Protein Expression and Purification 99 (2014) 10–17

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Cloning, expression, purification and three-dimensional structure prediction of haloalkane dehalogenase from a recently isolated *Ancylobacter aquaticus* strain UV5

Ajit Kumar, Balakrishna Pillay, Ademola O. Olaniran *

Discipline of Microbiology, School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal (Westville Campus), Private Bag X54001, Durban 4000, South Africa

ARTICLE INFO

Article history:
Received 30 January 2014
and in revised form 1 March 2014
Available online 15 March 2014

Keywords:
Haloalkane dehalogenase (DhlA)
Ancylobacter aquaticus UV5
1,2-Dichloroethane

ABSTRACT

Haloalkane dehalogenase (DhlA) converts 1,2-dichloroethane (1,2-DCA) to 2-chloroethane in the genus *Ancylobacter* and *Xanthobacter autotrophicus* GJ10 (*Xa*DhlA) and allows these organisms to utilise 1,2-DCA and some other halogenated alkanes for growth. The DhlA encoding gene (*dhlA*) was PCR-amplified from the genomic DNA of a recently isolated *Ancylobacter aquaticus* UV5 strain, cloned and overexpressed in *Escherichia coli* BL21 (DE3). The recombinant enzyme was purified by using Amicon ultra-15 centrifugal filter units, an anion-exchange QFF column followed by a gel-filtration column (Sephacryl HR100). Enzyme activity was determined by using 1,2-DCA as a substrate. Three-dimensional structure of the enzyme was predicted using SWISS-MODEL workspace and the biophysical properties were predicted by submitting the amino acid sequence of DhlA on ExPASy server. DhlA (M_r 35 kDa) exhibited optimum activity at temperature 37 °C and pH 9.0. The enzyme retained approximately 50% of its activity after 1 h of incubation at 50 °C, and showed moderate stability against denaturing agent urea. The DhlA displayed a K_m value of 842 μM and k_{cat}/K_m ratio of 168 mM^{-1} min^{-1} for its substrate 1,2-DCA. This DhlA was found to belong to the α/β hydrolase family with a catalytic triad composed of Asp-His-Asp in its active site. This is the first study reporting on the characterisation and reaction kinetics of purified DhlA from *A. aquaticus* UV5 indigenous to contaminated site in Africa.

Introduction

Haloalkane dehalogenases (HLDs)[1] have been found to be expressed in a wide range of bacteria [1–3]. They hydrolyse halogenated compounds by catalysing the cleavage of carbon–halogen bonds to form the corresponding alcohol and halide ion products [4]. A haloalkane dehalogenase (DhlA, EC = 3.8.1.5) encoded by *dhlA* gene in *Xanthobacter autotrophicus* GJ10 (*Xa*DhlA), genus *Ancylobacter* and many other bacteria is the first of four enzymes involved (together with a second dehydrogenase, an aldehyde dehydrogenase and haloalkanoate dehalogenase) in the catabolic pathway of 1,2-dichloroethane (1,2-DCA) [5–9]. This enzyme is classified as SSG-1 depending on the substrates specificity groups (SSG) [10] and into the family HLH-1 based on the sequence similarity [11]. As a potential bioremediation candidate, *Xa*DhlA is under consideration for use in decontaminating soil and groundwater rather than treatment with the *X. autotrophicus* strain itself. Therefore, in recent years much effort has been directed towards isolating new microorganisms that possess unusual catabolic activities and can be used for degradation of 1,2-DCA [12–14,5–9]. In a previous study in our laboratory, five indigenous bacterial strains belonging to the genus *Ancylobacter* (DH2, DH5, DH12, UV5 and UV6) were isolated from effluent samples collected from different wastewater treatment works in Durban, KwaZulu-Natal region of South Africa [15]. Three of the isolates were observed to degrade 1,2-DCA more efficiently than the previously characterised *Ancylobacter aquaticus* AD27 [12], however the catalytic mechanism of the enzymes produced by these organisms were not elucidated. In this study, the DhlA from one of isolates, *A. aquaticus* UV5 was cloned, overexpressed, purified and characterised to gain a better understanding of the catalytic action of this enzyme on 1,2-DCA. The three-dimensional structure of the DhlA from this organism was also predicted using bioinformatics tools to have an insight into its catalytic mechanism for possible exploration of its use in bioremediation strategies.

* Corresponding author. Tel.: +27 31260 7400; fax: +27 31260 7809.
E-mail address: olanirana@ukzn.ac.za (A.O. Olaniran).

[1] *Abbrevations used:* HLDs, haloalkane dehalogenases; 1,2-DCA, 1,2-dichloroethane; SSG, substrates specificity groups; NCBI, National Centre for Biotechnology Information; CV, column volumes.

http://dx.doi.org/10.1016/j.pep.2014.03.003
1046-5928/

Materials and methods

Media, bacterial culture and growth conditions

A. aquaticus UV5 strain (NCBI Accession No. FJ572208.1) was previously isolated from effluent sample collected from SAPPI wastewater treatment plant, Durban, South Africa as described elsewhere [15]. A loopful of glycerol stock culture of the organism was streaked onto nutrient agar plate and incubated at 30 °C for 72 h, thereafter; a single colony of the isolate was further inoculated into nutrient broth and grown at 30 C for 24 h. The culture was centrifuged at 10,000 rpm for 5 min and pelleted cells were used for genomic DNA isolation using ZN Fungal/Bacterial DNA MiniPrep Kit (ZYMO Research, South Africa). SeaKem® LE Agarose for DNA gels was purchased from Lonza, USA. All the reagents used were of analytical grade.

Cloning, expression and purification of recombinant DHL

The DhlA encoding gene *dhlA* (sequence ID: gb|FJ573165.1|), was amplified from genomic DNA of *A. aquaticus* UV5 using primer pair DhlF-5′-CGCGGATCCAATGATAAATGCAATTCGCAC-3′ and DhlR-5′-CGCAAGCTTCTATTCTGTCTCGGCAAAGTG-3′. The primers were manually designed according to the *dhlA* gene sequence obtained from National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/nuccore/ FJ573165.1). The restriction sites for *Bam*HI and *Hind*III (as underlined above) were inserted into forward and reverse primers, respectively. The concentration of reagents for each PCR reaction (50 μl) were as follows: primers, 1 μM each; dNTPs, 200 μM; $MgCl_2$, 1.5 mM, 10 ng of template DNA and 1 U of Phusion Hot Start II High-Fidelity DNA polymerase (Fermentas, Thermo Scientific, South Africa). The PCR conditions include: 95 °C for 5 min (1-cycle); 95 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min (30-cycles) and final extension for 5 min at 72 °C. Subsequently, the amplified 933 bp fragment was ligated into an expression vector, pET21b (Novagen, USA) using *Bam*HI and *Hind*III (Fermentas, Thermo Scientific, South Africa) restriction sites. The ligation products were then transformed into chemically competent *Escherichia coli* DH5α cells (Invitrogen). The ligated product pET21b.DhlA was isolated using a GeneJET Plasmid Miniprep Kit (Cat. No. K0502, Thermo Scientific, South Africa) and again transformed into chemically competent *E. coli* expression cells BL21 (DE3). The *E. coli* expression cells BL21(DE3) were grown at 37 °C in 1 l LB medium containing 100 μg/ml ampicillin and induced with 1 mM IPTG at A_{600nm} = 0.6. The cells were harvested after a 16 h incubation period at 30 °C by centrifugation at 10,000 rpm for 10 min. About 5 g of wet cells were re-suspended in 10 ml of 50 mM Tris buffer (pH 9.0) and lysed on ice using Sonic Ruptor 400 Ultrasonicator (OMNI International) (6 cycles each for 15-s pulse). The lysate was obtained by centrifuging the lysis solution at 10,000 rpm for 10 min. The recombinant DhlA enzyme was purified by ultramembrane filtration followed by column chromatography. The cell lysate (10 ml) was first passed through Amicon Ultra-15 Centrifugal Filter Unit (MW cut off 50 kDa, Cat. No. UFC905024) at 5000×*g*. The flow-through (10 ml) containing proteins below M_r of 50 kDa then loaded onto Amicon Ultra-15 Centrifugal Filter Unit (MW cut off 10 kDa, Cat. No. UFC901024) and centrifuged at 5000×*g*. About 1 ml of concentrate was collected in an Eppendorf tube and the flow-through was thrown out. These steps helped remove proteins above M_r of 55 kDa and below M_r of 10 kDa along with concentrating the proteins above M_r of 10 kDa to 1 ml. The Amicon ultra-15 centrifugal filter units were purchased from EMD Millipore Corporation, Billerica, MA, USA. A 5 ml anion exchange QFF column was equilibrated with 5 column volumes (CV) (1-CV = 5 ml) of 20 mM Tris buffer (pH 9.0) and 2 mg (1 ml) of total protein was loaded onto it. The unbound proteins were washed with 5-CV of 20 mM Tris buffer (pH 9.0). The proteins bound to the matrix were eluted with 10 CV of a 0–1.0 M linear gradient of NaCl in 20 mM Tris buffer (pH 9.0). The eluted proteins were collected as 2 ml fractions by using AKTA purifier100-P950 automated fraction collector. The fractions showing haloalkane dehalogenase activity (please see Section "Haloalkane dehalogenase activity assays" for activity assay) were pooled together and concentrated by using a new Amicon Ultra-15 Centrifugal Filter Unit (MW cut off 10 kDa). One millilitre of 0.5 mg total protein then was loaded on a 53 ml (=1-CV) gel filtration column manually packed with Sephacryl HR100 matrix (purchased from Sigma–Aldrich, St. Louis, MO, USA) and pre-equilibrated with 2-CV of 20 mM Tris buffer (pH 9.0). The proteins were eluted with 2-CV and collected as 2 ml factions. The fractions showing haloalkane dehalogenase activity were again pooled together and concentrated by using a new Amicon Ultra-15 Centrifugal Filter Unit (MW cut off 10 kDa). All the chromatography purification steps were performed using AKTA purifier100 machine (Amersham Pharmacia) at a flow rate of 5 ml/min for anion exchange and 0.5 ml/min for gel-filtration. At each purification step, a small volume of samples and fractions was concentrated by acetone precipitation (Supplementary material 1) and loaded on 12% SDS–PAGE to confirm the purity and homogeneity of the proteins [16].

Haloalkane dehalogenase activity assays

Haloalkane dehalogenase activity assay was performed in 50 mM Tris sulfate buffer (pH 9.0) containing 5 mM 1,2-DCA and enzyme (0.5 μM final concentration) in a total reaction mixture volume of 1 ml. After incubation for 30 min at 37 °C, 200 μl of 250 mM ferric ammonium sulfate ($Fe(NH_4)(SO_4)_2{\cdot}12H_2O$) prepared in 6 M nitric acid was added followed by the addition of 400 μl of saturated mercuric thiocyanate dissolved in absolute ethanol. The development of a red colour is an indicative of the presence of free halide which was measured spectrophotometrically at 460 nm [7]. Varying NaCl concentration (10—100 μM) was used to generate a standard curve and fitted linear by using software ORIGIN 8 pro (evaluation version) for the estimation of chloride ion in the medium. Total protein was quantified following the method of Bradford [17], using bovine serum albumin as standard. One unit of enzyme activity was defined as the amount of enzyme that catalysed the formation of 1 μM of Cl^{-1}/min in the reaction mixture under standard assay conditions. *X. autotrophicus* GJ10 was used as a positive control while *E. coli* BL21 (DE3) lysate was used as a negative control.

Kinetic analysis for the determination of K_m, and k_{cat}

The kinetic parameters for the substrate hydrolysis were determined by measuring the initial rate of enzymatic activity. The Michaelis–Menten constant (K_m) was determined from the Lineweaver–Burk plot by applying the Michaelis–Menten equation. For the Lineweaver–Burk analysis, enzyme (0.5 μM) was incubated with 1,2-DCA (0.05–10 mM) at 37 °C and pH 9.0 for 30 min. The reciprocals of the rate of substrate hydrolysis ($1/V$) were plotted against the reciprocals of the substrate concentrations ($1/[S]$), and the K_m values were determined by fitting the resulting data using ORIGIN 8 pro (Evaluation version). V_{max} was also determined from the Lineweaver–Burk plot. The catalytic constant of the enzyme substrate reaction (k_{cat}), also referred to as the turnover number, represents the number of reactions catalysed per unit time by each active site and was determined by the equation, $k_{cat} = V_{max}/[E]_t$, where V_{max} is the maximum velocity and $[E]_t$, is

the total enzyme concentration. Catalytic efficiency was calculated by the equation, k_{cat}/K_m.

Catalytic properties DHL

The optimum pH and temperature for DhlA activity were determined by performing standard enzyme assay using, 1,2-DCA as the substrate [7]. The effect of pH on enzyme activity was examined at 37 °C over a pH range of 3.0–10.0 using sodium citrate (pH 3.0–6.0), sodium phosphate (pH 6.0–8.0), and Tris–Cl (pH 8.0–10) buffers (50 mM). The effect of temperature on the enzyme activity was investigated at the optimum pH 9.0 in the temperature range of 25–50 °C. The thermal stability of the enzyme was examined by determining the residual activity of the enzyme after incubation of the enzyme (0.5 μM) for designated time periods (0–5 h) at three different temperatures (30 °C, 37 °C and 50 °C). Further procedure is described in figure legends. The stability of the enzyme in the presence of 1% and 5% (w/v) detergents; Tween 80, Triton X-100, SDS; and denaturing agent urea (4 M and 8 M) was investigated by measuring the residual activity after incubation of the enzyme (0.5 μM) with each compound for 60 min at 37 °C and pH 9.0. Blank samples were prepared by incubation of the compound with the buffer solution instead of the enzyme under the same conditions. Positive control experiments were performed in the absence of each compound under the same conditions. To investigate the effect of divalent cations on the enzyme activity, 1 mM and 5 mM of each divalent cation ($CaCl_2$, $CuSO_4$, $FeSO_4$, $MnCl_2$, $MgCl_2$, and $ZnSO_4$) was separately added to the enzyme (0.5 μM) and incubated for 1 h at 37 °C and pH 9.0. The effect of EDTA on the enzyme activity was measured by adding 1 mM or 5 mM EDTA to the enzyme (0.5 μM) and incubated for 60 min at 37 °C and pH 9.0. The residual activities were measured using the standard enzyme assay as described above.

Template based structure prediction

The DhlA (311 aa) was searched on NCBI protein database using keywords, "haloalkane dehalogenase". Homology search was carried out using PSI-BLAST in NCBI (non-redundant) database using ORF of the protein as query sequence. Biophysical properties of the protein were determined using ExPASy server. The local sequence alignment was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Structure prediction of the enzymes was carried out using SWISS-MODEL workspace, (http://swissmodel.expasy.org/workspace/index.php?func=modelling_simple1&userid=USERID&token=TOKEN) [18–20].

Statistics

All the kinetic parameters were determined by fitting the data using software ORIGIN 8 pro. For the kinetic analysis and rate constant determinations, the assays were carried out in triplicate, and the average value was considered throughout this work. *P* value of less than 0.05 was considered to indicate statistical significance.

Results and discussion

Over expression and purification of DhlA

The PCR-amplified *DhlA* gene was inserted into the expression vector pET21b and successfully transformed into chemically competent cells of *E. coli* Bl21 (DE3). Cell lysate of IPTG-induced *E. coli* Bl21 (DE3) exhibited the presence of prominent protein band with an apparent M_r of about 35 kDa (Fig. 1), consistent with that of the *dhlA* gene product. The samples collected after ultrafiltration passed through 5 ml anion exchange QFF anion exchange column

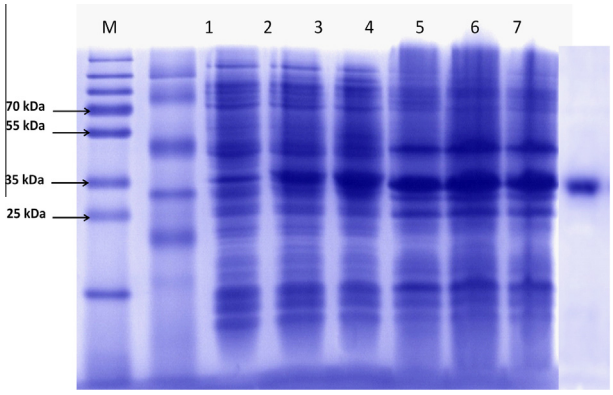

Fig. 1. Expression and purification of DhlA. Lane M shows protein marker, lane not labelled shows another protein marker and lane 1 shows the uninduced lysate of DhlA. Lanes 2–6 show the expression of DhlA after 1–3 h, 5 h and 16 h, respectively. Lane 7 shows the purified 35 kDa DhlA.

showed that fractions from B8-C1 dehalogenated 1,2-DCA (Supplementary material 2). The fractions pooled, concentrated and loaded on gel-filtration column showed that fraction A9 and A10 dehalogenated 1,2-DCA (Supplementary material 3). Fractions A9 and A10 loaded on 12% SDS–PAGE confirmed the purity and homogeneity of the protein. The recombinant DhlA enzyme was purified 28.1-fold to apparent homogeneity in four steps with a yield of 5% (Table 1). The purified DhlA showed a specific activity of 92.3 U/mg protein for the hydrolysis of the substrate (1,2-DCA). The single band of the protein on SDS–polyacrylamide gel (Fig. 1) confirms the apparent homogeneity and monomeric nature of the purified enzyme. The *A. aquaticus* UV5 strain was reported to degrade 1,2-DCA with less efficiency than the widely reported *A. aquaticus* AD27 in flasks containing MSM media [15]. The specific dehalogenase activity of the wild type DhlA measured in the crude extract of the isolate was reported to be 310 mU/mg protein and 210 mU/mg protein at pH 7.5 and 9.0, respectively, which is much lower than that reported for DhlA from *X. autotrophicus* [9]. DhlA from *X. autotrophicus* was purified 37-fold [21] and exhibited a value of 6 U/mg much lower than DhlA reported in this study. Crude extract of *A. aquaticus* AD20 showed a specific activity of 290 mU/mg protein which increased to 503 mU/mg protein after the purification of the enzyme [22]. The specific activity of the recombinant DhlA obtained in this study is about 18-fold higher than the DhlA activity previously reported for *A. aquaticus* AD20 [22].

Catalytic properties of DhlA

The recombinant DhlA from *A. aquaticus* UV5 strain displayed optimum activity at 37 °C (Fig. 2A) and pH 9.0 (Fig. 2B), which is similar to that of the wild type DhlA from *A. aquaticus* and *X. autotrophicus* [9,21,22]. The enzyme retained approximately 53%, 16%

Table 1
Table for DhlA Purification. A total of 1 l culture was processed for the purification. The cells were harvested by centrifugation at 10,000 rpm (About 5 g wet cells) and re-suspended in 10 ml of 50 mM Tris buffer (pH 9.0) and lysed by sonication.

Purification step	Protein (mg)	Total activity (U)	Sp. act. (U/mg)	Yield (%)	Purification factor
Cell Lysate (10 ml)	740	2430	3.28	100	1
UM55 kDa	264	1570	5.94	65	1.8
UM10 kDa	56	630	11.2	26	3.4
Anion Ex QFF	5.2	150	28.8	6	8.8
Sephacryl HR100	1.3	120	92.3	5	28.1

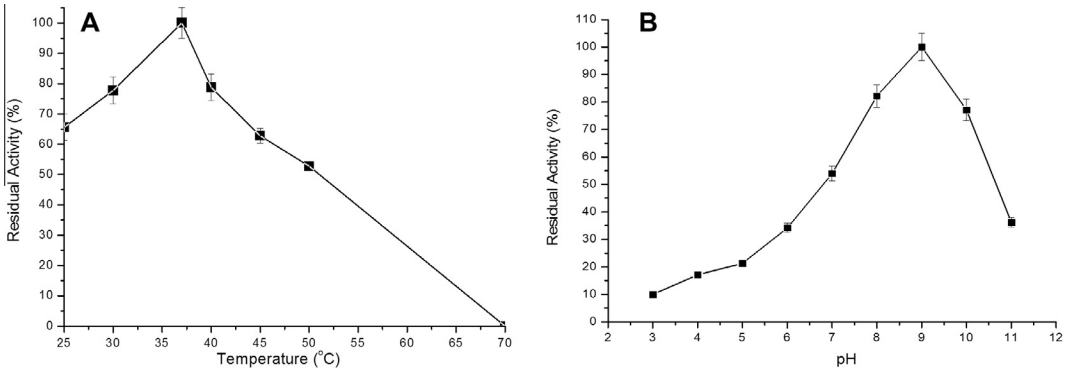

Fig. 2. Optimum temperature and pH for DhlA activity. (A) Optimum temperature of DhlA was determined by performing the standard enzymes assays at different temperatures in 50 mM Tris buffer (pH 9). (B) Optimum pH of DhlA was determined by performing the standard enzymes assays in different buffers at 37 °C (Please see Section "Haloalkane dehalogenase activity assays" for details).

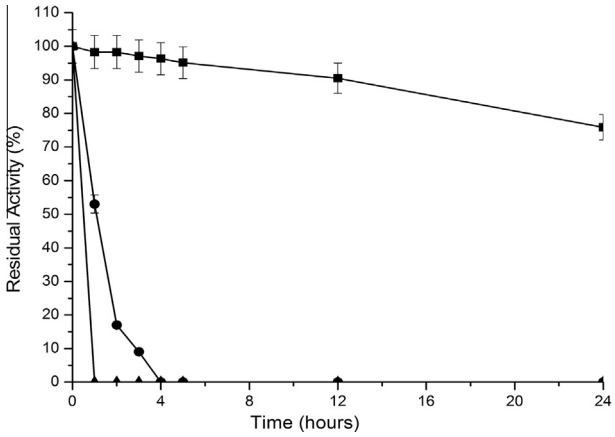

Fig. 3. Temperature stability of DhlA (5 μM) was determined by incubating at 37 °C (■), 50 °C (●) and 70 °C (♦). 100 μl of samples were removed after the designated time periods (0–5 h) and activity was determined in 1 ml reaction mixture (Final concentration of DhlA was 0.5 μM) .

Table 2
Residual activity (%) of DhlA in the presence of different reagents and metal ions.

Regents and metal ions		Residual activity (%)
Tween 80	1%	74
	5%	25
Triton X100	1%	65
	5%	17
Urea	4 M	21
	8 M	7
SDS	1%	24
	5%	6
EDTA	1 mM	100
	5 mM	100
Ca^{2+}	1 mM	100
	5 mM	100
Cu^{2+}	1 mM	100
	5 mM	100
Fe^{2+}	1 mM	100
	5 mM	100
Mn^{2+}	1 mM	100
	5 mM	100
Mg^{2+}	1 mM	100
	5 mM	100
Zn^{2+}	1 mM	100
	5 mM	100

and 9% of its activity after incubation at 50 °C for 1 h, 2 h and 3 h, respectively, and rapidly drops to 0% (Fig. 3). Wild type *Xa*DhlA exhibited no activity after 30 min incubation at 50 °C and at a pH of 7.5 [9,22]. The enzyme was stable in the presence of Tween 80, TritonX-100 and SDS, retaining 74%, 65%, and 24% of its activity, respectively, at 1% concentration as well as retaining 25%, 17% and 6% of its activity at 5% concentration of the respective compounds. Also, the enzyme retained 21% and 7% of its activity in the presence of 4 M and 8 M urea, respectively. The presence of metal ions like Ca^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+}, and Zn^{2+} in the reaction did not affect the activity of the enzyme, with 100% residual activity measured ($P < 0.05$) (Table 2). Results obtained in this study suggest that the purified DhlA possesses some stability against denaturing agents that can otherwise affect protein folding. It may be possible that enzyme denatured at 8 M urea was re-folded again upon dilution in the assay buffer and showed activity. The DhlA exhibited a K_m value of 842 μM for 1,2-DCA (Fig. 4) which is almost similar to 1.1 mM reported for *Xa*DhlA [22]. The kinetic properties of DhlA (Table 3) revealed a k_{cat}/K_m ratio of 168 mM^{-1} min^{-1} for 1,2-DCA dehalogenation. Studies on 1,2-DCA degrading organisms showed that the kinetic properties of DhlA have consequences for the K_s (Monod constant) of the organism. A low k_{cat} (turnover number), a low enzyme concentration, and a high K_m of the DhlA lead to a high K_s for 1,2-DCA utilisation [13]. At low substrate concentration ($S \ll K_m$), the rate of substrate removal by an enzyme is given by

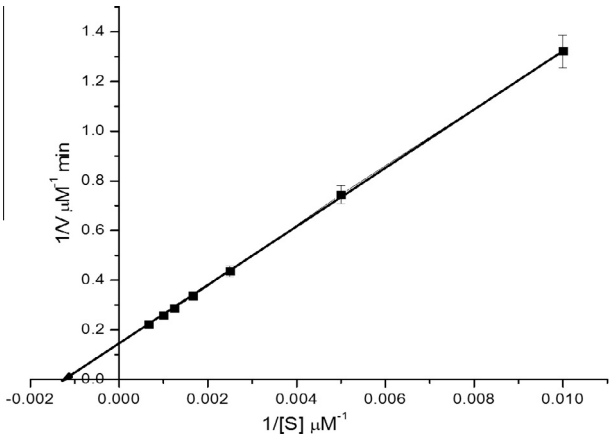

Fig. 4. Lineweaver–Burk plot for DhlA. Enzyme (0.5 μM) was incubated with 1,2-DCA (0.05–10 mM) at 37 °C and pH 9.0 for 30 min. The reciprocals of rate of substrate hydrolysis (1/*V*) were plotted against the reciprocals of the substrate concentrations (1/[*S*]), and the K_m values were determined by fitting the resulting data using ORIGIN 8 pro (evaluation version).

Table 3
Kinetic and predicted biophysical properties of DhlA.

Kinetic property	Value
K_m (μM)	842
V_{max} (μM/min)	7.1
k_{cat} (min^{-1}) = V_{max}/Et	142
Catalytic efficiency (k_{cat}/K_m) (mM^{-1} min^{-1})	168
Predicted biophysical properties	
Number of amino acids	310
Molecular mass (Da)	35144
pI	4.65
Total negatively charged residues	44
Total positively charged residues	27
Extinction coefficient (if all Cys reduced)	49,390 M^{-1} cm^{-1}
Instability index	41.8 (classified as instable)

k_{cat}/K_m (selectivity) and a higher value will lead to lower steady state substrate concentration and improved removal in bioreactors [23]. Both in natural environments and in treatment systems, the production of increased levels of DhlA with higher affinity would yield reduced concentrations of toxicants [23]. DhlA from *A. aquaticus* AD25 indicate the imperfect nature of the enzyme with the k_{cat} of only 6 s^{-1}, and the k_{cat}/K_m of 85,000 mM^{-1} min^{-1} for 1,2-DCA dehalogenation [13]. This is significantly higher than the K_s value of the organism, which indicates that the rate of 1,2-DCA conversion obtained at a substrate concentration equal to the K_m of the enzyme is higher than the rate needed for growth at $0.5_{\mu max}$. The k_{cat} for 1,2-DCA in this study was shown to be 2.36 s^{-1}, and the k_{cat}/K_m is 168 mM^{-1} min^{-1} which is much lower than the previously reported data [13]. This means that the recombinant DhlA characterised in this study is more ideal for 1,2-DCA degradation. Furthermore, the information on the K_m and catalytic efficiency of the DhlA can be useful in engineering the enzyme for industrial applications. Rate acceleration towards model compounds has been achieved by modifying the substrate binding site in DhlA. The halide release is the rate limiting step in the catalytic cycle of DhlA for some substrate; therefore mutations that remove cap domain interactions can increase the rate of the enzyme activity by facilitating the conformational change which allows halide dissociation from the active site [24].

Comparison of amino acid sequence homology

Homologues of DhlA were searched using BLASTP in NCBI (non-redundant) database [25–27]. DhlA (NCBI: ACL93467.1) protein sequence homology shows that this enzyme has evolved very distinctly i.e. from other species like *X. autotrophicus* (Fig. 5). Among the other reported DhlA, maximum similarity (97% identity) was found with DHL from *X. autotrophicus* and *A. aquaticus* [28–29]. The recombinant DhlA characterised in this study also showed 64% identity to haloalkane dehalogenase from *Vibrio* sp. HENC-03 (Accession No. WP 009704546.1) and 54% identity to putative DhlA from uncultured bacterium WWRS-2005 (Accession No. AAZ48944.1). The enzyme also showed 52% and 51% identity to the DhlA from *Congregibacter litoralis* KT71 and *Erythrobacter litoralis* HTCC2594, respectively. Hypothetical proteins from *Microbulbifer variabilis* (Accession No. WP 020415083.1), *Blastomonas* sp. AAP53 (Accession No. WP 017670922.1) and *Actinoplanes globisporus* (Accession No. WP 020518532.1) also have about 50% similarity to the DhlA in this study.

Template based structure and prediction of the catalytic mechanism

A 1.15 Å structure of DHL from *X. autotrophicus* (PDB: 1B6G) was used as a template for homology modelling. The model

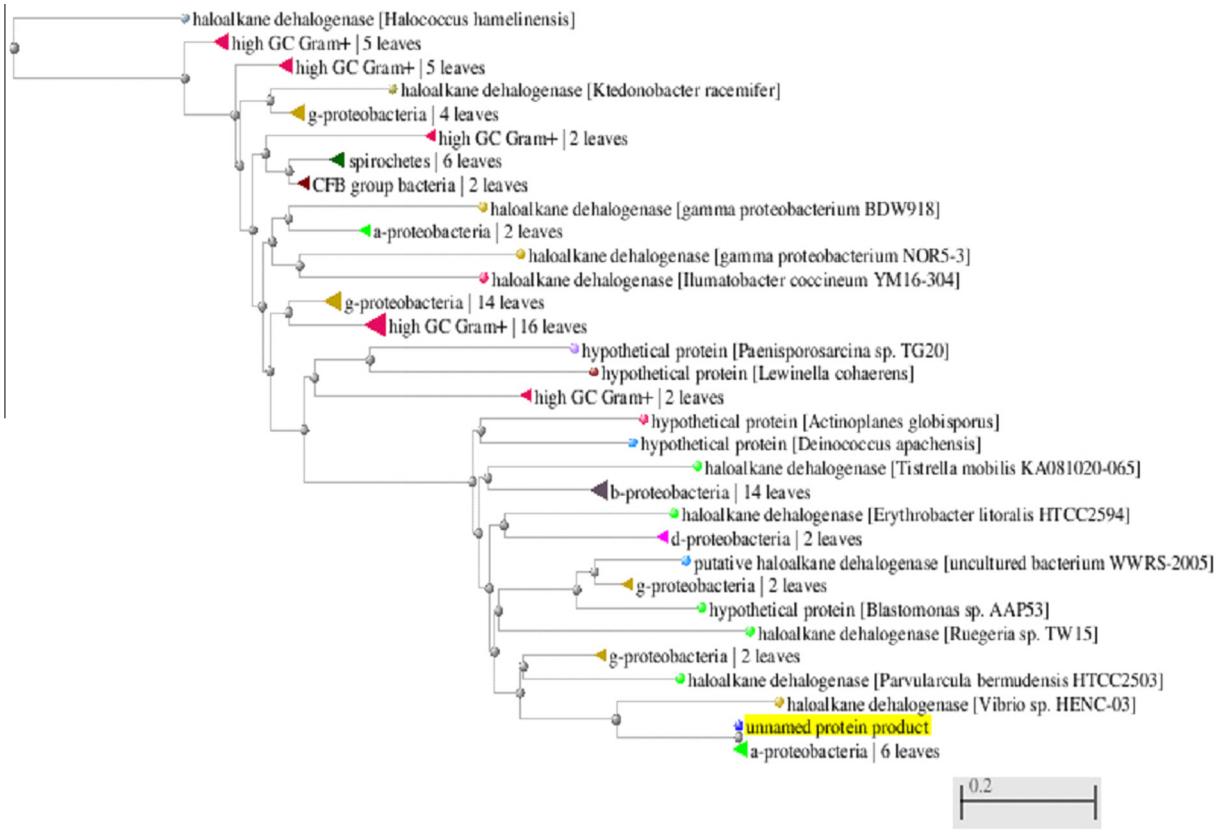

Fig. 5. NCBI protein sequence blasted distance tree for DhlA. Highlighted with yellow colour is the DhlA in this study. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

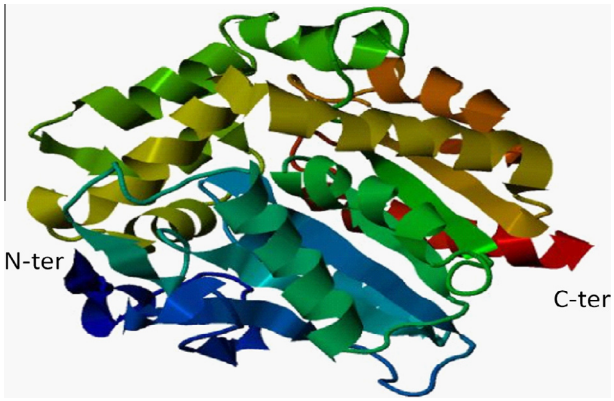

Fig. 6. Predicted tertiary structure of DhlA (from *Ancylobacter aquaticus* UV5 strain) deduced from PDB 1B6G (1.15 Å structure of DHL from *Xanthobacter autotrophicus*) and N- and C-terminal domains of the monomers are marked. α-Helices and β-sheets are presented in different colours. The model was built by homology modelling at SWISS-MODEL workspace using DhlA amino acid sequence (from *Ancylobacter aquaticus* UV5 strain) as the template. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

showed 97% sequence identity with the purified recombinant DhlA in this study (Figs. 6 and 7). All the residues involved in the catalytic site can be predicted from the structure as both 1B6G. Crystal structures are available for both the wild and mutant type DhlA [28–36]. DhlA showed a conserved domain for α/β hydrolase family which consist of a core domain with a canonical α/β-hydrolase fold and the cap domain with a principal helical structure [36–37]. The active site lies between the core domain and the cap domain and is formed by a catalytic pentad composed of a catalytic triad and two halide binding residues [38]. The active site is a blocked cavity located between these two domains and the catalysis is mediated by an Asp-His-Asp catalytic triad, functionally similar to the classical catalytic triad in serine proteases [32]. However, the nucleophilic amino acid is an aspartate rather than a serine. One of the oxygen molecules of the aspartate carboxylate group attacks the carbon atom to which the halogen is bound, replacing it by an SN_2 substitution mechanism. Two tryptophan residues, Trp125 and Trp175, form a halide-binding site and are involved in leaving group (chloride) stabilisation during this step [38]. The resulting covalent intermediate is an ester where the oxygen is provided by the enzyme, unlike in serine hydrolases, where the carbonyl oxygen of the ester intermediate comes from the substrate. The covalent alkyl enzyme intermediate, to which the halide is still bound, is hydrolysed by a water molecule that is activated by His289. The role of the second aspartic acid of the catalytic triad (Asp260) is to stabilise the positive charge that develops on His289 during hydrolysis of the ester, but it also has a structural role. The non-reacting carboxylate oxygen of Asp124 remains hydrogen bonded to the main chain amides of Glu56 and Trp125, which form an oxyanion hole [32]. The last step is release of halide from the active site [39]. This catalytic cycle was identified by soaking dehalogenase crystals with substrate at different pH values and solving the X-ray structures of the intermediates that accumulated [39].

```
DhlA.fasta                  MINAIRTPDQRFSNLDQYPFSPNYLDDLPGYPGLRAHYLD   40
1B6G_A|PDBID|CHAIN|SEQUENCE MVNAIRTPDQRFSNLDQYPFSPNYLDDLPGYPGLRAHYLD   40
Consensus                   m nairtpdqrfsnldqypfspnylddlpgypglrahyld

DhlA.fasta                  EGNSDAEDVFLCLHGEPTWSYLYRKMIPVFAESGARVIAP   80
1B6G_A|PDBID|CHAIN|SEQUENCE EGNSDAEDVFLCLHGEPTWSYLYRKMIPVFAESGARVIAP   80
Consensus                   egnsdaedvflclhgeptwsylyrkmipvfaesgarviap

DhlA.fasta                  DFFGFGKSDKPVDEEDYTFEFHRNFLLALIERLDLRNITL  120
1B6G_A|PDBID|CHAIN|SEQUENCE DFFGFGKSDKPVDEEDYTFEFHRNFLLALIERLDLRNITL  120
Consensus                   dffgfgksdkpvdeedytfefhrnfllalierldlrnitl

DhlA.fasta                  VVQDWGGFLGLTLPMADPSRFKRLIIMNACLMTDPVTQPA  160
1B6G_A|PDBID|CHAIN|SEQUENCE VVQDWGGFLGLTLPMADPSRFKRLIIMNACLMTDPVTQPA  160
Consensus                   vvqdwggflgltlpmadpsrfkrliimnaclmtdpvtqpa

DhlA.fasta                  FSAFVTQPADGFTAWKYDLVTPSDLRLDQFMKRWAPTLTE  200
1B6G_A|PDBID|CHAIN|SEQUENCE FSAFVTQPADGFTAWKYDLVTPSDLRLDQFMKRWAPTLTE  200
Consensus                   fsafvtqpadgftawkydlvtpsdlrldqfmkrwaptlte

DhlA.fasta                  AEASAYAAPFPDTSYQAGVRKFPKMVAQRDQACIDISTEA  240
1B6G_A|PDBID|CHAIN|SEQUENCE AEASAYAAPFPDTSYQAGVRKFPKMVAQRDQACIDISTEA  240
Consensus                   aeasayaapfpdtsyqagvrkfpkmvaqrdqacidistea

DhlA.fasta                  ISFWQNDWNGQTFMAIGMKDKLLGPDVMYPMKALINGCPE  280
1B6G_A|PDBID|CHAIN|SEQUENCE ISFWQNDWNGQTFMAIGMKDKLLGPDVMYPMKALINGCPE  280
Consensus                   isfwqndwngqtfmaigmkdkllgpdvmypmkalingcpe

DhlA.fasta                  PLEIADAGHFVQEFGEQVAREALKHFAETE            310
1B6G_A|PDBID|CHAIN|SEQUENCE PLEIADAGHFVQEFGEQVAREALKHFAETE            310
Consensus                   pleiadaghfvqefgeqvarealkhfaete
```

Fig. 7. CLUSTAL O (1.1.0) multiple protein sequence alignment for DhlA (shown as dhlA.fasta) with 1B6G (shown as 1B6G_A|PDBID|CHAIN|SEQUENCE). The sequence homology of amino acids (the degree of similarity between sequences of amino acids) is highlighted in yellow colour. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Predicted biophysical properties of DhlA

Biophysical properties of the DhlA (Table 3) as determined using the ExPASy server show an apparent M_r of 35 kDa with a theoretical pI of 4.65. The acidic nature of the protein is congruent with a higher content of acidic amino acids than basic amino acids. The instability index of DhlA is computed to be 41.8 and is classified as unstable. DhlA showed high content of aromatic amino acids (Trp, Tyr), resulting in high extinction coefficient value (49390 M^{-1} cm^{-1}). The aromatic amino acids like Trp, Tyr, Phe significantly contribute to the three-dimensional structure of proteins. Thus, the high number of aromatic amino acids in DhlA as compared to other amino acids suggests that the DhlA structure is highly hydrophobic in nature.

Conclusion

DhlA from a recently isolated *A. aquaticus* UV5 strain was cloned, overexpressed, purified and characterised to have a detailed understanding of the mechanism of 1,2-DCA degradation by this organism. Along with characterising the enzyme, three-dimensional structure of DhlA was predicted by using bioinformatics tools to have an insight into its catalytic mechanism. DhlA from this organism was found to belong to the α/β hydrolase family containing a catalytic triad composed of Asp-His-Asp in the active site as determined by accessing the reported catalytic sites of very closely related Dhls from other species. This is the first report on the reaction kinetics of purified DhlA from *A. aquaticus* strain indigenous to contaminated sites in Africa and may be helpful in further exploration of catalytic mechanisms for possible exploitation in bioremediation strategies.

Acknowledgments

This study was supported by the National Research Foundation (NRF), South Africa. The post-doctoral research fellowship awarded to Ajit Kumar by the University of KwaZulu-Natal is acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pep.2014.03.003.

References

[1] D.B. Janssen, Biocatalysis by dehalogenating enzymes, Adv. Appl. Microbiol. 61 (2007) 233–252.
[2] Z. Prokop, Y. Sato, J. Brezovsky, T. Mozga, et al., Enantio selectivity of haloalkane dehalogenases and its modulation by surface loop engineering, Angew. Chem. Int. Ed. Engl. 49 (2010) 6111–6115.
[3] Y. Sato, M. Monincova, R. Chaloupkova, Z. Prokop, Y. Ohtsubo, K. Minamisawa, M. Tsuda, J. Damborsky, Y. Nagata, Two rhizobial strains, *Mesorhizobium loti* MAFF303099 and *Bradyrhizobium japonicum* USDA110, encode haloalkane dehalogenases with novel structures and substrate specificities, Appl. Environ. Microbiol. 71 (2005) 4372–4379.
[4] D.B. Janssen, Evolving haloalkane dehalogenases, Curr. Opin. Chem. Biol. 8 (2004) 150–159.
[5] D.B. Janssen, D. Jager, B. Witholt, Degradation of n-haloalkanes and α, ω-dihaloalkanes by wild-type and mutants of *Acinetobacter* sp. strain G J70, Appl. Environ. Microbiol. 53 (1987) 561–566.
[6] D.B. Janssen, S. Keuning, B. Witholt, Involvement of a quinoprotein alcohol dehydrogenase and an NAD-dependent aldehyde dehydrogenase in 2-chloroethanol metabolism in *Xanthobacter autotrophicus* GJ10, J. Gen. Microbiol. 133 (1987) 85–92.
[7] D.B. Janssen, E. Van der Pries, J. Ploeg, B. Kazemier, P. Terpstra, B. Witholt, Cloning of 1,2-dichloroethane degradation genes of *Xanthobacter autotrophicus* GJ10, and expression and sequencing of the dhlA gene, J. Bacteriol. 171 (1989) 6791–6799.
[8] D.B. Janssen, A. Scheper, B.W. Witholt, Biodegradation of 2-chloroethanol and 1,2-dichloroethane by pure bacterial cultures, in: E.H. Houwink, R.R. Van der Meer (Eds.), Innovations in Biotechnology, Elsevier, Amsterdam, 1984, pp. 169–178.
[9] D.B. Janssen, A. Scheper, L. Dijkhuizen, B. Witholt, Degradation of halogenated aliphatic compounds by *Xanthobacter autotrophicus* GJ10, Appl. Environ. Microbiol. 49 (1985) 673–677.
[10] T. Koudelakova, E. Chovancova, J. Brezovsky, M. Monincova, A. Fortova, J. Jarkovsky, J. Damborsky, Substrate specificity of haloalkane dehalogenases, Biochem. J. 435 (2011) 345–354.
[11] E. Chovancova, J. Kosinski, J.M. Bujnicki, J. Damborsky, Phylogenetic analysis of haloalkane dehalogenases, Proteins 67 (2) (2007) 305–316.
[12] A.J. Van den Wijngaard, J. Prins, A.J.A.C. Smal, D.B. Janssen, Microbial growth on 2-chloroethylvinylether by *Ancylobacter aquaticus* AD25 and AD27, Appl. Environ. Microbiol. 59 (1993) 2777–2783.
[13] A.J. Van den Wijngaard, R.D. Wind, D.B. Janssen, Kinetics of bacterial growth on chlorinated aliphatic compounds, Appl. Environ. Microbiol. 59 (1993) 2041–2048.
[14] R. Scholtz, A. Schmuckle, A.M. Cook, T. Leisinger, Degradation of eighteen 1-monohaloalkanes by *Arthrobacter* sp. strain HA1, J. Gen. Microbiol. 13 (3) (1987) 267–274.
[15] A. Govender, B. Pillay, Characterization of 1,2-dichloroethane (DCA) degrading bacteria isolated from South African waste water, Afr. J. Biotechnol. 10 (55) (2011) 11567–11573.
[16] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[17] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.
[18] K. Arnold, L. Bordoli, J. Kopp, T. Schwede, The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling, Bioinformatics 22 (2006) 195–201.
[19] T. Schwede, J. Kopp, N. Guex, M.C. Peitsch, SWISS-MODEL: an automated protein homology-modeling server, Nucleic Acids Res. 31 (2003) 3381–3385.
[20] N. Guex, M.C. Peitsch, SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modelling, Electrophoresis 18 (1997) 2714–2723.
[21] S. Keuning, D.B. Janssen, B. Witholt, Purification and characterization of hydrolytic haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10, J. Bacteriol. 163 (1985) 635–639.
[22] A.J. Van den Wijngaard, K. van der Kamp, J. van der Ploeg, B. Kazemier, F. Pries, D.B. Janssen, Degradation of 1,2-dichloroethane by facultative methylotrophic bacteria, Appl. Environ. Microbiol. 58 (1992) 976–998.
[23] D.B. Janssen, J.R. van der Ploeg, F. Pries, Genetics and biochemistry of 1,2-dichloroethane degradation, Biodegradation 5 (1994) 249–257.
[24] J.P. Schanstra, I.S. Ridder, G.J. Heimeriks, R. Rink, G.J. Poelarends, K.H. Kalk, B.W. Dijkstra, D.B. Janssen, Kinetic characterization and X-ray structure of a mutant of haloalkane dehalogenase with higher catalytic activity and modified substrate range, Biochemistry 35 (1996) 13186–13195.
[25] S.F. Altschul, J.C. Wootton, E.M. Gertz, R. Agarwala, A. Morgulis, A.A. Schaffer, Y.K. Yu, Protein database searches using compositionally adjusted substitution matrices, FEBS J. 272 (2005) 5101–5109.
[26] S.F. Altschul, T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.
[27] H.J. Rozeboom, J. Kingma, D.B. Janssen, B.W. Dijkstra, Crystallization of haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10, J. Mol. Biol. 200 (3) (1988) 611–612.
[28] I.S. Ridder, H.J. Rozeboom, B.W. Dijkstra, Haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 refined at 1.15 Å resolution, Acta Crystallogr. D Biol. Crystallogr. 55 (7) (1999) 1273–1290.
[29] X. Liu, B.L. Hanson, P. Langan, R.E. Viola, The effect of deuteration on protein structure: a high-resolution comparison of hydrogenous and perdeuterated haloalkane dehalogenase, Acta Crystallogr. D Biol. Crystallogr. 63 (9) (2007) 1000–1008.
[30] K.H. Verschueren, S.M. Franken, H.J. Rozeboom, K.H. Kalk, B.W. Dijkstra, Refined X-ray structures of haloalkane dehalogenase at pH 6.2 and pH 8.2 and implications for the reaction mechanism, J. Mol. Biol. 232 (3) (1993) 856–872.
[31] K.H. Verschueren, F. Seljee, H.J. Rozeboom, K.H. Kalk, B.W. Dijkstra, Crystallographic analysis of the catalytic mechanism of haloalkane dehalogenase, Nature 363 (6431) (1993) 693–698.
[32] A.J. Oakley, M. Klvana, M. Otyepka, Y. Nagata, M.C. Wilce, J. Damborsky, Crystal structure of haloalkane dehalogenase LinB from *Sphingomonas paucimobilis* UT26 at 0.95 Å resolution: dynamics of catalytic residues, Biochemistry 43 (4) (2004) 870–878.
[33] J. Newman, T.S. Peat, R. Richard, L. Kan, P.E. Swanson, J.A. Affholter, I.H. Holmes, J.F. Schindler, C.J. Unkefer, T.C. Terwilliger, Haloalkane dehalogenases: structure of a *Rhodococcus* enzyme, Biochemistry 38 (49) (1999) 16105–16114.
[34] J. Marek, J. Vevodova, I.K. Smatanova, Y. Nagata, L.A. Svensson, J. Newman, M. Takagi, J. Damborsky, Crystal structure of the haloalkane dehalogenase from *Sphingomonas paucimobilis* UT26, Biochemistry 39 (46) (2000) 14082–14086.
[35] S.M. Franken, H.J. Rozeboom, K.H. Kalk, B.W. Dijkstra, Crystal structure of haloalkane dehalogenase: an enzyme to detoxify halogenated alkanes, EMBO J. 10 (6) (1991) 1297–1302.
[36] D.L. Ollis, E. Cheah, M. Cygler, B. Dijkstra, F. Frolow, S.M. Franken, M. Harel, S.J. Remington, I. Silman, J. Schrag, J.L. Sussman, K.H.G. Verschueren, A. Goldman, The α/β hydrolase fold, Protein Eng. 5 (1992) 197–211.

[37] M. Silberstein, J. Damborsky, S. Vajda, Exploring the binding sites of the haloalkane dehalogenase DhlA from *Xanthobacter autotrophicus* GJ10, Biochemistry 46 (32) (2007) 9239–9249.
[38] G.H. Krooshof, I.S. Ridder, A.W. Tepper, G.J. Vos, H.J. Rozeboom, K.H. Kalk, B.W. Dijkstra, D.B. Janssen, Kinetic analysis and X-ray structure of haloalkane dehalogenase with a modified halide-binding site, Biochemistry 37 (1998) 15013–15023.
[39] E.Y. Lau, K. Kahn, P.A. Bash, T.C. Bruice, The importance of reactant positioning in enzyme catalysis: a hybrid quantum mechanics/molecular mechanics study of a haloalkane dehalogenase, Proc. Natl. Acad. Sci. USA 97 (2000) 9937–9942.