine.

The protein belongs to the amidohydrolase superfamily which also houses the next two enzymes of the atrazine catabolic pathway: hydroxyatrazine ethyl-aminopropylohydrolase (AtzB) and N-isopropylammelide N-isopropylamino-hydrolase(AtzC) (de Souza *et al.*, 1996). Other members of amidohydrolase super family are triazine deaminase, hydantoinase, melamine deaminase, cytosine deaminase and phosphotriesterase.

Alkane hydroxylase (AlkB, AlkM)

Aliphatic hydrocarbon is introduced into the environment in large quantities both by human activities and by natural processes. Plants, for instance, can produce (odd-length) n-alkanes as part of mixtures of waxes.

Thus, they cannot be regarded as xenobiotic compounds, even though most cases of contamination of surface soils with high levels of alkanes are caused by industrial processing of petroleum.

In many different environments bacteria have been exposed to these compounds, and one would expect that evolution of enzymes that can hydroxylate alkanes has occurred over a longer period of time than with dehalogenases that act on exotic compounds.

Conclusion

Bioremediation is an effective treatment process that use organisms through their enzymatic activities. However, slow growth of microorganisms or difficulties in the control and maintain the optimal condition

for the microbial growth is disadvantages of biodegradation process. The direct application of enzymes in the environmental treatment process has been quite limited due to loss of enzyme activity, and therefore, novel methods of enzyme stabilization are developed. Different enzymes used against different substrate but many of them has proved to be wide substrate specificity too,its make them an effective target in the design of enzyme systems for bioremediation.

Microbes and their enzymes are responsible for breakdown and for that reason they are directly involved in biogeochemical cycles, and can be very useful in the development of bioremediation technology.

Xenobiotic halogenated substrates as a carbon sources was degraded by dichlormethane dehalogenase (DcmA), haloalkane dehalogenases (DhlA, DhaA,LinB) and atrazine chlorohydrolase (AtzA). Indeed, the dehalogenase genes are often associated with integrase genes, invertase genes, or insertion elements and they are usually localized on mobile plasmid. The diversity of such specialized dehalogenase act on xenobiotic seems to be more restricted then those act on other like alkanes. Presence of large number of unexplored functional sequences make the scope of creation of microbial system has enormous potential for scaling and growth.

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