



Purification and Characterization of a Novel *p,p'*-DDT Dehalogenase from *Aeromonas* sp. strain MY1

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ABSTRACT: Although dichlorodiphenyltrichloroethane (DDT) occupies a prominent position for its recalcitrance in the environment which led to its ban by the Stockholm Convention. This pesticide is still unregulated used in low and middle-income countries, which contributes to the global burden of this hazardous contaminant. In the recent time, biological approach has been proven to be effective and environmentally safer for the decontamination of the residual DDT. In this research, we reported a gel-purified *p,p'*-DDT dehalogenase from *Aeromonas* sp. strain MY1, revealing a single band with a molecular weight of approximately 37 kDa. The dehalogenase was unique for its capability to release chloride ions from *p,p'*-DDT substrate with optimum dechlorination activity at pH 8.0 and 35°C. The enzyme followed Michaelis-Menten's kinetics and measurement of its initial velocities with various *p,p'*-DDT concentrations showed a K_m of 27.05 $\mu\text{mol L}^{-1}$ and V_{max} of 476.19 $\mu\text{mol L}^{-1} \text{min}^{-1}$. However, the enzyme lost its dechlorination activity in the presence of Ag^{2+} and Hg^{2+} . This dehalogenase could pave a way for the effective decontamination of *p,p'*-DDT contaminated environment, suggesting its potentials for *p,p'*-DDT bio-cleansing applications.

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The environmental widespread and realization of the devastating ecological and health impacts of halogenated aromatic compounds, particularly organochlorine pesticides in which 1-chloro-4-[2,2,2-trichloro-1-(4chlorophenyl)ethyl]benzene (DDT) occupies a prominent position for its recalcitrance, led to the ban of this compound and other Persistent Organic Pollutants (POPs) by the Stockholm Convention in 2001. However, DDT is still unregulated used for disease vector control in many parts of low and middle-income Africa, the Pacific, and Asia (van den Berg *et al.*, 2017; Abdul Kader, 2019). The inherent environmental persistence demonstrated by DDT and its metabolites made them traceable in ambient air, domestic water supply, and other food varieties (Bussolaro *et al.*, 2012; Anand, 2019; Wu *et al.*, 2019; Mendes *et al.*, 2019; Sheldon *et al.*, 2019; Thompson *et al.*, 2019). Some reports on environmental and human toxicity showed that DDT intake is still occurring even in countries that fully respect the Stockholm Convention (Sudharshan *et al.*, 2012). In many foods, the levels of DDT in many cases exceeded the U.S. Environmental Protection Agency permitted daily threshold (Sheldon *et al.*, 2019). Ogbeide *et al.* (2015) detected DDT in domestic water samples from Nigeria. DDT has been known to be associated with several human health implications,

ranging from endocrine disruption, muscular dysfunction, and probable carcinogenicity (Mnif *et al.*, 2011; Hadara *et al.*, 2016; Piazza and Urbanetz, 2019; Truong *et al.*, 2019; Cohn *et al.*, 2019). Thus, still, there is an urgent need to remove the residual DDT from the environment to ensure the safety of the ecosystem. DDT and its metabolites can be removed from the polluted sites by both physical and biological decontamination approaches. However, due to the relative chemical stability of DDT, the physical approach remains ineffective (Foght *et al.*, 2001). Thus, the biological approach has been used and proven to be effective, safer, and environmentally friendly in the decontamination of the residual DDT (Fang *et al.*, 2010; Mwangi *et al.*, 2010; Abdul Hamid *et al.*, 2011). In recent times, microbial dehalogenases that cleavage carbon-halogen bonds have been captivating the interest of the scientific community for their potentials in biotechnological applications in the decontamination of halogenated organic pollutants (Liang *et al.*, 2019). Most of the microbial dehalogenases that have been reported are known to catalyze the cleavage of halogen atoms from the halogenated aliphatic compounds (Magnuson *et al.*, 2000; Abdul Hamid *et al.*, 2011; Jugder *et al.*, 2016; Jugder *et al.*, 2017). However, only a few dehalogenases were reported to dehalogenate the

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halogenated aromatic contaminants. This research focused on the partial purification and characterization of *p,p'*-DDT-dehalogenase from *Aeromonas* sp. strain MY1 isolated from pesticide-contaminated sites.

MATERIALS AND METHODS

Aeromonas sp. strain MY1: The *Aeromonas* sp. strain MY1 was the source of the enzyme (*p,p'*-DDT dehalogenase). The organism was isolated from pesticide-contaminated soil located at Phase I, Kadawa Irrigation Site, Hadejia-Jama'are River Basin, Kano State, Nigeria. The isolate was previously identified as *Aeromonas* sp. strain MY1 by a research team at the Bio-cleansing Annex, Center for Biotechnology Research, Bayero University, Kano, Nigeria. The strain was found to grow in the *p,p'*-DDT enrichment medium (Murtala *et al.*, 2020), and its 16S ribosomal RNA partial gene sequence can be found in the public databases of the National Center for Biotechnology Information (NCBI) under the universal accession number MN530936.

Enzyme purification: The enzyme extraction and purification protocols were carried out by modification of Motosugi *et al.* (1982) enzyme purification procedure. All protocols were carried out at 4 °C unless otherwise specified.

Cells harvest: An LB broth containing 20 g BactoTryptone, 10 g yeast extracts, and 20 g NaCl in 2 L of distilled and sterilized water was prepared as a large-scale medium to rapidly grow the strain MY1. For the growth of the isolate, a large and sterilized (4 L) flask was used for inoculation. The strain MY1 was used to inoculate 2 L LB medium and incubated overnight at 37°C and 200 rpm for 24 h. Before the inoculation, 0.1 mg mL⁻¹ of *p,p'*-DDT was added into the growth medium as an inducer of dehalogenases. After getting sufficient growth in 24 h, the bulk medium was centrifuged using 1200R Nuve™ (Turkey) refrigerated centrifuge at 4 °C and 10,000 rpm for 15 min. The pellet obtained was re-suspended in 50 mL of 50 mM potassium phosphate buffer (pH 7.5) and re-centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was again reconstituted with 50 mL of 50 mM potassium phosphate buffer (pH 7.5) kept at -20 °C for further protocols.

Enzyme extraction: To release the cellular contents, the strain MY1 cells were homogenized using a battery-operated motor and pestle (Pellet Pestle Motor) in 50 mL tubes as follows: bacterial cells (20 g wet weight) were suspended in 20 mL of 50 mM potassium phosphate buffer (pH 7.5) containing 0.1 mM phenylmethylsulphonyl fluoride (PMSF) as a

protease inhibitor and 0.1 mM dithiothreitol (DTT) as enzymes stabilizer. The cell suspension was then homogenized for 2 min in an ice water bath. Then, 10 mL of phosphate buffer was added into the homogenate and re-homogenized for 2 min. The homogenate was further mixed using a vortex mixer (vortex IR N2400-0001, Star Lab, Italy) by pulse vortexing after the addition of 5 mL potassium phosphate buffer. The homogenate was then centrifuged at 10,000 rpm for 10 min using a refrigerated centrifuge (ROTINA 380, Hettich, Germany) at 4 °C. The supernatant was carefully decanted in a tube while the cellular debris was discarded. The supernatant was stored in an -20 °C refrigerator for partial protein purification.

Activation of DEAE-cellulose: This activation protocol was established by the manufacturer, Sigma-Aldrich, United Kingdom. The dry resin (8 g) was suspended in 50 mL distilled water and allowed to settle for 30-45 min. The volume of resin was measured and regarded as the Column Volume CV (20 mL) that was used for measuring the washing solutions. The suspended resin was filtered and re-suspended in 40 mL of 0.1 M NaOH containing 0.5 M NaCl for 10 min. Then the slurry formed was poured into a Buchner funnel (60 mL) while applying GENTLE suction and allowing a flow of 10 mL buffer in 5 min. The slurry was further washed with 40 mL of 0.1 M NaOH containing 0.5 M NaCl again. The washing step was repeated using 0.5 M NaCl (without 0.1 M NaOH) and the washing cycle was further repeated using 0.1 M HCl containing 0.5 M NaCl. The final washing step was done using 70 mL deionized water until the effluent pH value was 5. The resin was suspended in 40 mL of 1 M NaCl and the pH of the slurry was adjusted to 7.5 with NaOH and allowed to stand for 2 h. The resin was then filtered and 100 mL of distilled water was passed through the resin on the filter. Then the resin was re-suspended with 40 mL of 500 mM sodium phosphate buffer (pH 7.5) and filtered. The resin was removed from the funnel and re-suspended with 100 mL of 50 mM buffer and filtered again. This was followed by re-suspension of resin with 40 mL of 50 mM sodium phosphate buffer. A small portion of the suspension was filtered and the pH of the filtrate was measured. The pH was within the range of 0.15 units of the 50 mM buffer, making the resin to be ready for use. Later, the resin was packed into a column of 20 × 100 mm capacity. Gravity flow of buffer was applied for washing resin and sample elution.

DEAE-cellulose: The supernatant containing the enzymes (30 mL) was applied onto the activated DEAE-cellulose column (20 × 100 mm) equilibrated

with 50 mM potassium phosphate buffer (pH 7.5). The column was washed with the buffer (pH 7.5), then elution was carried out with a linear gradient of 50 to 500 mM potassium phosphate buffer, pH 7.5 in the mixing chamber; flow rate 30 mL/h; each fraction, 5 mL. The enzyme was eluted at a particular buffer concentration. Active fractions were concentrated by adding ammonium sulfate (70% saturation) and then dialyzed against 5 mM potassium phosphate buffer (pH 7.5).

Gel filtration: A 5g of Sephadex G-100 was suspended in 50 mM potassium phosphate buffer (pH 7.5) for 72 h, it was degassed, and packed in a glass column (300 × 25 mm), and equilibrated with 50 mM potassium phosphate buffer (pH 7.5). The proteins (enzymes) solution obtained from ion-exchange (DEAE-cellulose) was applied to a Sephadex column and then eluted with the same buffer at a flow rate of 0.5 mL/min.

SDS-PAGE electrophoresis: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using CS-550V Cleaver Scientific SDS-PAGE electrophoretic system (United Kingdom) with a serial number 150603 034. A ready-made TruPAGE Precast Gels 4-12% from Sigma-Aldrich, UK (10 cm × 10 cm – 0.1 cm × 12 wells) were used for loading the Bio-Rad medium-range protein ladder, crude and partially purified protein samples as described by Laemmli, (1970). The gel was visualized by using a calibrated G-800 Densitometer (Bio-Rad).

Bradford assay: Bradford assay was performed to prepare a standard curve using bovine serum albumin and also to determine the protein concentration (Bradford, 1976). The standard curve was prepared using various concentrations of albumin mixed with 5 mL of Bradford reagent. The mixture was shaken and the absorbance was read using 6705 UV/Vis Spectrophotometer, (JENWAY, Cole-Parmer Ltd., UK) at 595 nm. The sample protein was also prepared by mixing 100 µL of the sample and 5 mL of Bradford reagent and the absorbance was taken at 595 nm.

Determination of *p,p'*-DDT dehalogenase activity

Chloride ion detection assay: The activity of the partially purified dehalogenase from strain MY1 towards dechlorination of *p,p'*-DDT was measured by its ability to release chlorine ions from the substrate. The chloride ion detection assay described by McGuinness *et al.* (2007) was used to quantitate the number of chloride ions released.

One unit of dehalogenase activity is the amount of enzyme that catalyzed the release of 1 micromolar of chloride ion per litre per minute ($\mu\text{mol L}^{-1} \text{min}^{-1}$).

Optimum pH and temperature: To determine the optimum pH and temperature of the *p,p'*-DDT dehalogenase, the enzyme preparation was incubated at various pH values (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5) and different temperatures (20, 25, 30, 35, 40 and 45°C) under shaking (100 rpm) for 16 h as described in the chloride detection assay protocol.

Effect of heavy metals on *p,p'*-DDT dehalogenase activity: To determine the effect of selected metal activators/inhibitors on the activity of the dehalogenase from strain MY1 on dechlorination of *p,p'*-DDT, the assay mixtures comprised metals (Fe^{2+} , Zn^{2+} , Cu^{2+} , Ag^{2+} and Hg^{2+}) at a final concentration of 1 mM as described by Abdul Hamid *et al.* (2011). Then incubated accordingly, and the chloride ions released was determined.

Statistical analysis: The activity of *p,p'*-DDT-dehalogenase from strain MY1 was performed in triplicates and the data represent the means together with standard deviation (\pm SD) of three independent experiments each. Analysis of variance (ANOVA) and student's t-test were used to analyze the data using OriginPro 8.5 software and MS-excel analytical tool pack. The significance was determined at a 95% confidence limit.

RESULTS AND DISCUSSION

Purification of *p,p'*-DDT dehalogenase from *Aeromonas sp.* strain MY1: The *p,p'*-DDT dehalogenase from strain MY1 was isolated from cells of cultures that were grown in the presence of *p,p'*-DDT as an inducer, and LB broth as a nutrient source as described in the methodology section. There was no *p,p'*-DDT dehalogenase activity when the cells were grown in the LB broth, indicating that the enzyme is an inducible enzyme, produced in the presence of *p,p'*-DDT in the growth medium. The partial purification of *p,p'*-DDT dehalogenase from strains MY1 was determined by DEAE-cellulose and Sephadex gel filtration. The purity of the enzyme was confirmed using SDS-PAGE, which also determined the approximate molecular weight of the enzyme.

Determination of molecular weight: The SDS-PAGE analysis of *p,p'*-DDT dehalogenase from strain MY1 revealed a single band with a molecular weight of approximately 37 KDa (Figure 1). This dehalogenase was thought to be a monomeric protein. Many researchers reported various bacterial dehalogenases with molecular weights close to that of *p,p'*-DDT dehalogenase from strain MY1 (Keuning *et al.*, 1985;

Jugder *et al.*, 2017;). Although some literature documented many bacterial monomeric dehalogenases with much high molecular weights than that of *p,p'*-DDT dehalogenase (Müller *et al.*, 2004; Abdul Hamid *et al.*, 2011).

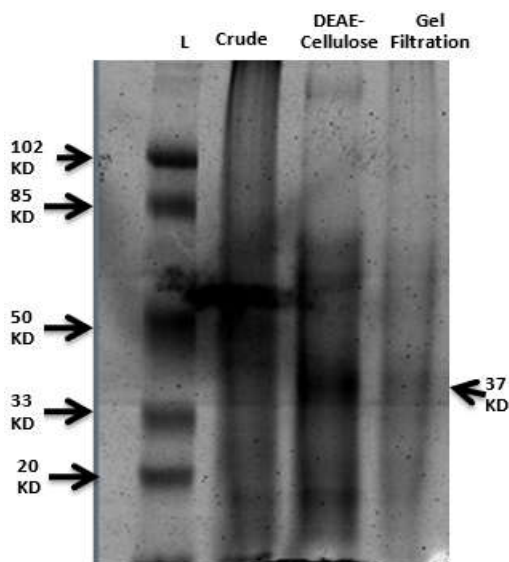


Fig 1: SDS-PAGE showing purification steps of *p,p'*-DDT dehalogenase from *Aeromonas* sp. strain MY1. L represents a BioRad medium-range protein ladder.

Determination of purification fold and protein activity of the dehalogenase: The DEAE-cellulose fraction of the *p,p'*-DDT dehalogenase from strain MY1 produced 47 mg of the partially purified enzyme and enzyme activity of 124 (Unit) and percentage yield of 52%. The Sephadex gel-purified *p,p'*-DDT dehalogenase yielded 20 mg of the purified enzyme with a percentage yield of 40%. The specific activity of the dehalogenase towards *p,p'*-DDT was found to be 4.8 (Unit/mg) (Table 1). From the total protein in the crude extract of *p,p'*-DDT induced cells, the dehalogenase protein contributed to nearly 1.4% of the total protein contents.

Characterization of *p,p'*-DDT dehalogenases purified from strain MY1

Effect of pH on the activity of *p,p'*-DDT dehalogenases purified from strain MY1: The effect of pH on the activity of *p,p'*-DDT dehalogenase was determined using the Sephadex gel-purified fraction of the enzyme. The *p,p'*-DDT dehalogenase from strain MY1 has optimum activity at pH 8.0. However, the enzyme demonstrated activity within a broad pH range of 6.0 to 8.5 (Figure 2).

Table 1: Purification of *p,p'*-DDT dehalogenase from *Aeromonas* sp. strain MY1

	Volume (mL)	Protein (mg)	Activity (Unit)	Specific Activity (Unit/mg)	Purification (fold)	Yield (%)
Crude Extract	20	1430	237	0.17	1	100
DEAE cellulose	4	47	124	2.64	15.5	52
Gel filtration	2	20	96	4.8	28	40

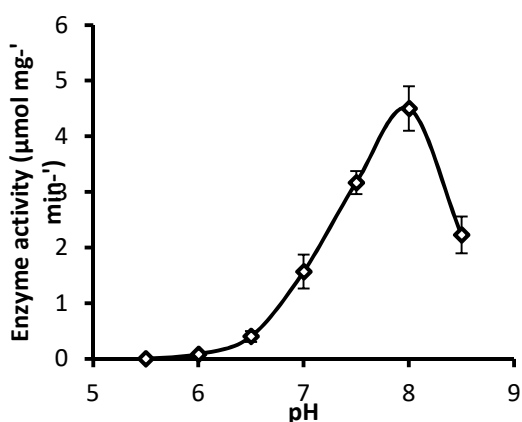


Fig 2: Effect of pH on the activity of *p,p'*-DDT dehalogenase from strain MY1 towards *p,p'*-DDT dechlorination. The experiments were conducted in triplicate.

The effect of temperature on the activity of dehalogenase from strain MY1 was determined at a range of temperatures between 20 to 50°C. A dramatic decrease in the activity of the enzyme was observed outside the temperature range of 20 to 40°C. However,

the dehalogenase demonstrated an optimum dehalogenation activity at 35°C (Figure 3).

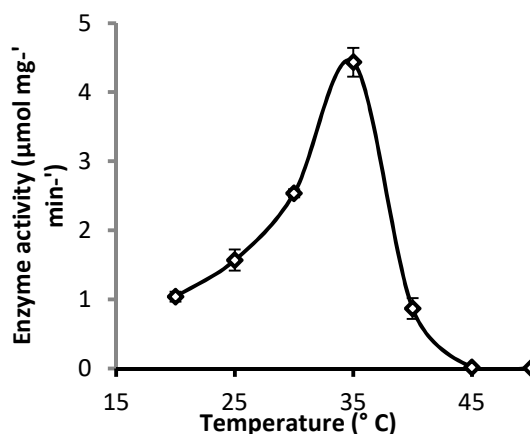


Fig 3: Effect of temperature on the activity of *p,p'*-DDT dehalogenase from strain MY1 towards *p,p'*-DDT dechlorination. The experiments were conducted in triplicate.

The dehalogenase demonstrated an optimum activity at a pH of 8.0 and a temperature of 35°C. This is not surprising by considering the environmental

conditions of the tropical region where strain MY1 was isolated. Alteration of pH or temperature, the dechlorination capacity of the enzyme is lowered. Temperature and pH are vital to microbial protein stability and functions since any alteration may result in the denaturation of functional proteins and consequent inactivation. It is therefore pertinent to ascertain the desired temperature and pH for optimum activity of the enzyme for effective application in bioremediation. Buryska *et al.* (2018) and Liang *et al.* (2019) reported some bacterial dehalogenases with a similar range of pH and temperature range.

Kinetic parameters of p,p'-DDT dehalogenase purified from strain MY1: The *Aeromonas* sp. strain MY1 was found to inducibly produced *p,p'*-DDT dehalogenase when grown on *p,p'*-DDT supplemented growth medium. The study of this *p,p'*-DDT dehalogenase is vital due to the spatial location of the chlorine substituent in dictating the susceptibility of chlorinated aromatic compounds, especially the persistent *p,p'*-DDT to degradation by microbial enzymes. Chlorine atom is known to interfere with the activity of some enzymes to degrade chlorinated aromatic compounds.

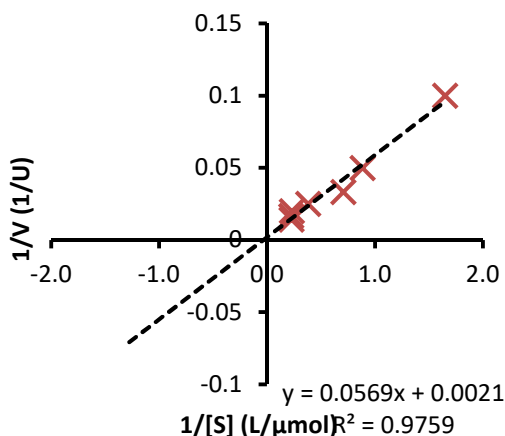


Fig 4: The Lineweaver-Burk plot of gel-purified *p,p'*-DDT dehalogenase from strain MY1. With the *p,p'*-DDT substrate, the enzyme followed Michaelis-Menten's kinetics

This interference was thought to be stemming from both steric and electrostatic interactions demonstrated by chlorine substituent, making the chlorinated aromatic compounds more resistant to degradation and more persistent in the environment (Copley, 1997). The success of the degradation of any chlorinated aromatic compound depends largely on the removal of chlorine substituent which paves way for further degradation via the opening of the aromatic rings and enhancing the susceptibility of the compound for complete degradation. The kinetic parameters of *p,p'*-DDT dehalogenase from strain MY1 were determined.

The K_m and V_{max} values were derived from the computed Lineweaver-Burk plot (Figure 4). With the *p,p'*-DDT substrate, the enzyme followed Michaelis-Menten's kinetics and measurement of initial velocities with various *p,p'*-DDT concentrations showed a K_m of $27.05 \mu\text{mol L}^{-1}$ and V_{max} ($476.19 \mu\text{mol L}^{-1} \text{min}^{-1}$) (Table 2). Keuning *et al.* (1985) and Kurihara *et al.* (2003) reported a dehalogenase that also followed Michaelis-Menten's behaviour.

Table 2: The K_m and V_{max} of gel-purified *p,p'*-DDT dehalogenases from strain MY1

Enzyme	K_m ($\mu\text{mol/L}$)	V_{max} ($\mu\text{mol L}^{-1} \text{min}^{-1}$)
<i>p,p'</i> -DDT dehalogenase	27.05	476.19

Effect of heavy metals on the p,p'-DDT dehalogenase activity: Metal ions modulate enzyme activity by serving as either activators or inhibitors. Fe^{2+} , Zn^{2+} , Cu^{2+} , Ag^{2+} and Hg^{2+} were tested for their effects on the activity of the *p,p'*-dehalogenase from strain MY1. The dehalogenase showed loss of activity after incubation with 1 mM Ag^{2+} and Hg^{2+} , demonstrating no residual activity concerning Ag^{2+} . Interestingly, there was no loss of activity in the presence of 1 mM Fe^{2+} , Zn^{2+} , and Cu^{2+} respectively (Figure 5).

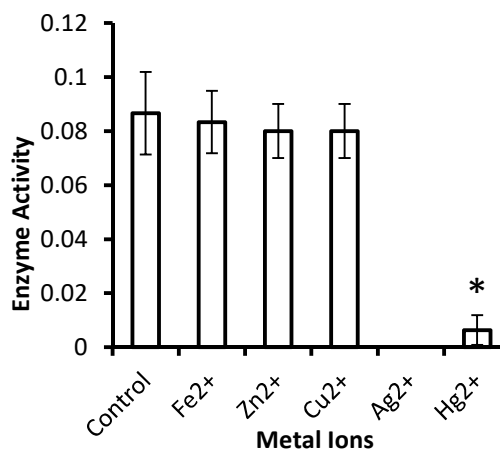


Fig 5: Effect of metal ions on the activity of *p,p'*-DDT dehalogenase from strain MY1 towards *p,p'*-DDT. The results are expressed as the number of chloride ions released in $\mu\text{m.g}^{-1}$ of the enzyme. The results are means \pm SD of three independent triplicate determinations. Values with '*' indicate a significant difference (at $P < 0.05$) when compared with their respective control.

The enzyme demonstrated no residual activity for Ag^{2+} and a very negligible activity for Hg^{2+} . Ag^{2+} and Hg^{2+} are well-known thiol reagents capable of inhibiting the catalytic activity of the thiol-containing enzyme's active sites (Keuning *et al.*, 1985; Warner *et al.*, 2005; Abdul Hamid *et al.* 2011; Agarwal *et al.*, 2017). This suggested that the active site of *p,p'*-DDT dehalogenase from strain MY1 may contain a thiol group and the probable participation of the group in

the enzyme's catalytic strategy. Abdul Hamid *et al.* (2011) observed a similar scenario in 3-chloropropionate dehalogenase from *Pseudomonas* sp. B6P. Interestingly, there was no loss of activity in the presence of 1 mM Fe²⁺, Zn²⁺, and Cu²⁺ respectively. However, these metals did not produce any stimulatory effect on the dehalogenase activity.

Conclusion: Following Michaelis-Menten's behaviour, the *p,p'*-DDT dehalogenase from *Aeromonas* sp. strain MY1 was unique for its capability to release chloride ions from *p,p'*-DDT substrate. Although the enzyme has lost its dehalogenase activity in presence of metallic thiol reagents, the dehalogenase could still pave way for the effective decontamination of *p,p'*-DDT contaminated environment, suggesting its potentials for *p,p'*-DDT bio-cleansing applications.

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