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# DEGRADATION OF MILLIMOLAR CONCENTRATION OF THE HERBICIDE DALAPON (2,2-DICHLOROPROPIONIC ACID) BY *RHIZOBIUM* SP. ISOLATED FROM SOIL

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## ABSTRACT
*The herbicide Dalapon is widely used in agricultural areas and is persistent in ground water. A Rhizobium sp. was able to grow at 0.2 mM 2,2-dichloropropionic acid (2,2DCP), which was 100-fold lower than the concentration of the substrate routinely used. Apparently, no new dehalogenases are required to allow growth on this low concentration of 2,2DCP as judged by electrophoretic mobility of dehalogenase proteins in native-PAGE analysis and protein separation by anion-exchange column chromatography. The kinetic analysis suggested that the known dehalogenases were able to act efficiently on low concentrations of haloalkanoic acids. The amount of each dehalogenase, from cells grown on low substrate concentration was different compared to that seen at 20 mM 2,2DCP due to complex regulatory controls, which respond to the growth environment.*

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**Keywords:** degradation, low substrate, oligotrophs, halogenated, Dalapon, 2,2DCP, dehalogenase

## Introduction
The study of oligotrophs and slow-growing organisms is an important area in environmental microbiology (19). Oligotrophs are ubiquitous in the environment and have been isolated from soil, rivers, lakes, oceans and tap water lacking organic substances (26). Two types of oligotrophs have been identified. Those that are able to grow only on a very low concentration of carbon are called obligate oligotrophs and those that are able grow at both low and high concentrations of organic substances are called facultative oligotrophs.

A detailed study was carried out on the enteric bacterium *Enterococcus faecalis*. The natural habitat of this bacterium is in the intestine of humans and animals. The ability of *Enterococcus faecalis* to survive in an oligotrophic environment such as in tap water was shown (9). Many oligotrophic bacteria can be isolated from clinical materials including urine, sputum and vaginal discharges. However, the clinical significance of such oligotrophic bacteria is uncertain (25).

The slow growth of oligotrophic bacteria is possibly due to the slow process of transport of nutrients into the cell. Dissolved nutrients entering the aquatic environment are assimilated into microorganisms due to permeability and active transport through the cytoplasmic membrane. In some oligotrophic bacteria there is a cellular appendage (prostheca) whose function is to increase the surface area of the cell and cytoplasmic membrane for increasing the transport of substances into a cell. A biochemical study on the prostheca of *Asticcacaulis biprosthecum* revealed that it did not contain DNA, RNA, NADH-oxidase and NADH-dehydrogenase. This highlights the specialised function of prostheca connected with the active transport of nutrients (13). Some oligotrophic bacteria show inhibition of growth in rich nutrient media (16). One reason may be the action of toxic metabolic products, particularly hydrogen peroxide which forms in a number of metabolic reactions.

Xenobiotic chemicals that enter the environment are mostly from chlorinated aliphatic acid compounds. Many of these pollutants are present in natural waters only at very low concentrations. However, such concentrations are still sufficient to be a public health and environmental concern (17). Biodegradation of low substrate concentrations of xenobiotic compounds has been a neglected field of research. However, some of the reported cases are the degradation of herbicide (2,4-dichlorophenoxy acetic acid) at 2.2 parts per billion and mineralisation of nanomolar concentrations of herbicide dichlobenil and its persistent metabolite 2,6-dichlorobenzamide by *Aminobacter* spp. (21). In addition, the carbon of very low concentrations of xenobiotics is often not converted to biomas carbon, thus other carbon sources may be required for cell growth under these conditions (23).

The bacterial degradation of low concentrations of 1,3-dichloro-2-propanol (1,3-DCP) has been reported. The isolate was identified as *Agrobacterium* sp., which was able to dehalogenate 1,3-DCP with a Km value of 0.1 mM (7). However, to the best of our knowledge, there is no report in the current literature for degradation of low concentration of haloalkanoic aliphatic acids and whether it involves a new kind of dehalogenase enzyme.

*Rhizobium* sp. was reported to grow on 2,2-dichloropropionic acid (2,2DCP), D,L-2-chloropropionic acid (D,L2-CP) and D,L-2-bromopropionic acid (D,L2-BP) as sole sources of carbon and energy (2). However, growth did not occur on 2,2DCP and D,L2-CP at concentrations in excess of 50 mM or 20 mM respectively,

suggesting toxicity of these compounds to the organism (17). The normal 2,2DCP concentration used to grow *Rhizobium* sp. is 20 mM. Most of the dehalogenase producing bacteria contain more than one dehalogenases. The only organism so far reported to make all three forms of dehalogenase is a *Rhizobium* sp. (18). Dehalogenase L (DehL) has been shown to be specific for the L-isomer monochloropropionate (L2-CP) and also to act on dichloroacetate (DCA) but not on 2,2DCP. Dehalogenase E (DehE) is a non-stereospecific dehalogenase acting on D,L2-CP, 2,2DCP, DCA, monochloroacetate (MCA) and trichloroacetate (TCA) (5, 11, 22). Dehalogenase D (DehD) has been shown to act only on D-isomer monochloropropionate (D2-CP) and MCA with no activity towards 2,2DCP or DCA. For each dehalogenase, the lactate product from D,L2-CP has the opposite stereochemical form to that of the substrate (18).

The aim of the present study was to investigate the ability of *Rhizobium* sp. to grow at millimolar concentration of 2,2DCP and to determine whether growth at this substrate concentration involves new kind(s) of dehalogenase enzyme. To the best of our knowledge, this is the first reported case showing degradation of haloaliphatic acid at low substrate concentration.

## Materials and Methods

### Cell growth

The cell inoculum was prepared by growing *Rhizobium* sp. in 20 mM lactate minimal medium. Then 15 ml of overnight culture were centrifuged and the cells washed twice with minimal medium before inoculation into 100 ml minimal medium supplied with 0.2 mM of 2,2DCP. Growth was monitored by measuring the amount of chloride ions released at appropriate time intervals (3). An uninoculated flask treated in the same way was used as a control. This is important in order to make sure the chloride measured in the growth medium was due to the cells' using the 2,2DCP rather than to the auto-degradation of the substrate in the growth medium.

For larger scale preparation (for use in enzyme analysis), cells were grown in 8 flasks each, with 500 ml of medium containing 0.2 mM 2,2DCP as a carbon source. As a control *Rhizobium* sp. grown in 20 mM 2,2DCP was also prepared for use in further analysis.

### Assay for halide ion

Measurement of free halide released during the dehalogenation reaction was carried out by an adaptation of the method of Bergman and Sanik (3). Sample (1 ml) was added into 100 µl of 0.25 M ammonium ferric sulphate in 9 M nitric acid and mixed thoroughly. To this was added 100 µl mercuric thiocyanate-saturated ethanol and the solution was mixed by vortexing. The colour was allowed to develop for 10 min and measurements were made at $A_{460nm}$ in a Pye-Unicam SP1750 Series spectrophotometer. Halide concentration was determined by comparing the absorbance of the test sample against a standard curve of known concentrations of halide.

### Preparation of cell-free extracts

Cell-free extracts were prepared from bacterial cells in mid- to late-exponential phase of growth. Cells from 100 ml culture were harvested by centrifugation at 10,000 *g* for 10 min at 4 °C. The cell pellets were resuspended in 20 ml of 0.1 M Tris-acetate buffer, pH 7.6, and centrifuged at 10,000 *g* for 10 min at 4 °C. The cells were then resuspended in 4 ml of 0.1 M Tris-acetate buffer, pH 7.6, and maintained at 0 °C for ultrasonication in an MSE Soniprep 150W ultrasonic disintegrator at a peak amplitude $\lambda$ = 10 µm for 30 s. Sonication of *Rhizobium* cell suspensions was generally carried out for three 30 s periods, with 30 s cooling between each sonication. Unbroken cells and cell wall material were removed by centrifugation at 20,000 *g* for 15 min at 4 °C. Cell-free extracts were ultracentrifuged at 120,000 *g* for 90 min at 4 °C for use in protein purification.

### Preparation of DehE, DehL and DehD

#### Preparation of cell-free extracts containing DehE

*Escherichia coli* strain BL21 (DE3) carrying plasmid (pJS771) containing the *dehE* gene was grown aerobically in a 100 ml LB/amp overnight at 30 °C. Then 15 ml to 20 ml of the culture ($A_{680\,nm}$ = 1.60) was transfered into 500 ml LB/amp in a 2 L flask. This was incubated on an orbital shaker at 30 °C. Absorbance was checked periodically until $A_{680\,nm}$ = 0.3 - 0.4, when 50 µl of 0.1 M IPTG was added to give a final concentration of 0.01 mM. The cell culture was then incubated at 20 °C overnight before harvesting by centrifugation at 10,000 *g* for 15 min at 4 °C. The cell pellets were washed in 20 ml of 0.1 M Tris-acetate buffer, pH 7.6, and centrifuged as before to recover the pellet. The supernatant was discarded and the pellet was kept at -20 °C until use. The extract was prepared as described before.

#### Preparation of cell-free extracts containing DehL and DehD

*Escherichia coli* K-12 strain NM522 carrying the plasmid pSC3 or pSC4 the containing *dehD* and *dehL* gene, respectively, were grown in a 10 ml LB/amp culture overnight at 37 °C. Then cells from 2 ml of the culture were washed with sterile PJC before inoculating into 20 mM D,L2-CP minimal medium containing 0.05 % yeast extract plus 0.3 mM IPTG. Cells were grown at 30 °C and harvested at late logarithmic phase ($A_{680nm}$ 0.7 to 0.8). Cells were harvested and the extracts prepared as already described.

### Assay for dehalogenase activity

Dehalogenase activity was determined as total chloride released at 30 °C in an incubation mixture containing 0.1 M Tris-acetate buffer (pH 7.6) (4700 µl), 0.1 M halogenated aliphatic acid (50 µl) and distilled water and enzyme to a final volume of 5000 µl. Following 5 min equilibration at 30 °C, the reaction was initiated by adding cell-free extract. Samples (1.0 ml) were removed at appropriate intervals and assayed for halide ions.

### Non-denaturing polyacrylamide gel electrophoresis

In non-denaturing PAGE, the enzyme extract remains active. Gels were prepared based on the method of Hardman and Slater (8). Resolving gels containing 12 % bis-acrylamide in 375 mM $Tris.SO_4$ (pH 8.8) were polymerized by the addition of 0.05 % ammonium persulphate and 0.05 % TEMED. Stacking gels were formed from 4 % bis-acrylamide in 125 mM $Tris.SO_4$ (pH 6.8).

Gels were left overnight at 4 °C before being used to allow the ammonium persulphate to decompose completely. Gels

were run using a Mini-Protean II gel system from Biorad in 25 mM Tris, 19 mM glycine buffer (pH 8.3) at a constant voltage of 200 V and temperature 4 °C for 1 h. Samples were prepared as in the case of cell extracts and mixed with 0.1 volumes of sample buffer (0.1 % bromophenol blue; 10 % glycerol; 100 mM DTT in 50 mM Tris-acetate, pH 6.8). The gel was run until the dye front reached the bottom of the gel. Gels were then stained for dehalogenase activity.

**Dehalogenase activity staining**

The gel was incubated in 50 mM Cl-free halogenated substrate for 15 min at 30 °C. The substrate was carefully removed and the gel was then placed in a 0.1 M $AgNO_3$ solution and incubated in the dark until bands appeared on the gel due to the precipitation of AgCl. The gel was then washed with distilled water to remove the $AgNO_3$ and fixed by washing in 5 % acetic acid for 10 min. The gel was finally washed with distilled water and stored in the dark and photographed as required.

**Fast protein liquid chromatography (FPLC) – Pharmacia system**

Protein purifications were carried out at 4 °C in a cold room. Anion exchange chromatography (MonoQ HR 5/5) was performed using a linearly increasing gradient of phosphate ions. The FPLC system used two buffers: one containing low salt concentration (Buffer A) and the other containing high salt concentration (Buffer B). Depending on the dehalogenase buffer A contained 5 mM or 20 mM phosphate buffer (pH 8), 1 mM EDTA, 10 % glycerol, 1 mM DTT, and buffer B contained 100 mM or 200 mM phosphate buffer, 1 mM EDTA, 10 % glycerol, 1 mM DTT. These buffers are then mixed via a gradient controller to give a linear gradient of 5 mM to 100 mM or 20 mM to 200 mM of phosphate. Samples for purification were prepared as cell-free extracts and approximately 5 mg of protein were applied to the MonoQ column per run. The column was run at a flow rate of 1 ml/min. Fractions (usually 1 ml) were collected and then assayed to determine which contained maximal dehalogenase activity. Active fractions were stored in ice until use.

## Results and Discussion

**Growth of *Rhizobium* sp. on 0.2 mM 2,2-dichloropropionate (2,2DCP)**

To our knowledge, there is only one reported case associated with hydrolytic dehalogenation which involves the genus *Rhizobium*. The halidohydrolase from this organism was able to attack chlorosubstituted *s*-triazine (4). Rhizobia is a collective name of the genera *Rhizobium, Sinorhizobium, Mesorhizobium* and *Bradyrhizobium*. *Agrobacterium* sp. from the *Rhizobium* subdivision was reported capable of complete mineralisation of 2,3-dichloro-1-propanol (6). Generally, it is quite rare for organisms from *Rhizobium* sp. to be involved in hydrolytic dehalogenation. However, this kind of organism is always associated with bacteria of agronomic importance because they form nitrogen-fixing symbioses with leguminous plants and the majority of genes for nitrogen fixing symbioses seem to be present on the chromosome of *Mesorhizobium* and *Bradyrhizobium* (14, 15, 24).

An initial experiment was carried out to establish whether *Rhizobium* sp. was able to grow on 0.2 mM 2,2DCP. A typical growth was measured with a doubling time of approximately 12 h. It was obtained that *Rhizobium* sp. grown on 20 mM 2,2DCP has similar behaviour to that at low concentration, with a doubling time of approximately 11 h, which was more or less the same doubling time for growth at low concentration. The time for harvesting cells was set between mid and late logarithmic phase similar to that for growth at low concentration.

This study established the ability of *Rhizobium* sp. to grow on low concentration of substrate, 0.2 mM 2,2DCP, 100x lower than the normal growth concentration. 2,2DCP was chosen because it is widely used as a herbicide (Dalapon). *Rhizobium* sp. was able to grow on 0.2 mM 2,2DCP with a doubling time of approximately 12 h, similar to the growth rate obtained at 20 mM 2,2DCP, indicating that the organism could be considered as a facultative oligotroph.

**Analysis of cell-free extract from 0.2 mM 2,2DCP-grown bacteria by enzyme assay**

**Table 1** shows the dehalogenase specific activity for crude extracts prepared from cells grown on 0.2 mM 2,2DCP and on 20 mM 2,2DCP. The substrates chosen were based on the specificity of the three dehalogenases the organism is known to produce. Dibromoacetate (DBA) is a substrate for DehL and DehE; 2,2DCP is a substrate for DehE; MCA is a substrate for DehD and DehE; and D,L2-CP is a substrate for all three dehalogenases. The results suggested that similar dehalogenase enzymes (DehL, DehE and DehD) were produced under the two growth conditions. The dehalogenase specific activity using DBA as substrate for cells grown in 20 mM 2,2DCP was twice that seen for growth at 0.2 mM 2,2DCP. Since from the 2,2DCP measurement the amount of DehE in each growth was

**TABLE 1.**

Dehalogenase specific activity for crude extracts from cells grown at low and high concentration

| Substrates | 0.2 mM 2,2DCP-grown bacteria (µmol$Cl^-$/min/mg protein) | 20 mM 2,2DCP-grown bacteria (µmol$Cl^-$/min/mg protein) |
|---|---|---|
| Dibromoacetate (DBA) | 0.66 ± 0.01 | 1.40 ± 0.02 |
| 2,2-dichloropropionate (2,2DCP) | 0.065 ± 0.01 | 0.055 ± 0.01 |
| Monochloroacetate(MCA) | 0.13 ± 0.03 | 0.11 ± 0.01 |
| D,L-2-chloropropionate (D,L2-CP) | 0.22 ± 0.01 | 0.23 ± 0.02 |

Values are the means of triplicate measurements.

the same, we assumed that there must be more DehL in the cells grown on 20 mM 2,2DCP.

Such assays of crude extract may not show the presence of any additional dehalogenase(s) in cells grown on low concentration, so the crude extracts were checked by native-PAGE analysis.

**Separation of DehL, DehE and DehD by native-PAGE**

The gel electrophoresis technique provides an efficient way for separating mixtures of proteins with different electrophoretic mobility to enable extracts to be analysed. The separation depends on the size of the protein and the charge of the protein. However, proteins with similar charge and size will not be separated by this procedure.

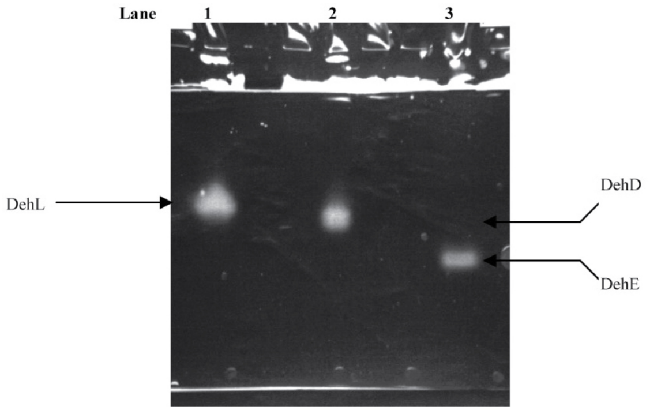


**Fig. 1.** Native-PAGE analysis of dehalogenase activity. Lane 1: DehL; Lane 2: DehD; Lane 3: DehE.

**TABLE 2.**

Estimated charge for dehalogenase enzymes from amino acid composition

| | **DehL** | **DehE** | **DehD** |
|---|---|---|---|
| **Amino acid** | **Residues** | **Residues** | **Residues** |
| ARG | 26 | 21 | 30 |
| LYS | 8 | 8 | 3 |
| ASP | 14 | 12 | 12 |
| GLU | 17 | 25 | 11 |
| **Total negative** | 31 | 37 | 23 |
| **Total positive** | 34 | 29 | 33 |
| **Overall** | +3 | -8 | +10 |

In preliminary experiments DehL, DehD and DehE were run on a non-denaturing gel prepared using Tris.$SO_4$ buffer, pH 8.8. The gel was then incubated with 50 mM D,L2-CP followed by staining the gel with 0.5 % (w/v) silver nitrate solution. D,L2-CP was used because all three dehalogenases could react with this substrate. The results showed that DehD and DehL migrated to the same position and DehE moved faster (**Fig. 1**). The native molecular weights were close to each other (DehL: 60,000 Da; DehE: 62,000 Da; DehD: 58,000 Da) so that could not account for the different electrophoretic mobility. However, from their amino acid composition, DehE shows an overall negative charge, whereas DehL and DehD apparently bear positive charges (**Table 2**). This may be the reason why DehE moved faster than the other two enzymes. Although the theoretical charge for DehL and DehD was positive, they must still have negative charges on the protein surface because both proteins moved into the gel but the extent of negative charge could not be calculated from the amino acid composition. It is impossible to know the actual charge for DehL and DehD but presumably it is less than for DehE because DehL and DehD moved more slowly.

Attempts were made to separate DehL and DehD by running the proteins in non-denaturing gels prepared from Tris.$SO_4$ buffer, pH 7.8 and pH 6.8. However, both DehL and DehD still moved in the same relative positions seen in **Fig. 1**. and so could not be separated.

**Native-PAGE analysis of the cell-free extract from *Rhizobium* sp. grown on 0.2 mM 2,2DCP and on 20 mM 2,2DCP**

Dehalogenases produced during growth on 0.2 mM and 20 mM 2,2DCP were analysed by electrophoretic mobility by non-denaturing PAGE and protein separation by ion exchange chromatography to investigate whether growth at low concentration may involve a new dehalogenase enzyme. Cell-free extracts of *Rhizobium* sp. grown on 0.2 mM and 20 mM 2,2DCP were prepared for non-denaturing PAGE. After electrophoresis, the gels were incubated with 2,2DCP, DBA and MCA and then stained for halide ions.

The result showed that, when incubated with 2,2DCP, a single band seen for the 0.2 mM 2,2DCP-grown cells was in the same position as the DehE of the 20 mM 2,2DCP grown cell-extract (**Fig. 2a**). This suggests DehE was produced when cells were grown on 20 mM and 0.2 mM 2,2DCP. However, a single band from the cells grown at low concentration might also indicate the presence of a different dehalogenase that migrated to the same position as DehE. However, other dehalogenases might not have been detected on the gel if the high concentration of substrate used (50 mM) was inhibitory. To check this possibility, a non-denaturing gel was incubated with 0.2 mM 2,2DCP. However, no bands were seen for either of the extracts.

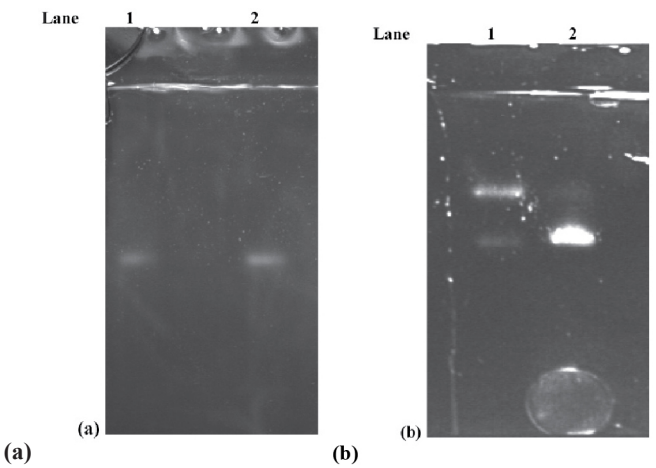


**Fig. 2.** Native-PAGE of dehalogenase activity towards 2,2DCP **(a)** and DBA **(b)**. Lane 1: cell-free extract from bacteria grown on 20 mM 2,2DCP**;** Lane 2: cell-free extract from bacteria grown on 0.2 mM 2,2DCP.

The presence of other known dehalogenases could be detected using other substrates such as DBA and MCA. DBA is a specific substrate for DehE and DehL and MCA is specific for DehE and DehD. The gel incubated with DBA showed two distinct bands, with different intensities between faster and slower bands for the two growth conditions (**Fig. 2b**). Two possible explanations for this might be that the relative amount of DehL was less than the amount of DehE when cells were grown at low concentration, or that a new dehalogenase might be present. The results for cells grown on high concentration might suggest that the relative amount of DehL is more than DehE.

The electrophoretic patterns of dehalogenase activity staining using DBA showed that cells grown on low concentration and on high concentration produced the same electrophoretic band pattern suggesting that the same dehalogenases were produced but the relative intensity of the bands was apparently different. However, it is difficult at this stage to explain the reason why the intensity of the faster band was different from the slower band in extracts prepared from cells grown at the lower 2,2DCP concentration compared to those grown at the higher 2,2DCP concentration. Probably further investigations need to be carried out to determine the N-terminal sequence of each dehalogenase that was present at growth on low 2,2DCP concentration to check its identity.

There was no difference in the band pattern between the slower and the faster bands in the gel incubated with MCA (data not shown). The band patterns from these figures could not be compared directly with the specific activities calculated in **Table 1** because different buffer conditions were used in the non-denaturing gel and in the enzyme assay.

As a control experiment, gels incubated with distilled water remained clear when stained for chloride or bromide ion, suggesting that the bands seen were due to enzyme–substrate interaction. To further characterise the dehalogenase enzyme(s) present, apparent Km values were measured for crude extracts prepared from cells grown at 0.2 mM and 20 mM 2,2DCP.

The non-denaturing gel electrophoresis shows that there was more than one dehalogenase enzyme produced during growth on high and low 2,2DCP concentration. This observation was similar to the previous investigation by Leigh (17), when *Rhizobium* sp. grown on 20 mM 2,2DCP as the sole carbon and energy source revealed three dehalogenases, although DehL and DehD do not play any role in 2,2DCP degradation. In other cases variation in the growth conditions influenced the nature of dehalogenase production (8). According to Allison et al. (1) different inducers gave a different pattern of induction compared to 2,2DCP and it was seen in the current investigation that different amounts of dehalogenases were observed when growth was at high and low 2,2DCP concentration.

**Apparent $K_m$ values using crude extract prepared from cells grown on 20 mM and 0.2 mM 2,2DCP**

The apparent $K_m$ values for extracts prepared from cells grown at the high and low concentration using DBA as substrate were determined. The $K_m$ value was 0.18 mM ± 0.05 in both cases. The $K_m$ values are closer to the known $K_m$ of crude DehL for DBA (0.19 mM ± 0.07) rather than to the value for crude DehE (0.89 mM ± 0.21) (10). However, the reason why the apparent $K_m$ appears to indicate the presence of DehL rather than of DehE could be investigated by looking at the relative amount of each individual enzyme produced under the two growth conditions. This could be achieved by separating them on an anion exchange column assuming no loss of activity occurred.

**Measurement of the dehalogenase enzyme profile for cells grown on 0.2 mM and 20 mM 2,2DCP**

Conditions for the separation of a mixture of DehL, DehE and DehD by anion exchange chromatography were established first before attempting to separate extracts from cells grown on low and high 2,2DCP concentration. Cell-free extracts of DehL, DehE and DehD were prepared in low ionic strength buffer, 0.01 M Tris-acetate, pH 7.6, and were mixed together before running on a MonoQ 5/5 column over a 5 mM to 100 mM sodium phosphate gradient. The fractions were collected and dehalogenase activity was measured in each fraction using D,L2-CP as substrate.

Three peaks of dehalogenase activity were present in this profile, as expected. The enzyme elution depended on the phosphate buffer concentration: for DehL (47 mM), DehE (72 mM) and DehD (31 mM). Under this condition, all three dehalogenase enzymes were successfully separated based on their charges. Since DehE protein has more negative charges as shown in **Table 2**, it will be retained more firmly on the column and was eluted last.

DehD and DehL also carried negative charges, otherwise they would not separate using this technique. However, DehD was eluted earlier than DehL and DehE, presumably due to the fact that it has less negative charges compared to the other two enzymes. The negative charge on the surface of DehL was presumably less than that on the surface of DehE. For that reason DehL was eluted before DehE from the MonoQ column. For analysis of extracts obtained from *Rhizobium* sp. grown at 0.2 mM and 20 mM 2,2DCP, a similar technique was utilised.

**Purification of dehalogenase enzyme from cells grown on 0.2 mM and 20 mM 2,2DCP**

An extract from *Rhizobium* sp. grown on 0.2 mM 2,2DCP was prepared in 0.01 M Tris-acetate buffer, pH 7.6, and applied to a MonoQ column. Protein fractions eluted were screened using D,L2-CP as substrate to detect the presence of DehL, DehE and DehD. The analysis showed that fraction 9 (DehD), fraction 13 (DehL) and fraction 16 (DehE) showed activity. These fractions were then analysed further using DBA as a substrate for fraction 13 (DehL) and fraction 16 (DehE) and MBA for fraction 9 (DehD), to determine the amount for each dehalogenase enzyme present. The amount of each enzyme was calculated and is shown in **Table 3** with DehL (0.2 U) twice as much as DehE (0.12 U) enzyme.

The Km values with DBA as a substrate were measured for fraction 13 (DehL) and fraction 16 (DehE). The results showed that the Km for fraction 13 (0.10 mM ± 0.01) was similar to the $K_m$ for purified DehL (0.27 mM ± 0.09) as reported by Huyop (10), and the $K_m$ for fraction 16 (0.47 ± 0.09 mM) was similar

to the $K_m$ for purified DehE (0.88 ± 0.14 mM) as identified by Huyop (10), supporting the identification of the activities. This result suggested that growth on low concentration of 2,2DCP did not produce any new dehalogenase. **Table 3** shows that the amount of DehL produced was more than of DehE and this possibly could explain the reason for the apparent Km with the crude extract being close to that of DehL. However, this finding still does not explain why the intensity of the faster band was stronger than that of the slower band from cells grown at low 2,2DCP concentration as seen in **Fig. 2b**.

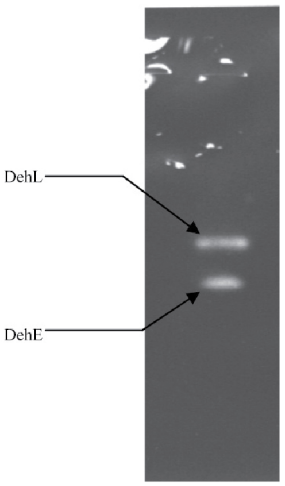


**Fig. 3.** Native-PAGE analysis of crude extract from *Rhizobium* sp. grown on 0.2 mM 2,2DCP extracted in 0.01 M Tris-acetate buffer, pH 7.6.

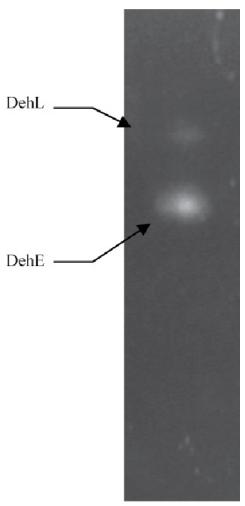


**Fig. 4.** Native-PAGE analysis of crude extract from *Rhizobium* sp. grown on 0.2 mM 2,2DCP extracted in 0.1 M Tris-acetate buffer, pH 7.6.

To further investigate this matter, the crude extract from growth on 0.2 mM 2,2DCP that was run on the MonoQ column was subjected to non-denaturing PAGE and stained for dehalogenase activity with DBA. The results showed that the faster and the slower bands stained to the same intensity in this case (**Fig. 3**). This was in contrast to the result seen in **Fig. 2b** where the intensity of the faster band was stronger than that of the slower band. The only difference between the two conditions was the concentration of buffer used to make the crude extracts. For **Fig. 2b**, 0.1 M Tris-acetate buffer, pH 7.6, was used and for **Fig. 3**, 0.01 M Tris-acetate buffer, pH 7.6, and the extract was ultra-centrifuged.

To see whether the difference was due to the different concentration of buffer used to make the extract, another batch of cells from the same growth experiment on 0.2 mM 2,2DCP was resuspended in 0.1 M Tris-acetate buffer, pH 7.6, sonicated and ultracentrifuged similar to the sample preparation for the MonoQ column. When this extract was run on a non-denaturing PAGE gel it showed stronger intensity in the faster band compared to the slower band (**Fig. 4**) similar to the earlier results shown in **Fig. 2b**. This suggested that the band pattern seen on the gel depended on the concentration of the buffer used to make the extract. However, the crude extract was not prepared in 0.1 M Tris-acetate buffer, pH 7.6, for the MonoQ column because all three dehalogenases would not then bind to the column.

Using the extract that was prepared in 0.01 M Tris-acetate pH 7.6 buffer, the apparent $K_m$ value was determined using DBA as a substrate to see whether the extract prepared in this different buffer affected the $K_m$. The $K_m$ value of 0.21 mM ± 0.09, was close to the $K_m$ for the same substrate using DehL enzyme (0.19 mM ± 0.07) as reported earlier by Huyop (10).

However, the $K_m$ (0.21 mM ± 0.09) was only slightly more than the $K_m$ for DBA in the cell-free extract prepared in 0.1 M Tris-acetate buffer, pH 7.6 (0.18 mM ± 0.05). This analysis suggests that preparing the cell-free extract in 0.1 M Tris-acetate buffer, pH 7.6, or in 0.01 M Tris-acetate buffer, pH 7.6, did not significantly affect the apparent $K_m$.

As a comparison an extract from *Rhizobium* sp. grown on 20 mM 2,2DCP was analysed in an identical way. The specific activity of the cell-free extract prepared in 0.01 M Tris-acetate pH 7.6 buffer was checked using D,L2-CP as a substrate (Specific activity: 0.20 μmol$Cl^-$/min/mg protein) to make sure that the cell-free extract contained dehalogenase activity before loading on a MonoQ column. The fractions were

**TABLE 3.**

Dehalogenase activities in cells grown on 0.2 mM 2,2DCP and 20 mM 2,2DCP

| **Enzymes** | **Activity, U/ml (Growth on 0.2 mM 2,2DCP)** | **Activity, U /ml (Growth on 20 mM 2,2DCP)** |
|---|---|---|
| DehL | 0.20 ± 0.03 | 0.78 ± 0.01 |
| DehE | 0.12 ± 0.04 | 0.22 ± 0.01 |
| DehD | 0.29 ± 0.02 | 0.56 ± 0.02 |

Values are the means of triplicate measurements.

screened using D,L2-CP to detect the most active fractions. The active fractions were eluted at 39 mM (fraction 9), 54 mM (fraction 13) and 73 mM (fraction 16) sodium phosphate buffer corresponding to DehD, DehL and DehE, respectively. They were then further analysed by enzyme assay using DBA and MBA as substrates to determine the amount of each enzyme present. The results showed that the relative amount of DehL produced was 3.5 times more than that of DehE (**Table 3**). The $K_m$ value determined using DBA as a substrate for fraction 13 (0.20 mM ± 0.06) was close to the $K_m$ of purified DehL (0.27 mM ± 0.09) (10) and the Km for fraction 16 (0.57 mM ± 0.08) was close to the Km of purified DehE (0.88 mM ± 0.14) (10).

The dehalogenase profiles from growth on 0.2 mM and 20 mM 2,2DCP suggested that different amounts of each dehalogenase enzyme were produced in the two growth conditions (**Table 3**). The data indicated that DehL was more abundant than DehE for both growth conditions and as a result the apparent Km measured in the cell-free extract was likely to represent primarily the activity of DehL. The different amounts of each dehalogenase enzyme produced were possibly due to different gene expression at high and low substrate concentration.

Analysis by column chromatography strongly suggests that DehL was more abundant than DehE in each growth condition. This might explain why the apparent Km values using the crude extract were close to the Km of DehL for DBA. In addition, to support this finding, previous evidence from *Pseudomonas putida* PP3 has shown that the nature of the growth substrate does affect the relative proportions of two dehalogenases produced by that organism as judged by the observed activity ratios (20).

## Conclusions

The present investigation did not show any new dehalogenase when *Rhizobium* sp. was grown at low haloalkanoic acid concentration. The low Km value for 2,2DCP of DehE (0.12 mM ± 0.02) as reported by Huyop (10) would seem to make that enzyme suited to dealing with low concentrations of growth substrate and no other enzyme would appear to be necessary. Different growth conditions might also suggest a complex regulatory control in *Rhizobium* sp., which responded to the growth environment (12).


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