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Purification and characterization of dehalogenase from *Bacillus cereus* SN1 isolated from cow dung

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ABSTRACT

Aims: This study was aimed to characterize a dehalogenase derived from *Bacillus cereus* SN1 isolated from cow dung.
Methodology and results: Cell-free extract of *Bacillus cereus* SN1 was purified using ion exchange and gel filtration chromatography. Fraction B2 of gel filtration gave the highest enzyme specific activity (0.155 µmol Cl^-/min/mg). The results of SDS-PAGE showed the enzyme was 25 kDa in size. The enzyme reached its optimum activity at 30 °C at pH 6, and was inhibited by Mercury(II) sulfate ($HgSO_4$). The K_m and k_{cat} values were 0.2 mM and 1.22/sec, respectively. The partial dehalogenase gene sequence was amplified using Group I dehalogenase primers. The amplified gene sequence was designated as DehSN1.
Conclusion, significance and impact of study: Dehalogenase from *Bacillus cereus* strain SN1 revealed new characteristics of dehalogenase protein. The findings indicated that the DehSN1 dehalogenase is a promising candidate for further studies as a bioremediation agent for agricultural applications.

Keywords: Cow dung; dehalogenase; 2,2-dichloropropionic acid; *Bacilluscereus* SN1; bioremediation.

INTRODUCTION

Halogenated compounds that originate from natural and/or industrial processes are often environmental pollutants. One such compound is 2,2-dichloropropionic acid (2,2DCP), commercially known as Dalapon, is a biocide used for agricultural weed management (Häggblom *et al*., 2000). The dependence of the agricultural sector on biocides for weed control is projected to increase along with the growth of global population (Varshney and Mishra, 2008). 2,2DCP is highly toxic and has been known to be highly resistant to degradation and therefore, can persists in the environment for long periods (Van Pée and Unversucht, 2003; Islam and Tanaka, 2004). The recalcitrance of such halogenated compound may be a local problem, however, it is a global concern, as the persistence of this hazardous substance is detrimental to human health (Birnbaum and Fenton, 2003; Hayes *et al*., 2006; Qing Li *et al.*, 2006). It was recently shown that 2,2DCP liberated through man-made effluents has been found in high concentrations in marine food sources, for instance, fishes and prawns that makes up a significant proportion of the human diet (Duarte *et al*., 2009; Edbeib*et al*., 2017). Worryingly, natural bioaccumulation processes further concentrate such a pollutant in marine food sources found further up the food chain (Chua *et al*., 2014; Edbeib*et al*., 2017).

In view of the numerous shortcomings associated with the long-term use of biocides loaded with 2,2DCP, such as resistance towards biotic and abiotic degradation as well as being carcinogenic and toxic to living beings (Edbeib*et al*., 2016; 2017), development of greener and safer means to eradicate such a toxic substance merits special consideration. It is known that halogenated compounds, i.e. 2,2DCP, prevailing in the environment can be neutralized by microbiological degradation. Dehalogenase (EC.3.8.1) is a class of enzymes that specifically act on halogenated compounds. These enzymes are naturally produced by certain microorganisms which cleaves the carbon-halogen bonds of halogenated compounds. The fragmented substance is subsequently transformed into environmentally benign

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products, such as pyruvate or hydroxyacids (Jing *et al.*, 2008a). A variety of bacterial species that carry dehalogenase genes have been isolated from various environmental sources (Edbeib *et al*., 2016; Satpathy *et al*., 2017). The isolated genes of diverse microbial origins were found to encode for the expressions of different types of dehalogenases *viz.* haloalkane dehalogenase, halohydrin dehalogenase, haloacetate dehalogenase, dichloromethane dehalogenase and $_{D}$- and $_{L}$-haloacid dehalogenase (Fetzner and Lingens, 1994). These enzymes are essential for the survival of microorganisms, which enable the utilization and conversion of halogenated compounds into substrates for microbial growth and propagation (Huyop and Nemati, 2010).

Herein, a new dehalogenase from a *Bacillus cereus* SN1 isolate was purified and characterized for its physicochemical properties. The assessments were carried out for better insight into the dehalogenation characteristics of the enzyme that could prospectively be useful for the bioremediation of contaminated environments.

MATERIALS AND METHODS

Preparation of cell free-extract

Bacillus cereus SN1 was isolated from cow dung in a rural area in the state of Kelantan, Malaysia. The sample was transferred aseptically into four 20 mL Luria Bertani (LB) and cultured overnight in a rotary shaker at 30 °C with agitation at 150 rpm. An approximately, 5 mL of the culture broth was inoculated into eight 2 L flasks containing 500 mL of LB. The flasks were incubated in a rotary shaker incubator at 30 °C, 150 rpm for 24 h before 2 mL of 20 mM 2,2DCP was added into the culture medium. At the late exponential phase, cell pellets were centrifugally (10,000 × g, 10 min, 4 °C) harvested and resuspended in 60 mL of 0.1 M Tris-acetate buffer (pH 7.5), followed by another round of centrifugation (10,000 × g, 10 min, 4 °C). The cells were then resuspended in a 4 mL 0.1 M Tris-acetate buffer and maintained at 0 °C prior to ultrasonication. The cells were then broken on ice using a BRANSON Digital Sonifier (USA) at 10% amplitude for 3 cycles of 30 sec sonication followed by 30 sec of cooling down. Finally, any unbroken cells and cell wall materials were removed by centrifugation (12,000 × g, 15 min, 4 °C). The supernatant was used for further analysis or stored at −10 °C.

Assay for dehalogenase activity

Dehalogenase activity was determined by detecting the liberated chloride ion in the growth medium. Measurement of the liberated chloride ion was carried out in accordance with the method of Bergman and Sanik (1957). A volume of the sample (1 mL) was added to a mixture containing 100 µL of 0.25 M ammonium ferric sulfate in 9 M nitric acid. After thorough mixing, 100 µL of mercuric thiocyanate–saturated ethanol was added and the solution was vortexed. The color was allowed to develop for 10 min at room temperature and measured at A_{460nm} using a UV-VIS spectrophotometer Shimadzu UV-1280 (Japan). Enzyme activity (IU) was defined as the amount of enzyme that catalyses the formation of 1 µmol halide ion/min.

Enzyme purification

The cell-free extract was purified by ion exchange chromatography. The binding buffer (20 mM Tris, pH 8) and elution buffer (20 mM Tris + 1 M NaCI, pH 8) were prepared and sterile Milipore Express filter with 0.22 µm size was used for the vacuum filtration step. A 1 mL Hi-trap QHP column was used in the ion exchange chromatography. Cell-free extract (5 mL) was filtered using a 0.22 µm Milipore Express filter and injected into the AKTA Purifier apparatus (GE Amersham AKTA Purifier 10, Germany). The sample was then loaded onto a Hi-Trap column pre-equilibrated with binding buffer at a flow rate of 1 mL/min. Ion exchange chromatography was run for 1 h and the bound protein was isocratically eluted using an elution buffer at a flow rate of 1 mL/min. 1 mL of each fraction was collected and tested for dehalogenase activity. The fraction showing the highest dehalogenase activity was used for further purification by gel filtration.

Gel filtration was carried out using Superdex 200 10/300 GL (Sigma-Aldrich). The column was pre-equilibrated with 0.1 M Tris-acetate buffer (pH 8), then eluted with the same buffer supplemented with 1 M NaCI. For molecular weight determination the column was calibrated using molecular weight standards from Sigma (USA), the relative molecular weights of these being: thyroglobulin 669 kDa, ferritin 440 kDa, aldolase 158 kDa, ribonuclease A 137 kDa, conalbumin 75 kDa, ovalbumin 43 kDa, carbonic anhydrase 29 kDa. A protein sample (0.5 mL) from the ion exchange chromatography was loaded onto the gel filtration column and was run for 1 h. Each fraction (an approximately 1 mL) was collected and checked for dehalogenase activity. The peak with the highest dehalogenase activity was first checked for homogeneity by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), pooled and kept at 4 °C for future use.The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as the standard.

Protein analysis

The protein samples were mixed with protein gel loading buffer containing SDS, and boiled at 95 °C for 10 min. The sample was then loaded in pre-formed wells and electrophoresed at 150 V for 72 min. The protein bands were visualized by staining with Coomassie brilliant Blue R- 250 and destained with Coomassie Gel Destain solution.

Characterization of physicochemical properties

Purified enzyme, was incubated for 10 min at various temperatures (25 – 50 °C) in order to examine the effect

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of the temperature. Aliquot (1 mL) of each sample was removed at 5 min intervals for the enzyme assay. For assessment of thermostability, the enzyme samples were incubated at 30 °C for 30 min. For examining the optimal pH, a 50 mM concentration of different types of buffers was used in the assay, as indicated in the following; potassium acetate (pH 4.0 to 6.0), phosphate buffer (pH 6.0 to 8.0), and glycine buffer (pH 8.0 to 10.0) (Jesenská *et al*., 2005). To determine the effects of inhibitors, such as ethylenediaminetetraacetic acid (EDTA), $HgSO_4$, and N-ethylmaleimide, the assay mixtures comprising of the inhibitors (1 mM) were incubated at 30 °C for 10 min. The reaction was stopped and the concentration of liberated chloride ions were determined (Jing *et al*., 2008a). Substrate specificity of the purified dehalogenase towards various substrates such as 2,2DCP, $_{D,L}$-2- bromopropionic acid, monochloroacetic acid, $_{D,\ L}$-2-chloropropionic acid and 3-chloropropionic acids were tested using the optimized condition.

Determination of K_m and V_{max}

Assessment of the influence of substrate concentrations on the purified dehalogenase was performed in 0.1 M Tris-acetate buffer (pH 7.5). The assay was monitored for concentrations ranging from 0.1 and 5 mM of 2,2DCP (Jing *et al*., 2008a). Values of K_m and V_{max} for 2,2DCP were determined from the Lineweaver–Burk double reciprocal plot of the substrate-saturation curve.

High-performance liquid chromatography (HPLC) analysis

HPLC analysis was used to determine pyruvate (dehalogenation product of 2,2DCP). The samples were prepared in a mixture containing enzyme dehalogenase and 2,2DCP and was subjected to filtration *via* nitrocellulose 0.2 µm filters (Sartorious). The HPLC equipment, Waters 2690 Alliance (USA) equipped with separations module was used in this study. Samples were resolved using a Hi-Plex H 300 mm x 7.7 mm column (Agilent Technologies, USA) at a flow rate of 0.6 mL/min equipped with a photo diode array detector set and read at an absorbance 210 nm.

Amplification of putative dehalogenase gene

The amplification of the putative dehalogenase gene was conducted using the universal deh primers. Group I comprised of dehIfor1 (5'-ACGYTNSGSGTGCCNTGGGT-3') and dehIrev2 (5'-SGCMAKSRCNYKGWARTCACT-3'), while Group II primers contained the dehIIfor1 (5'-TGGCGVCARMRDCARCTBGARTA-3') and dehIIrev1 (5'-TCSMADSBRTTBGASGANACRAA-3') (Hill *et al*., 1999). Genomic deoxyribonucleic acid (DNA) prepared from SN1 strain grown on 20 mM 2,2DCP minimal medium was used as the DNA template.

The polymerase chain reaction (PCR) consisted of mixed PCR green master mix (25 µL), forward primer (5 µL), reverse primer (5 µL), DNA template (5 µL) and nuclease free water (10 µL). The PCR thermo cycle used for amplifying Group I dehalogenase genewas: Initial denaturation: 94 °C (2 min) 1 cycle, Stage 1 (20 cycles): denaturation: 92 °C (1 min), annealing: 70 °C (30 sec), elongation: 72 °C (30 sec), Stage 2 (20 cycles): denaturation at 92 °C for 20 sec, annealing at 51 °C for 30 sec, elongation at 72 °C for 30 sec and final hold at 4 °C.

Amplification of Group II dehalogenase gene,was carried out as follows: Initial denaturation at 94 °C for 10 min, amplification step (36 cycle) includes denaturation at 92 °C for 45 sec, followed by annealing at 55 °C for 2 min and elongation at 72 °C for 45 sec. Final elongation 72 °C for 10 min and storage at 4 °C for infinite time. The PCR products were sent to First BASE Laboratories (Malaysia) Sdn. Bhd. for DNA sequencing services. Sequencing results was translated into amino acid sequence by the EXPASY tools and compared with other known dehalogenase using MultAlin open software (Corpet, 1988).

RESULTS

Purification of dehalogenase DehSN1

Cell-free extract was prepared from *Bacillus cereus* strain SN1 from a culture grown on 2,2DCP as the sole carbon and energy source. Cells were harvested at late logarithmic phase and dehalogenase activity of the cell-free extract was measured using a standard procedure (Huyop *et al.,* 2004). Figure 1 illustrates the peak for every fraction depending on absorbance (mAU). As can be seen, C3 fraction was the highest peak followed by the less resolved fractions, B11 and B12, respectively. All fractions were assayed for dehalogenase activity and it was revealed that only C3 fraction exhibited the highest dehalogenase specific activity of 0.136 µmol Cl^-/min/mg. Enzyme activity in fractions B11, B12, A8 and A9 were found insignificant (< 0.001 µmol Cl^-/min/mg), hence the fractions were discarded. Therefore, the C3 fraction was subjected to gel filtration chromatography to determine the native molecular weight of DehSN1. The elution fractions from C3 were collected and the native molecular weight was calculated using the equation of the standard curve. B2 fraction, gave the highest enzyme specific activity at 0.155 µmol Cl^-/min/mg and the native molecular weight was found to be 50 kDa. From the subunit molecular weight of a pure DehSN1 25 kDa (Figure 2), this suggests that the DehSN1 protein is a dimer.

Table 1 summarizes the steps involved in purifying DehSN1 derived from *B. cereus* SN1. DehSN1 was purified to a 126-fold with 92% yield (Table 1). The enzyme activity obtained from IEX indicated a 96% increment from the concentrated cell-free extract. For further analysis only, a pure protein from gel filtration step was used.

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Table 1: Purification table of dehalogenase DehSN1

Purification step	Total protein (mg)	Total activity U (µmol Cl^-/ min)	Specific activity (µmol Cl^-/ min/mg)	Yield (%)	Purification (fold)
Cell-free extract unconcentrated	41.70	0.061	0.00146	100	1
Cell-free extract concentrated	9.11	0.060	0.00659	98	5
Ion exchange chromatography	0.36	0.058	0.16022	95	109
Gel filtration	0.30	0.056	0.18461	92	126

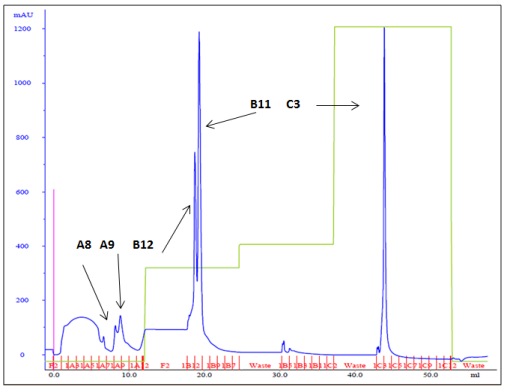

Figure 1: Ion exchange chromatography showing A8, A9, B12, B11 and C3 peaks. Each peak was tested for the highest dehalogenase enzyme activity.

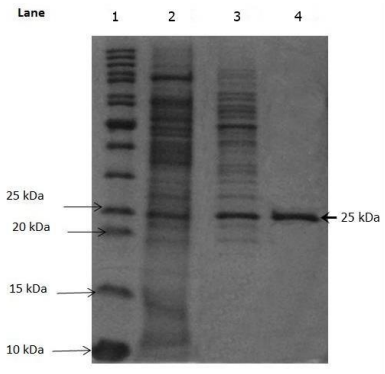

Figure 2: SDS–PAGE of DehSN1 protein. Lane 1: Protein marker unstained protein ladder (FERMENTAS); Lane 2: Unpurified protein (Cell-free extract) (6.9 µg); Lane 3: DehSN1 protein purified with ion exchange chromatography (7.2 µg); Lane 4: DehSN1 protein purified using gel filtration chromatography (4 µg).

Characterisation of DehSN1

The effect of pH on activity of the purified DehSN1 was determined by assaying under standard condition set between pH 5 to pH 9. Figure 3 illustrates that DehSN1 exhibited maximum activity at pH 6 (enzyme specific activity 0.211 µmol Cl^-/min/mg), hence indicating the optimal working pH of the enzyme. Nonetheless, activity of DehSN1 declined drastically beyond the optimal pH, to reach the lowest point at 0.055 µmol Cl^-/min/mg.

Figure 4 illustrates the effects of various temperatures on the activity of DehSN1 assayed at temperatures, 25 °C, 30 °C, 40 °C, and 50 °C. The optimum temperature for DehSN1 activity was attained at 30 °C with a corresponding specific activity of 0.382 µmol Cl^-/min/mg), beyond which enzyme activity started to decline.

The impact of some inhibitors upon DehSN1 activity was analyzed (Table 2). It was evident that DehSN1 was strongly inactivated by $HgSO_4$ but was less inactivated by N-ethylmaleimide and EDTA, respectively (Table 2).

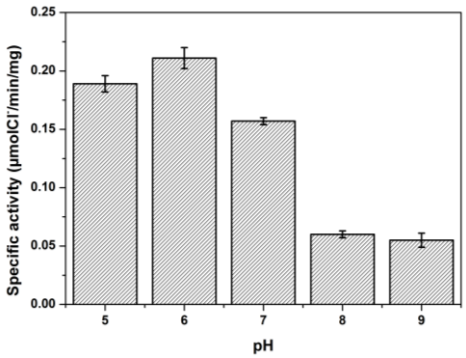

Figure 3: Effect of pH on the activity of DehSN1.

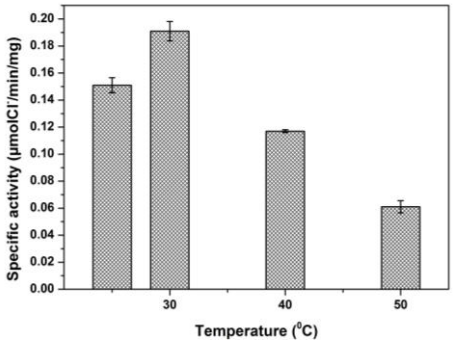

Figure 4: Effect of temperature on the activity of DehSN1 protein.

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Table 2: Effects of inhibitors on enzyme activity.

Reagent	Remaining activity (%)
None	100
EDTA	88
$HgSO_4$	0
N-ethylmaleimide	93

Substrates Specificity

Table 3 summarizes the specific activity for DehSN1 towards the various substrates. The results revealed that $_{DL}$-2-bromoropropionic acid showed the highest enzyme specific activity compared to other substrates. However, no activity was recorded for 3-chloropropionic acid. The data also indicated that DehSN1 is highly specific towards 2,2DCP.

Table 3: Dehalogenase activity towards different substrate.

Substrate	Specific activity ($\mu molCl^-$/min/mg)	Relative activity (%)
2,2-dichloropropionic acid	0.195	100
$_{DL}$-2-bromopropionic acid	0.151	77
Monocloroacetic acid	0.102	52
$_{DL}$-2-chloropropionic acid	0.138	71
3-chloropropionic acid	Not Detected	Not Detected

Kinetics of the Enzyme DehSN1

On the basis of a Lineweaver–Burk calculation, the K_m value of DehSN1 for 2,2DCP was determined to be 0.2 mM, while the V_{max} value was 0.0294 µmol/min. The $k_{cat}$of the enzyme for 2,2-dichloropropionic acid was calculated to be 1.22/s.

HPLC analysis of dehalogenation product of 2,2DCP

Figure 5a and 5b showed the HPLC profiles of all controls, pure 2,2DCP (t_R 14.84 min) and pure pyruvate (t_R 11.16 min) respectively. Figure 5c illustrates the initial peak area of 2,2DCP was 13562470 unit (t_R 14.8 min) while pyruvate 3882572 unit (t_R 11.17 min). Dehalogenase enzyme was then added into reaction tube, and allowed for the reaction takes place. Figure 5d shows the peak area of 2,2DCP decreased to 721960 unit and the peak area for pyruvate increased to 9578840 unit after 10 min of incubation suggesting that biotransformation of 2,2DCP into pyruvate had occurred.

Partial Amplification of Putative Dehalogenase Gene

The 500-bp PCR amplified DNA fragment of *dehSN1* obtained using a Group I primer was sent for DNA sequencing. However, no band was detected for the dehalogenase gene related to the Group II primer. The deduced amino sequence of DehSN1 encoded for 144 amino acids (Figure 6). Subsequently, the amino acid sequence of DehSN1 was compared to that of DehE from *Rhizobium* sp. RC1 (Stringfellow *et al*., 1997) (Figure 7). Pairwise alignment between the amino acids of DehSN1 and DehE showed that the sequences shared 75% identity. Certain amino acids within the sequence were conserved, with residues Asn33, Tyr117, Cys42, Ala120 and Asp136 being highly conserved.

DISCUSSIONS

Purification of dehalogenase DehSN1

Cell-free extract of bacteria strain SN1 grown on 20 mM 2,2DCP was prepared and subjected to purification using Ion Exchange Chromatography-IEX Hi-Trap column. Fraction C3 gave the highest peak and displayed the strongest enzyme activity. Subsequent treatment by gel-filtration chromatography showed that the molecular weight of native enzyme protein was 50 kDa while the protein subunit revealed by SDS-PAGE was 25 kDa. The result indicates that the SN1 was indeed a protein dimer. Dimeric protein units of other dehalogenases is not uncommon and have been reported by several researchers. Cairns *et al*., (1996) described the DehL of *Rhizobium* sp. RC1 had a native molecular mass of 61 kDa with the corresponding subunit size of 31 kDa. Conversely, HadL from *P. putida* strain AJ1 showed a native molecular mass of 79 kDa and a subunit size of 26 kDa. Tsang *et al*., (1988) found that the purified enzyme of *Pseudomonas cepacia* MBA4 designated as dehalogenase IVa, exhibited a relative molecular weight of 45 kDa, while the SDS-PAGE was 23 kDa.

Characterization of DehSN1

The naturally poly-ionic nature of an enzyme invariably causes its three-dimensional structure to be susceptible to fluctuations in the distribution of charges, fluctuations in the concentrations of H^+ and OH^- ions, on the surface of its protein as well as within its active site (Illanes, 2008). Since every enzyme has its own pH optimum values, identifying the optimum pH value is, therefore, crucial. An imbalance in these charges may cause the enzyme to lose its active conformation due to pH-induced folding events (Batumalaie *et al*., 2017). Furthermore, exposure to the extreme ends of the pH spectrum tend to destabilize the protein structure of an enzyme (Walsh, 2002).

In this study, the dehalogenating activity of the DehSN1 dehalogenase was at its highest at pH 6 (specific enzyme activity 0.211 µmol Cl^-/min/mg), indicating the enzyme prefers moderately acidic condition (Figure 4). This revelation was particularly interesting as the reported optimum pH for many known bacterial dehalogenases have been typically in the slightly alkaline regions (Jing, 2007; Hamid *et al*., 2011). The optimum pH of the DehSN1 dehalogenase activity was lower than that of the

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Methylobacterium sp. HJ1(pH 7.2) (Jing e*t al*., 2008a). Under an optimal pH, the conformation of the enzyme is ideal. Beyond this point, any changes in pH of the surrounding environment can lead to improper substrate binding and adversely affect the rate of enzyme reaction. The data indicate that DehSN1 is probably useful for applications requiring a pH that is closely acidic or neutral.

Aside to changes in pH, increment in temperature can cause the reacting molecules (enzymes and substrates) to gain more kinetic energy, thus increasing the probability of successful collisions along with the rate of the enzyme reaction. Considering the above findings, the optimum temperature for DehSN1 was affirmatively 30 °C, similar to as previously reported by Hamid *et al*., (2011) for *Pseudomonas* sp. B6P dehalogenase. Conversely, the optimum temperature of DehSN1 was lower than dehalogenase from *Methylobacterium* sp. HJ1 (35 °C) (Jing *et al*., 2008a). The study notes that the DehSN1 is highly sensitive to temperatures exceeding 35 °C. This is because the temperature threshold was exceeded, hence the surplus breaking of the ionic, intramolecular hydrogen bonds, van der Waals forces and hydrophobic interactions that stabilizes the enzyme tertiary structure is heightened. These changes increase structural over-flexibility and disrupt the active conformation of the enzyme which lower activity of DehSN1 (Manan *et al.*, 2018). On contrary, dehalogenase activity of DehSN1 was at its lowest at 50 °C (with enzyme specific activity 0.061 µmol Cl^-/min/mg), which is probably because of the thermal deactivation of the DehSN1 dehalogenase protein structure. It was evident that the DehSN1 protein was steadily distorted from its active form when the incubation temperature was increased from 30 to 50 °C. In addition, Wahab *et al*., (2014) stated that the protein folds begin to unravel excessively when the optimum temperature of an enzyme has been exceeded. The reason for this kind of behavior is because the molecules (DehSN1 and 2,2DCP) tend to move faster when the temperature is raised, concomitant with the observed increase in rate of the DehSN1 reaction (Marzuki *et al*., 2015; Manan *et al*., 2016; Isah *et al*., 2017; Manan *et al.*, 2018;) between the enzyme and the 2,2DCP molecules within the system. In contrast, the apparent low dehalogenase activity below 30 °C (Figure 5) was presumably due to the DehSN1 dehalogenase protein structure being too rigid and unable to unfold into its catalytically active form to catalyze the degradation of 2,2DCP (Isah *et al*., 2017).

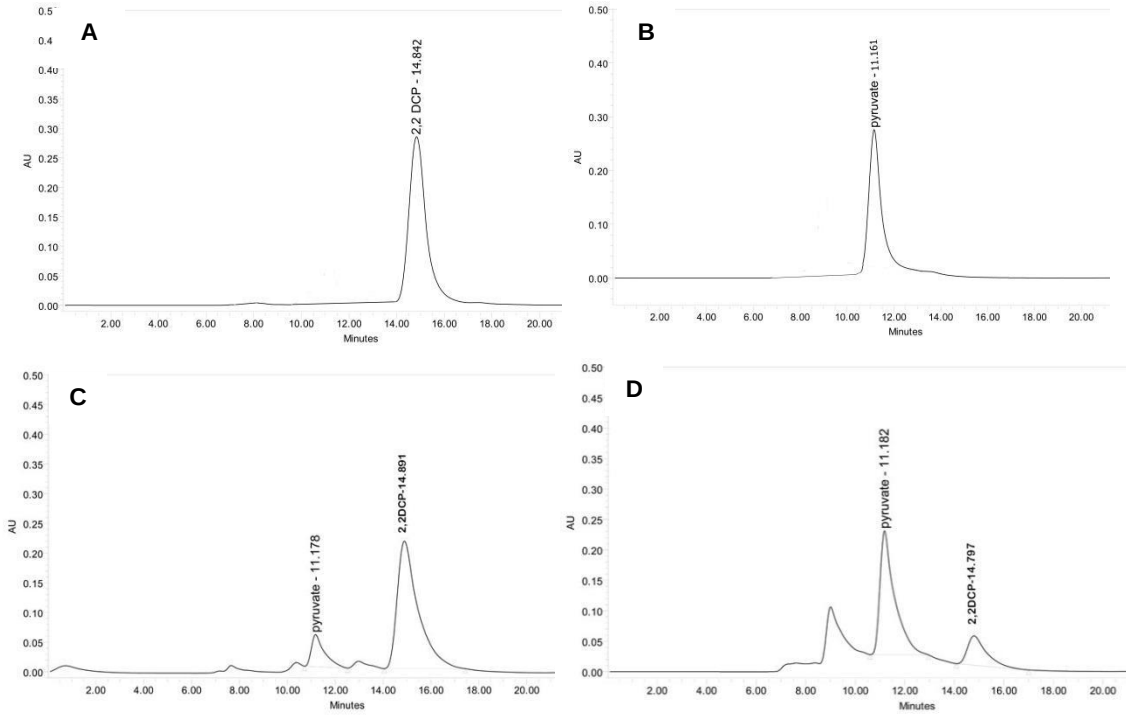

Figure 5: HPLC profiles of 2,2DCP degradation. (a) 20 mM of 2,2DCP standard showing retention time value of t_R: 14.90; (b) 20 mM of pyruvate standard showing retention time value of t_R:11.16; c) Initial amount of 2,2DCP (19 mM) (13562470 unit peak area) and initial amount of pyruvate (8 mM) (3882572 unit) d) Decreasing of peak area of 2,2DCP and increasing amount of peak area of pyruvate.

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      M  K  N  R  I  G  E  A  L  E  K  V  L  I  D  E  P  G   S  Q
1     atgaaaaacagaattggggaagcgttagaaaaagtattaattgatgaaccaggctcgcag      60

      H  I  R  K  Q  L  S  L  L  N  K  A  N  Y  S  I  S  T  I  H
61    cacattcgaaagcagctgagcttattaaataaagctaattattccatttcaacgatccat      120

      S  F  C  L  Q  V  I  R  G  Y  Y  Y  M  L  D  V  D  P  R  F
121   tcattttgtttacaagttattagaggatactattacatgcttgatgttgatcctcgtttc      180

      R  I  A  N  Q  T  E  N  E  L  L  K  E  E  V  L  D  D  I  L
181   cgcatagcgaatcaaacagaaaatgaactattaaaagaagaagtgctagatgacatatta      240

      E  E  E  Y  G  I  E  D  N  T  I  F  F  E  L  V  D  R  Y  T
241   gaagaagagtatggaattgaagataatacgatattctttgaacttgttgatcgttatacg      300

      S  D  R  S  A  D  D  D  L  Q  R  M  I  L  A  L  H  T  E  S
301   agtgaccgtagtgcagacgatgatttacaacgtatgattttagcgcttcatacagaatca      360

      R  A  H  P  N  P  E  K  W  L  D  K  L  V  E  A  Y  D  V  E
361   agagcacatccaaatccggaaaaatggcttgataaattagtagaagcatatgacgtagaa      420

      G  K  T  I
421   ggaaagacaatt
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Figure 6: DehSN1 gene encoded for 144 amino acids from 432 nucleic acid sequence.

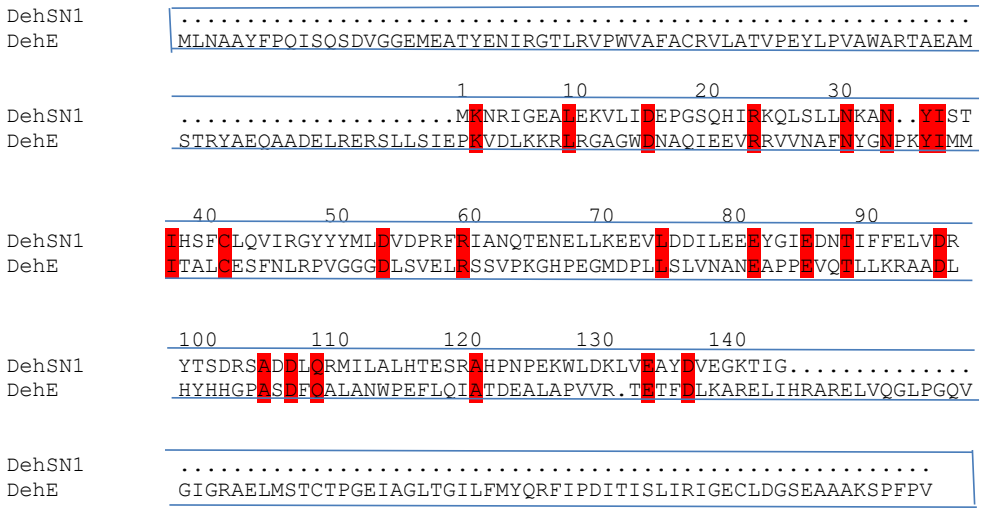

Figure 7: Pairwise between DehSN1 from *Bacillus cereus* SN1 and DehE from *Rhizobium* sp. RCI. Putative binding site residues were highlighted as labeled characters; boxed in red indicate conserved amino acids- Lys2, Leu9, Asp15, Arg23, Asn30, Asn33, Tyr36, Ile37, Ile38, Cys42, Asp54, Arg60, Leu75, Glu82, Glu86, Thr89, Asp96, Ala104, Asp106, Gln108, Ala120, Glu133 and Asp136.

As for enzyme inhibitors, their imminent function is to decrease enzyme catalyzed reaction rate by interrupting with the enzyme in certain ways, in which the impact may be permanent or temporary. This study had investigated three inhibitors, namely EDTA, $HgSO_4$, and N-ethylmaleimide. EDTA has been commonly used for depletion of ion; for instance, to deactivate enzymes that are metal-dependent, in two ways; in the form of assay for reactivity, and to reduce damages incurred upon DNA or proteins, whereas N-ethylmaleimide blocks vesicular transport (Dominguez and Ward, 2009). In this study, activity of DehSN1 was substantially inhibited by $HgSO_4$, but not by *N*-ethylmaleimide and EDTA. This is because; inhibitors ($HgSO_4$) reduces or even stop the catalytic activity of enzymes in biochemical reactions. Furthermore, dehalogenase enzyme E (DehE) derived from *Methylobacterium* sp. HJ1 was successfully inhibited by $HgSO_4$ (Jing *et al*., 2008a), and similar results were also found for DL-DEX and L-DEX (Liu *et al*., 1994).

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Substrates specificity

The substrate specificity of DehSN1 dehalogenase was assessed to identify the catalytic activities that may be related to its biological function. DehSN1 can act on 2,2-dichloropropionic acid, $_{DL}$-2-bromopropionic acid, monochloroacetic acid and $_{DL}$-2-chloropropionic acid. DehSN1 showed the highest activity on $_{DL}$-2-bromoropropionic acid (Table 3). Surprisingly, DEhSN1 could not act on 3-chloropropionic acid likely due to the misalignment of the haloacid for binding with the catalytic residues of the β−specific dehalogenase. Most dehalogenase can act on α-substituted halogenated aliphatic acids but rarely with β-halogenated substrates. In our case, the misalignment, may have interfered with the proper attainment of an optimum trajectory for 3-chloropropionic acid to bind to the active site of DEhSN1. The outcome of this study corroborates an earlier report by Jing *et al*. (2008a) for dehalogenase isolated from *Methylobacterium* sp. HJ1. Allison *et al*. (1983) claimed that *Rhizobium* sp. RC1 produced a dehalogenase that demonstrated higher activity on brominated compound as compared to chloro-substituted substrates, similar to that demonstrated by dehalogenase DEhSN1. This is probably due to brominated compounds possessing lower bromo-carbon bond energy (65.9 kcal/mol), in comparison to chloro-carbon bond (78.5 kcal/mol) (Goldman, 1972). A larger halogen atom is more susceptible to S_N2 attack associated with the longer carbon-halogen bond (Goldman, 1972).

Kinetic of enzyme DehSN1

The K_m value of DehSN1 was 0.2 mM for 2,2DCP, which value is relatively close to that reported for DehE from *Rhizobium* sp. RC1 for 2,2DCP (0.19 mM) (Huyop *et al*., 2004) but lower than a dehalogenase (K_m value 0.25 mM) isolated by Jing *et al*. (2008a) for 2,2DCP. A low K_m value (0.2 mM) obtained in this study was reflective of the high affinity of enzyme to the substrate. The data therefore affirmed that the DehSN1 dehalogenase produced by *B. cereus* SN1 was efficient in degrading 2,2DCP.

HPLC analysis of dehalogenation product of 22DCP

Pyruvate was detected by HPLC analysis suggesting the product of 2,2DCP dehalogenation produced, as reported by previous studies by Jing *et al*. (2008b) and Berry *et al*. (1979). It is hypothesized that pyruvate, a dehalogenation product of 2,2DCP might have functioned as carbon and energy sources for the SN1 strain. Berry *et al*. (1979), have shown that cell-free extracts prepared from bacteria grown on 2,2DCP as sole source of carbon readily converted this compound to pyruvate. The conversion of 2,2DCP to pyruvate may be taken as a measure of dehalogenase activity. Similarly, Jing *et al*. (2008b) colorimetrically estimated the formation of pyruvate using a method described by Friedman and Haugen (1943) and used an absorption coefficient of alkaline pyruvate 2,4-dinitrophenylhydrazone at A_{445nm} (Kornberg and Morris, 1965).

Amplification of putative partial dehalogenase gene

Group I dehalogenase successfully amplified a putative dehalogenase gene from *B. cereus* SN1, which categorically puts DehSN1 as a Group I dehalogenase. Group I dehalogenase genes are involve in the degradation of haloalkanoic acid such as 2,2DCP and $_{D, L}$-2-chloropropionic acid, inferred from the growth of bacterium SN1 in 2,2DCP minimal medium. Similar observations were reported for *P. aeruginosa* MX1 (Edbieb *et al*., 2016) and, in contrast DehGSI dehalogenase from *B. megaterium* belonged to Group II dehalogenase (Salim *et al*. 2011).

The high sequence identity between DehSN1 and DehE in the pairwise-alignment analysis further affirmed that the DehSN1 belongs to a Group I dehalogenase. Highly conserved amino acids viz. Asn33, Tyr36 and Ala120 in DehSN1 were equivalent amino acids to Asn114, Tyr117 and Ala187 in DehE that was reported by Hamid *et al*. (2013). Other conserved amino acids in DehSN1include Lys2, Leu9, Asp15, Arg23, Asn30, Ile37, Ile38, Cys42, Asp54, Arg60, Leu75, Glu82, Glu86, Thr89, Asp96, Ala104, Asp106, Gln108, Ala120, Glu133 and Asp136. Similarly, Asp189 found in DehE of *Rhizobium* sp. RC1 is known to involve in the haloacid degradation process (Hamid *et al*., 2013).

CONCLUSION

In conclusion, it was shown that cow dung is a good source for isolating novel enzymes. The data of this study implied that DehSN1 could be a novel enzyme and the enzyme could be a potential candidate for application as a bioremediation agent to clean up environments contaminated with halogenated substances. Further research into novel dehalogenases from cow dung microorganisms may help to broaden our understanding of these enzymes. It is a useful approach to provide novel dehalogenases for further application in green chemistry and the environment.

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