African Journal of Biotechnology Vol. 10(48), pp. 9725-9736, 29 August, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.934
ISSN 1684–5315 © 2011 Academic Journals

Review

A review on non-stereospecific haloalkanoic acid dehalogenases

Tengku Haziyamin Tengku Abdul Hamid[1], Azzmer Azzar Abdul Hamid[1] and Fahrul Huyop[2]*

[1]Department of Biotechnology, Faculty of Science, International Islamic University, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia.
[2]Department of Industrial Biotechnology, Faculty of Biosciences and Bioengineering, Universiti Teknologi Malaysia, 81300 Skudai, Johor, Malaysia.

Accepted 1 July, 2011

Haloalkanoic acid dehalogenases remove halides from organic haloacids and have potential as bioremediation agents. DehE from *Rhizobium* sp. RC1, DehI from *Pseudomonas putida* PP3 and D,L-DEX 113 from *Pseudomonas* sp. 113 are non-stereospecific dehalogenases that invert the configurations of D- and L- carbons bound to a halogen. The kinetics of DehE has been partially characterized and brominated compounds have greater specificity constant values than do the corresponding chlorinated compounds. The sequence of DehE is similar to that of DehI; therefore, the two enzymes may have similar structures and functions. The three-dimensional structure of DehI is known and its reaction mechanism was inferred from its structure and a mutagenesis study of D,L-DEX 113. Aspartate residues at positions 189 and 194 in DehI and D,L-DEX 113 were predicted to be involved in catalysis. These residues activate a water molecule that directly attacks the chiral carbon. Because DehE and DehI are sequentially related, delineating the structure of DehE is important to ascertain if the catalytic residues and reaction mechanism are the same for both enzymes. A structural prediction, sequence-homology modeling and a site-directed mutagenesis study of DehE might help achieve this goal.

Key words: Haloalkanoic acids, non-stereospecific dehalogenase, DehE, *Rhizobium* sp. RC1, enzyme kinetics, protein structure prediction, site-directed mutagenesis.

INTRODUCTION

Many man-made xenobiotic compounds have been abundantly dispersed in the environment and are difficult to eliminate as they are not easily degraded. One class of xenobiotic compounds is formed by volatile halogenated organic compounds, which, as relatively inert compounds, remain in the atmosphere for long periods. These compounds are harmful to the health of humans. For example, the herbicide Dalapon that contains 2,2-dichloropropionic acid (2,2DCP) as its active ingredient was introduced by Dow Chemical Company in 1953.

Many microorganisms (van Pee, 1996; Slater et al., 1995; Leigh et al., 1986; Allison et al., 1983) can break down halogenated compounds by cleaving their carbon-halogen bonds via dehalogenase-catalyzed reactions and therefore, may aid in the removal of organohalides from the environment. Dehalogenases are classified as haloalkane dehalogenases, halohydrin dehalogenases, haloacetate dehalogenases, dichloromethane dehalogenases and D- and L-haloalkanoic acid dehalogenases (Allison, 1981; Fetzner and Lingens, 1994; Jing and Huyop, 2007; Ismail et al., 2008; Jing and Huyop 2008; Jing et al., 2008; Darus et al., 2009; Mesri et al., 2009; Thasif et al., 2009). Many microorganisms produce more than one dehalogenase, which may give a microorganism a survival advantage under fluctuating environmental conditions (Slater et al., 1997). However, why multiple dehalogenases exist in an organism is far from proven (Allison, 1981; Cairns et al., 1996).

Haloalkanoic acid dehalogenases have been grouped

*Corresponding author. E-mail: fzhutm@gmail.com Tel: +60 7 55 30065. Fax: +60 7 553 1112.

Table 1. Class 1D: D-isomer specific – inverts/substrate product configuration; Class 1L: L-isomer specific – inverts substrate/product configuration.

Organism-Dehalogenase	Reference
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	
Pseudomonas 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)
Xanthobacter autotrophicus strain GJ10 – DhlB	van der Ploeg et al. (1991)
Pseudomonas 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)

according to their dehalogenation mechanism or their substrate stereospecificity. Although, various dehalogenases have been grouped together, the classification may not indicate sequence similarity among the proteins. These enzymes differ in many ways, for example, pH optimum (Slater et al., 1979), size and subunit structure (Motosugi et al., 1982a; Allison et al., 1983; Tsang et al., 1988; Smith et al., 1990), electrophoretic mobility under non-denaturing conditions /substrate specificity (Hardman and Slater, 1981a, b). The majority of dehalogenases are inducible rather than constitutively expressed. Inducers for dehalogenases are not always the growth substrate, and regulation of expression is poorly understood (Allison et al., 1983; Huyop and Nemati, 2010).

Slater et al. (1997) classified haloalkanoic acid dehalogenases as hydrolytic dehalogenases, haloalcohol dehalogenases and cofactor-dependent dehalogenases. Hydrolytic dehalogenases are the most common dehalogenases and have been sub classified as 2-haloalkanoic acid hydrolytic dehalogenases and haloalkane hydrolytic dehalogenases. 2-Haloalkanoic acid dehalogenases are divided into class 1 (stereospecific) or class 2 (non-stereospecific) and further subdivided into Class 1D, Class 1L, Class 2I and Class 2R. Class 1D dehalogenases is less common than are 1L enzymes (Table 1). DehD from *Rhizobium* sp. RC1 is a Class 1D dehalogenase and it selectively inverts the D-configuration of the chiral carbon in D-isomeric substrates, for example, D-2-chloropropionic acid (D-2CP), to produce the L-configuration at the chiral carbon; whereas, class 1L dehalogenases remove the halide from an L–isomeric substrate, for example, L-2-chloropropionic acid (L-2CP) and then inverts the product configuration.

Class 2 dehalogenases are not substrate specific. Class 2I dehalogenases are distinguished by their abilities to dehalogenate both D- and L-isomers by a mechanism that involves the inversion of the substrate configuration (Table 2). *Pseudomonas putida* PP3 expresses two 2-haloalkanoic acid dehalogenases, namely DehI and DehII (Thomas, 1990), both of which are active against many halogenated compounds. Motosugi et al. (1982a, b) isolated *Pseudomonas* sp. 113 which can grow on both D- and L-2CP. According to its catalytic mechanism, the *Pseudomonas* sp. 113 dehalogenase D,L-DEX 113 defined a new class of dehalogenases as its mechanism does not involve an enzyme-substrate ester intermediate (Nardi-Dei et al., 1999). Instead, water directly attacks the α-carbon of a 2-haloalkanoic acid and displaces the halogen atom.

Brokamp and Schmidt (1991) isolated *Alcaligenes xylosoxidans* ssp. *denitrificans* ABIV from garden soil after repeatedly sub-culturing the organism in medium containing dichloroacetic acid. *A. xylosoxidans* ABIV has an inducible non-stereospecific hydrolytic dehalogenase and therefore, it can use different 2-haloalkanoic acids as the sole carbon source, for example, mono- or dichloroacetic acid and mono- or dichloropropionic acid. A sequence in the *A. xylosoxidans* ABIV genome (*dhlIV*) is homologous to a short segment of the D-specific dehalogenase *(hadD)* from *P. putida* AJ1. Restriction-enzyme patterns indicated that *dhlIV* and *dehI* from *P. putida* PP3 are similar genes. The DhlIV dehalogenation product of D- or L-chloropropionic acid is lactic acid that has an inverted configuration around its chiral carbon.

Both isomers of monochloropropionic acid (2CP) (Liu et al., 1994) are substrates of *Pseudomonas* sp. YL. 2-haloacid dehalogenase. The enzyme resembles the D,L-2-haloacid dehalogenase from *Pseudomonas* sp. 113 in its stereospecificity. On the basis of its pH optimum and activity staining, it was concluded that the *Pseudomonas* sp. YL 2-haloacid dehalogenase was capable of

Table 2. Class 2I: D and L isomers as substrates – inverts substrate product configuration; Class 2R: D and L isomers as substrates – retains substrate product configuration.

Organism - Dehalogenase	Native molecular mass (kDA)	Subunit molecular mass (kDA)	Reference
Class 2I: D and L isomer as substrate			
Pseudomonas strain 113 (DL-DEX)	68	34	Motosugi et al. (1982 a, b)
Pseudomonas putida strain PP3 – DehII	52	26	Weightman et al. (1982); Topping (1992)
Alcaligenes xylosoxidans ssp. denitrificants ABIV – DhIIV	64	32	Brokamp and Schmidt (1991); Brokamp et al. (1997)
Pseudomonas putida YL – 2-haloacid dehalogenase – D,L DEX YL	36	36	Liu et al. (1994)
Rhizobium sp. RC1 – DehE	64	32	Allison (1981); Huyop et al. (2004)
Class 2R: D and L isomer as substrates			
Pseudomonas putida strain PP3 – DehI			Weightman et al. (1982); Topping (1992)
Isolate K37 – HdIV			Murdiyatmo (1991)

dehalogenating D- and L-2CP.

DehE from *Rhizobium* sp. RC1 is a non-stereospecific dehalogenase that acts upon D,L-2CP, 2,2DCP, monochloroacetate and dichloroacetate. DehE inverts the configuration of the chiral carbon. However, according to Allison et al. (1983), this enzyme is sensitive to sulfhydryl-blocking reagents, which Slater et al. (1997) did not find. *Rhizobium* sp. RC1 plays a vital role in degrading halogenated compounds because it can use substrates of different stereo specificities. DehE inverts the configuration of D- and L-chiral carbons (Slater et al., 1995). Thus, dehalogenases, such as DehE, that are non-stereospecific are very useful for the degradation of halogenated compounds and for production of optically active 2-hydroxyalkanoic acids, which are important industrial reagents.

Class 2R dehalogenases differ from the Class 2I enzymes by their abilities to dehalogenate D- and L-isomers with retention of product configuration (Table 2). Murdiyatmo (1991) purified the enzyme HdIV from an unidentified isolate denoted strain K37 and sequenced its first 13 N-terminal amino acid residues. These 13 N-terminal amino acid residues correspond exactly to that encoded by the putative *dehI* open-reading frame beginning at the second encoded methionine (Slater et al., 1997). Between the first and second methionine codons, there is a strong Shine-Dalgarno sequence, separated by eight bases from the initiation codon, which is a separation considered to be optimal for transcription (Gold, 1988). DehI from *P. putida* PP3 is dimeric (Table 2, Weightman et al., 1979a, b; Topping, 1992). Recently, the crystal structure of DehI was solved and its catalytic mechanism established (Schmidberger et al., 2008). These investigators claimed that DehI inverted the configuration of the substrate chiral carbon, a finding that contrasts with the study by Topping (1992).

For this review, the catalytic activities of DehE and DehI are discussed because their amino acid sequences are similar and they therefore may have a similar structure and function. Structural studies using DehE should be useful. However, because not all proteins can be crystallized, a computationally derived model of the DehE structure would also be useful to examine the catalytic mechanism(s) of non-stereospecific haloalkanoic acid dehalogenases and to increase our understanding of their tertiary structures so that more stable dehalogenases may be produced for industrial applications.

DEHALOGENASE GENE ORGANIZATION

Dehalogenase gene organization in *Rhizobium* sp. RC1

The genetic organization of the *Rhizobium* sp. RC1 dehalogenases has been studied using mutant strains. Characterization of these mutants suggested that the dehalogenase genes are under the control of the regulatory gene *dehR*, which was proposed to encode a protein that positively regulates dehalogenase expression

Table 3. Dehalogenase synthesis by *Rhizobium* sp.RC1.

Mutant Strain	DehL	DehE	DehD
Type A	Absent	Absent	Absent
Type 1	Made inducibly	Made inducibly	Made inducibly
Type 2	Absent	Made constitutively	Absent
Type 3	Made constitutively	Absent	Made constitutively

DehL, Dehalogenase L; DehE, dehalogenase E; DehD, dehalogenase D

at the transcriptional level. Previously, Leigh (1986) suggested that the mode of regulation for the dehalogenase genes involves inhibition of their transcription when the *dehR* gene product is not bound to their promoter. The *Rhizobium* sp. RC1 dehalogenase genes are positively regulated by a promoter that controls *dehE* expression and a second promoter that controls *dehD* and *dehL* expression. Current investigation proved using cloned *dehR* controls *dehE* in *Escherichia coli* system (Huyop and Cooper, 2011).

Regulation of *Rhizobium* sp. RC1 dehalogenase synthesis

A *Rhizobium* sp. RC1 type A mutant produced by chemical mutagenesis could not use 2,2DCP or D,L-2CP as the sole carbon and energy source. The results of enzyme assays and PAGE indicated that dehalogenases were absent in this mutant. Plating on agar containing 2,2DCP or D,L-2CP and subsequent selection yielded three types of revertants. When 2,2DCP was used as the carbon source, mutants denoted types 1 and 2 were isolated. The type 1 mutant regained inducible production of the dehalogenases, that is, the wild-type phenotype was recovered and the three dehalogenases were inducible. The type 2 mutant constitutively produced DehE but not DehL and DehD. Using D,L-2CP as the selective medium, a mutant strain (type 3) that constitutively produced DehL and DehD, but not DehE was isolated. The characteristics of these mutants are summarized in Table 3, which were used by the authors to suggest a model for the regulation of dehalogenase gene expression in *Rhizobium* sp. RC1 (Figure 1).

The type A mutant was proposed to carry a mutation in the regulator gene that would cause the loss of expression of all the dehalogenases provided that all three genes are controlled by this regulator. To obtain the type 1 secondary mutant (with the wild-type phenotype) a reversion of the original mutation or a repressor mutation in the regulator gene must have occurred. Because the type 2 secondary mutant produced DehE constitutively, a mutation in its promoter region that controlled expression of DehE must have occurred, which resulted in the constitutive expression of DehE. The promoter controlling the expression of DehD and DehL would be unchanged so that the expression of those two dehalogenases would still be inhibited. Because the type 3 secondary mutant expressed DehD and DehL constitutively, a mutation in their promoter(s) must have occurred.

The relative locations of *dehD* and *dehL* have been confirmed by genomic DNA sequencing. *dehD* is located upstream of *dehL* with 177 bp of non-coding DNA between them (Cairns et al., 1996). The third Rhizobial dehalogenase gene, *dehE*, has also sequenced. However, this gene is not particularly close to *dehL* and *dehD* and its location relative to the other two is not known. A recent study suggested that a sequence upstream of *dehE* is an open-reading frame that encodes the dehalogenase regulatory gene, *dehR* (Huyop and Cooper, 2011). The amino acid sequence deduced from the *dehR* sequence has 70% sequence identity to that of the *P. putida* PP3 dehalogenase regulatory gene, suggesting that *dehR* is located close to *dehE*.

P. putida PP3 dehalogenase gene organization

P. putida PP3 produces DehI and DehII. DehI is most active against D,L-2CP, whereas DehII acts on monochloroacetate and dichloroacetate (Senior et al., 1976; Slater et al., 1979; Weightman et al., 1982). Thomas (1990) studied *dehI* in great detail. *dehI* is located in a mobile genetic element, is often inserted into targeted plasmids and subsequently, transferred into the chromosome of a second *P. putida* strain.

According to Topping (1992), expression of *dehI* is under the positive control of the adjacent regulatory gene *$dehR_1$*. Partial sequencing of these two genes indicated that the regulatory protein is an RNA polymerase σ-factor, 54-dependent activator protein. A putative -24/-12 promoter was identified immediately upstream of *dehI*. Topping (1992) confirmed the location of the *P. putida* PP3 dehalogenase genes and the function of their encoded proteins. The cloning, location and functional analysis of *dehI* and *$dehR_I$*, which are carried on the mobile element DEH, have been described (Topping, 1992). *dehI* is transcribed from a regulator promoter within DEH, *dehI* has been expressed in *E. coli* and *P. putida*. An activator of dehalogenase expression, *$dehR_I$*, is located next its cognate structural gene *dehI*. The genetic organization of the *P. putida* PP3 dehalogenases

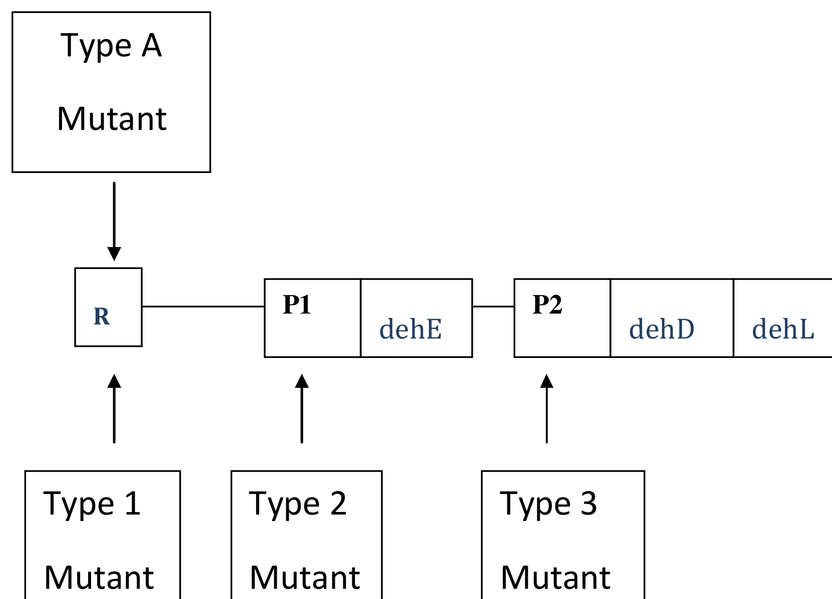


Figure 1. Proposed genetic organization and regulation for the *Rhizobium* sp. RC1 dehalogenase genes, R: regulator gene; controls all three dehalogenases *P1/P2*: Promoter regions *dehE, dehD, dehL*: structural genes for dehalogenases; Arrows: indicate sites of mutations. Type A mutant: no dehalogenases detected; Type 1 mutant: reversion to wild type; Type 2 mutant: constitutive production of DehE only (mutation in promoter P1); Type 3 mutant: constitutive production of DehL and DehD only (mutation in promoter P2).

is described in Figure 2.

ENZYMATIC CHARACTERIZATION OF *RHIZOBIUM* SP. RC1 DehE

DehE activity was measured against D,L-2CP between pH 6.1 and 10.5 and found not to be pH dependent, although, it was slightly more active between pH 9.1 and 10.5 (Leigh, 1986). No optimum pH was assigned to this enzyme. DehE was partially inactivated by 1 mM N-ethylmaleimide and 0.01 mM p-chloromecuribenzoate. DehE was more susceptible to N-ethylmaleimide (78% inhibition) and p-chloromecuribenzoate (85.2% inhibition) than were DehD and DehL (Leigh, 1986).

DehE acts more rapidly on trichloroacetic acid than on tribromoacetic acid (Huyop et al., 2004). Both compounds are inducers of the *Rhizobial* dehalogenases (Allison et al., 1983). Crude DehE (specific activity against D,L-2CP, 5.0 U/mg protein) acted on trichloroacetic acid and tribromoacetic acid with specific activities of 0.40 U/mg protein and 1.6 U/mg protein, respectively (Huyop et al.,2004). It has been reported that oxalic acid was a product of trichloroacetic acid dehalogenation but an assay of the reaction mixture for oxalic acid was negative (Stringfellow et al.,1997). Formic acid, the decarboxylation product of oxalic acid, was also not found. Identical results were obtained for tribromoacetic acid (Stringfellow et al., 1997).

DehE activity assay

In general, the enzyme assay was carried out at 30°C in a 5-ml mixture containing 0.09 M Tris-acetate (pH 7.5), substrate and enzyme (Huyop et al., 2004). Samples were removed at 5-min intervals and the amount of free halide was determined colorimetrically (Bergman and Sanik, 1957). Color was allowed to develop for 10 min at room temperature and then measured at A_{460}. Enzyme activity (1 U) was defined as the amount of enzyme that catalyzed the formation of 1 µmol halide ion/min. For substrate specificity and kinetics, two types of substrates were used- those suitable for growth of *Rhizobium* sp. RC1 and those acted upon by enzyme. DehE acted on all of the tested substrates and did not show any substrate specificity.

Expression and purification of DehE

The following was the work of Huyop et al., (2004). Cultivation of *E. coli* BL21 (DE3) that carried the pJS771 (*dehE*$^+$) vector was used for *dehE* expression. For DehE purification, a cell-free extract was prepared in 0.1 M Tris-acetate (pH 7.6). Approximately 6 mg protein (6 U as

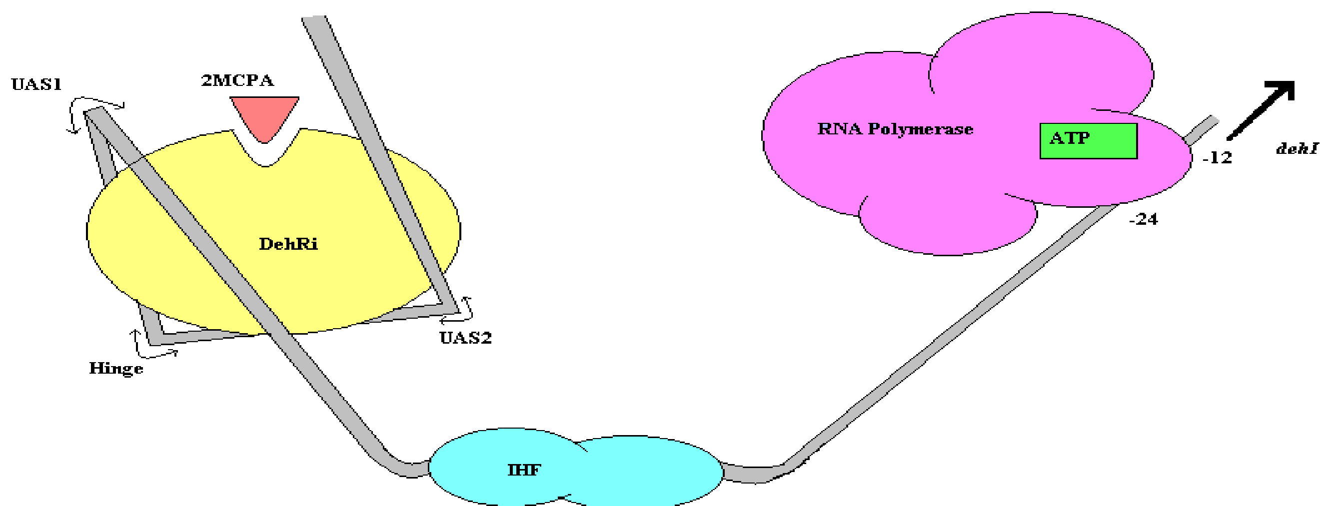


Figure 2. Schematic diagram of possible activation system of *dehI* by $DehR_I$. $DehR_I$ binds to upstream activation sites of *dehI* promoter (UAS_1 and UAS_2), causing DNA bending in the hinge region. RNA polymerase containing the σ^{54} subunit binds to the *dehI* -24 /-12 promoters. Integration host factor (IHF) binds to a specific site between the UAS regions and the promoter and mediates DNA bending such that contact is made between $DehR_I$ and the polymerase. In the presence of an inducer, such as 2MCPA, a conformational change occurs in $DehR_I$ enabling it activate the polymerase. Transcription is then initiated with accompanied hydrolysis of ATP (Adapted from Topping, 1992).

assessed with 2,2DCP as substrate) was loaded onto a MonoQ HR 5/5 anion-exchange column equilibrated with 20 mM sodium phosphate (pH 7.6) containing 1 mM EDTA, 1 mM dithiothreitol, 10%(m/v) glycerol and eluted with a 20- to 200-mM sodium phosphate gradient. DehE eluted in two fractions at ~80 mM sodium phosphate. The fractions contained 2.7 U and 2.9 U of enzyme and had specific activities of 2.1 and 2.9 U/mg, when 2,2DCP was used as the substrate. A 32-kDa protein band was evident upon SDS-PAGE for both MonoQ fractions. The molecular weight of purified, native DehE was also assessed by tandem Superose 12 chromatography (Pharmacia) that had been calibrated with molecular weight protein standards. The molecular weight was found to be 62 kDa, suggesting that DehE is a dimer in its native state (Huyop et al., 2004).

Enzyme kinetic analysis

An early investigation of the K_m values for DehE was carried out by Allison et al. (1983), but many of the reported values seemed surprisingly large and needed to be re-examined using cloned *dehE.* The K_m values for both chlorinated and brominated substrates are given in Table 4. The values are not significantly different for chlorinated and brominated propionate. However, the K_m values for chloro- and bromoacetates decreased as the number of halogens (one to three) in the compounds increased. The k_{cat} values for growth substrates varied, with 2,2DCP being the best substrate and D-2CP the worst. Catalytic efficiencies are best compared by examining the k_{cat}/K_m ratio, known as the specificity constant. k_{cat}/K_m values for the DehE substrates are listed in Table 4. Most of the brominated substrates have a larger specificity constant value than do their corresponding chlorinated substrates.

CHARACTERIZATION OF DehI FROM *P. PUTIDA* PP3

Weightman et al. (1979b) showed that *P. putida* PP3 produced two dehalogenases, which were separated by DEAE-Sephadex A50 chromatography and distinguishable by their electrophoretic mobilities through a non-denaturing polyacrylamide gel. DehI is most active against D,L-2CP (Slater et al., 1979). DehI is a regulated enzyme and can dehalogenate (albeit at lower rates) a variety of haloalkanoic acid compounds (Topping, 1992). DehI is sensitive to sulfhydryl-blocking agents (Weightman et al., 1982). Dithiothreitol stabilizes DehI in cell-free extracts (Weightman et al., 1979a, b). Although, DehI hydrolyzes D,L-2CP, the configuration of the chiral carbon is preserved (Weightman et al., 1982). It is a type 2I dehalogenase (Schmidberger et al., 2008). The calculated molecular weights for DehI and DehII are 46 and 52 kDa, respectively. The molecular weight of DehI was estimated as 33 kDa by SDS-PAGE (Topping, 1992). DehI was purified and further characterized by Park et al. (2003) who named it D,L-DEX 312. The enzyme has maximum activity at 30 to 40°C, pH 9.5 and is inactivated completely when incubated at 40°C for 35 min. D,L-DEX 312 catalyzed the hydrolytic dehalogenation of 2-chloropropionamide and 2-bromopropionamide, which identified it as the first enzyme found that dehalogenates 2-haloacid amides.

Table 4. Km and Kcat value for DehE (Adapted from Huyop et al., 2004).

Substrate for growth	Kcat (Sec^{-1})	Km	Specificity constant (M^{-1}sec^{-1})
D-2CP	08.16	5.20 x 10^4 M (0.52 mM)	1.56 x 10^4
D-2BP	20.66	4.60 x 10^4 M (0.46 mM)	4.40 x 10^4
L-2CP	13.43	4.10 x 10^4 M (0.41 mM)	3.27 x 10^4
L-2BP	13.43	2.90 x 10^4 M (0.29 mM)	4.60 x 10^4
D,L-2CP	10.03	3.50 x 10^4 M (0.35 mM)	2.86 x 10^4
D,L-2BP	12.40	2.20 x 10^4 M (0.22 mM)	5.64 x 10^4
2,2-DCP	5.58	1.90 x 10^4 M (0.19 mM)	2.94 x 10^4
D,L-2,3DCP	01.44	3.60 x 10^4 M (0.36 mM)	0.40 x 10^4
MCA	25.83	1.19 x 10^3 M (0.19 mM)	2.17 x 10^4
DCA	01.65	3.60 x 10^4 M(0.36 mM)	0.46 x 10^4
TCA	00.20	3.10 x 10^4 M (0.31 mM)	0.65 x 10^4
MBA	89.90	2.18 x 10^3 M (2.18 mM)	4.12 x 10^4
DBA	14.46	8.80 x 10^4 M (0.88 mM)	1.64 x 10^4
TBA	02.06	3.20 x 10^4 M (0.32 mM)	0.64 x 10^4

2CP: 2-chloropropionic acid; DCP: dichloropropionic acid; MCA: monochloroacetate; DCA: dichloroacetate; TCA: trichloroacetate; MBA: monobromoacetate; DBA: dibromoacetate ; TBA: tribromoacetate

AMINO ACIDS SEQUENCE COMPARISONS FOR DehE, DehI AND RELATED DEHALOGENASES

The deduced amino acid sequences of DehE (Accession number CAA75671) and DehI (Accession number AAN60470) have been deposited in the National Center for Biotechnology Information. Both dehalogenases contain 296 residues (Table 5). The sequences of both enzymes were submitted to www.expasy.org for analysis by ProtParam and ColorSeq (Gasteiger et al., 2005; Bechet et al., 2010). Both enzymes contain more negatively charged than positively charged residues. The theoretical pI value for both dehalogenases is ~5. Both enzymes are expected to be water soluble as their grand average of hydropathicity indexes has negative values. However, the value for DehE is more negative than that for DehI, even though they have the same number of hydrophilic residues. In addition, basic and acidic residues are uniformly dispersed in the DehI sequence. Conversely, DehE has more negatively charged than positively charged residues; thus, DehE may have patches of acidic areas on its surface. Using EMBOSS (http://www.ebi.ac.uk/Tools), a pairwise comparison of the DehE and DehI sequences indicated that they are 72% identical and 85% similar (Figure 3).

Lassmann and Sonnhammer (2005) also searched the NCBI database, with the DehE amino acid sequence as the query and found that the *A. xylosoxidans* ssp. DhlIV sequence is 72% identical and the D,L-DEX 113 sequence is 39% identical. Because these three enzymes are sensitive to sulfhydryl-blocking reagents, their protein sequences were examined to identify a consensus cysteine(s) (Figure 4). DhlIV and DehI contain two cysteines (positions 42 and 288), D,L-DEX 113 contains one cysteine (position 178) and DehE contains four cysteines residues (positions 42, 128, 256 and 288). Thus, DhlIV, DehI and DehE have conserved cysteines at positions 42 and 288, which are not found in D,L-DEX 113.

CATALYTIC MECHANISM OF NON-STEREOSPECIFIC HALOALKANOIC ACID DEHALOGENASES

DehE, DehI and D,L-DEX 113 catalyze the hydrolytic dehalogenation of both D- and L-2-haloalkanoic acids to produce the corresponding L- and D-2-hydroxyalkanoic acids. All three enzymes are similar to L-2-haloacid dehalogenases and D-2-haloacid dehalogenases in that they catalyze the hydrolytic dehalogenation of 2-haloalkanoic acids with inversion of the chiral carbon.

The gene encoding D,L-DEX 113 corresponds to 307 amino acid residues and the sequence is closely related to that of D-2-haloacid dehalogenase from *P. putida* AJ1, which acts specifically on D-2-haloalkanoic acids (Barth et al., 1992). The sequence identity is 23.5% for the two enzymes. Conversely, D,L-DEX 113 and the L-2-haloacid dehalogenases do not share substantial sequence identity. Because the sequence of D,L-DEX 113 is similar to that of D-2-haloacid dehalogenase, the two active sites are probably also similar. In total, there are 26 polar residues directly involve in the catalytic mechanism that affect the rate of D,L-DEX 113 (Nardi-Dei et al., 1997). However, only Thr65, Glu69 and Asp194 are critical for dehalogenation of D- and L-2-chloropropionate. It was concluded that the active site of D,L-DEX 113 is the same for both enantiomers. This conclusion was also reached by Schmidberger et al. (2008) for the active site of DehI.

Table 5. Amino acids composition.

DehE of *Rhizobium* sp. RC1 (residue per subunit)			DehI of *Pseudomonas putida* PP3 (residue per subunit)		
Amino acid	**Frequency**	**Percentage**	**Amino acid**	**Frequency**	**Percentage**
Alanine (A)	34	11.5	Alanine (A)	31	10.5
Arginine (R)	21	7.1	Arginine (R)	20	6.8
Asparagine (N)	10	3.4	Asparagine (N)	7	2.4
Aspartic acid (D)	12	4.1	Aspartic acid (D)	13	4.4
Cysteine (C)	4	1.4	Cysteine (C)	2	0.7
Glutamine (Q)	11	3.7	Glutamine (Q)	10	3.4
Glutamic acid (E)	25	8.4	Glutamic Acid (E)	22	7.4
Glycine (G)	20	6.8	Glycine (G)	19	6.4
Histidine (H)	5	1.7	Histidine (H)	5	1.7
Isoleucine (I)	16	5.4	Isoleucine (I)	14	4.7
Leucine (L)	31	10.5	Leucine (L)	38	12.8
Lysine (K)	8	2.7	Lysine (K)	10	3.4
Methionine (M)	8	2.7	Methionine (M)	8	2.7
Phenylalanine (F)	10	3.4	Phenylalanine (F)	9	3.0
Proline (P)	20	6.8	Proline (P)	21	7.1
Serine (S)	15	5.1	Serine (S)	19	6.4
Threonine (T)	14	4.7	Threonine (T)	15	5.1
Tryptophan (W)	4	1.4	Tryptophan (W)	3	1.0
Tyrosine (Y)	8	2.7	Tyrosine (Y)	11	3.7
Valine (V)	20	6.8	Valine (V)	19	6.4
Characteristic of amino acids residue					
Hydrophobic (non-polar)	113	38.2		113	38.2
Hydrophilic (polar)	116	39.2		116	39.2
Positive (basic)	34	11.5		35	11.8
Negative (acidic)	37	12.5		35	11.8
Aromatic	22	7.4		23	7.8
Hydroxyl	37	12.5		45	15.2
Number of amino acids	296			296	
Calculated molecular weight	65,351 Da			65,451 Da	

The catalytic mechanism of D,L-DEX 113 was assessed by an 18O-labeling experiment and a site-directed mutagenesis study (Nardi-Dei et al., 1997). For single- and multiple-turnover reactions by a large excess of D,L-DEX 113 in $H_2{}^{18}O$ with D- or L-2-chloropropionate as the substrate, the major product was ^{18}O-labeled lactate as shown by ion-spray mass spectrometry. Therefore, the oxygen of $H_2{}^{18}O$ directly attacked the α-carbon of the 2-haloalkanoic acid and displaced the halide (Figure 5a). The results of site-directed mutagenesis experiments indicated that Glu69 and Asp194 are crucial for the catalysis of D,L-DEX 113, even though Asp189 had been predicted to be a catalytic residue in DehI (Schmidberger et al., 2008). In addition, Asp 194 and 189 were in homologous positions. One of these may function as a catalytic base to activate the water molecule that attacks the substrate α-carbon. Unlike all known stereospecific dehalogenases, which have an active-site carboxylate that attacks the carbon bound to the halogen to form an ester intermediate (Figure 5b), D,L-DEX 113 and DehI do not form an ester intermediate during catalysis. It is therefore important to delineate the DehE catalytic mechanism; however, to date, DehE has not been subjected to a mutagenesis study similar to those performed for D,L-DEX 113 and DehI dehalogenases. Clarification of the catalytic mechanism of DehE would add credence to the proposed catalytic mechanism used by non-specific dehalogenases and allow for the creation of new products for industrial applications.

Structural study of DehI and protein crystallization

To date, only the crystal structure of DehI has been solved (Schmidberger et al., 2008), which showed the enzyme to be a homodimer. Each subunit contains two domains that are virtually structurally identical and are related to each other as a pseudo-dimer. Examination of

```
DehE   1  MLNAAYFPQISQSDVGGEMEATYENIRQTLRVPWVAFACRVLATVPEYLP  50
          | | |||||:|| || |||| ||| || ||||||||| |||||| | |||
DehI   1  MTNPAYFPQLSQLDVSGEMESTYEDIRLTLRVPWVAFGCRVLATFPGYLP  50

DehE  51  VAWARTAEAMSTRYAEQAADELRERSLLSIEPKVDLKKRLRGAGWDNAQI  100
           || | |||: ||||||||||||||||| | |   || ||  || |  :|
DehI  51  LAWRRSAEALITRYAEQAADELRERSLLNIGPLPNLKERLYAAGFDDGEI  100

DehE 101  EEVRRVVNAFNYGNPKYIMMITALCESFNLRPVGGGDLSVELRSSVPKGH  150
          | ||||  |||||||||:::|||| ||  :||||| : | ||| |:||||
DehI 101  EKVRRVLYAFNYGNPKYLLLITALSESMQMRPVGGAEVSSELRASIPKGH  150

DehE 151  PEGMDPLLSLVNANEAPPEVQTLLKRAADLHYHHGPASDFQALANWPEFL  200
          | |||||| || |  |  ||| |||| ||||||||||||||||||||  |
DehI 151  PKGMDPLLPLVDATKASTEVQGLLKRVADLHYHHGPASDFQALANWPKVL  200

DehE 201  QIATDEALAPVVRTETFDLKARELIHRARELVQGLPGQVGIGRAELMSTC  250
          || ||| |||| ||| :| | |||: || ||| ||||  |: | ||||
DehI 201  QIVTDEVLAPVARTEQYDAKSRELVTRAPELVRGLPGSAGVQRSELMSML  250

DehE 251  TPGEIAGLTGILFMYQRFIPDITISLIRIGECLDGSEAAAKSPFPVZ    297
          || |:|||||:|||||||| |||||:| | ||||| ||| |||||:|
DehI 251  TPNELAGLTGVLFMYQRFIADITISIIHITECLDGAEAASKSPFPIZ    297
```

Figure 3. Amino acid sequence comparison between DehE from *Rhizobium* sp. RC1 (Allison, 1981) and DehI from *P. putida* PP3 (Topping, 1992). (Percent identity 213/296 = 72% and similarity 251/296= 85%). Two dots indicate amino acid similarity.

the active site revealed the likely binding modes for both D- and L-substrates with respect to the key catalytic residues. Asp189 was predicted to activate a water molecule for nucleophilic attack of the substrate chiral carbon, resulting in an inversion of configuration for both D- and L-substrates. The DehI structure provides insight into its reaction mechanism and its ability to process both D- and L-substrates. Because the sequences of DehE and DehI are closely related, the structure of DehE may be modeled with DehI as the template.

Key catalytic residues

The sequence of D,L-DEX 113 is 40% identical to that of DehI. Nardi-Dei et al. (1997) mutated 26 conserved, polar/charged residues in D,L-DEX 113 to another similar amino acids in the same group for functional analysis. Of these, three were essential for catalytic activity: Thr65 (Thr62), Glu69 (Glu66) and Asp194 (Asp189) (the equivalent residue numbers for DehI are enclosed in parentheses). Mutation of Asp28 (Asp25), Glu250 (Glu245), Tyr120 (Tyr117), Thr219 (Thr214) and Asn117 (Asn114) resulted in diminished activity. Of the residues essential for activity, only Asp189 that is homologous to Asp194 in D,L-DEX 113 located at the active-site location of DehI. The Asp189 side chain is found at the edge of the cavity (Schmidberger et al., 2008) and is likely to be directly involved in catalysis. The three-dimensional structure of DehI helps rationalize the importance of residues of Asp189, which by analogy to those of D,L-DEX 113 diminish activity when mutated.

Examination of the three-dimensional structure of DehI suggests that Asp189 activates the water molecule with the assistance of Asn114. Asn114 and Asp189 are adjacent to each other and the sulfate ions and ideally positioned to interact with bound substrate. The theoretical pKa of the Asp 189 carboxylic acid in the DehI structure is 6.6 (Li et al., 2005), which is unusually high for an aspartic acid (pKa ~3.7). Given that the enzymatic activity of the Group I α-HA dehalogenases is optimal at approximately pH 9 (Motosugi et al., 1982b; Brokamp and Schmidt, 1991), an elevated pKa for Asp189 is consistent with it activating a water molecule by abstracting a proton. The theoretical pKa for Asp189 may be due in part to the presence of Asn114. Mutation of Asn114 to an aspartic acid decreases the theoretical Asp189 pKa to 5.4. This is also consistent with the experimental data of Nardi-Dei et al. (1999), which showed that mutation of the equivalent residue in D,L-DEX 113 reduced its activity. Figure 6 shows the reaction mechanism proposed for DehI. Asp189 and Asn114 act in concert as a base and activate an adjacent water molecule. The activated water molecule attacks the chiral substrate carbon and the halogen is held in the halide-binding site. An SN2 transition-state intermediate is formed, followed by release of the halide and formation of the inverted hydroxylated product. The reaction mechanism is consistent with that proposed by Nardi-Dei et al. (1997) with Asp189 a critical catalytic residue.

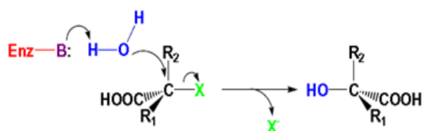
PROSPECTIVE STUDIES USING DehE

Non-stereospecific dehalogenases have been highlighted in this review because of their potential as bioremediation

```
         1                                                   50
  DehE   ...MLNAAYF PQISQSDVGG EMEATYENIR QTLRVPWVAF ACRVLATVPE
 DhlIV   ...MTNPAYF PQLSQLDVSG EMESTYEDIR LTLRVPWVAF GCRVLATFPG
  DehI   ...MTNPAYF PQLSQLDVSG EMESTYEDIR LTLRVPWVAF GCRVLATFPG
D,LDEX   MSHRPILKNF PQVDHHQASG KLGDLYNDIH DTLRVPWVAF GIRVMSQFEH
                  * **       *      *  *   *********   **

         51                                                 100
  DehE   YLPVAWARTA EAMSTRYAEQ AADELRERSL LSIEPKVDLK KRLRGAGWDN
 DhlIV   YLPLAWRRSA EALITRYAEQ AADELRERSL LNIGPLPNLK ERLYAAGFDD
  DehI   YLPLAWRRSA EALITRYAEQ AADELRERSL LNIGPLPNLK ERLYAAGFDD
D,LDEX   FVPAAWEALK PQISTRYAEE GADKVREAAI IPGSAPANPT PALLANGWSE
           * **         *****   ** ***  * *            *   *

         101                                                150
  DehE   AQIEEVRRVV NAFNYGNPKY IMMITALCES FNLRPVGGG. ..DLSVELRS
 DhlIV   GEIEKVRRVL YAFNYGNPKY LLLITALSES MQMRPVGGA. ..EVSSELRA
  DehI   GEIEKVRRVL YAFNYGNPKY LLLITALSES MQMRPVGGA. ..EVSSELRA
D,LDEX   EEIAKLKATL DGLNYGNPKY LILISAWNEA WHGRDAGGGA GKRLDSVQSE
           *           *******    * *  *     *  **

         151                                                200
  DehE   SVPKGHPEGM DPLLSLVNAN EAPPEVQTLL KRAADLHYHH GPASDFQALA
 DhlIV   SIPKGHPKGM DPLLPLVDAT KASTEVQGLL KRVADLHYHH GPASDFQALA
  DehI   SIPKGHPKGM DPLLPLVDAT KASTEVQGLL KRVADLHYHH GPASDFQALA
D,LDEX   RLPYGLPQGV EKF.HLIDPE AADDQVQCLL RDIRDAFLHH GPASDYRVLA
           * * * *       *      *   ** **     *   ** *****   **

         201                                                250
  DehE   NWPEFLQIAT DEALAPVVRT ETFDLKAREL IHRARELVQG LPGQVGIGRA
 DhlIV   NWPKVLQIVT DEVLAPVART EQYDAKSREL VTRARELVRG LPGSAGVQRS
  DehI   NWPKVLQIVT DEVLAPVART EQYDAKSREL VTRAPELVRG LPGSAGVQRS
D,LDEX   AWPDYLEIAF RDTLKPVALT TEFELTTSRI RKIAREHVRG FDGAGGVAWR
          **  * *      * **  *               * * * *   *  *

         251                                                300
  DehE   ELMSTCTPGE IAGLTGILFM YQRFIPDITI SLIRIGECLD GSEAAAKSPF
 DhlIV   ELMSMLTPNE LAGLTGVLFM YQRFIADITI SIIHITECLD GAEAASKSPF
  DehI   ELMSMLTPNE LAGLTGVLFM YQRFIADITI SIIHITECLD GAEAASKSPF
D,LDEX   DMADRMTPEE IAGLTGVLFM YNRFIADITV AIIRLKQAFG SAEDATENKF
               ** *  ***** *** * *** ***    *          * *    *

         301
  DehE   PVZ......
 DhlIV   PIZ......
  DehI   PIZ......
D,LDEX   RVWPTEKGZ
```

Figure 4. Multiple sequence alignment (Corpet, 1988) of *Rhizobium* sp. RC1 DehE with *A. xylosoxidans* ssp. *denitrificans* ABIV DhlIV (Brokamp et al., 1997), *P. putida* PP3 DehI (Topping, 1992) and *Pseudomonas* sp. strain 113 D,L-DEX (Nardi-Dei et al., 1997). *indicates sequence identity.

a.



b.

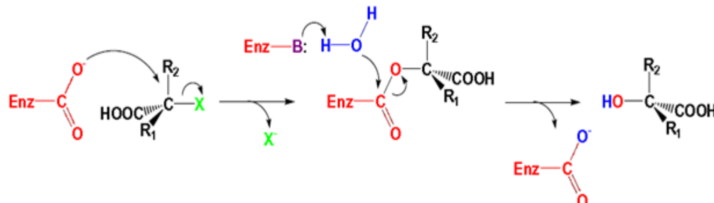


Figure 5. Reaction mechanisms of 2-haloacid dehalogenases (Adapted from Nardi-Dei et al., 1997): (a) a general base catalytic mechanism; (b) nucleophilic attack by an acidic amino acid residue followed by hydrolysis of the ester intermediate.

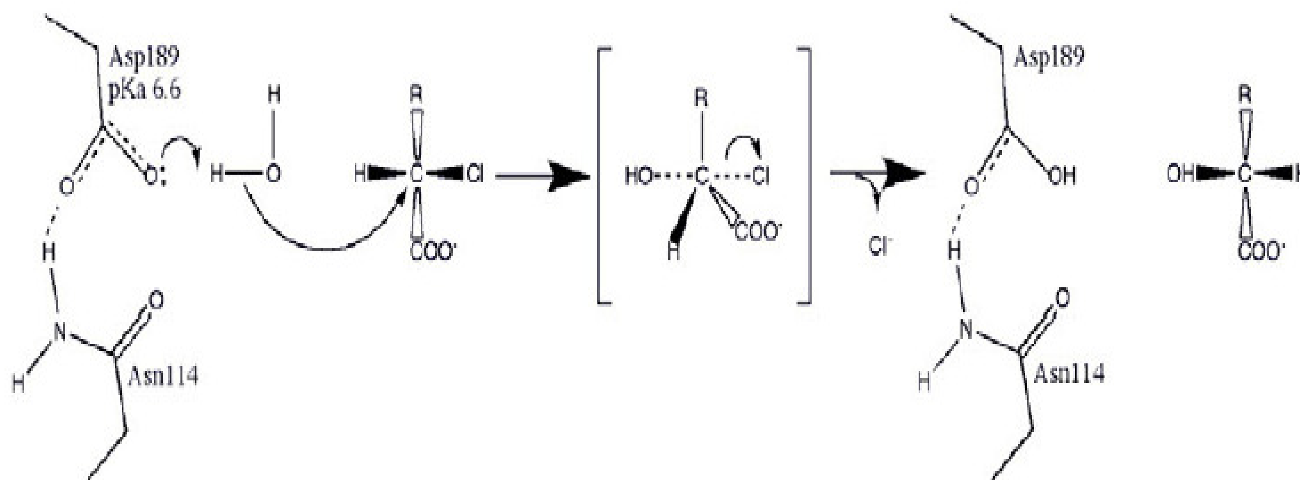


Figure 6. Keys of catalytic residues that involved in SN2 mechanism. (Adapted from Schmidberger et al., 2008).

agents. A mutagenesis study using D,L-DEX 113 identified residues important to catalysis. By aligning the 12 residues were identified that form the active site. Examination of the three-dimensional structure of DehI supports the sequence alignment study, with the key catalytic residues, Asp189 located in a cluster in the active site.

Because DehE from *Rhizobium* sp. RC1 is a homolog of DehI, it has been predicted to have the same catalytic residues and similar three-dimensional structure. To identify its catalytic residues, the conserved charged and polar residues should be subjected to site-directed mutagenesis. In addition, because a multiple sequence alignment using D-specific haloalkanoic acid dehalogenases revealed that Asn187 is probably responsible for the stereospecificity of DehE, by mutating this residue it may be possible to generate a form of DehE that targets only D-substrates. A mutated DehE that is specific for D-substrates would increase its commercial value because D-specific haloalkanoic acid dehalogenases are widely used in industry.

ACKNOWLEDGEMENTS

The author thanks the Malaysian Government for partly sponsor the work under research grant schemes FRGS 4F008 and GUP (QJ130000.7135.00H34).

REFERENCES

Allison N (1981). Bacterial degradation of halogenated aliphatic acids, PhD. Thesis, Trent Polytechnic, Nottingham, United Kingdom.

Allison N, Skinner AJ, Cooper RA (1983). The dehalogenases of a 2,2 dichloropropionate degrading bacterium. J. Gen. Microbiol.,129:1283-1293.

Barth PT, Bolton L, Thomson JC (1992). Cloning and partial sequencing of an operon encoding two *Pseudomonas putida* haloalkanoate dehalogenases of opposite stereospecificity. J. Bacteriol., 174: 2612-2619.

Bechet E, Gruszczyk J, Terreux R, Gueguen-Chaignon V, Vigouroux A, Obadia B, Cozzone AJ, Nessler S, Grangeasse C (2010). Identification of structural and molecular determinants of the tyrosine-kinase Wzc and implications in capsular polysaccharide export. Mol. Microbiol., 77: 1315-1325.

Bergman JG, Sanik J (1957). Determination of trace amounts of chlorine in naptha. Anal. Chem., 29: 241-243.

Brokamp A, Schmidt FRJ (1991). Survival of *Alcaligenes xylosoxidans* degrading 2,2-dichloropropionate and horizontal transfer of its halidohydrolase gene in a soil microcosm. Curr. Microbiol., 22: 299–306.

Brokamp A, Happe B, Schmidt FRJ (1997). Cloning and nucleotide sequence of a D,L-haloalkanoic acid dehalogenase encoding gene from *Alcaligenes xylosoxidans sp. denitrificans* ABIV. Biodegradation, 7(5):383-396.

Cairns SS, Cornish A, Cooper RA (1996). Cloning, sequencing and expression in *Escherichia coli* of two *Rhizobium sp.* genes encoding haloalkanoate dehalogenases of opposite stereospecificity. Eur. J. Biochem., 253:744-749.

Corpet F (1988). Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res., 16(22): 10881-10890.

Darus R, Pakingking Jr. RV, Shamsir MS, Huyop F (2009). Biodegradation of Monochlorocetic acid (MCA) by a presumptive *Pseudomonas* sp. Bacterium isolated from Malaysian paddy field. Israeli J. Aqua.– Bamidgeh, 61(3): p. 282.

Fetzner SR, Lingens F (1994). Bacterial Dehalogenases. Micro. Rev., 58(4): 641-685.

Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A (2005).*Protein Identification and Analysis Tools on the ExPASy Server;*(In) John M. Walker (ed): The Proteomics Protocols Handbook, Humana Press. pp. 571-607.

Gold L (1988). Post-translational regulatory mechanism in *E. coli.* Annu. Rev. Biochem., 57: 199-233.

Hardman DJ, Slater JH (1981a). Dehalogenases in soil bacteria. J. Gen. Microbiol., 123: 117-128.

Hardman DJ, Slater JH (1981b). The dehalogenase complement of a soil *Pseudomonad* grown in closed and open cultures on haloalkanoic acids. J. Gen. Microbiol., 127: 399-405.

Huyop F, Cooper RA (2011).Regulation of dehalogenase E (DehE) and expression of dehalogenase regulator gene (DehR) from Rhizobium sp. RC1 in *E.coli*. Biotechnol. & Biotechnol. Eq., 25(1): 2237-2242.

Huyop F, Nemati M (2010) Properties of dehalogenase from *Rhizobium* sp. RC1. Afr. J. Microbiol. Res., 4(25): 2836-2847.

Huyop F, Tan Yea Yusn, Ismail M, Wahab RA, Cooper RA (2004).

Overexpression and characterisation of non-stereospecific haloacid Dehalogenase E (DehE) of *Rhizobium sp.* Asia Pac. J. Mol. Biol. Biotechnol., 12(1&2): 15-20.

Ismail SN, Taha AM, Jing NH, Wahab RA, Hamid AA, Pakingking Jr. RV, Huyop F (2008). Biodegradation of monochloroaceticacid (MCA) by a presumptive *Pseudomonas* sp. strain R1 bacterium isolated from Malaysian paddy (rice) field. Biotechnol., 7(3):481-486.

Jing NH, Huyop F (2008). Enzymatic dehalogenation of 2,2-dichloropropionic acid by locally isolated *Methylobacterium sp*. HJ1. J. Biol. Sci., 8(1):233-235.

Jing NH, Taha AM, Pakingking Jr. RV, Wahab RA, Huyop F (2008). Dehalogenase from *Methylobacterium* sp. HJ1 induced by the herbicide 2,2-dichloropropionate (Dalapon). Afr. J. Microbiol. Res., 2:32-36.

Jing NH, Huyop F (2007). Dehalogenation of chlorinated aliphatic acid by *Rhodococcus* sp. Asia Pac. J. Mol. Biol. Biotechnol., 15(3):147-151.

Jones DHA, Barth PT, Byrom D, Thomas CM (1992). Nucleotide sequence of the structural gene encoding a 2-haloalkanoic acid dehalogenase of *Pseudomonas putida* strain AJ1 and purification of the encoded protein. J. Gen. Microbiol., 138: 675-683.

Kawasaki H, Toyama T, Maeda T, Nishino H, Tonomura K (1994). Cloning and sequence analysis of a plasmid encoded 2-haloacid dehalogenase gene from *Pseudomonas putida* no. 109. Biosci. Biotechnol. Biochem., 58: 160-163.

Kawasaki H, Tsuda K, Matsushita I, Tonomura K (1992) Lack of homology between two haloacetate dehalogenase genes encoded on a plasmid from *Moraxella species* strain B. J. Gen. Microbiol., 138: 1317-1323.

Lassmann T, Sonnhammer ELL (2005). Kalign - an accurate and fast multiple sequence alignment algorithm. BMC Bioinformatics. 6: 298.

Leigh JA (1986). Studies on bacterial dehalogenases. PhD thesis, Trent Polytechnic, Nottingham. United Kingdom.

Leigh JA, Skinner AJ, Cooper RA (1986). Isolation and partial characterisation of dehalogenase - deficient mutants of a *Rhizobium sp.* FEMS Microbiol. Lett., 36:163-166.

Leigh JA, Skinner AJ, Cooper RA (1988). Partial purification, stereospecificity and stoichiometry of three dehalogenases from a *Rhizobium species*. FEMS Microbiol. Lett., 49: 353-356.

Li H, Robertson AD, Jensen JH (2005). Very fast empirical prediction and rationalization of protein pKa values. Proteins, 61:704–721.

Liu J, Kurihara T, Hasan AKM, Nardi-Dei V, Koshikawa H, Esaki N, Soda K (1994). Purification and characterization of thermostable and non-thermostable 2-haloacid dehalogenases with different stereospecificities from Pseudomonas sp. Strain YL. Appl. Environ. Microbiol., 60: 2389-2393.

Mesri S, Wahab RA, Huyop F (2009). Degradation of 3-chloropropionic acid by *Pseudomonas* sp. B6P isolated from a rice paddy field. Annal. Microbiol., 59(3): 447-451.

Motosugi K, Esaki N, Soda K. (1982a). Purification and properties of 2-haloacid dehalogenase from *P. putida.* Agric. Biol. Chem., 46: 837-838.

Motosugi K, Esaki N, Soda K (1982b). Bacterial assimilation of D- and L-2-chloropropionates and occurrence of a new dehalogenase. Arch. Microbiol., 131: 179-183.

Murdiyatmo U (1991). Molecular genetic analysis of a 2-haloacid halidohydrolase structural gene. Phd. Thesis, Univ. Kent at Canterbury, Canterbury, United Kingdom.

Murdiyatmo U, Asmara W, Tsang JSH, Baines AJ, Bull AT, Hardman DJ (1992). Molecular biology of the 2-haloacid halidohydrolase from *Pseudomonas cepacia* MBA4. Biochem. J., 284: 87-93.

Nardi-Dei V, Kurihara T, Okamura O, Liu JQ, Koshikawa H, Ozaki H, Terashima Y, Esaki N, Soda K (1994). Comparative studies of genes encoding thermostable L-2-haloacid dehalogenase from *Pseudomonas* species strain YL, other dehalogenases and two related hypothetical proteins from *Escherichia coli.* Appl. Environ. Microbiol., 60: 3375-3380.

Nardi-Dei V, Kurihara T, Chung P, Esaki N, Soda K (1997). Bacterial D,L-2-haloacid dehalogenase from *Pseudomonas sp.* strain 113: Gene cloning and structural comparison with D and L-2haloacid dehalogenases. J. Bacteriol., 179(3): 4232-4238.

Nardi-Dei V, Kurihara T, Chung P, Masaru , Susumu T, Soda K, Nobuyoshi E (1999). DL-2Haloacid Dehalogenase from *Pseudomonas sp.* 113 is a new class of dehalogenase catalysing hydrolytic dehalogenation not involving enzyme-substrate ester intermediate. J. Biol. Chem., 274(30): 20977-20981.

Park C, Kurihara T, Yoshimura T,Soda K, Esaki N (2003). A new DL-2-haloacid dehalogenase acting on 2-haloacid amides: purification, characterization, and mechanism. J. Mol. Catal. B-Enzym., 23: 329-336

Schmidberger JW, Wilce JA, Weightman AJ, Whisstock JC, Wilce MCJ (2008). The crystal structure of DehI reveals a new α-Haloacid Dehalogenase fold and active-site mechanism. J. Mol. Biol., 378: 284-294.

Schneider B, Muller R, Frank R, Lingens F (1991). Complete nucleotide sequences and comparison of the structural genes of two 2-haloalkanoic acid dehalogenases from *Pseudomonas species* CBS3. J. Bacteriol., 173: 1530-1535.

Senior E, Bull AT, Slater JH (1976). Enzyme evolution in a microbial community growing on the herbicide Dalapon, London. 263:476-479.

Slater JH, Lovatt D, Weightman, AJ, Senior E, Bull AT (1979). The growth of Pseudomonas putida on chlorinated aliphatic acids and its dehalogenase activity. J. Gen. Microbiol., 114: 125-136.

Slater JH, Bull AT, Hardman DJ (1995). Microbial dehalogenation. Biodegradation 6:181-189.

Slater JH, Bull AT, Hardman DJ (1997). Microbial dehalogenation of halogenated alkanoic acids. Adv. in Micro. Physiol., 38: 133-176.

Smith JM, Harrison K, Colby J (1990). Purification and characterization of D-2-haloacid dehalogenase from *Pseudomonas putida* strain AJ1/23 J. Gen. Microbiol., 136: 881-886.

Stringfellow JM, Cairns SS, Cornish A, Cooper RA (1997). Haloalkanoate dehalogenase II (DehE) of a *Rhizobium sp.* Molecular analysis of the gene and formation of carbon monoxide from trihaloacetate by the enzyme. Eur. J. Biochem., 250: 789-793.

Thasif S, Hamdan S, Huyop F (2009). Degradation of D,L-2chloropropionic acid by bacterial dehalogenases that shows stereospecificity and its partial enzymatic characteristics. Biotechnology, 8(2): 264-269.

Thomas AWW (1990). Analysis of a mobile genetic element from *Pseudomonas putida* which encodes dehalogenase functions. PhD. Thesis, Univ. Wales, Cardiff, United Kingdom.

Topping AW (1992). An investigation into the transposition and dehalogenase functions of *DEH*, a mobile genetic element, from *Pseudomonas putida* strain PP3. PhD Thesis, Univ. Wales, Cardiff, United Kingdom.

Tsang JSH, Sallis PJ, Bull AT, Hardman DJ (1988). A monobromoacetate dehalogenase from *Pseudomonas cepacia* MBA4. Arch. Microbiol., 150: 441-446.

Van Pee KH (1996). Biosynthesis of halogenated metabolites by bacteria. Ann. Rev. Microbiol., 50:375-399.

Van Der Ploeg J, Van Hall G, Janssen DB (1991). Characterization of haloacid dehalogenase from *Xanthobacter autotrophicus* GJ10 and sequencing of the *dhlb* gene. J. Bacteriol., 173: 7925-7933.

Weightman AJ, Slater JH, Bull AT (1979a). Cleavage of the carbon-chlorin bond by *Pseudomonas putida*. Soc. Gen. Microbiol., 6:76-77.

Weightman AJ, Slater JH, Bull AT (1979b). The partial purification of two dehalogenases from *Pseudomonas putida* PP3. FEMS Microbiol. Lett., 6: 231-234.

Weightman AJ, Weightman AL, Slater JH (1982). Stereospecificity of 2-monochloropropionate dehalogenation by the two dehalogenases of *Pseudomonas putida* PP3: evidence of two different dehalogenation mechanisms. J. Gen. Microbiol., 131: 1755-1762.