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## Degradation of D,L-2-chloropropionic Acid by Bacterial Dehalogenases that Shows Stereospecificity and its Partial Enzymatic Characteristics

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**Abstract:** A *Pseudomonas* sp. strain S3, which can utilise a halogenated compound of D,L-2CP as sole carbon and energy source, catalyses the hydrolytic dehalogenation of both D- and L-isomers of 2-chloropropionic acid. Two kinds of dehalogenase enzymes were isolated from cells of *Pseudomonas* sp. strain S3. A thermostable L-specific dehalogenase (DehL) and non-thermostable D-specific dehalogenase (DehD) can be obtained when cells grown only in the presence of D,L-2CP. These inducible enzymes were then further characterised. The maximum activity of D-specific dehalogenase (DehD) enzyme on D-2CP was found at pH 9.5 at 35°C, whereas the L-specific dehalogenase is thermostable and retained its full activity upon heating at 55°C for 15 min. The pH and temperature optima for dehalogenation of L-2CP were 7.5 and 50°C, respectively.

**Key words:** 2-chloropropionic acid, dehalogenase, degradation, enzymatic characteristics

### INTRODUCTION

Halogenated organic compounds are widely used as herbicides and pesticides. They were found widely throughout the biosphere due to rapid developments in industry and agriculture. They can cause serious environmental pollution and health problems to the human population. Microbial catabolism of dehalogenase producing bacteria has been well studied by Hardman (1991), Leisinger and Bader (1993), Janssen *et al.* (1994), Olaniran *et al.* (2001, 2004), Jing and Huyop (2007), Jing *et al.* (2008) and Ismail *et al.* (2008). A variety of halogenated compounds such as halo acids, which are produced by chemical industries, are degraded through dehalogenation by microbial dehalogenases that involve carbon-halogen bond cleavage (Copley, 1998).

Dehalogenation reactions have been classified into different types based on their substrate specificities and the configuration of the products (Leigh *et al.*, 1988; Slater *et al.*, 1997; Huyop *et al.*, 2004, 2008a, b). Class 1D dehalogenases are less common in nature than 1L enzymes. Class D enzyme can dehalogenate selectively D-isomeric substrates such as D-2-chloropropionic acid (D-2CP) with inversion of product configuration and forms L-2-hydroxy acids (e.g., L-lactate). Class 1L removed halide from L-2CP inverting the product configuration becomes D-lactate or D-2hydroxy acids. This enzyme also reacts with sulfhydryl blocking

reagents. Enzymes in Class 2I were distinguished by their ability to dehalogenate both D- and L-isomers by a mechanism that inverts substrate product configurations. The enzymes were unaffected by sulphydryl blocking reagents which is in contrast to Class 2R. Enzymes from Class 2R differ from Class 2I enzymes in their ability to dehalogenated both D and L isomers with retention of product configuration.

Current study is important because derivatives of propionic such as 2-chloropropionic acid and lactic acid is used as building blocks in the synthesis of chiral agrochemicals. In some herbicides such as Fluazifop®, the desired herbicidal activity rely on one of the enantiomers. As a result it has become necessary to develop chemo-enzymatic methods using enantioselective dehalogenase for the preparation of this type of biologically active chemicals. Markets for optically active agrochemicals and pharmaceuticals drive the first commercial use of 2-haloalkanoate dehalogenase enzymes as reported by Swanson (1999). In addition to their application in biotransformations, dehalogenases play an important role in environmental protection by degrading halogenated pollutants that cause serious environmental problems.

Present investigation suggested that two different kinds of dehalogenases were found in cells of *Pseudomonas* sp. strain S3 grown on D,L-2CP. The main aim of this investigation is to seek information and to



characterise of both DehD and DehL enzymes. Both enzymes have great potential in biotransformations study. It will become economically attractive if the microorganisms themselves or cell-free extracts systems could be used as cheap catalysts. An investigation of their characteristics may shed light on this question.

## MATERIALS AND METHODS

**Chemicals:** Racemic mixture of D,L-2CP and 2,2DCP were obtained from Merck-Schuchardt Company. D- and L-2-chloropropionic acids were obtained from SIGMA Chemicals, USA. Other chemicals used were of analytical grade.

**Bacterial strains and growth conditions:** *Pseudomonas* sp. strain S3 was isolated from Paddy (rice) field agricultural area with D,L-2CP as the sole carbon and energy source. The cells were grown aerobically at 30°C in a 250 mL flask in 100 mL of a minimal medium. The liquid minimal medium was prepared as 10x concentrated basal salts containing  $K_2HPO_4 \cdot 3H_2O$  (42.5 g L<sup>-1</sup>),  $NaH_2PO_4 \cdot 2H_2O$  (10.0 g L<sup>-1</sup>) and  $(NH_4)_2SO_4$  (25.0 g L<sup>-1</sup>). The trace metal salts solution was a 10x concentrate that contained nitriloacetic acid (NTA) (1.0 g L<sup>-1</sup>),  $MgSO_4$  (2.0 g L<sup>-1</sup>),  $FeSO_4 \cdot 7H_2O$  (120.0 mg L<sup>-1</sup>),  $MnSO_4 \cdot 4H_2O$  (30 mg L<sup>-1</sup>),  $ZnSO_4 \cdot H_2O$  (30 mg L<sup>-1</sup>) and  $CoCl_2$  (10 mg L<sup>-1</sup>) in distilled water (Hareland *et al.*, 1975). Minimal media for growing bacteria contained 10 mL of 10x basal salts and 10 mL of 10x trace metal salts per 100 mL of distilled water and were autoclaved (121°C, for 15 min).

The different carbon sources (D-2CP, L-2CP, D,L-2CP and 2,2DCP) were neutralized with NaOH and filter sterilised separately using Millipore GV filter unit (pore size 0.22 µm) and added to the autoclaved salts medium to an appropriate final concentration. The extent of growth determined by measuring the absorbance at  $A_{680\text{ nm}}$  and the release of chloride. Cells were harvested by centrifugation at the late-logarithmic phase and stored as a frozen pellet at -70°C after they were washed twice with 0.1 M Tris-acetate buffer pH 7.5.

**Basic biochemical test and 16S rDNA sequencing analysis:** Colony S3 was subjected to classical test (Gram staining, motility, oxidase and catalase) for characterisation and tentative identification. Further test using 16S rDNA was carried out by PCR amplification of 16S rDNA of S3 organism. The PCR product was purified using PCR purification kit (Qiagen) and served as template for sequencing with the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer).

Sequencing was performed on a 373 PRISM DNA sequencer-ABI (First Base Laboratory, Malaysia). The nucleotide sequence was compared with those in the EMBL database (Stoesser *et al.*, 1998) using FASTA3 at the European Bioinformatics Institute (<http://www.ebi.ac.uk>) and with those from the Ribosomal Data base Project by using SIMILARITY RANK to identify closely related sequences (Maidak *et al.*, 1997).

**Dehalogenase enzyme assay and activity:** Activity of the enzyme was measured by determining the release of chloride indicated by a colorimetric method employing mercuric thiocyanate as previously reported by Bergman and Sanik (1957). For the standard assay, dehalogenase activity was determined as total chloride released at 30°C in an incubation mixture containing 0.1 M Tris-acetate buffer (pH 7.5) (4700 µL), 0.1 M halogenated aliphatic acid (50 µL) and distilled water and enzyme to a final volume of 5 mL. After 5 min equilibration at 30°C, the reaction was initiated by adding cell-free extract. Samples (1.0 mL) were removed at appropriate intervals and assayed for halide ions. Each assay was carried out in triplicates. The absorbance of the mixture was measured at  $A_{460\text{ nm}}$  and was proportional to the chloride ion concentration. Control experiment was similar to the assay procedure, except the cell-free extract was omitted from the reaction mixture. Specific activity is defined as the µmole of chloride liberated per milligram protein in 10 min under the stated conditions.

**Determination of the temperature and pH optimum:** To determine pH optimum, the cell-free extracts were mixed with equal amounts of 0.1 M Tris-acetate buffer from pH 5, 6, 7, 8, 9, 10 and 11. The reaction was started by addition of the substrate and after 10 min incubation chloride ions released was determined as described. Control experiment, lacking enzyme preparation was included in each set of assay to detect spontaneous halides released.

For the determination of the temperature optimum, cell-free extracts were incubated for 15 min at various temperature (35, 45, 50, 55 and 60°C) and 1 mL sample were removed at 5 min interval for the enzyme assay at 30°C as described before.

**Non-denaturing polyacrylamide gel electrophoresis (PAGE):** In non-denaturing PAGE, the enzyme extract remains active. Gel was prepared based on the method of Hardman and Slater (1981). Resolving gel containing 12% bis-acrylamide in 375 mM Tris- acetate pH 7.8 were polymerized by the addition of 0.05% ammonium



persulphate and 0.05% TEMED. Stacking gel was formed from 4% bis-acrylamide in 125 mM Tris- acetate pH 6.8. Gel was left overnight at 4°C before being used to allow the ammonium persulphate to decompose completely. Gel was run using a Mini-Protean II gel system from Biorad in 25 mM Tris, 19 mM glycine buffer (pH 8.3) at a constant voltage of 200 V and a temperature 4°C for 1 h. Samples were prepared as for cell extracts and mixed with 0.1 volumes of sample buffer (0.1% bromophenol blue; 10% glycerol; 100 mM DTT in 50 mM Tris-acetate pH 6.8). The gel was run until the dye front reached the bottom of the gel. Gel was then stained for dehalogenase activity.

The gel was incubated in 40 mM Cl<sup>-</sup> free halogenated substrate for 15 min at 30°C. Substrate was carefully removed and the gel was then placed in a 0.1 M AgNO<sub>3</sub> solution and incubated in the dark until bands appeared on the gel due to the precipitation of AgCl. The gel was then washed with distilled water to remove the AgNO<sub>3</sub> and fixed by washing in 5% acetic acid for 10 min.

## RESULTS

**Bacterial growth and cell morphology:** The bacteria grew well in liquid minimal media supplemented with 20 mM D,L-2CP as a carbon source with cell doubling time of 10 h. Growth above 20 mM of the same substrate was inhibited due to the toxicity of the substrate. Cell growth was not observed when the media supplied with 20 mM 2,2DCP as a carbon source. In contrast, cells were grown in liquid media using 20 mM of lactate as a carbon source in the presence of 2,2CDP, suggesting that 2,2DCP was not toxic to the cells. However, dehalogenase enzyme activity was not detected when cells grown in non-halogenated compound such as lactate and in the presence of 2,2DCP. Bacterium S3 was a Gram negative rod and in chains. The S3 was also subjected to a 16S rDNA gene analysis (Accession No. FJ 968758). The phenotypic and genotypic characterisation indicated that S3 belonged to the genus *Pseudomonas*.

**Substrate stereospecificity of cell free extracts:** The substrate stereospecificity was investigated. As shown in Table 1, cell free extracts prepared from cells grown in 20 mM D,L-2CP reacted with all substrates of D,L-2CP, D-2CP and L-2CP. The cell free extracts reacted with D,L-2CP and released to an equimolar amount of chloride ions. D-2CP and L-2CP were substrates for the cell free extracts as indicated by the chloride ion released.

**Substrate specificity:** The substrate specificities of the two putative dehalogenases in the cell free extracts is

Table 1: Stereospecificities of putative DehD and DehL in cell free extracts

Enzyme	Substrate (20 mM)	Chloride released (mM)
Cell free extracts	D-2CP	18
	D,L-2CP	19
	L-2CP	20

Table 2: Substrates specificities of cell free extracts from *Pseudomonas* sp. strain S3

Substrates	Cell free extracts (%)
Monobromoacetate	95
Monoiodoacetate	80
Monochloroacetate	100
L-2CP	110
D-2CP	100
2,2DCP	0

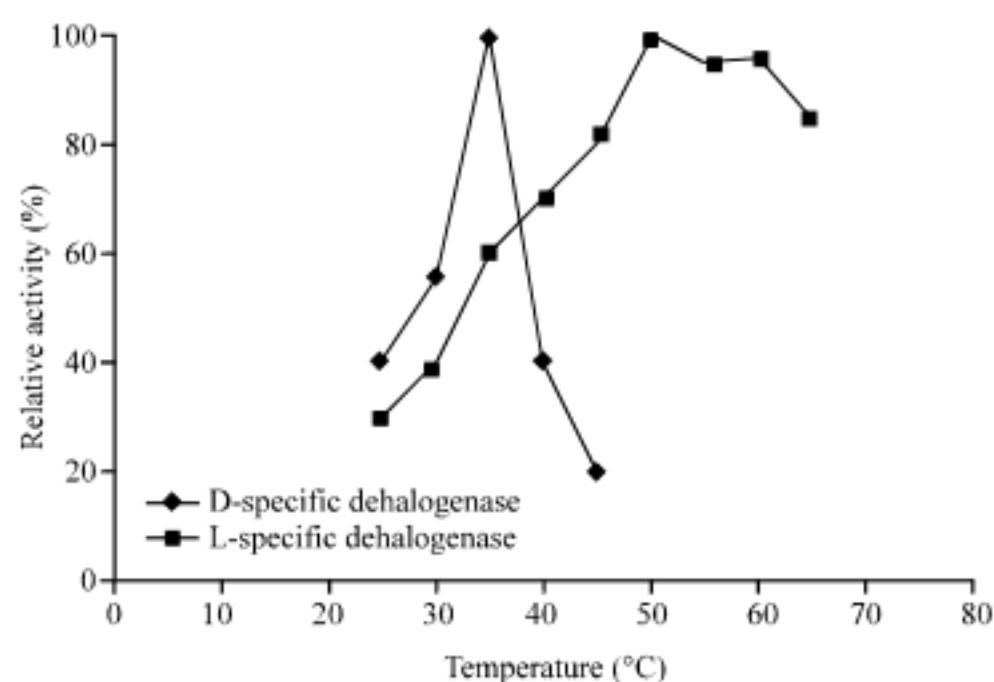


Fig. 1: Effect of temperature on the reaction velocity of cell free extract react with D-2CP and L-2CP measured at pH 9.5 and 8.5, respectively

shown in Table 2. All the monohaloacetates except monofluoroacetate and 2,2DCP were dehalogenated effectively by both enzymes. The Km values for D-2CP was 0.3 mM and L-2CP was 0.05 mM.

**Effects of pH and temperature:** The maximum activity for putative D-specific dehalogenase was found at pH 9.5, whereas putative L-specific dehalogenase showed its maximum activity at pH 8.5, when the initial halide ion formation from 1 mM D-2CP and L-2CP were measured at 30°C. L-specific dehalogenase was stable at pH 5 and pH 10 for 15 min at 30°C.

The effect of temperature was also examined. As shown in Fig. 1, the maximum activities of this extracts for D-2CP and L-2CP were observed at 35 and 50°C, respectively. Cell free extracts retained its full activity upon heating at 55°C for 15 min using L-2CP as substrate, whereas, using D-2CP the cell free extracts retained less than 10% activity under these conditions (Fig. 2). However, temperature above 55°C, the enzyme activity was totally lost. This indicates, possibly the putative L-specific dehalogenase has a such high thermostability.



**Non-denaturing polyacrylamide gel electrophoresis (NATIVE-PAGE) of cell-free extracts:** Activity stain PAGE utilizing Ag to precipitate free halide ions in regions of dehalogenase activity, has been employed to identify electrophoretically distinguishable dehalogenases. Using this technique, two kinds of dehalogenases were identified in cell free extracts migrated at different position (Fig. 3) when incubated with D- and L-2CP, respectively. This suggesting, both enzymes has different stereospecificity and molecular weight with D specific dehalogenase migrated faster than L specific dehalogenase. There was no electrophoretic mobility band pattern was located when gel incubated with 2,2DCP and distilled water.

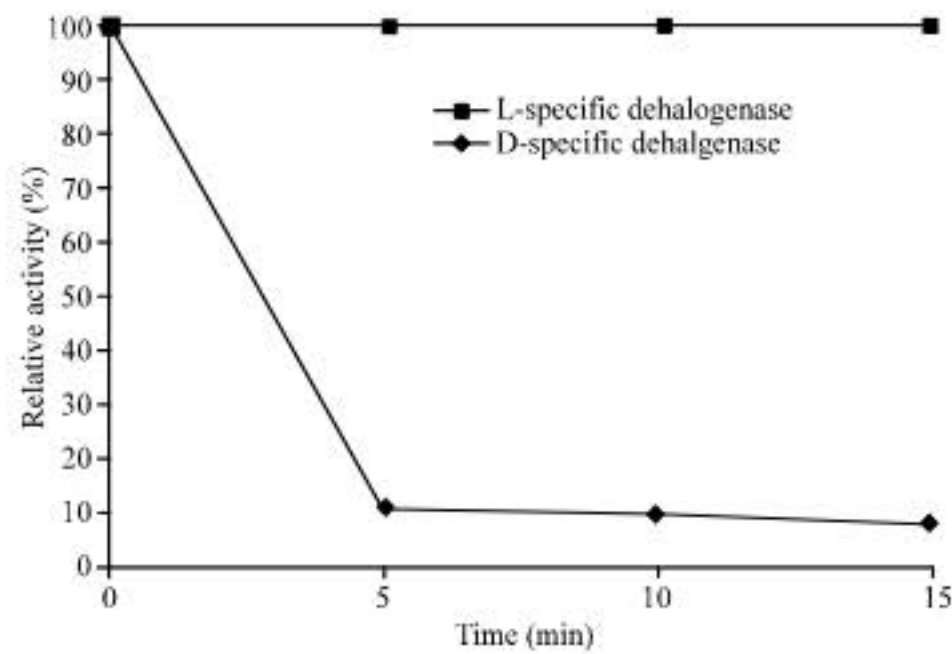


Fig. 2: Cell free extracts ( $0.8 \text{ mg mL}^{-1}$ ) was incubated at ( $55^\circ\text{C}$ ) for the indicated periods in  $0.1 \text{ mM}$  Tris-acetate buffer pH 7.5. After incubation, the remaining activity was measured by standard assay methods using D-2CP and L-2CP as substrates

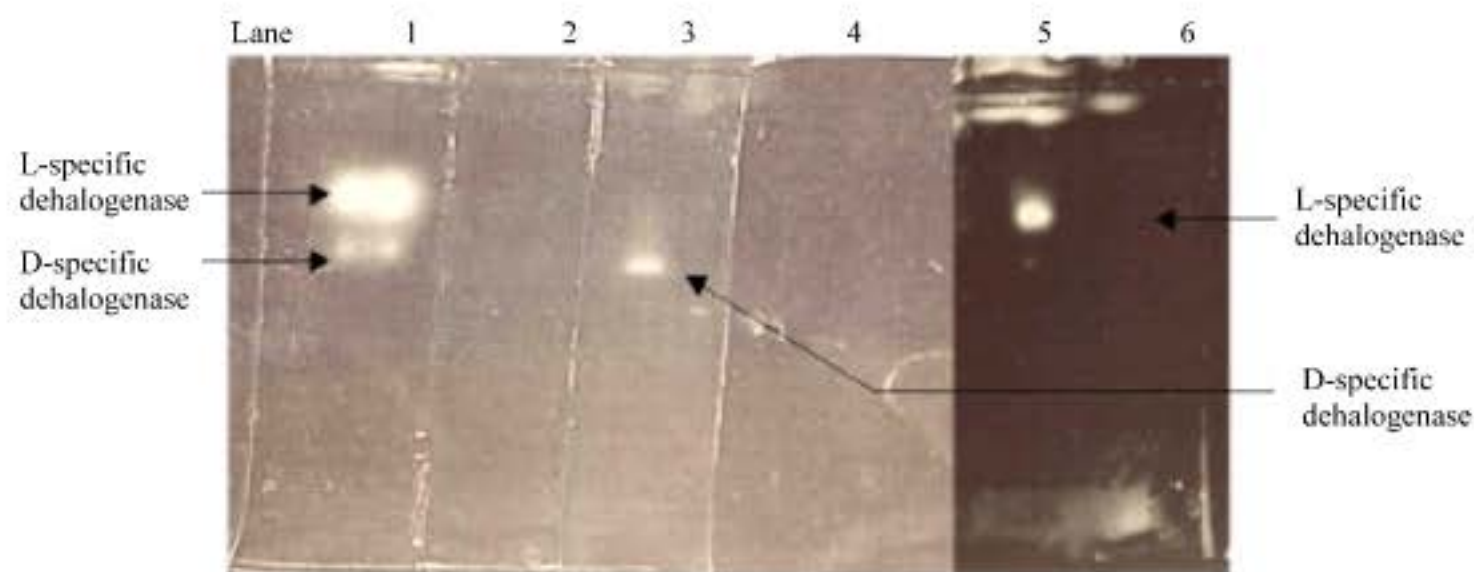


Fig. 3: Activity staining of non-denaturing polyacrylamide gel electrophoresis of a mixture of putatives D- and L-specific dehalogenases in cell free extracts ( $1.5 \text{ mg mL}^{-1}$ ). Lane 1: Gel incubated with  $50 \text{ mM}$  D,L-2CP, Lane 2: Control reaction gel incubated with distilled water, Lane 3: Gel incubated with  $50 \text{ mM}$  D-2CP only, Lane 4: Gel incubated with 2,2DCP, Lane 5: Gel incubated with  $50 \text{ mM}$  L-2CP only, Lane 6: Control reaction incubated with distilled water

## DISCUSSION

Two kinds of putative dehalogenases have been studied from *Pseudomonas* sp. strain S3 and characterized. Both enzymes were inducible enzymes when D,L2CP was supplied as a carbon source in the growth medium. D-specific and L-specific dehalogenases act on both D- and L-isomers of 2-haloacids indiscriminately (Table 1, 2) and L-specific dehalogenase is thermostable enzyme which acts on only L-isomer of 2-haloacids. However, no dehalogenase enzyme was detected when cells grown in  $20 \text{ mM}$  lactate minimal medium in the presence of 2,2DCP suggesting that 2,2DCP was not substrate-inducer for both dehalogenases.

The *Pseudomonas* sp. strain S3, D-specific dehalogenase resembles DehD from *Rhizobium* sp. RC1 (Cairns *et al.*, 1996) and HadD from *Pseudomonas putida* AJ1/23 (Smith *et al.*, 1990) in substrate stereospecificity and optimum pH (strain S3 pH 9.5; *Rhizobium* sp. DehD, pH 9.1-10.5; *Pseudomonas putida* AJ1/23, pH 9.5 ). The *Pseudomonas* sp. strain S3, DehL, belongs to the L-family along with those reported by Goldman *et al.* (1968), Little Williams (1971), Motosugi *et al.* (1982), Tsang *et al.* (1988), Jones *et al.* (1992), Schneider *et al.* (1991), Van der Ploeg *et al.* (1991), Kawasaki *et al.* (1994), Murdiyatmo *et al.* (1992), Nardi-Dei *et al.* (1994) and Cairns *et al.* (1996). However, properties of current enzyme was not studied for their basic molecular weight.

In current investigation, a high thermostability, high temperature optimum and strict specificity are characteristics of the *Pseudomonas* sp. strain S3. This is the first example of a thermostable DehL isolated from paddy (rice) field in Malaysia. DehL from previously reported *Rhizobium* sp. RC1 (Cairns *et al.*, 1996) is not thermostable upon heat treatment up to  $60^\circ\text{C}$ .



*Pseudomonas* sp. strain S3 DehL efficiently catalyse the dehalogenation of not only short carbon chain for example haloacetates and it is also has low Km values with high specific activities. In addition, L-specific dehalogenase is very useful for degradation of toxic 2-haloalkanoic acids in an environment and production of D-2-hydroxyalkanoic acids which are useful as chiral synthons.

For future research the enzymes will be purified and their molecular weight/subunit structures will be determined. The primary and tertiary structures and site directed mutagenesis of both dehalogenases of *Pseudomonas* sp. strain S3 are also important for future study.

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