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Review of Isolation of Potential Dehalogenase Marine Bacteria that can Degrade 2, 2-Dichloropropionate (2,2-DCP)

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Abstract
A 2, 2-dichloropropionic acid is categorized as organic herbicidal compounds under the class aliphatic. The common name of 2, 2-DCP is referred as 'Dalapon' as designated by both the British Standards Institution and Weed Science Society of America (Ashton and Crafts, 1973). In the meantime, 'Dalapon' is also considering the trademark of 2, 2-DCP sold as herbicide by the Dow Chemical Company. In addition, there are other trade names available in the market for instant Unipon, Basinex P, Dalacide and Radapon. The commercial name (Dalapon) refers to the herbicide of 2, 2-DCP in the form of the sodium salt and the products usually contain 85% sodium salt of 2, 2-DCP. The characteristic of 2, 2-dichloropropionate in its pure acid form is a colourless liquid with an acrid odor and as a sodium salt; it tastes salty and is a white to off-white powder that readily takes up water or moisture. 2, 2-DCP is stable under normal temperatures and pressures, nonflammable but may pose a slight fire hazard if exposed to heat or flame. Thermal decomposition of 2, 2-DCP will release corrosive fumes of hydrogen chloride or toxic chlorine gas. The molecular structure of 2, 2-DCP consist of three carbon compound with molecular formula $CH_3CH(Cl_2)COO$ (as shown in Figure 2.1). Furthermore, 2, 2-DCP or Dalapon was proven in several research to be non carcinogenic. This confirmed by feeding animals using Dalapon salt for a few months up to two years. It was clearly that no carcinogenic activates or mutation has been observed.

1.Introuduction

There is a carboxylic functional group (-COOH) and two chloride substituent at (Cα) position. The α-chlorination is particularly important within the halogenated propionic series because it results in herbicidal activity. Chlorination in other position alone does not result in such activity. Generally, the substitution of other halogens for chlorine reduces the herbicidal activity throughout the series. For the meantime, the presence of two chloride ions increases the resistance of the compound against biodegradation. Carboxylic group gives the compound the characteristic of acid. In addition, the presence of carboxylic group enables the sodium salt of 2, 2-DCP to take up water or moisture easily (Leasure, 1964).

In general, 2, 2-DCP in the form of the sodium salt (Dalapon) is categorized one of the best

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herbicides available for the purpose of controlling specific annual and perennial grasses. For instant, Bermuda grass, quack grass, Johnson grass and it is selective, that means it kills only the target plants while sparing other non target types of vegetation (Ashton and Crafts, 1973; EXTOXNET, 1993). In plants, 2, 2- DCP is taken up by leaves and will be translocated throughout the plant and even it is strong acid and protein precipitant, it is capable of producing an acute contact burn. The herbicide can cause widely effects on plants such as leaf chlorosis, leaf necrosis, growth inhibition and eventually death of the target plant (Ashton and Crafts, 1973).

Non Herbicidal Effects of 2, 2-DCP

2, 2-dichloropropionic acid can cause toxicological effects to animals as well as humans beside it effects on target plant. For humans, 2, 2-DCP is moderately toxic and it effects of acute exposure include loss of appetite, slowing of pulse, irritation eyes such as corneal damage, skin irritation, skin burns, gastrointestinal (GI) disturbances such as diarrhea or vomiting and also irritation of the respiratory tract. Some previous studies which done in dogs and rats showed increased kidney weights in animals fed very high daily doses. In one experiment, rats fed with 50mg/kg/day for two years and dogs fed with 1100mg/kg/day for one year both showed slight average increase in kidney weight (Occupational health Services, 1986). On the other hand, 2, 2-DCP is non toxic to birds and its very low toxicity to fish. As for the aquatic invertebrates, the toxicity is highly varied depending on the species (Pimentel, 1971).

Molecular Fate of 2, 2-DCP

The stability of 2, 2-DCP in higher plants and animals is quite stable but in soil it degrades rapidly (Ashton and Crafts, 1973). The microbial degradation in soil and leaching appear to be the important fate processes with respect to 2, 2-DCP (Howard, 1989). Even this compound does not readily bind or absorb to soil particles but it has the ability to mobile in all soil types and thus results in leaching. The speed of degradation is wholly dependent on soil conditions favorable to microorganisms, with temperature of 20 to 30°C and adequate soil moisture. The half life of 2, 2-DCP is one and a half to three days and 12 hours in human blood and blood system of dogs respectively. In natural water, 2, 2-DCP disappears by microbial degradation and hydrolysis (Howard, 1989). When microbial degradation is being absent, the half life of 2, 2-DCP by hydrolysis is several months in temperature below 25°C. Microbial growth under preferred conditions can decompose 2, 2-DCP by microorganisms and this will probably be completed within one month.

Biodegradation of Halogenated Aliphatic Acids

In general, many types of microorganisms are capable of utilizing halogenated organic compounds as a main carbon source for growth, and the organically bound halogen is liberated as the halide ion. The ability of degradation of these microorganisms refers to possess enzymes which are normally inducible known as dehalogenases that catalyze dehalogenation reactions (Bollag and Alexander, 1970; Hardman and Slater,

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1981)

Table 1: Examples of microorganisms involved in the biodegradation of chlorinated aliphatic hydrocarbons (Chaudhry and Chapalamadugu, 1991).

COMPOUND	MICROORGANISM	REFERENCE
2-chloropropionic acid	*Pseudomonas* sp.	Hardman *et al*. (1986),
2-monochloroacetic acid	*Alcaligenes* sp.	Hardman *et al*. (1986),
1,2-dichloroethane	Anaerobes	Bouwer and McCarty, (1983)
	Methane-utilizing bacteria	Yokata *et al*., (1986)
1-chlorobutane	Anaerobes and anaerobes	Wubbolts and Timmis, (1990)
1,2-dichloropropane	*Pseudomonas fluorescens*	Vandenbergh and Kunka, (1988)
1,9-dichlorononane	Anaerobes	Yokata *et al*., (1986)
Perchloroethane	*Methanosarcina* sp.	Fathepure *et al*.(1998),

Bacterial Degradation of 2, 2-DCP

Many researchers had been isolated different types of bacteria that able to decompose 2, 2-DCP as their sole carbon. Allison et al., (1983) had isolated Rhizobium sp. which was found to produce three dehaloganases. Slater et al., (1979) on the other hand isolated Pseudomonas putida PP3 that also produce three distinct dehalogenases. Schwarze et al., (1997) had also isolated several bacteria that able to produce dehalogenases. Jensen, (1957) proposed that some bacteria that capable of growing in 2, 2-DCP as a substrate will induce dehalogenase enzyme that catalyze a hydrolytic process of dechlorination at which point the resulting hydroxyl acid was dehydrogenated by the general respiratory mechanism and utilized as a source of energy. After that, this hypothesis was proven by Berry and Skinner (1976) with the Rhizobium sp. previous investigations reported that only Rhizobium sp. able to produce the three forms of dehalogenase and nothing clear about the physiological role for each enzyme. Furthermore, latest researches have been improved the previous analysis and agreed that the growth of Rhizobium sp. in 2, 2-DCP was slower (Doubling time 12 hours) while growth in D, L-2-bromopropionate was faster (Doubling time of 5 to 6 hours). This result leads to suggest that there is more than one dehalogenases in some of bacterial species.

Therefore, dehalogenation process is an important step to degrade of 2, 2- DCP. In this process, the dehalogenase enzyme will cleave the carbon chlorine bonds and replacing it by a hydroxyl group derived from water (Jensen, 1957; Schwarze et al.,1997). Pyruvate is the end

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product of this reaction which is a common intermediate in metabolic pathway and is readily metabolized by bacteria. According to Jensen, (1957) the degradation process of 2, 2-DCP can be summarized.

Basic Classification and Function of Dehalogenases

Many of soil bacteria that capable to degrade halogenated compounds had been isolated and it is well understood that only microorganisms producing dehalogenases are capable of growing on halogenated compounds as a sole of carbon an energy (Hardman and Slater, 1981). However, dehalogenases for isolated bacteria can be characterized according to their genetic and biochemical properties and they are different in terms of size and subunit structure, substrate specificities, electrophoretic mobility and steroselectivity (Schwarz et al., 1997). The significance of multiple dehalogenases is yet not to be investigated.

Table 2: Some bacteria that producing 2-haloalkanoic acid hydrolytic

GROUP	CLASS	BACTERIAL DEHALOGENASE	REFERENCES
2-haloalkanoic acid hydrolytic dehalogenase	1D	*Rhizobium* sp. -DehD	Leigh *et al*.,(1986)
	1L	*Rhizobium* sp. -DehL	Leigh *et al*.,(1986)
	21	*Rhizobium* sp. -DehE	Leigh *et al*.,(1986)
	2R	*Pseudomonas putida* strain PP3- DehI	Weighman *et al*.,(1982)

In general, dehalogenases can be classified in to three major grouping based on mechanisms of reactions or by substrate specificities. Slater et al, 1997 proposed three basic groups of dehalogenases classification which are: hydrolytic dehalogenase, halo alcohol dehalogenase and co-factor dependent dehalogenases. For instant, 2-haloalkanoic acid hydrolytic dehalogenases were the most common and well studied ones (Table 2). Many types of dehalogenases or abbreviation (Deh) have been found and characterized. For example, in Rhizobium sp. there is more than one dehalogenase present (Huyop et al., 2004). For instance, DehD acts exclusively on D-2-haloacids (e.g. D-2-chloropropionate) and forms the corresponding L-2-hydroxyacids (e.g. L-lactate). On the other hand, DehL acts only on L-isomer of D, L-2-CP and several other 2-haloacids and forms D-lactate. The DehL and DehD for Rhizobial are completely different by comparing their amino acids sequence and the amino acids sequence of DehE show little similarity to DehL(16%) and DehD (14%) (Huyop et al.,

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2008). In addition, DehE can catalyze both isomers of 2-haloacids. Furthermore, DehE gene might had evolved from dehD and dehL genes and gain new ability to react with 2, 2-DCPA (Huyop et al., 2008). In short, the functions of different bacterial dehalogenases are shown in Table 3.

Table 3: The functions of some microbial dehalogenases

TYPE OF DEHALOGENASE	FUNCTION	REFERENCES
Methylobacterium sp.HJ1	Shows greatest activity on 2, 2 DCP. Less able to degrade 2-Chloropropionate	Ng and Huyop, (2008)
Pseudomonas putida CBS3	Shows activity on L-2-chloropropionic acid and L-monochloroacetate	Schneider *et al.*, (1991)
Burkholderia cepacia MBA4	Able to degrade monochloroacetate and L-2-chloropropionic acid	Schmidberger *et al.*,(2008)
Rhizobium sp. Dehalogenase E	Acting on both L- and D-steroisomers. Acting on monochloroacetic acid, dichloroacetic acid, 2-chlorobut yric acid and 2,3-dichloropropionic acid	Stringfellow *et al.*, (1997)
Rhizobium sp. Dehalogenase D	Sterospecific for D-2-chloropropionic acid. Collectively acted on monochloroacetic acid, dichloroacetic acid, 2-chlorobutyric acid and 2,3- dichloropropionic acid	Huyop and Cooper, (2003)
Arthrobacter	Shows greater activity on 2, 2 dichloropropionic acid than 2-chloropropionic acid. Able to degrade dichloroacetate. No activity on 3- chloropropionate	Puat (2008)

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Generally, varieties of halogenated compounds which are produced by chemicals industries are degraded through dehalogenation. Dehalogenation is one form of biodegradation which enable the microorganisms in soil and water to utilize halogenated substances. Many investigations had been done of microbial degradation of halo aromatic and haloaliphatic compounds led to the identification of a diversity of dehalogenation mechanisms and dehalogenases (Janssen et al., 1994; Fetzner,
1998). There are two important aspects to study dehalogenases, first to understand the variety of α or β haloalkanoic dehalogenase (Hill et al., 1999).Second, several of isolated microorganisms able to produce multiple dehalogenases and this essential to control dehalogenase gene regulation (Huyop and Cooper, 2011). Many studies had been reported by Janssen et al., (1994), Leisinger and Bader, (1993), Hardman, (1991), Jing and Huyop, (2007), Jing et al., (2008) and Ismail et al., (2008). All these studies were explained the catabolism process of dehalogenase in different types of bacteria and variety of halogenated compounds for instance halo acids are degraded by microbial dehalogenases through dehalogenation process which involve carbon- halogen bond cleavage (Copley, 1998).
Dehalogenation reactions have been classified by (Leigh et al., 1988; Slater et al., 1997; Huyop et al., 2004, 2008a, b). The classification was based on their substrate specificities and the configuration of the products. Class 1L is more common than 1D enzyme in nature. Class 1 L removed halide from L-2CP inverting the product configuration becomes D-lactate or D-2hydroxy acids. This enzyme also reacts with sulfhydryl blocking reagents. Enzymes in Class 2I were distinguished by their ability to dehalogenate both D- and L-isomers by a mechanism that inverts substrate product configurations. Class D enzyme can dehalogenate selectively D- isomeric substrates such as D-2-chloropropionic acid (D-2CP) with inversion of product configuration and forms L-2-hydroxy acids (e.g., L-lactate). In addition, the enzymes from Class 2R differ from Class 2I enzymes in their ability to dehalogenated both D and L isomers with retention of product configuration. Furthermore, the presence of more than one dehalogenases in one microorganism is far from clear (Huyop and Nemati, 2010). Different types of bacterial strains had been isolated from diverse environment and these bacteria can produce more than one dehalogenase enzyme. For instance, Moraxella sp. strain B was isolated from industrial wastewater. It has two haloacetate dehalogenases H-1 and H-2. The specificity of these enzymes is different. H-1 acts better on monofluoroacetate than on monochloro- or monobromoacetate, but has little activity against monoiodoacetate.
On the other hand, H-2 acts on monochloro-, monobromo-, and monoiodoacetate, but not on monofluoroacetate (Kawasaki et al., 1981). The molecular masses estimated by SDS-PAGE were 33 kDa for H-1 and 26 kDa for H-
2. In general, many harmful halogenated aliphatic compounds can be released from commercial, agricultural and chemical industrial which produce large amounts of short chain halogenated aliphatic hydrocarbons which are used as degreasing agents, organic solvents and pesticides. The sources of these products are chlorinated or brominated alkanes and alkenes that contain one to three carbon atoms such as halogenated alkanoic acids (HAA), haloalkanes, trichloroethane and ethylene dibromide (EDB). Biodegradation process needs some requirements can be summarized as follow:

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I. Accessibility of a compound to microorganisms

Halo organic compounds may be adsorbed to particular matter in the soil (e.g. clays), thereby preventing potential microbial attack. Similarly, chemical complexing to other molecules leads to the same consequence.

II. Entry of a compound into an organism

The lack of penetration, coupled with the absence of suitable extra-cellular enzymes may result in a compound being resistant to biological attack. Specific transport mechanisms have evolved to facilitate the entry of naturally occurring compounds. However, on initial exposure to many unnatural halogenated molecules the uptake mechanisms are unlikely to function.

III. Induction of catabolic enzymes

Some important conditions in biodegradation process are the ability of organic compound to induce the synthesis of, and act as substrate for degradative enzymes (assuming that the enzymes are not produced constitutively). Microbial growth in general is unable to utilize xenobiotic haloorganic compounds as substrates, as they are too far removed from the main stream of catabolic pathways. Halogen substitution sufficiently alters a molecule's structure so as to reduce the rate of its transformation, and possible prevent its metabolism. In the instance, the fate of xenobiotics is to some extent determined by the degree of structural analogy between the synthetic compound and a natural compound for which catabolic function exist. Such structural analogies include comparable reactivities, together with analogous size and polarity of functional groups (Hughes, 1988).

IV. Aerobic or anaerobic environments.

Anoxic/aerobic environments can accumulate some compounds which would otherwise be degraded under different condition (Alexander, 1981).

The Impact of Pesticides in the Environment and Human Concerns

The pesticide or xenobiotic can be substance or mixture of substances that used to kill a pest. The subclasses of pesticides includes: herbicides, insecticides, fungicides, pediculicides, biocides and rodenticides (Gilden et al., 2010). According to Yuldeman et al., 1998, reported that many types of pesticides are harmful to human and the environment especially organochlorines which are very toxic. Pesticides are predisposed to harmfully affect human health even today since their persistent residues are often present in variety environments. The effect of pesticides on human health can cause endocrine disruptor properties, cancer, genetic mutation and behavioral changes, male sterility and birth defects. In addition, pesticides can affect on kidney, liver, nervous system and may cause allergies (Yuldeman et al.,

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1998). Even though most of the Southeast Asian countries as well as Malaysia, Thailand, Indonesia, and Vietnam have banned the use of these pesticide compounds since 1990s, but the residues of OCPs are still detected in waters and soil or sediments at the significant levels (Ibrahim et al., 2002).

Generally, herbicides used widely in industrial sites, clear waste ground and also use to kill plant material with which they come into contact. On the other hand, some plants are able to produce natural herbicides for instance, genus Juglans (walnuts). The synthetic of pesticides have been used widely because they provide many benefits to farmers and consumers. Actually, different factors can change the productivit y patterns, either directly or indirectly and include use of pesticides associated with more efficient agricultural practices. Table 4 indicates global pesticides sales as categorized by the major groups of chemicals of the world in 1997. All the pesticides represented here are mainly synthetic organic compounds. The global chemical pesticide market was about $ 31 billion in 1997 with a mature market growth of 1-2% per year (Drogui and Lafrance, 2010).

Table 4: Global chemical pesticides market (1997)

Product	**Sales, Billions of Dollar**	**Sales, Billions of Dollar**
Herbicides	14.7	47.6
Insecticides	9.1	29.4
Fungicides	5.4	17.5
Others	1.7	5.5
World Wide Total	**30.9**	**100.0**

Furthermore, organochlorine pesticides are the most toxic for the environment and the half- life of organochlorine can persist for long period in the soil more than 10 years (Ritter et al., 1995). Table 5 shows that major groups of chemical pesticides.

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The Chemistry of Halogenated Compound

It is good to understand the relationship between biological activity and chemical structure. For many years, the removal halogens from organic compound especially chlorine and fluorine has mesmerized microbiologists and chemists (Slater et al., 1995). In fact, the number of halogen per organic molecule considers the critical factor to degrade this compound. In addition, the species of microorganism that use in degradation process because not all microorganisms can degrade halogenated compound (Slater et al., 1997). The acidity of halogenated alkanoic acid compound also effects the growth of microorganism and some bacteria needs specific pH to reach optimum growth.

Thus, substituent along the alkanoic acid will affect its acidity and this phenomenon was called inductive effect. The inductive effect can be observed physically and chemically through the transmission of charge in the chain of a molecule by electrostatic induction. There for, the halogen group that has higher electro negativity, it will be able to pull the electrons of the molecule towards its self and this can cause the weakening of O-H bonds. In chemistry, the electro negativity can be defined as the ability for an atom to attract to itself an electron pair shared with another atom in a chemical bond. Moreover, the atom that containing negative electricity tends to migrate to the positive pole in electrolysis and assuming potential when in contact with a dissimilar substance. Furthermore, the inductive effect of halogen substituent can be more strong acids when the number of halogen substituent is increased. For instance, the halogenated compound such as monochloroacetic acid (MCA) and dichloroacetic acid (DCA) are less strong than tri chloroacetic acid (TCA). The halogen atoms are more electronegative than carbon atoms. So that, based on (Table 2.6), fluorine is the most electronegative of the halogen forms the strongest acid in the monochloroacetic acid, then comes the chlorine, bromine and iodine are less electronegative and form weaker acids (Huyop, 2008).

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Class of Chemical Pesticides	First Used	Examples	Types	Current Status	Effects
Organochlorines	1942	aldrin; chlordane; dieldrin; endrin; heptachlor; lindane ,methoxychlor; toxaphene; hexachlorobenzene (HCB); pentachlorophenol (PCP); DDT	insecticide, acaricide, HCB & PCP are fungicides	Lindane, Methoxychlor And Pentachlorophenol are registered in Canada. The other products have been discontinued in Canada, but they are still used in developing nations	Persistent, bioaccumula affective the ability to reproduce,develop, and to withstand environmental stress by depressing the nervous, endocrine and immune systems
Organophosphates	Very early 1940s	schradan;	insecticide, acaricide	Schradan was discontinued in 1964 and resulted in a move toward less toxic groups (e.g. malathion, parathion)	Non-persistent, systemic (cholinesterase-inhibiting), not very selective, toxic to human
Carbamates	First Appeared in 1930 but large-scale use in mid-1950s	parathion; malathion	fungicide , insecticide, acaricide	Aldicarb was discontinued in 1964; the others are registered in Canada Although carbamates share a mode of action with the organophosphates, their effects are reversible and they are biotransformed in-vivo	Non-persistent, cholinesterase-inhibiting, not very selective, toxic to birds and fish

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Table 6. Electro negativity in Pauling unit

Halogen Element	**Atomic Number**	**Electro negativity**
Fluorine	9	3.980
Chlorine	18	3.160
Bromine	35	2.960
2.960	53	2.660

In general, many of halogenated compounds in environment are xenobiotics especially chlorinated. These compounds are manufactured and use as pesticides. Leasure (1964) notes from different types of halogenated substrate that chlorinated substrates are best suited for herbicidal activity over the fluorinated, brominated and iodinated compounds. The acidic strength is indicated by acidic dissociation constant (Ka) and its expressed on a logarithmic scale. Therefore, stronger acids have smaller
value of pKa (Huyop, 2008) (Figure 2.4).

$$Pk_a = - \log e\, k_a$$

Figure 2.4: Calculation of Pka

According to Solomons (1994), the arrangement of groups around the carbon atom of halogenated compound is normally tetrahedral. In addition, high electronegative element will give a stronger carbon –halogen bond molecule (Wade,2003; Solomons, 1994).

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The Mechanisms of Dehalogenation

In general, different types of bacterial dehalogenases are capable to cleavage of halogen substituents from haloalkanes, haloacoholes, haloaromatics and haloalkanoic acids. According to the enzymatic cleavage of carbon –halogen bond, seven mechanisms of dehalogenation are known, namely, oxygenolytic, hydrolytic, reductive, and thiolytic dehalogenation, intermolecular nucleophilic displacement, dehydrohalogenation and hydration haloalkane dehalogenases are bacterial enzymes cleaving the carbon –halogen bond of halogenated aliphatic compounds by a hydrolytic mechanism. In this reaction, the water molecule serves as co-substrate during the catalysis and there is no proof indicating the involvement of co-factors or metal ions in the catalytic mechanism (Janssen et al., 1994). The classification of bacterial dehalogenases can be depends on their substrate specificities, molecular mechanisms, reaction kinetics and DNA or amino acid sequence information (Slater et al., 1995). Biodegradation process of numerous halogenated aromatic compounds has been reported under both conditions aerobic and anaerobic (Fetzner et al., 1998).

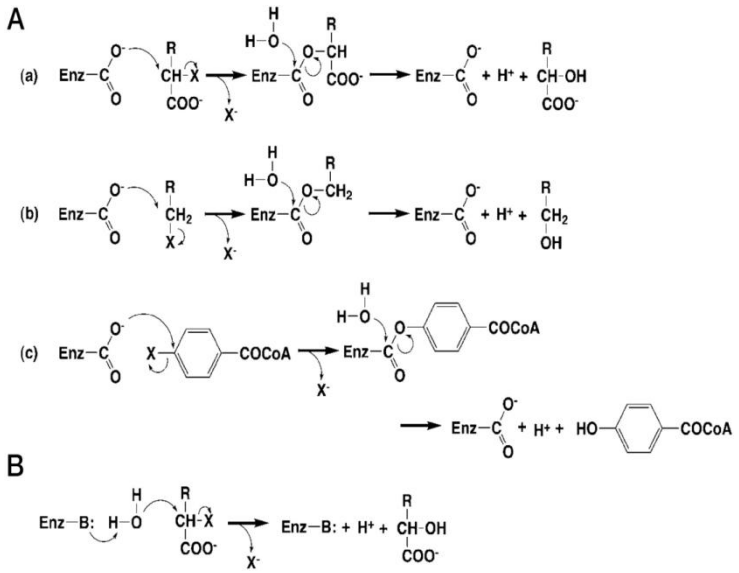

Figure 5: Reaction mechanisms of dehalogenases catalyzing the hydrolytic dehalogenation. Reaction A: Mechanisms of: a, l-DEX; b, haloalkane dehalogenase; and c, 4-chlorobenzoyl-CoA dehalogenase. Reaction B: Mechanism which does not involve an ester intermediate (Nardi et al.1999).

The most important group of dehalogenases is hydrolytic dehalogenases. They catalyze the cleavage of carbon–halogen bonds through a nucleophilic substitution by water to yield alcohol (Van Der Ploeg et al., 1991). Based on the substrate ranges, these enzymes have been grouped in to haloalkane dehalogenases and haloacid dehalogenases active against halogenated

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alkanes and halogenated alkanoic acid respectively (Kawasaki et al., 1981; Klages et al., 1983). The biodegradative mechanisms for halo-organic compounds have been reported in to three main mechanisms. Firstly, mineralisation of organic compound and this can prevent or reduce its persistence in the environment.
This includes breakdown of an organic compound into an inorganic state, and the conversion of the carbon –skeleton in to intermediary metabolite. Constitutes partial degradation is the second mechanism, which is clearly demonstrated by halo- aromatic compounds. For example, PCB's are composed of aromatic ring pairs which may possess one unsubstituted and a chloro-substituted ring. A single organism can sometimes use the unsubstituted nucleus as the growth substrate, whereas the halo-substituted ring will be excreted into the culture fluid as an organic end product.

Co-metabolism is the third mechanism of biodegradative. In this mechanism the microbial action that modifies the structure of a chemical, without deriving energy from the catabolism for microbial growth (Alexander, 1981; Baggi et al.,2005). The population involved in co-metabolism is assumed to grow on other substrate while performing the transformation and the lack of increase in population biomass is reflected by the inability of the co-metabolising microorganisms to utilize the chemical for biosynthetic purposes. Co-metabolism of halo-organic compounds does not result in complete mineralisation to inorganic halide, CO2 and H2O, but it does seemingly reduce toxicity in the environment, which indicates the ecological importance of this phenomenon. It has been suggested that co-metabolism may account for the degradation of many pesticides which do not sustain microbial growth (Alexander, 1981).

Generally, term recalcitrance is used for the many xenobiotics that endure for long periods in natural ecosystems in chemically unchanged states, owing to the inability of microorganisms to degrade them. In some cases, the toxic effect of the insecticide in the environment was reduced while complete mineralisation did not occur. Some products that are formed from such chemically related compounds may not subsequently be altered by these enzymes, and consequently accumulated, preventing further mineralisation and the generation of energy for microbial growth. This suggests that the toxicity of a compound is somewhat reduced by co-metabolism but not eliminated. Many of halogenated compounds, especially polychlorinated types are generally degraded slowly and regarded as recalcitrant molecules. This is a phenomenon discussed by many authors since considerations about the fates of these recalcitrant compounds by biological, chemical and physical agents are extremely important in understanding persisting toxological effects in the biosphere (Barriault et al., 1998; Kim and Picapdal 2001; Araoz and Viale, 2004; Xu et al., 2004).

Bacterial Identification by 16S rRNA Gene Analysis

The identification of microorganisms has been based on similarities in their morphological, developmental and nutritional characteristics. Nevertheless, with the rapid development of molecular biology, rRNAs sequencing is gaining wide acceptance in the identification of

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bacteria. This due to all self-replicating bacteria contains ribosomes, which are essential to protein synthesis. Nucleotide sequencing of the 16S rRNA has been proven to be extremely valuable for the study of bacterial evolution and phylogeny (Olsen and Woese, 1993). Different methods such as computer analysis and molecular biology can be used for obtaining 16S rRNA.

The DNA sequencing is one way to detect the identity of an organism. 16S rRNA gene is te most evolutionary conserved macromolecules in all living organism. Thus, 16S rRNA the most suitable molecule can be used in identification process. In other words, 16S rRNA is universal in all living things and large enough in providing significant number of nucleotides when comparing sequence (Ng, 2006). According to Weisburg and his colleagues (1990) they suggested that, the comparison of rRNA sequences is a great tool for deducing phylogentic and evolutionary relationships among bacteria, archaebacteria, and eukaryotic organisms. In addition, Fox and his colleagues (1992) stated that 16S rRNA (gens coding for rRNA) sequence comparisons were conducted with the following three psychrophilic strains: Basillus psychrophilius W16AT, W5, and Basillus globisporus W25. These strains exhibited more than 99.5% sequence identity and within experimental uncertainty could be regarded as identical. Their close taxonomic relationship was further documented by phenotypic similarities. On the contrary, some previous researches of DNA-DNA hybridization results established that these strains do not belong to the same species if current standards are used. These results give emphasis to important point that the effective identit y of 16S rRNA sequences is not necessarily a sufficient criterion to guarantee species identity.

Multiple Sequence Alignment

Multiple sequence alignment (MSA) is a tool that commonly used in bioinformatics study nowadays. The generation of alignment is very common tasks in computational sequence analysis and it used widely in sequence alignment of three or more biological sequences, generally protein, DNA, or RNA. In many cases, the input set of query sequences are assumed to have an evolutionary relationship by which they share a lineage and are descended from a common ancestor. From the resulting MSA, sequence homology can be inferred and phylogenetic analysis can be conducted to assess the sequences' shared evolutionary origins. Multiple sequence alignment is often used to assess sequence conservation of protein domains, tertiary and secondary structures, and even individual amino acids or nucleotides. However, homology is not assumed between aligned sequences. In such cases, residues aligned in the same column are typically assumed to be structurally equivalent. Multiple sequence alignment also refers to the process of aligning such a sequence set. Because three or more sequences of biologically relevant length can be difficult and are almost always time-consuming to align by hand, computational algorithms are used to produce and analyze the alignments. In multiple sequence alignment, either protein or nucleotide sequence can be chose to align. However, with the nucleotide sequence, it may not possible to distinguish conservative from non conservative substitution.
In general, widely used software in multiple sequence alignment called ClustalW and ClutsalX and the advantage of using ClutalW is that the sequences are downweighted according to how

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closely related they are with other sequences. Hence, it prevents large groups of similar sequences from dominating an alignment. Both ClustalW and ClutsalX are available free in internet. According to Wong (2004), phylogeny is the evolutionary process of an organism and its phylogenetic relationship among descendants. Phylogenetics means the field in studying the phylogeny of organism. However, the aim of studying phylogeny is to reconstruct the evolutionary process of different organisms.

Basic Concept of Phylogenetic Tree

A Phylogenetic tree or Phylogram, sometimes called the 'Tree of Life', shows the evolutionary relationships among various species that are believed to have a common ancestor. Each node with descendants represents the most recent common ancestor of the descendants, with edge lengths in our tree, corresponding to time estimates. Each node in a phylogenetic tree is called a taxonomic unit. Evolutionary relationship among genes and organisms can be elegantly illustrated by a phylogenetic tree, comparable to a pedigree showing which genes or organisms are most closely related. Phylogenetic trees are described this way because the various diagrams used for depicting these relationships resemble the structure of a tree and the terms referring to the various parts of these diagrams (i.e, root, stem, branch, node, and leaf) are also reminiscent of trees. External (terminal) nodes, the extant (existing) taxa, are often called operational taxonomic units (OTUs), a generic term that can represent many types of comparable taxa (e.g., a family of organisms, individuals, or virus strains of a single species; a set of related genes; or even genes regions). Similarly, internal nodes may be called hypothetical taxonomic units (HTUs) to emphasize that they are the hypothetical progenitors of OTUs. A group of taxa that belong to the same branch have a monophyletic origin and is called a cluster. The branching pattern- that is, the order of the nodes- is called the topology of the tree.

Phylogenetic Analysis Using Distance-Matrix Approach

Distance-matrix methods of phylogenetic analysis explicitly rely on a measure of "genetic distance" between the sequences being classified, and therefore they require an Multiple Sequence alignment as an input. Distance is often defined as the fraction of mismatches at aligned positions, with gaps either ignored or counted as mismatches (Mount, 2004). Distance methods attempt to construct an all-to-all matrix from the sequence query set describing the distance between each sequence pair. From this is constructed a phylogenetic tree that places closely related sequences under the same interior node and whose branch lengths closely reproduce the observed distances between sequences. Distance-matrix methods may produce either rooted or unrooted trees, depending on the algorithm used to calculate them. They are frequently used as the basis for progressive and iterative types of multiple sequence alignments. The main disadvantage of distance-matrix methods is their inability to efficiently use information about local high-variation regions that appear across multiple subtrees (Felsenstein, 2004).

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Conclusion

Generally, neighbor-joining method is a method that is connected to the cluster method but does not require the data to be ultrametric (Saitou and Nei, 1987). That means, neighbor-joining method is applying general data clustering techniques to sequence analysis using genetic distance as a clustering metric. The method is particularly suited for datasets comprising lineages with largely varying rates of evolution. In addition, it can be used in combination with methods that allow correction for superimposed substitutions. Neighbor-joining method keeps track of nodes on a tree rather than cluster of taxa. The tree is constructed by connecting the slightest distant pair of nodes in this modified matrix. However, at each stage in the process two terminal nodes are replaced by one new node. After many steps, the process is complete when two nodes remain, separated by a single branch. There are some advantages of using neighbor-joining method such as permits correction for multiple substitutions, let lineage with largely different branch lengths, and it is fast and thus suitable for large datasets. Moreover, the disadvantages of this method are: it gives only one possible tree, sequence information is reduced, and it strongly dependent on the model of evolution used.

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