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# The potential of a novel β-specific dehalogenase from *Bacillus cereus* WH2 as a bioremediation agent for the removal of β-haloalkanoic acids

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## ABSTRACT

**Aims:** This study aims to describe the biochemical and kinetic properties of a dehalogenase produced by a bacterium, *Bacillus cereus* WH2 (KU721999), that is uniquely adept at degrading a β-haloalkanoic acid, i.e., 3-chloropropionic acid (3-CP), and using it as the bacterium's sole carbon source. The bacterium was isolated from abandoned agricultural land in Universiti Teknologi Malaysia that was previously exposed to herbicides and pesticides.
**Methodology and results:** The *B. cereus* impressively removed 97% of 3-CP after 36 h of culturing. The intracellular WH2 dehalogenase of the bacterium was purified 2.5-fold and has an estimated molecular mass of 37 kDa. The highest activity of the dehalogenase was achieved under conditions of 30 °C and pH 7. The metal ions $Hg^{2+}$ and $Ag^{2+}$ substantially repressed the enzyme's activity, but the enzyme's activity was uninhibited by dithiothreitol (DTT) and EDTA. The WH2 dehalogenase showed a higher affinity for 3-CP ($K_m$ = 0.32 mM, $k_{cat}$ = 5.74 $s^{-1}$) than for 3-chlorobutyric acid (3-CB) ($K_m$ = 0.52 mM; $k_{cat}$ = 5.60 $s^{-1}$). The enzyme was ~1.6-fold more catalytically efficient ($k_{cat}/K_m$) in dehalogenating the three-carbon substrate 3-CP (17.8 $mM^{-1}$ $s^{-1}$) than the four-carbon 3-CB (11.2 $mM^{-1}$ $s^{-1}$).
**Conclusion, significance and impact of study:** The novel *B. cereus* bacterium isolated in this study may prove applicable as a bioremediation agent to cleaning environments that are polluted with β-halogenated compounds. Furthermore, such an approach to treat polluted environments is more sustainable and potentially safer than chemical treatments.

*Keywords:* Dehalogenase, *Bacillus cereus*, 3-chloropropionic acid, β-haloalkanoic acid, bioremediation

## INTRODUCTION

The use of herbicides, insecticides and fungicides in the agricultural sector to control unwanted plant growth (Aktar *et al.*, 2009) and the infestation of pests has increased in recent decades. This rise is primarily attributable to the agricultural sector being one of Malaysia's economic mainstays. Considering the widespread use of such chemicals in agricultural activities, greater liberation of pollutants (glyphosate, 2-4-D, diuron, paraquat and Dalapon) into the environment is expected. It is now a major public concern as such chemicals are highly detrimental to the well-being of humans and ecosystems due to their persistence in the environment. β-Chlorinated compound, such as 3-chloropropionic acid (3-CP) and 3-chlorobutyric acid (3-CBA), are mainly used as intermediates in the syntheses of pharmaceuticals and pesticides and are known for their carcinogenic and genotoxic effects on both animals and humans (Mishra and Sharma, 2011). The problem is further exacerbated by the fact that these chemicals have relatively high solubility in water, which increases their mobility into nearby water bodies, *viz.*, rivers, groundwater and the marine environment (Shahidul Islam and Tanaka, 2004). It is a matter of fact that these pollutants have been found to be prevalent in water resources, especially those located near rice fields (Mesri *et al.*, 2009). Hence, cleaning up environments that are contaminated with such substances has become biotechnologically challenging.

Global food sources are largely sources from water bodies, i.e., rivers and marine environments; the latter of these provides a considerable fraction of the human diet (Duarte *et al.*, 2009). Such ecosystems contain

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increasingly higher concentrations of β-chlorinated compounds; 3-CP can potentially increase the risk of food contamination. The quest for an alternative environmentally benign approach i.e., bioprospecting for effective microorganisms to naturally detoxify β-chlorinated compounds and their analogs, therefore deserves scientific consideration. Moreover, the high tendency of these compounds to bioaccumulate in living tissues and subsequently move higher up the food chain has been indicated (Barbarossa *et al.*, 2016; Olutona *et al.*, 2016). Aside from the added stability of 3-CP, the relative hydrophobicity of the compound also promotes its partitioning into the organic phase. This fractionation renders 3-CP difficult to remediate (Field and Sierra-Alvarez, 2004).

This study focused on bioprospecting to find competent soil microorganisms that produce a class of enzymes called dehalogenases, specifically the β-specific dehalogenase. This unique capability is the result of an evolutionary adaptation that genetically acclimatizes the microorganisms to their environment (Sinha *et al.*, 2009; Edbeib *et al.*, 2016) to ensure their survival. As a matter of fact, the incessant exposure to such toxic compounds, i.e., β-halogenated compounds, is the driving force (Field and Sierra-Alvarez, 2004; Muslem *et al.*, 2015) for such genetic flexibility. It allows the development of new traits in microbes for coping with the continual influx of such compounds into the ecosystem. This adaptability means that the isolates can only utilize β-halogenated compounds, such as 3−CP, as their sole source of carbon for their growth. By producing this specific enzyme, the isolate can transform the harmful 3−CP into a less toxic component, *viz.,* alcohols, halide ions and protons, with water as the sole co-substrate.

Earlier studies have reported on the thermal stability, pH dependence, kinetic parameters, mechanism of dehalogenation, electrophoretic mobility, or the inhibition by sulfhydryl-blocking agents of β-degrading bacterial dehalogenases (Lin *et al.*, 2011; Zhang *et al.*, 2013; Li and Shao, 2014). However, a comprehensive study that describes the biochemical characteristics of 3−CP-degrading dehalogenase remains lacking. In this present study, a novel *Bacillus cereus* strain, WH2, that produces a β-specific dehalogenase was isolated and identified. The WH2 dehalogenase of the *B. cereus* isolate was found to be competent in assimilating 3-CP as its sole carbon source for growth. The intracellular dehalogenase was then extracted, and the enzyme was purified to homogeneity and subsequently characterized. This present study was aimed at establishing the optimized conditions (temperature, pH and metal ions) for the maximum hydrolysis of 3-CP. The effects of inhibitors and substrate specificity were evaluated, and the kinetic constants of the WH2 dehalogenase were also established.

## MATERIALS AND METHODS

### Chemicals

The various halogenated compounds (monochloroacetic acid (MCA), dichloroacetic acid (DCA), D,L−2−chloropropionic acid (D,L-2-CP), 2,2-dichloropropionic acid (2,2-DCP), 2,2,3-trichloropropionic acid (2,2,3-TCP), 2,3-dichloropropionic acid (2,3-DCP), 2-chlorobutyric acid (2-CB), 3-chlorobutyric acid (3-CB), 2,2-dichlorobutyric acid (2,2-DCB), 2,2,3-trichlorobutyric acid (2,2,3-TCB) and 3-chloropropionic acid (3-CP)) used in this study were all of analytical grade and purchased from the Sigma Chemical Co. (St. Louis, USA). Salts, $K_2HPO_4{\cdot}3H_2O$, $NaH_2PO_4{\cdot}2H_2O$, $(NH_4)_2SO_4$, trace metal salts, nitriloacetic acid (NTA), $MgSO_4$, $FeSO_4{\cdot}7H_2O$, $MnSO_4{\cdot}4H_2O$, $ZnSO_4{\cdot}H_2O$ and $CoCl_2$ were purchased from the Sigma Chemical Co. (St. Louis, USA). Other chemicals such as Tris-acetate, ethylenediaminetetraacetic acid (EDTA), glycerol, ammonium sulfate $(NH_4)_2SO_4$ and sodium dodecyl sulfate-polyacrylamide gels were also bought from the Sigma Chemical Co. (St. Louis, USA).

### Microorganisms, culture conditions and growth analysis

The bacterial strain *Bacillus cereus* WH2 (accession number: KU721999) was collected from abandoned agricultural land located on the grounds of Universiti Teknologi Malaysia that had been extensively exposed to herbicides and pesticides. Isolation of the bacterium was performed by growing the isolates on a basal medium containing 3-CP (10 mM) as the sole carbon and energy source. The medium consisted of 10 × concentrated basal salts, which consisted of $K_2HPO_4{\cdot}3H_2O$ (42.5 g/L), $NaH_2PO_4{\cdot}2H_2O$ (10.0 g/L) and $(NH_4)_2SO_4$ (25.0 g/L). The 10 × trace metal salt solution comprised nitriloacetic acid (1.0 g/L), $MgSO_4$ (2.0 g/L), $FeSO_4{\cdot}7H_2O$ (120.0 mg/L), $MnSO_4{\cdot}4H_2O$ (30.0 mg/L), $ZnSO_4{\cdot}H_2O$ (30 mg/L) and $CoCl_2$ (10.0 mg/L) dissolved in distilled water (Hareland *et al.*, 1975). The minimal media for growing the bacteria comprised 10 mL of 10 × basal salts and 10 mL of 10 × trace metal salts per 100 mL of distilled water, which was autoclaved (121 °C, 15 min, 15 psi). The carbon source, 3−CP, was neutralized with NaOH prior to filter sterilization and subsequently added to the sterilized salts medium to reach a final concentration of 10 mM. The determination of bacterial growth was monitored by measuring the absorbance at 600 nm with a UV-VIS spectrophotometer (Perkin Elmer).

The growth was assessed by monitoring the depletion of the 3-CP in the liquid growth medium using high-performance liquid chromatography (HPLC). The samples from the growth medium were taken at appropriate time intervals and subjected to filtration through a 0.2 µm nitrocellulose filter (Sartorius) to remove bacterial cells and particulates prior to the HPLC analysis. The analysis was performed at a flow rate of 1.0 mL/min using isocratic elution with a mobile phase of aqueous potassium sulfate

(20 mM):acetonitrile (60:40). The fractions of the sample were monitored with a UV detector equipped with a ZORBAX Eclipse XDB-C18 (4.6 mm × 150 mm, 5 µM particle sizes).

**Preparation of cell-free extract**

Cells from a 100 mL culture were harvested by centrifugation at 10 000 ×*g* for 10 min at 4 °C. The harvested cells were suspended in Tris-acetate (0.1 M), EDTA (1 mM), and glycerol (10%, w/v), pH 7.5, and centrifuged (10 000 ×*g* for 10 min at 4 °C). The cell pellets were then resuspended in 4 mL of the same buffer and cooled in ice prior to sonication in an MSE Soniprep 150 W ultrasonic disintegrator (peak amplitude, λ = 10 microns, 30 sec). The unbroken cells and cell wall materials were removed by centrifugation (40 000 ×*g* for 30 min at 4 °C), and the supernatant was decanted and stored at 4 °C. The dehalogenase activity was determined colorimetrically and represented the concentration of $Cl^-$ ions that were released (Bergmann and Sanik, 1957), unless otherwise indicated.

**Dehalogenase activity assay and determination of protein content**

The protein content of the purified WH2 dehalogenase was estimated using a calibration curve that had been prepared from standard solutions of bovine serum albumin (BSA) (Sigma, USA) and Bradford reagent (Sigma, USA). The color development was monitored at 595 nm in a PG Instruments T60 UV/VIS Spectrophotometer using a solution without BSA as the blank (Bradford, 1976). The dehalogenase activity was estimated by determining the total number of $Cl^-$ ions released using a colorimetric method employing mercuric thiocyanate (Bergmann and Sanik, 1957); samples were measured at 30 °C in a mixture that consisted of 5 mL 0.09 M Tris-acetate (pH 7.5), 1 mM substrate and enzyme. The samples were removed at intervals, and the free $Cl^-$ ion was determined colorimetrically. The addition of the mercuric thiocyanate was to improve the detection limits to quantify the liberated $Cl^-$ ions in water by UV-visible spectroscopy. The $Cl^-$ ions present in the solution cause the mercuric thiocyanate salt to dissociate and the liberated thiocyanate ions bind to the Fe(III); this complex intensely absorbs at 460 nm. Hence, the concentration of released $Cl^-$ ions could be estimated (Cirello-Egamino and Brindle, 1995). One unit of enzyme activity (1U) is defined as the amount of enzyme required to catalyze the formation of 1 µmole halide ion/min.

**Protein purification and characterization**

Purification of the crude WH2 dehalogenase was carried out by ammonium sulfate precipitation executed at 4 °C, unless specified otherwise. The crude enzyme extract was precipitated by treatment with 20% ammonium sulfate [$(NH_4)_2SO_4$ (300 mg/mL) plus cell-free extracts (30 mL)] and 80% (w/v) [$(NH_4)_2SO_4$ (423 mg/mL) plus cell-free extracts (30 mL)] at various saturation levels and was left to stand for 4 h prior to centrifugation (40 000 ×*g*, 30 min, 4 °C). All the collected precipitates were resuspended in Tris-acetate buffer at (0.1 M, 6 mL, pH 7.5) and dialyzed overnight against 20 mM Tris-acetatebuffer (200 mL, pH 7.5) to remove residual $(NH_4)_2SO_4$. The purity of the purified dehalogenase was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The samples with the protein fractions were electrophoresed together with a pre-stained broad-range protein marker (Mark12 protein standard). All the concentrated fractions were subjected to protein and dehalogenase activity assays to establish the fraction with the most dehalogenase activity.

**Determination of the molecular weight of WH2 dehalogenase by SDS-PAGE**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (TGX Fastcast, Bio-Rad) was performed with a 5% stacking and a 12% separating gel to determine the molecular weight of the purified WH2 dehalogenase (Laemmli, 1970). A 50 µL buffer sample (0.05% bromophenol blue, 5% β-mercaptoethanol, 10% glycerol, and 1% SDS in 0.25 M Tris-HCl buffer, pH 6.8) was added to a 100 µL aliquot of protein samples in Eppendorf tubes, and the solution was boiled in a hot water bath for 5 min, cooled to room temperature and electrophoresed. The protein bands were visualized by staining with Coomassie Brilliant Blue G (Sigma) and destained in distilled water overnight. The molecular weight of the purified WH2 dehalogenase was determined by comparing its band to those of marker proteins (standard protein marker, 15-350 kDa; Sigma, USA).

**Characterization of purified WH2 dehalogenase**

*Effect of the temperature and optimum pH*

The effect of temperature on the activity of WH2 dehalogenase was evaluated at temperatures ranging from 20 to 55 °C; the protein was incubated for 10 min with stirring at 200 rpm for 30 min prior to the activity assay. An aliquot of the sample (1 mL) was withdrawn at 5 min intervals and assayed under standard conditions. The effect of pH was investigated by using various buffer systems (100 mM) acetate buffer (pH 4-6), potassium phosphate buffer (pH 6-8), Tris-Cl buffer (pH 8-9) and glycine-NaOH (pH 9). The samples were stirred at 200 rpm for 30 min. The reaction was started by the addition of the substrate, and after incubation for 10 min, the number of released $Cl^-$ ions were determined using the standard assay method (Bergmann and Sanik, 1957). A negative control that lacked enzyme was run in parallel with the assay mixtures to detect spontaneously released $Cl^-$ ions.

*Effects of metal ions and inhibitors*

The purified WH2 dehalogenase was treated with several types of metal ions ($Ca^{2+}$, $Mg^{2+}$, $Ag^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Hg^{2+}$ and $Co^{2+}$) and inhibitors (EDTA and DTT) (1 mM) before being subjected to activity assays under standard conditions.

**Substrate specificity**

The evaluation of substrate specificity for the purified WH2 dehalogenase was examined using MCA, DCA, D,L-2-CP, 3-CP, 2,2-DCP, 2,3-DCP, 2,2,3-TCP, 2-CB, 3-CB, 2,2,3-TCB and 2,2-DCB as substrates. All substrates were separately sterilized and aseptically added to a reaction mixture (5 mL) that consisted of the haloacid (0.1 mM) substrate and purified WH2 enzyme, and the product production was assayed under standard conditions (Bergmann and Sanik, 1957).

**Kinetics studies**

The kinetic measurements were carried out in Tris-acetatebuffer (0.1 M, 4.7 mL) by varying the concentration of 3-CP (2, 4, 6, 8 and 10 mM). The kinetic parameter $k_{cat}$ and the turnover numbers were estimated on the basis of one active site per 37 kDa subunit, while the value of $K_m$ was derived using the Michaelis-Menten plot from an experimental steady-state data (Eqn.1) (Souza *et al.*, 2015).

$$V1 = \frac{V_{max}[S]}{K_m + [S]} \quad \text{Eqn.1}$$

The $K_m$ (Michaelis constant) is defined as the concentration of substrate needed to reach half of the maximum velocity and is a measure of substrate affinity; $V_{max}$ is the maximum velocity and is directly proportional to the enzyme concentration. The value of $k_{cat}$ described the number of substrate molecules turned over per enzyme molecule per second, while the catalytic efficiency was determined by dividing the value of $k_{cat}$ by $K_m$.

**RESULTS**

**HPLC analysis of 3-CP degradation by WH2 dehalogenase**

In our evaluation, the growth medium of the bacterium was analyzed by high-performance liquid chromatography (HPLC) to monitor the depletion of the 3-CP and/or the generation of the $Cl^-$ ion end-product from the dehalogenation reaction. The chromatogram revealed a large decline in the initial 3-CP concentration ($t_R$ = 1.7 min) in the growth medium that was inoculated with *B. cereus* WH2. The concentration of 3-CP gradually declined until 97% of it was used, after a 36 h incubation period; this decrease was indicated by the large reduction in the area of the 3-CP peak in the chromatogram. This result affirms the feasibility of using the WH2 bacteria as a potential bioremediation agent to degrade 3-CP. We further carried out a time course assessment on the growth curve of the WH2 bacterial strain to compare with the 3-CP depletion in the growth medium. It was evident that the 3-CP was rapidly consumed by the WH2 bacteria as its carbon source during the exponential growth phase. This consumption was represented by the initial peak area being substantially reduced from 4363916 mAU*s (0 h) to 144940 mAU*s (36 h). The bacterial growth was monitored beyond 36 h, until the starvation phase was reached. Here, the study found that the utilization of 3−CP began to gradually decline (Figure 1).

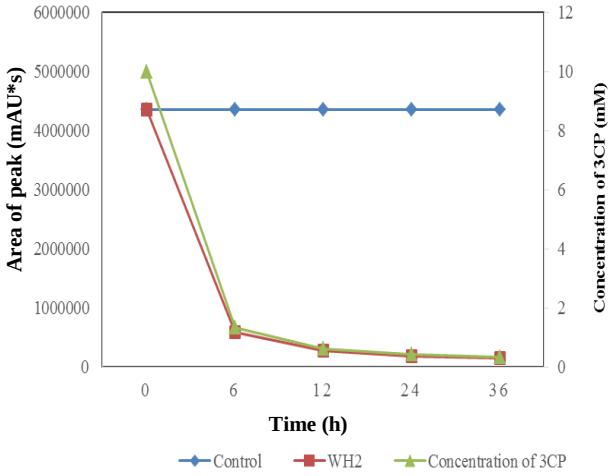


**Figure 1:** The growth curve of *B. cereus* WH2 *versus* the depletion of 3-CP.

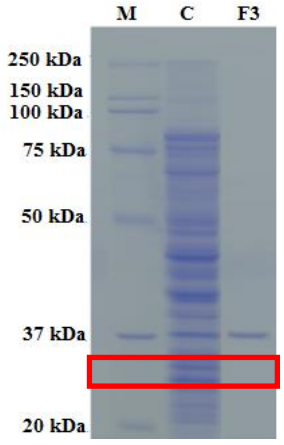


**Figure 2:** SDS-PAGE analysis of the purified WH2 dehalogenase; M, Protein Marker; C, Cell free extract; F3, (60-80% ammonium sulphate).

### Purification of the *B. cereus* WH2 dehalogenase

Ammonium sulfate precipitation of the crude WH2 dehalogenase precipitated the WH2 dehalogenase protein when the salt concentration was at 60 to 80% saturation. Each fraction showed only a single band at 37 kDa, suggesting that the isolated proteins were pure (Figure 2). Consequently, the protein precipitates were subjected to enzymatic assays using 3-CP as the substrate. All the protein fractions revealed good dehalogenase activity, thus confirming that the fractions contained the WH2 dehalogenase. The fractions were combined, and the pure WH2 dehalogenase protein was subsequently concentrated. In this study, the WH2 dehalogenase was successfully purified ~2.5-fold, with a specific activity of 3.75 U/mg (Table 1).

**Table 1:** Summary of the purification of Deh WH2 from *B. cereus* WH2.

| Fraction | Volume | Total activity (U/mL) | Total protein (mg/mL) | Specific activity (U/mg) | Purification fold | Yield (%) |
|---|---|---|---|---|---|---|
| Crude extract | 100 | 6.500 | 3.32 | 1.50 | 1.00 | 100 |
| 60-80% $(NH_4)_2SO_4$ purification | 6 | 4.45 | 1.19 | 3.74 | 2.49 | 68.50 |
| Dialysis | 6 | 3.20 | 0.85 | 3.75 | 2.50 | 49.23 |

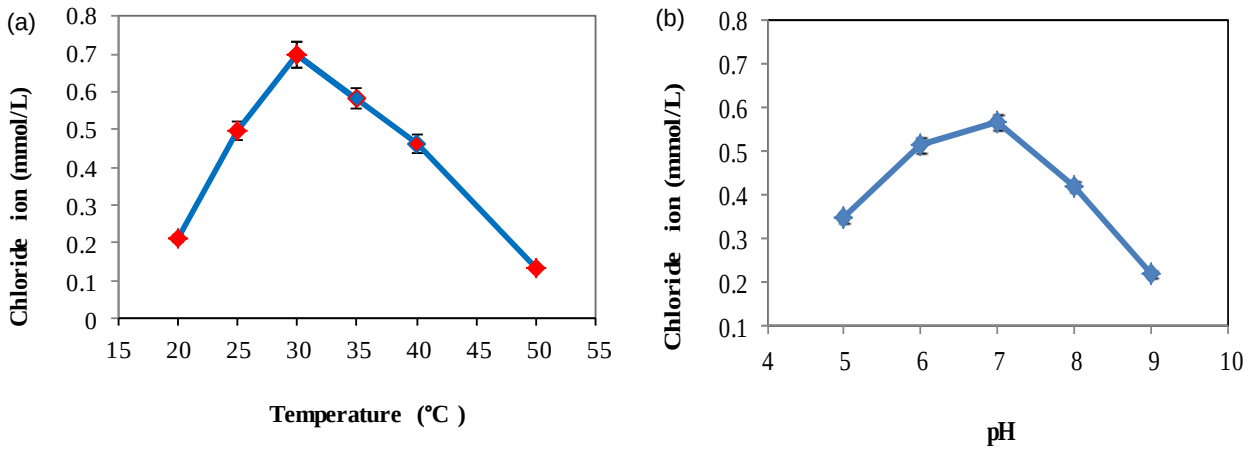


**Figure 3:** The effects of (a) temperature (ranging from 20-50 °C), and (b) pH (4-9) on chloride ion released catalyzed by the purified WH2 dehalogenase**.** The buffer used: 0.1 M Tris-acetate, 10% (w/v) glycerol.

### Effect of temperature and pH on dehalogenase activity

The results revealed that the activity of the pure WH2 dehalogenase increased from its value at 20 °C (0.2 mmol/L) to achieve its highest value at 30 °C (0.7 mmol/L); above this temperature, the activity decreased. The lowest activity was recorded at 50 °C (0.12 mmol/L) (Figure 3a).

Using a similar preincubation method, the effect of pH values from 4-9 on the activity of the dehalogenase was investigated at 30 °C. The WH2 dehalogenase was found to give its maximum activity (0.56 mmol/L) at pH 7 (Figure 3b).

### Effects of metal ions and inhibitors

The effect of different metal ions ($Ca^{2+}$, $Mg^{2+}$, $Ag^{2+}$, $Zn^{2+}$, $Hg^{2+}$, $Cu^{2+}$, and $Hg^{2+}$) on the activity of the pure Deh WH2 dehalogenase was assessed. This study found that supplementation with various metal ions did not improve the activity of Deh WH2. In fact, the WH2 enzyme was strongly inactivated by $Ag^{2+}$, $Hg^{2+}$, $Cu^{2+}$ and $Zn^{2+}$ with the resulting relative activities of 10.1, 29.5, 33.3 and 40.1%, respectively. In contrast, supplementation with $Ca^{2+}$ and $Mg^{2+}$ were found to be less deactivating and produced greater relative activities, corresponding to 80 and 83%, respectively, (Figure 4).

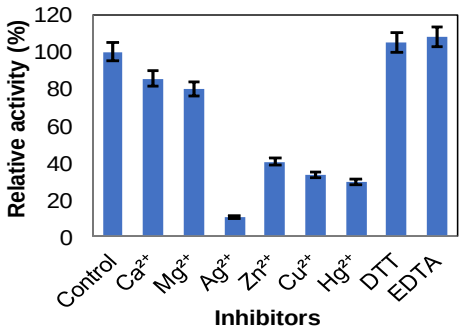


**Figure 4:** The effects of metal ions and inhibitors (1 mM each) on the relative activity of the purified WH2 dehalogenase. The buffer used: 0.1 M Tris-acetate, 10% (w/v) glycerol at 40 °C.

The effects of common enzymatic inhibitors (DTT and EDTA) on the dehalogenation of 3−CP that was catalyzed by the pure WH2 dehalogenase was also studied. To our surprise, supplementing the reaction mixtures with DTT (a reducing agent) and EDTA (a chelating agent) did not significantly affect the catalytic activity of the WH2 dehalogenase. The relative activities for the DTT- (102%) and EDTA-treated samples (107%) were found to be marginally higher to the negative control (Figure 4).

**Table 2:** The cell free extracts dehalogenase activity toward different substrates. The buffer used: 0.1 M Tris-acetate, 1 mM EDTA, 10% (w/v) glycerol at 40 °C.

| Substrates | Specific Activity (μmol $Cl^-$ $min^{-1}$ $mg^{-1}$ protein) |
|---|---|
| **Two-carbon acid** | |
| Monochloroacetic acid | 0 |
| Dichloroacetic acid | 0 |
| Trichloroacetic acid | 0 |
| **Three-carbon acids** | |
| 2-chloropropionic acid | 0 |
| 3-chloropropionic acid | 4.7 |
| 2,2-dichloropropionic acid | 0 |
| 2,3-dichloropropionic acid | 3.6 |
| 2,2,3-trichloropropionic acid | 0 |
| **Four-carbon acids** | |
| 2-chlorobutyric acid | 0 |
| 3-chlorobutyric acid | 3.6 |
| 2,2,3-trichlorobutyric acid | 1.2 |
| 2,2-dichlorobutyric acid | 0 |

**Substrate specificity**

The WH2 dehalogenase was unable to hydrolyze most of the assessed chlorinated haloacids, *viz.,* monochloroacetic acid, dichloroacetic acid, 2-chloropropionic acid, 2,2-dichloropropionic acid and 2,2,3-trichloropropionic acid. This conclusion was inferred from the fact that no liberated $Cl^-$ ions were detected in the reaction mixtures. The exceptions, however, were the four-carbon acids, 2,2,3-trichloropropionic acid (1.2 μmol $Cl^-$ $min^{-1}$ $mg^{-1}$ protein) and 3-chlorobutyric acid (3.6 μmol $Cl^-$ $min^{-1}$ $mg^{-1}$ protein), and the three-carbon acids, 2,3-dichloropropionic acid (3.6 μmol $Cl^-$ $min^{-1}$ $mg^{-1}$ protein) and 3-CP (4.7 μmol $Cl^-$ $min^{-1}$ $mg^{-1}$ protein), which was hydrolyzed the fastest (Table 2). Notably, the successful dechlorination of the mono- or di-chlorinated substrates was specific only for those substrates whose Cl atom was located at the β-position of the haloacids.

**Kinetics studies**

The kinetic parameters $k_{cat}$, and $K_m$ for substrates 3-CP and 3-CB were determined by steady-state kinetic analysis, and the $K_m$ values of the two substrates were compared to determine the relative affinity of the WH2 dehalogenase for each substrate. Our assessment shows that the WH2 dehalogenase has a higher affinity for 3-CP than for 3-CB, corresponding to $K_m$ values of 0.32 and 0.52 mM, respectively. The corresponding $k_{cat}$ values for these two substrates are 5.74 and 5.60 $s^{-1}$, respectively. Dividing the values of $k_{cat}$ by the values of $K_m$ revealed that the WH2 dehalogenase is ~1.6-fold more efficient in degrading the shorter, three-carbon substrate 3-CP (17.8 $mM^{-1}$ $s^{-1}$) than the four-carbon substrate 3-CB (11.2 $mM^{-1}$ $s^{-1}$). This result affirms that the WH2 dehalogenase has a greater preference for the shorter substrate, 3-CP (Table 3).

**Table 3:** Kinetic parameters of the Deh WH2.

| Substrate [S], (mM) | $K_m$ (mM) | $V_{max}$ (μM $Cl^-$ $min^{-1}$ $μM^{-1}$ enzyme) | $k_{cat}$ ($s^{-1}$) | Catalytic efficiency $k_{cat}$/ $K_m$ ($mM^{-1}$ $s^{-1}$) |
|---|---|---|---|---|
| 3−CP | 0.32 | 0.00574 | 5.74 | 17.8 |
| 3−CB | 0.52 | 0.0056 | 5.60 | 11.2 |

**DISCUSSION**

Considering the low natural abundance of β-chloroalkanoate-degrading bacteria and the inability of α-chloroalkanoate-degrading bacteria to degrade β-chloro substituted compounds (Mesri *et al.*, 2009; Muslem *et al.*, 2015), further attempts to isolate β-chloro-degrading bacteria are necessary. In this context, this study believes that the biotechnological route, i.e., bioremediation to clean environments that are contaminated with such compounds, is potentially a safer and environmentally friendly avenue in comparison to chemical methods. As a matter of fact, earlier studies conducted by several researchers agreed with our perspective on the removal of such toxic compounds (Hamid *et al.*, 2011; Janssen *et al.*, 2005; Mesri *et al.*, 2009; Muslem *et al.*, 2015). This study showed that the novel *B. cereus* WH2 was a potentially useful bioremediation agent to degrade β-chlorohaloalkanoic acids, i.e., 3-CP. Since such compounds are extensively used in various xenobiotic activities, especially in the industrial and agricultural sectors, the continuous liberation of high quantities of these compounds into the environment is an issue that we need to address immediately. In this milieu, using the β-specific *B. cereus* WH2 bacterium to carry out *in situ* neutralization of such pollutants may prove beneficial; thus, developing a further understanding of the biochemical properties of the dehalogenase that the *B.*

*cereus* WH2 bacterium produces has scientific relevance. Interestingly, the pure WH2 dehalogenase (3.75 U/mg) showed higher specific activity than a pure dehalogenase from the *Pseudomonas* sp. B6P bacterium (1 µmol/mg) that was grown in the same substrate (Hamid *et al.*, 2011) (Table 1). This results suggests that the WH2 dehalogenase was possibly the more efficient enzyme in the bioremediation of 3-CP.

The temperature of an enzymatic reaction is one of the known key factors that can affect the activity of an enzyme (Wahab *et al.*, 2016). Hence, it was crucial that such an aspect was assessed in this work with respect to the dehalogenating ability of the WH2 dehalogenase. In this regard, the activity of the WH2 dehalogenase here (Figure 3a) was comparable to that of dehalogenases that were isolated from *Rhodococcus* sp. (Jing and Huyop, 2007; Jing *et al.*, 2010; Hamid *et al.*, 2011) but was more efficient than the dehalogenase from the *Pseudomonas* sp. B6P bacterium (Mesri *et al.*, 2009). The dehalogenase of the B6P bacterium degraded the 3-CP at a considerably slower rate (1 µmol/L) at an identical optimum temperature as the WH2 dehalogenase (30 °C). This observation may be explained in terms of the kinetics of an enzyme reaction whereby catalysis at an optimum temperature would suitably raise the kinetic energy within a system to an energy level equivalent to the activation energy of the reaction (Marzuki *et al.*, 2015a; Manan *et al.*, 2016; Isah *et al.*, 2017). In this study, the molecules (WH2 dehalogenase and 3-CP) tend to move faster when the temperature is elevated. This acceleration causes the rate of the reaction to increase with the increased collisions (Marzuki *et al.*, 2015a; Manan *et al.*, 2016; Isah *et al.*, 2017) between the enzyme and the 3-CP molecules within the system. Aside from this factor, the elevation of the reaction temperature would also impart other benefits, *viz*., an improved diffusion process, better integration and mutual solubility of the substrate and enzymes, and a reduced viscosity to promote the better integration of the reaction mixture ( Marzuki *et al.*, 2015a; Manan *et al.*, 2016; Isah *et al.*, 2017). Hence, the rate of the WH2 dehalogenase to hydrolyze the 3-CP is increased and subsequently resulted in greater dehalogenation activity. In contrast, the observably low dehalogenase activity below 30 °C was presumably due to the WH2 dehalogenase protein structure being too rigid and being unable to achieve the catalytically active form (Marzuki *et al.*, 2015b; Isah *et al.*, 2017) to catalyze the degradation of 3-CP. The dehalogenating activity of the enzyme was at its lowest at 50 °C (0.12 mmol/L), which is possibly brought about by the onset of the thermal deactivation of the WH2 dehalogenase protein structure. It was evident that the WH2 protein was gradually distorted from its active form when the incubation temperature was increased from 30 to 50 °C, which is consistent with the concomitant drop in catalytic activity (Figure 3a). It has been described that beyond the optimum temperature of an enzyme, the protein folds begin to unravel excessively (Wahab *et al.*, 2014) and becomes increasingly flexible (Snellman and Colwell, 2004). This change, consequently, causes the partial loss of its global, active conformation (Wahab *et al.*, 2014); thus, the activity of the enzyme begins to decline. Hence, the lower dehalogenating ability of the WH2 dehalogenase at the higher temperatures was therefore justified.

The study also profiled the effect of long-term exposure to solutions of various pH on the stability of the WH2 dehalogenase. According to a report by Illanes (2008), the catalytic behavior of enzymes is highly dependent on the surrounding pH. Being naturally poly-ionic, enzymes are, therefore, vulnerable to fluctuations in the charge distributions on the surface and also within the active sites. In this study, the dehalogenating activity of the WH2 dehalogenase was its highest at pH 7 (0.57 mmol/L) (Figure 3b). This revelation was particularly interesting as the reported optimum pH for many known bacterial dehalogenases has been somewhat closer to pH 9 (Hamid *et al.*, 2011). Judging from the relatively sharp peak in the pH-dependent activity profile (Figure 2b), the working pH for the WH2 dehalogenase (pH 7) was not as broad as that reported for a dehalogenase from *Pseudomonas* sp. B6P. The latter has an optimum pH of 7.5 but was consistent in catalyzing the degradation of 3-CP over a broader pH range. This behavior is different from that of the WH2 dehalogenase, whose activity dropped drastically to 0.4 and 0.2 mmol/L at pH 8 and 9, respectively. It is worth mentioning here that the optimum pH of the WH2 dehalogenase activity was lower than that of the *Rhodococcus* sp. that was isolated in two earlier studies (pH 7.6 and 8.0) (Hamid *et al.*, 2011). Notably, the activity of the WH2 dehalogenase was low at pH 9 (2.0 mmol/L) (Figure 3b), presumably due to the enzyme partially losing its catalytically active form at high pH. Under such conditions, the deprotonation of the surface amino acids is increased to produce a greater number of negatively charged and neutral surface amino acids. This change eventually causes extensive structural alterations and the irreversible disruption of the poly-ionic three-dimensional structure of the WH2 dehalogenase. In contrast, a solution with very low pH values (pH 4-5) can substantially reverse the trend in the ionization state of the WH2 protein structure that occurs at high pH values and also distorts the enzyme structural protein. The correct orientation and the binding of the substrate to the active site of the enzyme is, therefore, less frequently achieved. Essentially, both extreme conditions inactivated the WH2 dehalogenase (Figure 3b).

According to the body of literature, metal ions have been known to interact ionically with enzymes, which invariably could improve the conformation of the enzyme to enhance catalysis or distort its active conformation (Snellman and Colwell, 2004), which reduces enzyme activity. The finding of this study is consistent with a report that described that β-haloacid-degrading enzymes are generally sensitive to metal ions such as $Ag^{2+}$ and $Hg^{2+}$ (Hamid *et al.*, 2011). Pertinently, the least inactivation of the dehalogenase activity of the WH2 dehalogenase was observed in reaction mixtures that were supplemented with $Ca^{2+}$ (Figure 4). In comparison to previous works, $Ca^{2+}$ was found to be the most common

metal ion that improved enzyme activity, as the metal ion was thought to be involved in the stabilization of the tertiary structure of enzymes (Snellman and Colwell, 2004). The results in this study strongly affirm this theory. Based on this finding, the WH2 dehalogenase produced by the bacterium *B. cereus* was not a metallo-enzyme, as the dehalogenating activity of the enzyme was retained even without the addition of metal ions. As a matter of fact, the study found that the WH2 dehalogenase functioned exceedingly well in reactions that were void of any metal ions. Similarly, additives such as DTT have been known to cause disruptions to the inter- and intramolecular disulfide bonds in enzymes (Maulik *et al.*, 2009). EDTA, in contrast, affects enzymes differently, by chelating metal ions sited near the active site, making them unavailable for coordination with the enzyme (Dosanjh and Kaur, 2002). However, variations in their effects on the rate of enzyme reactions have been described (Lee *et al.*, 2015). Interestingly, this study found that both DTT and EDTA marginally improve the dehalogenating activity of the WH2 dehalogenase (Figure 4). Such an unusual effect may not occur in other enzyme systems. Most importantly, this unique characteristic of the WH2 dehalogenase may prove beneficial. Often, enzyme preparations incorporate other substances that may be of a similarly deactivating nature as DTT or EDTA. Hence, these results imply that the activity of the WH2 dehalogenase was unaffected by the low concentrations of DTT and EDTA. This aspect is key when considering the WH2 dehalogenase as a green bioremediation reagent for the *in situ* treatment of halogen-polluted environments.

The substrate specificity of the purified WH2 dehalogenase was assessed to identify the catalytic activities that may be related to its biological function. Pertinently, its absolute inactivity towards all α-located haloacids and the strict preference of the dehalogenase to degrade β-located Cl atoms in haloacids indicates that the WH2 dehalogenase is rather unique (Table 2). This characteristic of this dehalogenase is comparable to that of the previously reported dehalogenases of *Rhodococcus* sp. (Bollag, 1974; Jing and Huyop, 2007; Jing *et al.*, 2008; Mesri *et al.*, 2009; Jing *et al.*, 2010). Correspondingly, the inability of α-haloalkanoic dehalogenases to utilize β-substituted 3-CP as their organisms' sole carbon source was presumably due to the misalignment of the haloacid for binding with the catalytic residues of the α−specific dehalogenase. This misalignment, in turn, interferes with the proper attainment of an optimum trajectory for the substrate to bind to the active site. Our previous study has explicitly indicated that an effective interaction between the dehalogenase and the substrate, i.e., 3-CP, occurs within a narrow region in the interior of the active site (Hamid *et al.*, 2013). Hence, the relocation of the Cl atom of the α-haloacid (i.e., 2-CP) to the β-carbon (i.e., 3-CP), an additional one carbon further away from the C=O, will theoretically result in the ill-fitting of the 3-CP into the substrate-binding region of an α-specific dehalogenase. The nucleophilic (−OH), or catalytic, serine is rendered incapable of a nucleophilic attack on the Cl at the β-carbon. Under such circumstances, the vital charge relay process that occurs in the catalytic triad (serine, aspartate and arginine), which initiates the mechanism of Cl hydrolysis in the active site (Hamid *et al.*, 2015; Adamu *et al.*, 2016), is halted. Such a phenomenon would be consistent with the observed inability of the WH2 dehalogenase to catalyze the hydrolysis of all types of α-haloacids (Table 2). Hence, the inability of the β-haloalkanoic dehalogenase, i.e., the WH2 dehalogenase of *B. cereus,* to degrade α-chlorinated haloacids, i.e., the monochloroacetic, dichloroacetic and trichloroacetic acids, in this study is justifiably supported.

Crucially, the study of enzyme kinetics is mainly important for two basic reasons: i) for explaining how enzymes work and ii) for predicting how enzymes behave in living organisms. This study revealed that the dehalogenation reaction catalyzed by the WH2 dehalogenase followed a typical rate-limiting step (mass-transfer limited) that is observed in most enzymes, i.e., the step is not diffusion-limited. Moreover, diffusion-limited enzymes, which possess what is also known as kinetic perfection, are rare among biological enzymes (Bar-Even *et al.*, 2011). The observably low $K_m$ (0.32 mM) of the *B. cereus* WH2 dehalogenase for 3-CP indicates the high affinity of the enzyme towards the substrate (Table 3). Justifiably, the low value of $K_m$ that is seen here agrees with the observed rapid growth of the *B. cereus* WH2 bacteria in the 3-CP minimal media that was used throughout this study. Hence, these findings confirm the efficacy of the *B. cereus* bacterium and, specifically, the WH2 dehalogenase that it produces, to degrade β-haloacids.

## CONCLUSION

This study describes the purification and characterization of a dehalogenase from the soil bacterium WH2, which was isolated from agricultural land in the UTM. A taxonomic analysis identified the WH2 as *Bacillus cereus*, and interestingly, the bacterium was capable of utilizing the β-haloacid 3-CP. A noteworthy point to mention here is that the WH2 dehalogenase successfully degraded ~97% of 10 mM 3-CP in a shake flask culture. In view of its effectiveness, the enzyme was purified by ammonium sulfate precipitation to afford a monomer of 37 kDa. The activity of the purified WH2 dehalogenase reached its pH and temperature optima at pH 7 and 30 °C. Remarkably, the activity of the dehalogenase was improved when DTT or EDTA was added into the reaction mixture, while metal ions, *viz.,* $Mn^{2+}$, $Fe^{3+}$ and $Mg^{2+}$, did not adversely affected the dehalogenating activity of the WH2 dehalogenase. In contrast, $Hg^{2+}$ and $Ag^{2+}$ strongly inhibited the activity of the WH2 dehalogenase, thus indicating that the use of *B. cereus* and/or the WH2 dehalogenase for the bioremediation of environments that contain high concentrations of such metal ions may not be suitable. Kinetically, the activity of the WH2 dehalogenase followed the Michaelis-Menten model and showed a lower $K_m$ value for 3-CP (0.32 mM) than for the four-carbon

substrate 3-CB (0.52 mM). It can be construed that the WH2 dehalogenase has a greater preference for a three-carbon haloacid as its substrate than the longer four-carbon chain homolog.

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