REVIEW
DOI: 10.5504/BBEQ.2011.0143 A&EB
AGRICULTURE AND ENVIRONMENTAL BIOTECHNOLOGY

D-SPECIFIC DEHALOGENASES, A REVIEW

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ABSTRACT

Bacterial dehalogenases cleave carbon-halogen bonds with different stereo-configurations. Rhizobium sp. RC1 produces a D-haloalkanoic-specific dehalogenase (DehD) that can cleave the carbon-halogen bond of halogenated organic pollutants. The sequence identity for DehD and HadD from Pseudomonas putida AJ1 is 27%, and 20 amino acid residues are highly conserved in these two enzymes. Therefore, DehD and HadD may have active sites that contain the same charged catalytic residues. The activity of DehD rapidly increases with increasing pH and is optimum at pH 9.5. It also has lower K_m values and higher k_{cat} values for various substrates than do other D-specific dehalogenases. Because DehD catalyzes the hydrolytic dehalogenation of D-haloalkanoic acids with inversion around the chiral carbon, it has potential industrial applications. Site-directed mutagenesis of DehD can be exploited for industrial production of chemicals, pharmaceutical and medical applications, and in environmental remediation.

Biotechnol. & Biotechnol. Eq. 2012, **26**(2), 2817-2822

Keywords: dehalogenases, D-specific dehalogenase, DehD, *Rhizobium* sp. RC1, catalytic residues

Introduction

Enzymes involved in the conversion of organohalogen compounds have potential applications in environmental technologies and the chemical industry (18, 23, 24). For the catabolism of recalcitrant halogenated organic compounds in the biosphere, dehalogenation is regarded as the key first step (12). The use of dehalogenases in industrial processes is well established (34), and potentially can be used for the biodegradation of environmentally toxic halogenated compounds such as man-made chemicals used as refrigerants, fire retardants, paints, solvents, herbicides and pesticides (8). Certain dehalogenases initiate the breakdown of halogenated organic compounds by cleaving the carbon–halogen bond with the inversion the configuration of the chiral carbon (13, 27, 30, 35).

Rhizobium sp. RC1 was originally isolated by Skinner and his colleagues at Nottingham University (5). Allison et al. (2) and Leigh et al. (22) showed that *Rhizobium* sp. was a fast growing, Gram-negative microorganism. *Rhizobium* sp. RC1 was found to produce three kinds of dehalogenases: D, E, and L as reported by Leigh et al. (22) and Cairns et al. (7). This review focuses on the putative structures and functions of dehalogenases specific for D-2-haloacids.

Dehalogenase specificities

Originally, dehalogenases were classified according to their substrate specificities (31). However, Hill et al. (12) grouped these enzymes according to their amino acid sequence similarities. Consequently, certain group I dehalogenases degrade both D- and L-haloacids (6, 36), whereas others process only D-enantiomers or L-enantiomers. Group II α-dehalogenases specifically degrade substrates containing a halogen bound to α-carbon that has an L-configuration resulting into inversion of configuration of the chiral carbon (30). Group III dehalogenases, unlike other groups, degrade only D-enantiomers.

Type-D dehalogenases

Group III dehalogenases, or type-D dehalogenases, specifically act on D-enantiomers. They have been isolated from *Rhizobium* sp. RC1 (DehD) (7) and *Pseudomonas putida* AJ1 (HadD) (32, 33). To date, DehD has not been studied in depth, and its sequence identity to other dehalogenases is not significant. Therefore, its structure and function should be characterized to understand its relationship with other dehalogenases.

Dehalogenase D from *Rhizobium* sp. RC1

Cloning of DehD

Cairns et al. (7) cloned a gene encoding DehD into *Escherichia coli* K-12, strain NM522. *Rhizobium* sp. RC1 genomic DNA was first digested with *Eco*RI and ligated into pUC19 to form a gene library. Transformants that were ampicillin resistant and could survive on D,L-2-CP and on D-2-CP were selected. A clone denoted pSC1 that harbored a 6.5-kb *Eco*R1 insert was purified from one of these transformants. To locate the dehalogenase gene(s), subclones (pSC248 and pSC2) were produced and were characterized according to their expression of dehalogenase activity and growth on 2-chloropropionic acid. Finally, pSC3 was constructed from pSC2 and endowed *E. coli* with the ability to grow in the presence of D-2CP, but not L-2CP, as the sole carbon and energy source (7), suggesting that pSC3 contained the complete *dehD*.

TABLE 1

k_{cat}, K_m, and specificity constant for DehD

S/N	Substrate	k_{cat} (sec^{-1})	K_m (mM)	Specificity constant ($M^{-1}\cdot sec^{-1}$)
1	D-2-chloropropionic acid (D-2-CP)	7.45	0.06	1.12×10^5
2	D-2-bromopropionic acid (D-2-BP)	187.53	0.48	3.90×10^5
3	D,L-2-chloropropionic acid (D,L-2-CP)	6.28*	0.04*	1.46×10^5*
4	D,L-2-bromopropionic acid (D,L-2-BP)	193.33*	0.4*	4.83×10^5*
5	D,L-2,3-dichloropropionic acid (D,L-2,3-DCP)	29.58*	0.38*	7.78×10^4*
6	Monochloroacetic acid (MCA)	4.25	0.25	1.70×10^4
7	Monobromoacetic acid (MBA)	362.50	0.67	5.41×10^5

S/N indicates serial number. Each value is the mean of three measurements. * indicates values corrected for the D-isomer concentration. K_m is Michaelis-Menten constant. The k_{cat} value is equivalent to the number of substrate molecules converted to product in a given unit of time for a single enzyme molecule when the enzyme is saturated with substrate (14).

Purification of DehD

DehD was purified by anion-exchange column chromatography (14). A cell-free extract of *E. coli* that expressed cloned DehD activity was prepared in 0.01 M Tris-acetate, pH 7.6. Protein (2.8 mg, 4.3 U enzymes) was applied to a MonoQ HR 5/5 anion exchange column equilibrated with 5 mM sodium phosphate, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, 10% (ml/v) glycerol and eluted with 15 ml of a 5–100 mM sodium phosphate gradient at a flow rate of 1 ml/min. The analysis of purity was performed using extracts prepared from NM522 (pSC219) grown on glycerol with ampicillin + IPTG. SDS/PAGE of this extract showed that, the peak fractions from the gel filtration step, accounted for about 40%) each of DehD and DehL (dehalogenase specific for L-enantiomers) of the total protein. The 29-kDa and 31-kDa bands were well separated from several weaker bands. The extracts prepared from induced NM522 (pSC3) showed the strong 29-kDa protein band, which was therefore attributed to DehD (7).

Subunit composition, nucleotide and amino acid sequences of DehD

Subunit molecular masses of dehalogenases were estimated using methods described by Laemmli (21). A construct pSC3 from this extract endowed *E. coli* with the ability to grow on D-CP, but not with L-CP, as a sole carbon and energy source, implying that the pSC3 subclone had only the complete DehD encoding gene. SDS-PAGE analysis of extract from induced *E. coli* NM522:pSC3 (*dehD*$^+$) showed an intense 29-kDa protein band. Gel filtration chromatography indicated the presence of a 58 ± 5 kDa protein in the extract. Therefore, native DehD is a homodimer (7). The nucleotide and amino sequences of DehD are found under the accession number CAA63793.1 in NCBI protein database (http://www.ncbi.nlm.nih.gov/protein/).

Kinetics of DehD

The K_m and k_{cat} values for DehD and various substrates have been determined (**Table 1**). Huyop and Cooper (14) confirmed that DehD acted only on D-carbon-containing substrates and was inactive against L-2-chloropropionic acid (L-2CP), L-2-bromopropionic acid (L-2BP), 2,2-dichloropropionic acid (2,2-DCP), dichloroacetate (DCA), trichloroacetate (TCA), dibromoacetate (DBA), and tribromoacetate (TBA) (**Table 2**). DehD dehalogenated one carbon in D,L-2,3-DCP, presumably carbon 2, because it did not react with 3-chloropropionate (1), suggesting that carbon 3 in D,L-2,3-DCP was not dehalogenated. The total amount of chloride released indicated that the D,L-2,3-DCP preparation contained equal amounts of the L- and D-isomers as had been found for D,L-2-chloropropionic acid (D,L-2-CP).

TABLE 2

K_m values for different substrates using pure DehD enzyme

S/N	Halogenated compound	K_m (mM)
1	D-2-chloropropionic acid (D-2-CP)	0.06±0.01
2	D-2-bromopropionic acid (D-2-BP)	0.48±0.09
3	L-2-chloropropionic acid (L-2-CP)	-
4	L-2-bromopropionic acid (L-2-BP)	-
5	D,L-2-dichloropropionic acid (D,L-2-CP)	0.04±0.01*
6	D,L-2-bromopropionic acid (D,L-2-BP)	0.4±0.04*
7	2,2-dichloropropionic acid (2,2-DCP)	-
8	D,L-2,3-dichloropropionic acid (D,L-2,3-DCP)	0.38±0.11*
9	Monochloroacetic acid (MCA)	0.25±0.04
10	Dichloroacetic acid (DCA)	-
11	Trichloroacetic acid (TCA)	-
12	Monobromoacetic acid (MBA)	0.67±0.17
13	Dibromoacetic acid (DBA)	-
14	Tribromoacetic acid (TBA)	-

S/N indicates serial number. Each value is the mean of three measurements ± standard deviation, - indicates values not detected, * indicates values corrected for the D-isomer concentration (14).

Optimum temperature and pH of DehD

When a crude cell-free extract prepared from *Rhizobium* sp. grown on 2,2-DCP was incubated at 40 ºC, its dehalogenase activity against 2,2-DCP, D,L-2-CP, monochloroacetate (MCA), and DCA decreased (1). However, there has been no

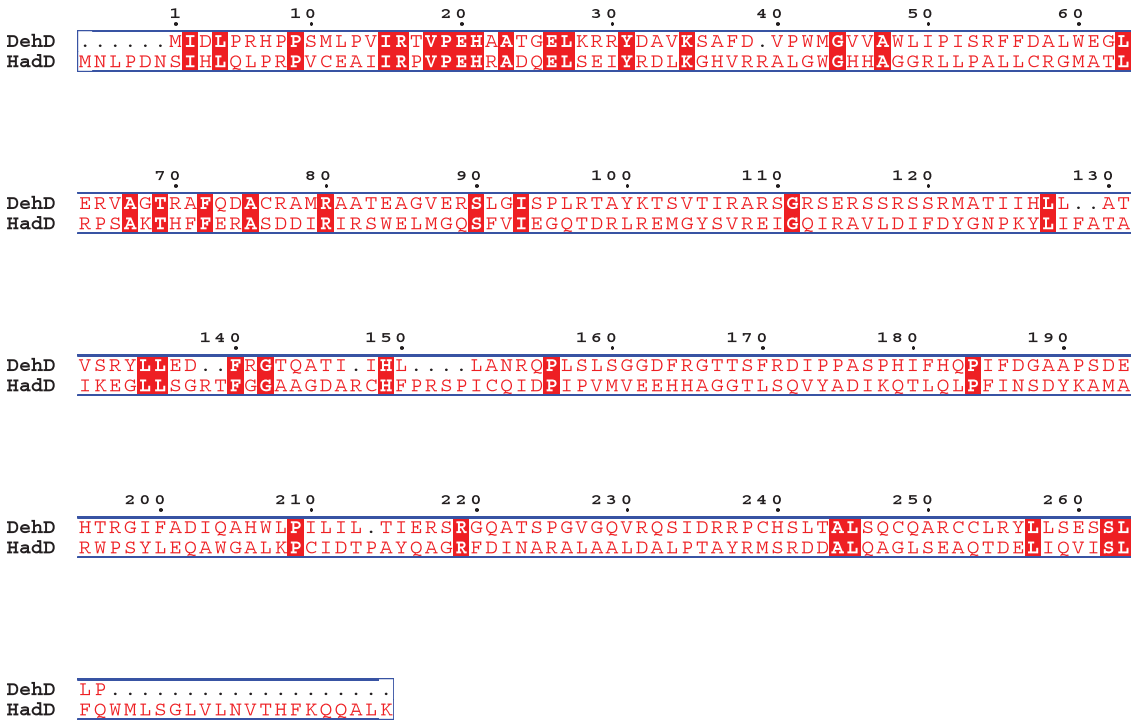

Fig. 1. Amino acid sequence alignment of DehD and HadD. Residues conserved in both sequences are shown in red with white characters.

systematic study correlating DehD activity with temperature and pH.

Enzyme assay for DehD

DehD activity was assayed by Cairns et al. (7) and Huyop and Cooper (14). The specific activity of DehD (7, 14) and HadD (32) were stereospecific for D-isomers. The K_m values for MCA and D,L-2-BP for DehD were 0.25 and 0.4 mM, respectively. The activity of HadD was determined. The K_m values at pH 7.5 for MCA and D,L-2-BP were 27.5 and 1.99 mM respectively (32), much higher than that of DehD; indicative of a better industrial biocatalyst (14).

The Haloalkanoic Acid Dehalogenase D from *P. putida* AJ1/23

Cloning, nucleotide sequencing and purification of *hadD* from *P. putida* AJI/23

D-specific dehalogenase gene from chromosomal DNA of *P. putida* AJ1/23 which encodes *hadD*, was inserted in the broad-host-range IncQ vectors TB107 and pTB244 was shotgun cloned into *E. coli* K-12 strain C600. The resulting *E. coli* chromosomal DNA was digested with *Sst*I, *Sst*II, *Hind*III, or *Xho*I and ligated into pTB107 plasmids that had been cut with the same enzymes. Clone selection and *hadD* selection are described by Barth et al. (4). The sequence of HadD is found under accession number AAA25831.1 in NCBI protein database.

HadD has been purified to homogeneity (32). It has a relative molecular mass of 134 kDa and is composed of four identical subunits, each with a relative molecular mass of 31.8 ± 0.5 kDa, suggesting that the structure is a tetramer (16, 32). Using an ion chromatographic assay, the enzyme was found to breakdown short-chain 2-halocarboxylic acids (4).

Physical properties of HadD

SDS-PAGE analysis of HadD showed it is a protein with molecular weight of 33.6 kDa. Electrophoresis through a non-denaturing gel revealed a band at approximately 130 kDa with D-specific dehalogenase activity (4). The HadD contains 301 residues. The enzyme has a theoretical pI of 7.15, which is similar to its measured pI value of (7.54) (4). The number of negatively charged residues is 33 (11%), and the number of positively charged residues, excluding histidine, is 33 (11%). The total number of aromatic residues (Phe, Trp, and Tyr) is 31 (10.4%). At neutral pH, nuclear proteins tend to have a larger percentage of positively charged residues. The aliphatic index of HadD is 92.16, the instability index is 42.77, and the grand average hydropathy is –0.191 (10, 20).

K_m values and optimum temperature and pH for HadD

A pH activity profile using D,L-2-chloropropionate as the substrate, and K_m values for D,L-2-chloropropionate, chloroacetate, and D,L-2-bromopropionate, have been determined for HadD (32, 33). Dehalogenase activity was measured between 1.0 and 10 mM D,L-2-chloropropionate and between pH 6.0 and 10.5. A K_m of 2.2 mM for D-2-chloropropionate was determined assuming that the amount of D-2-chloropropionate present was half that of the added D,L-2-chloropropionate. The K_m value for D-2-CP was nearly constant between pH 6.0 and 10.0. Dehalogenase activity with certain other substrates was also determined at pH 7.5. At pH

7.5, the K_m value for chloroacetate was 27.5 mM and 1.99 mM for D,L-2-bromopropionate.

The highest temperature at which HadD was active was 50 °C at pH 9.5, and it was rapidly inactivated at higher temperatures (33). The enzyme was stable between 30 °C and 35 °C at pH 9.5, but it lost activity below pH 5. There was also a rapid loss of activity at this pH above 40 °C. At 56 °C, there was rapid inactivation of the enzyme accompanied by visible protein precipitation (32, 33)

Comparison of the DehD and HadD sequences and their amino acid compositions

Multiple sequence alignment using Multalin (**Fig. 1**) (9) showed that DehD and HadD share 27% identity. Sequences Ile2[8], Leu4[10], Pro9[15], Ile15[21], Arg16[22], Val18[24], Pro19[25], Glu20[26], His21[27], Ala23[29], Glu26[32], Leu27[33], Tyr31[37], Lys35[41], Gly44[51], Ala47[54], Leu53[70], Ala67[74], Thr69[76], Pro72[79], Ala75[82], Arg80[87], Ser90[97], Ile93[100], Gly111[118], Leu128[135], Leu136[145], Leu137[146], Phe140[151], Gly142[153], His149[161], Pro156[172], Pro184[200], Pro209[225], Arg219[236], Ala244[261], Leu245[262], Leu257[274], Ser262[279], and Leu263[280] are highly conserved (numbers in brackets are those for HadD). The functional roles of these residues are expected to be the same for both enzymes.

TABLE 3

Comparison of amino acid residues of DehD and HadD

Amino acid	Residues in DehD		Residues in HadD	
	Frequency	Percentage (%)	Frequency	Percentage (%)
Ala (A)	27	10.2	32	10.6
Arg (R)	30	11.3	24	8.0
Asn (N)	1	0.4	6	2.0
Asp (D)	12	4.5	18	6.0
Cys (C	5	1.9	5	1.7
Gln (Q)	10	3.6	17	5.6
Glu (E)	11	4.2	15	5.0
Gly (G)	16	6.0	22	7.3
His (H)	9	3.4	10	3.3
Ile (I)	19	7.2	21	7.0
Leu (L)	26	9.8	33	11.0
Lys (K)	3	1.1	9	3.0
Met (M)	5	1.9	8	2.7
Phe (F)	10	3.8	11	3.7
Pro (P)	18	6.8	17	5.6
Ser (S)	26	9.8	16	5.3
Thr (T)	17	6.4	11	3.7
Trp (W)	4	1.5	5	1.7
Tyr (Y)	4	1.5	9	3.0
Val (V)	12	4.5	12	4.0

The amino acid compositions of DehD (265 residues) and HadD (301 residues) are compared in **Table 3**. There are marked differences in molecular weight, theoretical pI value, aliphatic index, and the grand average hydropathy value. As aliphatic indices of a globular protein increase, their thermostability also increases (15). Proteins with an instability index less than 40 may not be stable *in vitro* (11). The high-frequency residues for DehD are Arg (30 residues, 11.3%), Ala (27 residues, 10.2%), and Leu and Ser (26 residues each, 9.8%). For HadD, the high-frequency residues are Leu (33 residues, 11%), Ala (32, 10.6%), and Arg (24, 8.0%). At neutral pH, nuclear proteins tend to have a high relative percentage of positively charged amino acids (3). The percentage of Ala in the enzymes is therefore approximately the same. This could account for their similarity in structure. There is only one Asn (0.4%) in DehD, and the amino acid that occurs the least number of times in HadD is Cys (5 residues, 1.7%; **Table 3**). The large number of Arg, Leu, and Ser residues in DehD and Leu and Arg residues in HadD may be of structural and functional importance.

Asp199 in HadD of *P. putida* AJ1 (its equivalent in DehD of *Rhizobium* sp. RC1 is Ala189) has been implicated in attacking the chloride at the chiral carbon of haloalkanoic acid and inverting its configuration. Asn134 in HadD of *P. putida* AJ1 (its equivalent in DehD of *Rhizobium* sp. RC1 is Thr124) assists Asp199 in activating a water molecule (29). However, these residues are not conserved in DehD of *Rhizobium* sp. RC1, and thus the catalytic mechanism for this DehD remains unclear.

Mechanism of D-Specific Dehalogenase Action

The general mechanism for dehalogenation of D,L-2-chloropropionate with inversion of configuration was initially proposed by Weightman et al. (36), who proposed that the active site has a positively charged pocket that binds the carboxylate residue usually close to the basic group which promotes nucleophilic attack. This is in agreement with the known substrate diversity and pH range of D-haloacid dehalogenases. A similar reaction mechanism for the DehD that catalyzes dehalogenation of D-2-chloropropionate was also proposed by Kurihara et al. (19) (**Fig. 2**).

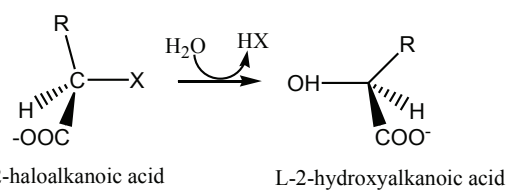

Fig. 2. Reaction catalyzed by 2-haloacid dehalogenases. R, alkyl group; X, halogen.

Because the amino acid sequence of DL-DEX 113 from *Pseudomonas* sp.113 is similar to that of the HadD from *P. putida* AJ1 that specifically acts on D-2-haloalkanoic acids, Barth et al. (4) inferred that the reaction mechanism of the DehD from *Rhizobium* sp. RC1 is also similar to that of DL-

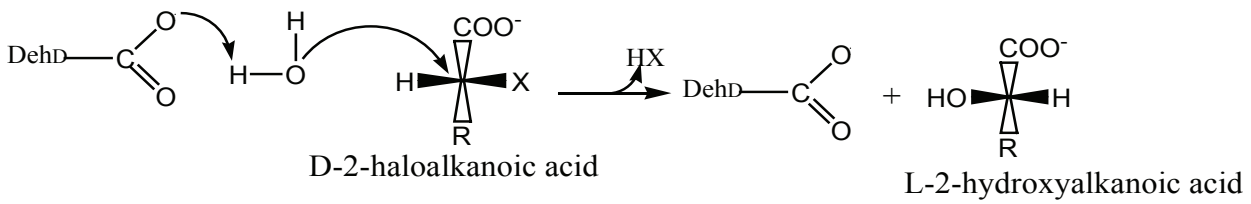

Fig. 3. Proposed reaction mechanism for DehD. An Asp indirectly attacks the carbon bond to the halogen via an activated water molecule. An ester intermediate is not formed.

DEX 113 (**Fig. 3**). Nardi-Dei et al. (25) observed that an enzyme activated water molecule directly attacks the α-carbon of the 2-haloalkanoic acid to displace the halogen atom. This was the first example of an enzymatic hydrolytic dehalogenation that proceeds without producing an ester intermediate. The reaction mechanism of the DehD from *Rhizobium* sp. RC1, however, remains unclear—specifically, how the active-site residues mediate hydrolytic dehalogenation (28).

Proposed Catalytic Site of Dehalogenase D (DehD) That Underlies the Enzyme's Action

The positively charged pocket for the binding of the carboxylate residue was proposed at the active site and found close to the basic group that promotes nucleophilic attack (36), which proceeds via an S_N2 mechanism (27, 30, 31), as is observed for the HadD of *P. putida* AJ1/23 (4). Lys and Arg residues were proposed to be at the active site (22). The amino acid sequence of DL-DEX from *P. putida* strain 113 is similar to HadD of *P. putida* AJ1 that specifically acts on D-2-haloalkanoic acids (4). Charged and polar residues (Thr65, Glu69, and Asp194) were found to be essential for dehalogenation of D-2-haloalkanoic acids, although only Thr65 is conserved with HadD (26). The involvement of these residues in dehalogenation of D-haloalkanoic acids was investigated. The residues Thr65 [Thr62], Glu69 [Glu66], and Asp194 [Asp189] are conserved among D-haloalkanoic acid dehalogenases and the DehI group of enzymes (residues in brackets) (29), suggesting that these polar residues are found in the active site and play a key role in dehalogenation of D-2-haloalkanoic acids.

DehD from *Rhizobium* sp. RC1 is 27% identical to HadD from *P. putida* AJ1/23. Therefore, we propose that both dehalogenases that process D-haloalkanoic acids share the same active site and possibly the same catalytic residues. The role of polar residues in the binding site of *Rhizobium* sp. RC1, though not a close homolog of HadD, needs to be determined by site-directed mutagenesis.

Conclusions and Future Perspectives

Protein function and structure are directly related. Crystallographic or nuclear magnetic resonance spectral determination of protein structures is more expensive and time consuming than is gene sequencing, which means that the number of sequences currently available greatly exceeds the number of protein structures. Thus, sequence information must be used to identify evolutionary relationships and/or predict common structures and functions (17). Several computational algorithms can be used to infer protein structure and identify functional residues from amino acid sequences alone. This review summarizes the available information concerning the structures and functions, purifications, physical properties, and reaction mechanisms of the D-2-haloalkanoic dehalogenases DehD from *Rhizobium* sp. RC1 and HadD from *P. putida* AJ1/23. These enzymes share only 27% catalytic residue similarity and for this reason they could not have common catalytic activities due to their non-conservation. Therefore, extrapolation of DehD catalytic residues from it could be a flaw. DehD from *Rhizobium* sp. RC1 is a unique protein among other dehalogenases reported so far. Crystallization of DehD to better elucidate its structure and catalytic residues is consequently recommended. The residues involved in catalysis and substrate binding in DehD should be subjected to a mutagenesis study that may result in an increase in activity because this enzyme may be potentially useful for bioremediation.

Acknowledgements

The authors would like to thank the Malaysian Government and Universiti Teknologi Malaysia (MOHE/FRGS Vot 4F008/J130000.7835.4F008) for partially sponsoring this work.

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