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Microbial isolation and degradation of selected haloalkanoic aliphatic acids by locally isolated bacteria: A review

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ABSTRACT

The liberation of halogenated compounds by both natural processes and man-made activities has led to extensive contamination of the biosphere. Bioremediation *via* the dehalogenation process offers a sustainable way to eliminate such hazardous contaminants. Whereas, a large number of natural soil microorganisms (i.e., bacteria and fungi) that have been isolated are capable of degrading and detoxifying such contaminants, information on the preferred types of halogenated compounds that they catalyze is lacking. In this review, we discuss those microorganisms that have the potential to perform bioremediation of such environmental contaminants. We also present a method for isolating novel dehalogenase-producing microorganisms from cow dung.

Keywords: cow dung, bioremediation, dehalogenase, naturally occurring halogenated compound

INTRODUCTION

Unmanageable hazardous wastes from industrial and agricultural sectors are sources of environmental pollutants that are often mutagenic, carcinogenic and toxic. These substances can harm the environment and have deleterious effects on human health. Pesticides are frequently used in the agricultural sector to increase the yield of vegetables, fruits and other food products, although they can also have negative effects on humans and the environment. For example, young children exposed to pesticides can develop neuro-developmental retardation (Liu and Schelar, 2012). In addition, the continuous use of herbicides in the agricultural sector leads to their gradual accumulation in the soil. These substances subsequently become mobile when it rains or as a result of irrigation practices and eventually leach into bodies of water such as groundwater, rivers and the sea, where their highly water-soluble nature and mobility may prove harmful to aquatic life (Aktar *et al.*, 2009). Fortunately, it is possible to eliminate traces of herbicides and pesticides from the environment by applying the method of bioremediation, a process that occurs naturally in the environment using *in situ* microorganisms that transform hazardous halogenated pollutants into non-toxic materials (Randhawa and Kullar, 2011). In this review, we describe the specific characteristics of known dehalogenase-producing bacteria isolated from local environments. We also outline an approach to isolate new microorganisms from cow dung.

Basic principles of the dehalogenation process

Mechanistic studies show that dehalogenases hydrolytically cleave the carbon-halogen bond of Dalapon, resulting in the formation of hydroxyalkanoics from monosubstituted compounds (Figure 1) (Foy, 1975). Hydrolytic dehalogenation means the halogen substituent is replaced in a nucleophilic substitution reaction by a hydroxyl group derived from water molecules. The degradation pathway includes 2,2dichloropropionic acid (2,2DCP) as reported by Kearney *et al.* (1964) using radio-labeled ^{14}C-dalapon.

The catalytic mechanism of dehalogenation involves a nucleophilic attack by the aspartate residue in the active site of the dehalogenase. Based on structural studies, aspartate 189 (Asp189) is the key residue in the mechanism of dehalogenation (Schmidberger *et al.*, 2008). The mechanism begins with the Asp189, activating a water molecule to donate a hydroxyl group and initiating a nucleophilic attack on the α-carbon of the substrate via an S_N2 displacement reaction (Figure 2). The mechanism by which water molecules are activated to initiate nucleophilic attack on the chiral center of a substrate is proved by observations that reaction products show an

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inverted configuration from a D- to L- isomer or vice versa (Nardi-Dei *et al.*, 1999).

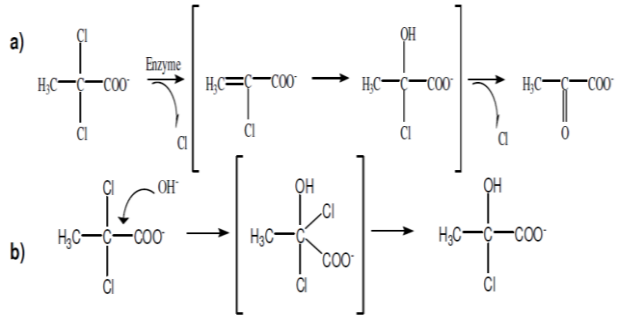

Figure 1: a) Dehalogenation of 2,2-DCP by an *Arthrobacter* sp. **b)** Enzyme-catalyzed nucleophilic substitution of one of the chlorine substitutes on the α-carbon with a hydroxyl group. This mechanism is proposed to explain the removal of the first chlorine from 2,2-DCP by an *Arthrobacter* sp. dehalogenase (Kearney *et al.*,1964).

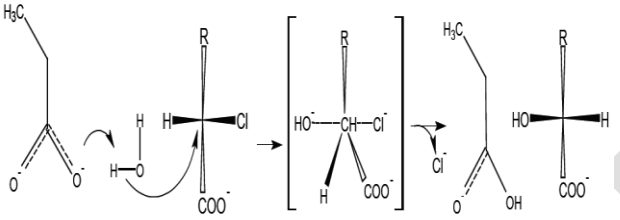

Figure 2: Hydrolytic dehalogenation mechanism of breaking down carbon-chlorine bond. Aspartate activates the water molecule for a nucleophilic attack, displacing a chloride ion via an S_N2 displacement reaction (Schmidberger *et al*., 2008)**.**

Dehalogenase classification

Dehalogenases are categorized as hydrolytic enzymes. Dehalogenases are divided into Class 1 (stereospecific) or Class 2 (non-stereospecific) enzymes and are further subdivided into Class 1D, Class 1L, Class 2I and Class 2R (Table 1) as reported by Slater *et al.* (1997). In contrast, Hill *et al.* (1999) assigned dehalogenase gene families into groups I and II. Group I dehalogenases act on only D-2-chloropropionic acid (D-2CP) or on both D- and L-2-chloropropionic acid (L-2CP), whereas group II dehalogenases are stereospecific, dechlorinating only L- but not D-2CP. DehE and DehD dehalogenases from *Rhizobium* sp. RC1 are categorized as group I dehalogenases**.** The DehE enzyme catalyzes the hydrolytic dehalogenation of D,L-haloalkanoic acid and also effectively dehalogenates all of the monohaloacetic acids except for monofluoroacetic acid (MFA). In contrast, DehD specifically acts on D-haloalkanoic acid, monochloroacetic acid (MCA) and monobromoacetic acid (MBA), but not on dichloroacetic acid (DCA) or trichloroacetic acid (TCA) (Alomar *et al.*, 2014).

Utilization of selected halogenated compounds by locally isolated bacteria

Halogenated organic compounds, a class of xenobiotics, are one of the largest and most problematic groups of environmental pollutants. Continuous exposure to halogenated compounds in the environment may provide evolutionary pressure to generate increased diversity among dehalogenase-producing microorganisms. Thus microbes isolated from various locations have been characterized for their ability to degrade these compounds (Table 2). (For an overview of the microbial degradation process of xenobiotics and the catabolic genes involved in microbial transformation of xenobiotic compounds, see Agrawal and Shahi, 2015.)

Dehalogenation by Rhodococcus sp.

Jing and Huyop (2007a) investigated a bacterium isolated from an agricultural field at Universiti Teknologi Malaysia (UTM) and identified it as *Rhodococcus* sp. HJ1. This strain**,** HJ1, was characterized using 16S rRNA sequencing and biochemical analysis. The bacterial species is able to utilize 3-chloropropionic acid (3CP) as its sole source of carbon and energy. The cells were grown in minimal medium supplied with possible intermediates of 3CP metabolism; acrylic acid and 3CP acid were utilized readily at similar rates. There is no evidence that 3-hydroxypropionic acid serves as an intermediate in the metabolism of β-chloropropionic acid (Jing and Huyop, 2007a). Further characterization by Jing and Huyop (2008) indicated that HJ1 is able to utilize 3CP as its sole source of carbon and energy, with cells doubling every 17.1 h. HJ1 can grow only on β-substituted haloalkanoate and not on α-substituted substrates (D,L-2-chloropropionate, 2,2-dichloropropionate, 2,3-dichloropropionate and bromopropionate). The utilization of 3CP was observed by the depletion of 20 mM 3CP in the growth medium as determined by high-performance liquid chromatography analysis of the medium on day 1 and day 2**,** thus indicating that 3CP is fully utilized by *Rhodococcus* sp HJ1. Dehalogenase specific activity associated with this species was 0.013 μmol Cl^-/ml/min/mg protein in cell-free extract as determined by measuring the chloride ion release.

Crude extracts from HJ1 cultures show dehalogenase activity with various halogen-substituted organic acids, with the highest activity observed using 3-chloroproporionic acid as a substrate (Jing *et al*., 2008a). This enzyme follows Michaelis-Menten kinetics and has a K_m for 3-chloropropionic acid of 0.2 mM. Maximum activity occurs at pH 7.6 at 30°C. The enzyme activity in cell-free extracts is unaffected by addition of ethylene diaminetetraacetic acid ordithiothreitol or by Mn^{2+} and Zn^{2+} ions but is reduced by $HgCl_2$ (70%) and $Pb(NO_3)_2$ (80%). The dehalogenase activity of HJ1 is thus inducible

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and specific for catalyzing the removal of only β-substituted dehalogenase. This suggests that the enzyme mechanisms of HJ1 dehalogenase are very specific (Jing *et al*., 2008a).

Table 1: Class of dehalogenase.

Class	Organism	Dehalogenase	References
Class 1D: D-isomer specific	*Pseudomonas putida* strain AJ1	HadD	Barth *et al.* (1992); Smith *et al.* (1990)
	Rhizobium sp.RC1	DehD	Leigh *et al.* (1986, 1988)
Class 1L: L-isomer specific	*P. putida* strain AJ1	HadL	Jones *et al.* (1992)
	Pseudomonas sp. strain CBS3	DehCI	Schneider *et al.* (1991)
	Pseudomonas sp. strain CBS3	DehCII	Schneider *et al.* (1991)
	*Xanthobacterautotrophicus*strain GJ10	DhlB	van der Ploeg *et al.* (1991)
	P. putida strain 109	Deh109	Kawasaki *et al.* (1994)
	P. cepacia strain MBA4	Hd1IVa	Murdiyatmo *et al.* (1992)
	Moraxella sp. strain B	DehH2	Kawasaki *et al.* (1992)
	Pseudomonas sp. strain YL-	L-DEX	Nardi-Dei *et al*.,(1994)
	Rhizobium sp. RC1	DehL	Leigh (1986); Cairns (1996)
Class 2I: D-andL-isomers as substrate (inverts substrate product configuration)	*Pseudomonas* strain 113	DL-DEX	Motosugi *et al.* (1982 a, b)
	P. putida strain PP3	DehII	Weightman *et al.* (1982); Topping (1992)
	Alcaligenes xylosoxidans ssp. *denitrificans* ABIV	DhIIV	Brokamp and Schmidt (1991); Brokamp *et al.* (1997)
	Rhizobium sp. RC1	DehE	Allison (1981); Huyop *et al.* (2004)
Class 2R: D- and L-isomers as substrate (retains substrate product configuration)	*P. putida* strain PP3	DehI	Weightman *et al.* (1982); Topping (1992)
	Isolate K37	HdIV	Murdiyatmo (1991)

Table 2: Bacteria that can grow on halogenated substrates.

Bacterium	Source Isolate	Substrate for Growth	Cell Doubling Time (h)	Reference
Rhodococcus sp. HJ1	UTM agricultural soil	Acrylic acid 3-chloropropionic acid	-	Jing and Huyop (2007a)
Methylo bacterium sp.	UTM agricultural soil	2,2-dichloropropionic acid	23	Jing and Huyop (2007b)
Burkholderia cepacia MBA4	Soil	Monochloroacetic acid (MCA)	-	Yu *et al.* (2007)
Pseudomonas sp. R1	Rice paddy field	Monochloroacetic acid (MCA)	13–14	Ismail *et al.* (2008)
Rhodococcus sp.	UTM agricultural field	3-chloropropionic acid	17.1	Jing and Huyop (2008a).
Rhodococcus sp. HJ1	Soil	3-chloropropionic acid	-	Jing *et al.* (2008a)
*Methylo bacterium*sp. HJ1	Agricultural soil	2,2-dichloropropionic acid	14	Jing *et al.* (2008b)
Pseudomonas sp. B6P	Rice paddy field	3-chloropropionic acid	-	Mesri *et al.* (2009)
Pseudomonas sp. strain S3	Rice paddy field	D,L-2-chloropropionic acid	-	Thasif *et al.* (2009)
Pseudomonas sp. strain S3	Rice paddy field	D,L-2-chloropropionic acid	-	Hamid *et al.* (2010a)
Citrobacter sp. AZZ2	Volcanic area,GunungSibayak	2,2-dichloropropionic acid	15	Hamid *et al.* (2010b)
Bacillus sp. strain TW1	Kuala Terengganu water treatment and distribution plant	Monochloroacetic acid (MCA)	13	Zulkifly *et al.* (2010)
Aminobacter sp. SA1	Soil	2,2-dichloropropionic acid D,L-2-chloropropionic acid	7	Amini *et al.* (2011)
Pseudomonas sp. B6P	Rice paddy field	3-chloropropionic acid	-	Hamid *et al.* (2011)
Bacillus sp.	Volcanic area,GunungSibayak	2,2-dichloropropionic acid	8	Roslan *et al.* (2011)
Bacillus megaterium GS1	Volcanic area,GunungSibayak	2,2-dichloropropionic aid	-	Salim *et al.* (2011)
Labrys sp. strain Wy1	Soil from a Melaka rubber estate	2,2-dichloropropionic acid	33.44	Wong and Huyop (2011)

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Serratia marcescens sp. SE1	Soil surrounding lake water located at the UTM	2,2-dichloropropionic acid	5	Abel *et al.* (2012a)
Ralstonia solanacearum strain MK 121002,	Gut of pond-reared rohu (*Labeorohita*)	2,2-dichloropropionic acid	7.2	Abel *et al.* (2012b)
Acinetobacter baumannii strain MK121007			23.3	
Chromo-bacterium violaceum strain MK121009			6.2	
Enterobacter cloacae	Soil from rubber estate in Melaka	2,2-dichloropropionic acid	7.48	Wong and Huyop (2012)
Burkholderia sp. KU-25			6.16 6.96 11.59 12.24 11.44	
E. cloacae MN1	Soil from seaside area of Tigbauan, IIoilo, Philippines	2,2-dichloropropionic acid	10	Nemati *et al.* (2013)
Arthrobacter sp. S1	Contaminated soil in an area in Bacolod City, Phlippines	2,2-dichloropropionic acid	5	Bagherbaigi *et al.* (2013)
		D,L-2-chloropropionic acid	7	
		3-chloropropionic acid	10	
Arthrobacter sp. strain D2	Soil contaminated with herbicides and pesticides	Monochloroacetic acid (MCA)	7	Alomar *et al.* (2014)
Arthrobacter sp. strain D3			7	
Labrys sp. strain D1			26	
Arthrobacter sp. strain D2	Soil contaminated with herbicides and pesticides	Monobromoacetic acid 2,2-dichloropropionic acid D,L-2-chloropropionic acid L- 2-chloropropionic acid D-2-chloropropionic acid Glycolate	-	Alomar *et al.* (2014)
Burkholderia sp. HY1	Mud from UTM agricultural area	2,2-dichloropropionic acid	42.15	Hadeed *et al.* (2014)
Bacillus sp.	Marine sediment	2,2-dichloropropionic acid	39.60	Khosrowabadi and Huyop (2014)
Rhodococcus			36.60	
Lysinibacillus			30.71	
Microbacterium			41.23	
Aminobacter			36.70	
Raoutellaornithilolytica	Wastewaterfrom Tioman Island	2,2-dichloropropionic acid	23.11	Niknam *et al.* (2014)
Bacillus sp. H4	Marine sponge *Gelliodes*sp.	3-chloropropionic acid	56.82	Sufian *et al.* (2015)
Terrabacter terrae JHA1	Soil from UTM agricultural area	2,2-dichloropropionic acid	-	Almaki *et al.* (2016)
Pseudomonas aeruginosa MX1	Seawater at Desaru Beach	2,2-dichloropropionic acid	44	Edbeib *et al.* (2016)

Dehalogenation by Methylobacterium sp.

Methylobacterium sp. HN2006B isolated from a UTM agricultural area is able to grow on 2,2DCP as its sole source of carbon. The bacterium was grown on 2,2-dichloropropionate and D,L-2-chloropropionate with a doubling time of 23 and 26 h, respectively (Jing and Huyop, 2007b), whereas *Methylobacterium* sp. HJ1 could grow on 2,2-dichloropropionate two times faster than strain HN2006B (Jing *et al.*, 2008b). Jing *et al.* (2008c) further characterized the dehalogenase enzyme from HJ1 and found that the protein is non-stereospecific and can act on both isomers of D-and L-2-chloropropionic acid.

Degradation at a low concentration of halogenated compounds

Huyop and Cooper (2012) studied the possibility of growth of *Rhizobium* sp.RC1 in the presence of low concentrations of halogenated compounds as its sole carbon and energy source. The degradation of low concentrations of 2,2DCP was achieved with a cell doubling time of 12 h. *Rhizobium* sp. RC1 was able to grow in the presence of 0.2 mM 2,2DCP, which is 100-fold lower than the concentration of the substrate routinely used (20 mM), with a cell doubling time of 11 h.

Growth at low concentrations was also reported by Zulkifly *et al.* (2010) and Amini *et al.* (2011). Zulkifly *et al.* (2010) isolated bacteria from the Kuala Terengganu water treatment and distribution plant in Malaysia. *Bacillus* sp. strain TW1 was identified by morphological and biochemical analyses and by PCR amplification of its 16S rRNA gene. TW1 was isolated because of its ability to grow in the presence of 0.5 mM monochloroacetic acid (MCA), which is 10-fold lower than typical MCA concentrations when used as the sole carbon and energy source (Zulkifly *et al*., 2010). These growth conditions resulted in a maximum chloride ion release of 0.32 µmol Cl mL^{-1}.

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Another soil microorganism, identified as *Aminobacter* sp. SA1 by partial biochemical and 16S rRNA sequencing, was isolated on 2,2DCP as the sole carbon and energy source. This bacterium has the ability to degrade low concentrations of 2,2DCP (up to 1 mM) with a doubling time of 7 h for these cells (Amini *et al.*, 2011).

Monochloroacetate (MCA)

MCA is one example of a halogenated acetic acid. 2,2-DCP is used as a herbicide, whereas MBA has been used for a preservation agent. Monochloroacetate or monochloroacetic acid and MBA belong to the same acetate group. MCA is a halogenated acetic acid belonging to the same acetate group as MBA, which is used as a preservative.

A bacterial strain tentatively identified as *Pseudomonas* sp. R1 was isolated from a paddy (rice) field and can degrade MCA at concentrations ranging from 5 to 40 mM. *Pseudomonas* sp. R1 thus could be used as a biological agent for the biodegradation of MCA in contaminated agricultural area (Ismail *et al.*, 2008). *Arthrobacter* sp. strains D2 and D3 and *Labrys* sp. strain D1, all of which are capable of degrading 20 mM MCA, were also isolated from soil contaminated with herbicides and pesticides (Alomar *et al.*, 2014). All three isolates were able to grow on MCA as the sole source of carbon and energy with concomitant chloride ion release into the growth medium. Strains D2 and D3 grew four times faster than D1. Strain D2 also grows in 10 mM MBA, 2,2DCP, D,L-2-chloropropionic acid, L-2-chloropropionic acid (L-2CP), D-2CP or glycolate as its sole source of carbon and energy (Alomar *et al.*, 2014).

Degradation of β-chloro-substituted haloalkanoic acids (3CP)

There have been few reports on the degradation of β-chloro-substituted haloalkanoic acids such as 3CP. Such studies would be useful to understand the diversity of dehalogenase enzyme functions. The position of the halogen substituent is important in governing the susceptibility of halogenated aliphatic acids to degradation by microbial dehalogenase enzymes. For example, dehalogenases that act on an α-carbon chloride, as is present in 2,2-DCP, are common as compared with those that act on β-substituted halogenated aliphatic acids. *Pseudomonas* sp. B6P dehalogenase is specific for β-substituted halogenated aliphatic acids but is unable to dehalogenate α-halogenated substrates (Mesri *et al.*, 2009). Hamid *et al.* (2015) suggested that most of the common dehalogenases that act on α-halogenated substrates show strong binding ability for 2,2DCP, D-2CP and L-2CP but less affinity for 3CP, a β-substituted halogenated aliphatic acid. An *in silico* analysis indicated that the S188V mutation of DehE improves substrate specificity toward 3CP. By replacing S188 with a valine residue, the inter-molecular distance reduced and stabilized bonding of the carboxylate of 3CP to hydrogens of the substrate-binding residues.

Yusn and Huyop (2009) described the first isolated putative gene involved in 3CP degradation. The putative dehalogenase gene, designated as *deh*, was expressed in *Escherichia coli*. If the gene is cloned and well expressed it will be potentially useful thus can be used in plant transformation studies. Hamid *et al.* (2011) reported the properties of dehalogenases from 3CP-degrading bacteria. The enzyme have a monomer of 56,000 Da, stable between pH 5 to 8 and its activity was not affected by metal ions, however was inhibited by Hg^{2+} and Ag^{2+}.Therefore, 3CP dehalogenase from *Pseudomonas* sp. B6P is distinctive for its substrate specificities as compared with other known dehalogenase enzymes**.** Sufian *et al.* (2015) isolated a 3CP-degrading bacterium designated as strain H4 from the marine sponge *Gelliodes* sp. that is capable of degrading 3CP as its sole carbon and energy source. In liquid medium, the doubling time for strain H4 was 56.82 ± 0.1 h, whereas the maximum chloride ion release was 2.03 ± 0.01 mM. Strain H4 is closely related to *Bacillus aryabhattai* B8W22 (Sufian *et al*., 2015).

D,L-2-chloropropionic acid (D,L-2-CP)

A *Pseudomonas* sp. strain S3, which can utilize the halogenated compound D,L-2CP as its sole carbon and energy source, catalyzes both D- and L-isomers of 2-chloropropionic acid (Thasif *et al.*, 2009). *Pseudomonas* sp. strain S3 was isolated from a paddy (rice) field using D,L-2CP as the sole carbon and energy source (Thasif *et al.*, 2009). It catalyzes the hydrolytic dehalogenation of both D- and L-isomers of 2-chloropropionic acid (Hamid *et al.*, 2010a). *Arthrobacter* sp. strain D2 isolated from soil contaminated with herbicides and pesticides also grows in 10 mM of D,L-2CP (Alomar *et al.*, 2014).

Degradation of 2,2DCP and 3CP

A bacterium identified as *Arthrobacter* sp. S1 by 16S rRNA was isolated from contaminated soil in an area in Bacolod City, Philippines. This was the first description of an *Arthrobacter* that can utilize α-halocarboxylic acid (αHA) 2,2-DCP and D,L-2CP as well as β-halocarboxylic acid 3CP as its sole carbon source with cell doubling times of 5±0.2, 7±0.1 and 10±0.1 h, respectively (Bagherbaigi *et al.*, 2013). A comparative analysis of the deduced amino acid sequence of dehalogenase from S1 indicated that <15% of the amino acids were identical among group I and group II dehalogenases, suggesting that the putative dehalogenase is completely distinct from both α-haloacid and β-haloacid dehalogenases.

Dehalogenase thermostability

All cellular components, including proteins, nucleic acids and lipids, must be heat stable for an organism to grow at high temperatures. Raven and Johnson (2001) suggest that thermophilic bacteria produce thermo stable proteins

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that can be more readily crystallized to obtain stable enzymes for structural and functional studies.

Hamid *et al.* (2010b) isolated an unknown strain, AZZ2, from a volcanic area in Gunung Sibayak, Indonesia, which was determined to be a *Citrobacter* sp. based on BLASTn analysis of 16S rRNA sequences. *Citrobacter* sp. AZZ2 grows well on 2,2DCP as its sole source of carbon and energy. In addition, Roslan *et al.* (2011) isolated *Bacillus megaterium* GS1 from the surrounding volcanic area at the foot of Gunung Sibayak, Indonesia. This bacterium can grow at higher temperature rather than at normal temperature of 25–30°C; the isolated bacterium grew best at 40°C but failed to grow at 60°C. The isolate grew rapidly on 2,2DCP, with a doubling time of 8 h.

The putative dehalogenase gene DehGSl was identified by direct sequencing and analysis of PCR-amplified genomic DNA from *Bacillus megaterium* GS1. Comparative analysis of the sequence data indicated that DehGSl is related to group II l-specific dehalogenases with an overall 25% amino acid sequence identity (Salim *et al.*, 2011).

Naturally occurring halogenated compounds

The halogenated compound monofluoroacetic acid (MFA) can be found in the leaves of the South African plant *Dichapetalum cymosum* (Gifblaar) (Marais, 1944). MFA, which is very toxic to vertebrates, has been used to control pests; it is also responsible for numerous cattle deaths from errant grazing. The young leaves of this plant are most toxic during the spring and autumn. The lethal oral dose for cattle and humans is 0.15 mg and 0.5 mg (kg bodyweight)$^{-1}$, respectively; 20 g of fresh leaves may kill a sheep. Although MFA is harmless when ingested, it becomes very toxic when converted to monofluorocitric acid in the body. Fluorocitrate inhibits the enzyme aconitase, which is a mitochondrial acetate carrier. A subsequent increase in the level of citrate in tissues and in the blood leads to acidosis, a general metabolic imbalance. The citrate build-up also inhibits the enzyme phospho-fructokinase and, hence, inhibits glucose metabolism. Because of this drastic reduction of cellular respiration, death usually occurs as a result of acute heart failure within 24 h of eating the plant (Hendriks, 2012). Pertinently, recent studies have supported the hypothesis that the microflora in the digestive tract of cattle may develop special adaptations to counteract the effects of halogenated chemicals in their diet (Singh and Fulekar, 2007; Hendriks, 2012; Camboim *et al.*, 2012a,b; Arunkumar and Chandrasekaran, 2013). Bacteria found in the digestive tract of herbivores that regularly fed on *D. cymosum*are effective in treating cows affected by the toxicity of monofluoroacetate (Hendriks, 2012; Camboim *et al.*, 2012a; Camboim *et al.*, 2012b). We hypothesized that free-range cows in Malaysia may consume leaves that contain naturally occurring halogenated compound. Therefore, the isolation of bacteria from the dung of these cows may lead to the characterization of useful dehalogenases.

Studies on cow dung to treat pollutants

Several studies have analyzed the degradation of pollutants by using cow dung (Table 3). Singh and Fulekar (2007) reported that the cow dung consortium, which includes bacteria, fungi and actinomycetes, is effective in degrading phenol (up to 500 mg/L phenol in 120 h). Some of the bacteria found in cow dung were *Pseudomonas* sp., *Streptococcus* sp., *Sarcina* sp. and *Escherichia* sp., and the fungi included *Penicillium* sp., *Rhizopus* sp. and *Mucor* sp.

A study by Obire *et al.* (2008) examined the fungi isolated from cow dung for the potential to degrade petroleum waste, whereas Joshi and Pandey (2011) screened cow dung for petroleum-degrading bacteria with the ability to biodegrade and biotransform petroleum products such as xylene, benzene, toluene, kerosene and diesel oil. Several fungi (*Alternaria* sp., *Aspergillus* sp., *Cephalosporium* sp., *Cladosporium*sp., *Geotrichum* sp., *Monilia* sp., *Mucor* sp., *Penicillium* sp., *Rhizopus* sp., *Sporotrichum* sp., *Thamnidum* sp., *Candida* sp., *Rhodotorula* sp. and *Torulopsis* sp.) and bacteria (*Bacillus* sp., *Proteus* sp. and *Pseudomonas* sp.) were identified in these two studies. Singh and Fulekar (2009) analyzed the bioremediation of benzene, toluene and *o*-xylene (BTX) with a cow dung microbial consortium using the batch scale process. Benzene and toluene were completely degraded at a concentration of 100 mg/L, and 97% of *o*-xylene was degraded at a concentration of 50 mg/L.

Ibiene *et al.* (2011) studied the influence of organic fertilizers such as spent mushroom substrate, cow dung and poultry droppings on the bioremediation of hydrocarbon-contaminated soil. Various microorganisms such as *Bacillus*, *Pseudomonas*, *Klebsiella*, *Proteus*, *Flavobacterium*, *Clostridium*, *Micrococcus* and *Acinetobacter*, as well as the fungi *Penicillium*, *Aspergillus*, *Saccharomyces*, *Rhizopus*, *Fusarium* and *Mucor* were isolated. Therefore, it was concluded that cow dung, spent mushroom substrate and poultry droppings are effective nutrient sources for bioremediation.

Orji *et al.* (2012) used cow dung as a source of limiting of nutrients for the bioremediation of petroleum hydrocarbon–polluted mangrove swamps. Bahadure *et al.* (2013) and Umanu *et al.* (2013) studied the effectiveness of cow dung, goat manure and spent fruit residues on the degradation of fresh unused motor oil and spent motor oil in soil. Cow dung was most effective on artificially contaminated soil, whereas spent fruit residue resulted in a greater decrease in total hydrocarbon from naturally contaminated soil. Adams *et al.* (2014) showed that cow dung is effective in the remediation of soil contaminated with spent automotive oil at an automobile mechanic workshop. The microorganisms identified included *Bacillus* sp., *Staphylococcus* sp., *Pseudomonas* sp., *Flavobacterium* sp., *Arthrobacter* sp., *Enterobacter* sp., *Trichoderma* sp., *Mucor* sp. and *Aspergillus* sp.

Ikuesan *et al.* (2015) identified crude oil–degrading microorganisms among various bacteria and fungi isolates. Greater crude oil consumption was attributed to

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the presence of enzyme systems that effectively degrade hydrocarbons and the presence of catabolic genes involved in hydrocarbon degradation in the microorganisms.

Geetha and Fulekar (2008) characterized the microbial consortium within cow dung slurry for bioremediation of soil contaminated with pesticides. Some of the pesticides tested were chlorpyrifos, cypermethrin, fenvalerate and trichlopyrbutoxyethyl ester. Bioremediation of pesticides was more effective given the greater nutrient availability and larger microbial population of the cow dung slurry and soil pesticide mix under controlled environmental conditions.

Boricha and Fulekar (2009) analyzed the physical, chemical and microbial characteristics of cow dung for its potential use in bioremediation of hazardous pesticides. To isolate and identify potential microorganisms, the microbial consortium of cow dung was exposed to different concentrations of the pesticide cypermethrin such as 10 mg/L, 25 mg/L, 50 mg/L and 100 mg/L using the scale-up process technique. Results showed that the potential organism resistant to higher concentration of pesticide.

Arunkumar and Chandrasekaran (2013) investigated the ability of cow dung slurry to degrade endosulfan, which is used in agricultural practice as an organochlorine pesticide. Carbon, nitrogen, phosphorus, sulfate, calcium, chloride, sodium, potassium, magnesium and larger microbial population were present in cow dung slurry and soil. Therefore, they can affect the bioremediation of pesticides under controlled environmental conditions. In this system, endosulfan degradation increased as time increased.

Khan and Manchur (2015) analyzed the ability of cowdung slurry to promote biodegradation of the pesticides namely Carbofuran, Diazinon Chlorpyrifos and Fenvalerate. Ojonoma and Udeme (2014) reported that soil polluted with palm oil mill effluent can be treated using chicken droppings and cow dung. Yadav and Thakare (2015) tested the effect of cow dung on saline-contaminated soil. Ultimately, there was a slight increase in pH and a decrease in the electrical conductivity of soil samples, suggesting that cow dung can be effective in treating moderately saline soil.

In summary, many researchers have used cow dung and the presence of various microbes in it as a source of biodegradation and bioremediation. As cow dung can be slightly acidic, as reported by Whalen *et al.* (2000) and Yadav and Thakare (2015), it would be interesting to further study the isolation of various microflora from cow dung and their ability to degrade halogenated compounds and possibly produce novel dehalogenases.

Table 3: Degradation of environmental pollutants by cow dung constituents.

Characterized Cow Dung Microorganisms	Pollutants	Reference
Pseudomonas sp., *Streptococcus* sp., *Sarcina* sp., *E. coli*, *Penicillium* sp., *Rhizopus* sp., *Mucor* sp.and *Nocardia* sp.	Phenol	Singh and Fulekar (2007)
Nocardia sp., *Mucor* sp., *Phizopus stolonifer*, *Aspergillus*, *Penicillium*, *Streptococcus*, *Sarcina* sp. and *Fecal streptococcus*	Pesticides (chlorpyrifos, cypermethrin, fenvalerate, trichlopyrbutoxyethyl ester)	Geetha and Fulekar (2008)
Alternaria sp., *Aspergillus* sp., *Cephalosporium* sp., *Cladosporium* sp., *Geotrichum* sp., *Monilia* sp., *Mucor* sp., *Penicillium* sp., *Rhizopus* sp., *Sporotrichum* sp., *Thamnidum* sp., *Candida* sp., *Rhodotorula* sp. and *Torulopsis* sp.	Petroleum	Obire *et al.* (2008)
Pseudomonas sp., *Actinomycetes* sp., *Cellulomonas* sp., *E. coli*, *Flavobacterium* sp., *Serratia* sp., *Nocardia* sp., *Sarcina* sp., *Salmonella* sp., *Staphylococcus aureus*, *Alcaligens* sp., *Bacillus* sp. and fungi	Pesticide (cypermethrin)	Boricha and Fulekar (2009)
Pseudomonas sp., *Streptococcus* sp., *Sarcina* sp., *E. coli*, *Penicillium* sp., *Rhizopus* sp., *Mucor* sp., *Aspergillus* sp. *and Nocardia* sp.	Benzene, Toluene and *o*-xylene (BTX)	Singh and Fulekar (2009)
Bacillus, *Pseudomonas* and *Proteus* sp.	Petroleum-based products (xylene, benzene, toluene, kerosene, diesel oil)	Joshi and Pandey (2011)
Bacillus, *Pseudomonas*, *Klebsiella*, *Proteus*, *Flavobacterium*, *Clostridium*, *Micrococcus*, *Acinetobacter*, *Penicillium*, *Aspergillus*, *Saccharomyces*, *Rhizopus*, *Fusarium* and *Mucor*	Hydrocarbon	Ibiene *et al.* (2011)
Bacillus sp.,*Citrobacter* sp., *Micrococcus* sp., *Vibrio* sp., *Flavobacterium* sp.and *Corynebacterium* sp. Fungi included *Rhizopus* sp., *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., *Saccharomyces* sp. and *Mucor* sp.	Hydrocarbon	Orji *et al.* (2012)
Penicillium chrysogenum, *Aspergillus* sp., *Pseudomonas aeruginosa*, *Bacillus* sp., *Alcaligenes faecalis*, *Morganella* sp.and *Serratia* sp.	Motor oil	Umanu *et al.* (2013)

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P. aeruginosa, *Bacillus* sp.and *Micrococcus roseus*	Palm oil effluent	Ojonoma and Udeme (2014)
Bacillus sp., *Staphylococcus* sp.,*Pseudomonas* sp., *Flavobacterium* sp., *Arthrobacter* sp., *Enterobacter* sp., *Trichoderma* sp., *Mucor* sp.and *Aspergillus* sp.	Hydrocarbon	Adams *et al.* (2014)
Klebsiella pneumoniae, *P. aeruginosa*, *E. coli*, *Enterobacter* sp., *Pseudomonas pseudomallei*, *Staphylococcus hominis*, *Bacillus subtilis*, *Klebsiella edwardsii*, *Actinomyces bovis*, *Acinetobacter calcoaceticus* and *Alcaligenes faecalis.* Fungi were *Aspergillus flavus*, *Aspergillus glaucus*,*Penicillium citrium*, *Aspergillus niger*, *Scopulariopsis brevicaulis*, *Fusarium* sp., *Aspergillus terreus* and *Meyerozyma* spp.	Crude oil	Ikuesan *et al.* (2015)
Bacillus cereus, *Serratia*, *Pseudomonas*, *Salmonella*, *Sarcina*, *Flavobacterium*, *Agrobacterium*, *Plesiomonas*, *Achromobacter*, *Aspergillus*, *Mucor*	Pesticides (carbofuran, diazinon, chlorpyrifos, fenvalerate)	Khan and Manchur (2015)

Future research

Several studies indicate that the dung of a cow may prove to be a rich source of pollutant-degrading microflora. The diet of ruminant animals consists mostly of forage, which may contain naturally occurring halogenated compounds that might be reduced to non-toxic levels by bacterial dehalogenases in the rumen and digestive tract. In addition to functioning as an inexpensive way to supply limiting nutrients to crops, cow dung also has been shown to contain microorganisms with potential applications for bioremediation of several types of pollutants. Studies using microorganisms from cow dung to degrade 2,2DCP and other halogenated pollutants have not been carried out. Cow dung, a cost-effective organic fertilizer, may be useful for bioremediation of many other halogenated contaminants in agricultural areas. The long-term intention of bioremediation strategy is to develop a cost-effective and environmentally friendly approach. Cow dung is cost effective and also relatively environmentally friendly.

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