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Biochemical activities of 1,2-dichloroethane (DCA) degrading bacteria

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One of the most widely produced industrial solvents is 1,2-dichloroethane (DCA), a known carcinogen, toxic to both terrestrial and aquatic ecosystems. Annual production in the United States, Japan and Europe alone are in excess of thirteen thousand metric tons. Entry into the environment is mainly due to poor handling, accidental spillages and illegal dumping. Five indigenous DCA degrading bacterial isolates capable of completely degrading DCA under aerobic conditions recently isolated from South African waste water treatment facilities, were found to belong to the genus *Ancylobacter*. The specific activities of the enzymes in DCA catabolism were compared with previously characterized DCA degrading bacterial isolates using crude cell lysates and different intermediates within the catabolic route as substrates. The catabolic route by which DCA is degraded was found to be similar to previously characterized isolates of *Ancylobacter* as well as *Xanthobacter autotrophicus* GJ10. The specific activities of all the enzymes within the catabolic route of the South African isolates were found to be lower when compared with previously characterized isolates. Polyacrylamide gel electrophoresis of crude cell lysates did not indicate over-expression of the hydrolytic dehalogenase as observed in a previously characterized isolate of *Ancylobacter*. Although, the overall specific activities of each of the enzymes in the DCA catabolic route were found to be lower in this study, these isolates may still have potential use in the bioremediation of DCA contaminated sites in South Africa.

Key words: 1,2- Dichloroethane, halogenated hydrocarbon, dehalogenase.

INTRODUCTION

At present halogenated aliphatic compounds constitute an important class of environmental pollutants within both terrestrial and aquatic ecosystems and various microorganisms have evolved that, are able to degrade some of these compounds and use them as sole sources of carbon and energy (van Hylckama et al., 2001). Microorganisms capable of degrading these chemicals have been isolated from contaminated sites and the genes and enzymes involved in their degradative pathways have been extensively studied (Fetzner and Lingens, 1994; Janssen et al., 2001). The catabolic pathway involved in the complete mineralization of 1,2-dichloroethane was first identified by Janssen et al.

(1985) in *Xanthobacter autotrophicus* GJ10 (Figure 1).

The first and usually most important step in the mineralization of chlorinated aliphatic compounds is known to be dehalogenation. Enzymes that cleave the carbon-halogen bond of haloaliphatics are generally referred to as dehalogenases and these may be present in either aerobes or anaerobes (Fetzner and Lingens, 1994). Thus, numerous haloalkane dehalogenase homologs have been detected in both known haloalkane degraders as well as in strains that are not known to degrade halogenated compounds (Jesenska et al., 2002). The roles of the haloalkane dehalogenase homologs in the latter strains are not known.

The dehalogenase from *X. autotrophicus* GJ10 allows this organism to use a number of short chain aliphatic compounds as sole carbon and energy sources. This constitutive haloalkane dehalogenase (DhlA) catalyses the hydrolytic dehalogenation of halogenated $C_2 - C_4$

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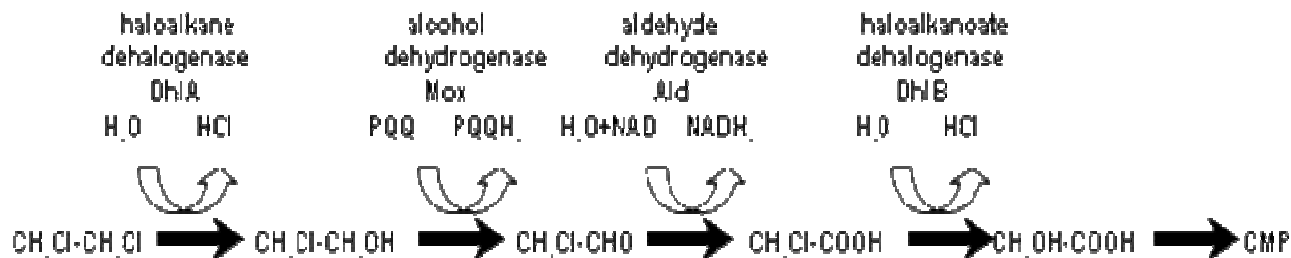
Figure 1. Catabolic route for the metabolism of DCA in *X. autotrophicus* GJ10, CMP (central metabolic pathway), (Adapted from, Janssen et al., 1985).

alkanes such as chlorinated, brominated and iodated compounds to the corresponding haloalcohols (Keuning et al., 1985). It has been found that the haloalkane dehalogenase catalyses the hydrolytic release of halide from haloalkanes, resulting in the replacement of the halogen substituent by a hydroxyl group (Keuning et al., 1985). Other hydrolytic haloalkane dehalogenases belonging to the same family that have been extensively studied include LinB from *Sphingomonas paucimobilis* (Nagata et al., 1993) and DhaA from both *Rhodococcus erythropolis* NCIMB13064 (formerly *Rhodococcus rhodochrous* NCIMB13064) (Poelarends et al., 2000) and *Pseudomonas pavonaceae* 170 (formerly *Pseudomonas cichorii* 170) (Poelarends et al., 1998). The hydrolytic dehalogenase of *X. autotrophicus* GJ10 (DhlA) is also conserved within the facultative methylotrophs *Ancylobacter aquaticus* AD20, AD25 and AD27 (van den Wijngaard et al., 1992). It has also been recently found that, *Xanthobacter flavus* UE15 possesses an identical hydrolytic dehalogenase (Song et al., 2004).

The oxidation of 2-chloroethanol to chloroacetate in *X. autrophicus* GJ10 is catalysed by two different dehydrogenases. It was found that the chloroethanol dehydrogenase was a pyrrolo-quinoline quinone containing alcohol dehydrogenase (Mox) and due to its broad substrate specificity was active with 2-chloroethanol. Several methylotrophic bacteria have been identified that produces quinoprotein alcohol dehydrogenases which require ammonia or amines for activity and are able to catalyse the oxidation of several primary alcohols (Janssen et al., 1987). It was also shown that, 2-chloroethanol was converted to chloroacetaldehyde by a phenazine methosulfate-linked alcohol dehydrogenase by *A. aquaticus* AD25. In addition to 2-chloroethanol, the inducible alcohol dehydrogenase of this isolate was able to convert 2-bromoethanol, methanol and ethanol (van den Wijngaard et al., 1992).

The highly reactive and potentially toxic intermediate, chloroacetaldehyde is then converted to monochloroacetic acid (Janssen et al., 1985). An inducible NAD-dependent chloroacetaldehyde dehydrogenase that catalyses the conversion of chloroacetaldehyde to monochloroacetic acid in *X. autotrophicus* GJ10 was first identified by Janssen et al. (1987). It was later reported that, *X.*

autotrophicus GJ10 produced at least three different aldehyde dehydrogenases of which one was plasmid-encoded (van der Ploeg et al., 1994). Bergeron et al. (1998) later identified a linear plasmid-based chloroacetaldehyde dehydrogenase gene (*aldA*) and a chromosomal homolog (*aldB*) in *X. autotrophicus* GJ10. Although, isolates of *A. aquaticus* have a similar degradative route to *X. autotrophicus* GJ10 (van den Wijngaard et al., 1992), the aldehyde dehydrogenase genes have not been identified.

X. autotrophicus GJ10 has been shown to convert monochloroacetic acid to glycolate which enters into the cell's central metabolic pathway enabling it to completely utilize DCA as a sole carbon and energy source (Janssen et al., 1985). The haloacid dehalogenase gene (*dhlB*) has been cloned and sequenced (van der Ploeg et al., 1994). Characterisation of the enzyme has revealed that these enzyme catalyses the cleavage of carbon-halogen bonds through a nucleophilic substitution by water to yield free halide and is therefore, characterized as a hydrolytic dehalogenase.

The aim of this study was to determine whether the South African isolates of *A. aquaticus* possess a similar degradative pathway when compared with *X. autotrophicus* GJ10 and two previous characterized isolates of *A. aquaticus*. Intermediates of the DCA degradative pathway of *X. autotrophicus* GJ10 were used as substrates for each of the assay procedures. Polyacrylamide gel electrophoresis (PAGE) was also carried out in order to determine whether any of the enzymes were over-expressed.

MATERIALS AND METHODS

Bacterial isolates

Table 1 lists the haloalkane degrading bacteria used in this study. *X. autotrophicus* GJ10, *A. aquaticus* AD25 and AD27 were used as controls in this study.

Growth of bacterial cultures

The culture medium used was described previously (Janssen et al., 1985) with the exception that, vitamin solution was replaced with 30

Table 1. Haloalkane degrading bacteria used in this study.

Bacterial strain	Source
A. aquaticus DH2	This study
A. aquaticus DH5	This study
A. aquaticus DH12	This study
A. aquaticus UV5	This study
A. aquaticus UV6	This study
A. aquaticus AD25	D. B. Janssen*
A. aquaticus AD27	D. B. Janssen*
X. autotrophicus GJ10	D. B. Janssen*

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mg/l yeast extract. The new medium was referred to as MMY. The carbon source (1,2-dichloroethane) was added to a final concentration of 5 mM. Batch cultures were grown in 250 ml serum bottles that were filled to one-third their volume and were closed gas tight with Teflon-lined screw caps to prevent evaporation of volatile substrates. Large volumes of cultures were grown (2 L) at 30°C with rotary shaking. The five South African isolates as well as *A. aquaticus* AD25, AD27 and *X. autotrophicus* GJ10 were grown to late log phase.

Preparation of crude cell lysates and protein quantification

Cells were harvested by centrifugation (10 min at 10 000 x *g*) and re-suspended in 100 ml TEM buffer (10 mM Tris sulphate, 1 mM β-mercaptoethanol and 1 mM EDTA, pH 7.5). Cells were pelleted by centrifugation (10 min, 10 000 x *g*) and resuspended in 10 ml TEM buffer. Bacterial cells were lysed by sonication. A crude extract was obtained by centrifugation at 4°C for 30 min at 40 000 x *g*. The crude extract was stored at 4°C to prevent degradation of proteins and subsequent loss in activity (van der Wijngaard et al., 1992).

Total protein was quantified according to the Bradford method (Bradford, 1976). A protein standard curve was constructed using varying concentrations of BSA (bovine serum albumin) fraction V (Roche Biochemicals) in 0.15 M NaCl as standards. Based on the regression equation, the total protein within the lysate was determined.

Assay procedures

In order to determine whether the South African isolates possessed a catabolic route that was similar to that of *X. autotrophicus* GJ10 and other *Ancylobacter* isolates (van den Wijngaard et al., 1992) various intermediates in the pathway were used as substrates. The assays used, not only determined whether the various reactions were catalysed but also determined the quantity of substrate utilized. All assays were performed in triplicate and the averages of triplicate assays are indicated.

Haloalkane dehalogenase

The colorimetric method used for the detection and quantification of halides has been described by Bergmann and Sanik (1957). The method used for this assay was adapted from Janssen et al. (1985) and Keuning et al. (1985). The assay is based on the insolubility of $HgCl_2$, which is formed and precipitated when Cl^- is mixed with

Hg(SCN). This releases SCN^-, which forms a red complex with Fe^{2+}. The $Fe(SCN)_2$ complex thus, formed is stable for at least 1 h and measured at 460 nm. It should be noted that the calibration curve is not linear and the assay can only be used for samples containing 0.01 to 2 mM of chloride. As a precaution, samples should always be freshly prepared and to prevent evaporation of substrates, stocks should be made in tightly closed vials containing only a small volume gas phase.

To prepare free chloride calibration curves 0.5 ml of standard solutions of 0 to 2 mM of potassium chloride in H_2O were mixed with 1.8 ml diluted reagent I (0.25 M $NH_4Fe(SO_4)_2$ in 9 M HNO_3). Reagent I was diluted eight fold with H_2O before use. Colour development was initiated by the addition of 0.2 ml of reagent II (saturated solution of $Hg(SCN)_2$ in absolute ethanol) and mixing. Spectrophotometric readings were taken at 460 nm using a standard UV/VIS spectrophotometer (Pharmacia). Water was used as the blank. Data was then transferred to a spreadsheet and regression analysis was performed to construct a calibration curve.

The substrate solution (30 ml) containing 5 mM DCA (Aldrich) in 50 mM Tris.SO$_4$ (pH 7.5) was prepared in a 30 ml serum bottle with a Teflon lined screwcap and 3 ml was aliquoted into 5 tubes. A separate set of 5 tubes was also prepared containing 1.8 ml diluted halide reagent I. Reactions were started by adding 200 μl of the crude cell lysate to the tubes containing the substrate solution which was placed into a circulating water bath at 30°C. Samples were taken after 1 min (t= 0) by removing 500 μl of the incubation mixture and adding it to the first tube containing reagent I followed by the addition of 0.2 ml of reagent II. Sampling was repeated in the same way after different time intervals and added to the different tubes containing reagent I, followed by the addition of reagent II. Readings were taken at 460 nm using standard UV/VIS spectrophotometer (Pharmacia) using water as the blank. To ensure accuracy, all extinctions were below 0.4 absorbance units. The amount of free chloride and dehalogenase activities were determined from the chloride standard curves and protein standard curves. One unit is defined as the amount of enzyme that produces 1 μmol of product per min under the conditions used. It should be noted that, 2 moles of halide are produced per mole of substrate for DCA because this assay is used to measure both the hydrolytic dehalogenase and haloacetate dehalogenase activity (Janssen et al., 1987).

Alcohol dehydrogenase

In order to measure the haloalcohol dehydrogenase activity of the cell free extracts of the South African isolates of *Ancylobacter*, 2-chloroethanol-dependant reduction of 2,6-dichloroindophenol (DCIP) was followed spectrophotometrically at 600 nm in a coupled fashion with phenazine methosulphate (PMS) as an artificial electron acceptor (Janssen et al., 1987).

Stock solutions of the following reagents were prepared prior to spectrophotometric analysis: 0.5 M KH_2PO_4, pH8.0; 10 mM PMS; 10 mM DCIP; 100 mM NH_4Cl and 10 mM NaCN. In a 1.5 ml quartz cuvette, each of the components were added to a final volume of 1 ml and concentration of 83 mM KH_2PO_4; 15 mM NH_4Cl; 0.09 mM NaCN; 0.1 mM DCIP; 0.22 mM PMS and 5 mM 2-chloroethanol (Aldrich). Crude cell lysate (100 μl) was then added and the final volume brought up to 1 ml with water and mixed by inversion. The spectrophotometer was set to auto zero and the activity was measured in "timedrive" mode at 30°C immediately after adding the substrate to the cuvette. The decrease in absorbance was measured at 600 nm for 10 min (ε_{DCIP} = 21.9 mM^{-1}cm^{-1} at 600 nm).

The activity was calculated by obtaining the initial slope of the absorbance (1 to 3 min) and dividing the result by 21.9 mM^{-1}cm^{-1} to get the change in concentration. This change in concentration was converted to activity by multiplying it by 10 to account for the amount of crude cell lysate in the cuvette (100 μl in this case). This

result was then converted to specific activity by dividing it by the protein concentration. The activity was corrected by subtracting the value for zero activity. One unit of enzyme activity is defined as 1 µmol substrate converted per minute (Janssen et al., 1987).

Aldehyde dehydrogenase

Aldehyde dehydrogenase activity of the crude cell lysate was measured spectrophotmetrically at 340 nm by following the 2-chloroacetaldehyde-dependent reduction of NAD^+ to NADH (Janssen et al., 1987). The reaction mixture contained (in a final volume of 1 ml), 100 mM sodium pyrophosphate (pH 8.75), 1 mM NAD, 1 mM dithiothreitol, 10 mM β-mercaptoethanol, 5 mM chloroacetaldehyde (Aldrich), and 100 µl crude cell lysate. After the spectrophotometer was set to autozero, the activity was measured in "timedrive" at 30°C. The decrease in absorbance was measured at 340 nm for 10 min (ε_{NAD+} = 6.22 $mM^{-1}cm^{-1}$ at 340 nm). The activity was calculated by obtaining the initial slope of the absorbance (1 to 3 min) and dividing the result by ε = 6.22 $mM^{-1}cm^{-1}$ to get the change in concentration. This change in concentration was converted to activity by multiplying it by 10 to account for the amount of crude cell lysate in the cuvette (100 µl). This result was then converted to specific activity by dividing it by the protein concentration. The activity is corrected by subtracting the value for zero activity. One unit is defined as the activity catalyzing the formation of 1 µmol NADH per minute (Janssen et al., 1987).

Chloroacetate dehalogenase

The assay used to determine chloroacetate dehalogenase activity was similar to the procedure used to determine hydrolytic dehalogenase activity except that 1,2-dichloroethane was replaced with 5 mM monochloroacetic acid (Aldrich). One unit is defined as the amount of enzyme that produces 1 µmol of halide per min under the conditions used. It should be noted here that, 1 mole of halide is produced per mole of substrate for monochloroacetic acid (Janssen et al., 1987).

SDS-PAGE analysis

The comparison of the individual protein components was determined by SDS-PAGE on 10% (w/v) polyacrylamide according to the method of Laemmli (1970). Each well was loaded with 100 µg/ml crude cell lysate in order to determine if the 37 kDa hydrolytic dehalogenase was over-expressed in any of the isolates. The reference protein sizes were 205, 116, 97, 66, 55, 45, 36, 29, 24, 20, 16.2 and 6.5 kDa (Broad Range Marker - Amersham).

RESULTS

Protein quantification

A protein concentration range was measured between 0.0025 to 0.5 mg/ml using the Bradford method at an absorbance of 595 nm. These values were used to construct the linear standard curve from which the linear regression equation, $f(x) = 1.6968x + 0.3622$ was generated. Crude cell lysates were obtained following cell disruption by sonication and the total amount of protein present in the crude cell lysates was calculated using the linear regression equation. The amount of protein in

crude cell lysates would later be used to determine the specific activity of different enzymes involved in the DCA catabolic pathway.

Haloalkane dehalogenase activity

Haloalkane dehalogenase activity was first calculated by quantifying the amount of chlorine liberated in solution during enzymatic cleavage of the carbon chlorine bond of DCA. Chloride ions in solution were measured by firstly determining the absorbance at 460 nm of different concentrations of KCl using the assay procedure described and generating a standard curve. The generated regression, equation $f(x) = 4.558x2 + 0.908x + 0.042$ was used to determine the amount of chlorine released by each of the isolates at different time intervals in the halide release assay. The rate of halide release by each of the isolates over 20 min was then obtained from the regression equation generated from the graph constructed of free halide versus time.

The hydrolytic dehalogenase specific activity of each of the isolates was calculated by determining the amount of protein that is required to liberate 1 µmol of chlorine per minute under the conditions used. It was found that, *A. aquaticus* AD25 (Figure 2) showed the highest activity (190.351 mU/mg protein) toward DCA followed by *A. aquaticus* AD27 (131.271 mU/mg protein). Three of the South Africa isolates of *A. aquaticus* (DH2, DH5 and DH12) had higher activities compared with *X. autotrophicus* G10 which had an activity of 66.979 mU/mg of protein (Figure 2). *A. aquaticus* AD25 had approximately 3 fold higher activity when compared with *X. autotrophicus* GJ10 and approximately 2.5 to 3 fold higher activity when compared with the South African isolates of *Ancylobacter* (Figure 2).

2-Chloroethanol dehydrogenase activity

The haloalcohol dehydrogenase specific activity (Figure 2) when 2-chloroethanol was used as the substrate in the assay procedure was found to be highest in *X. autotrophicus* GJ10 (10.5 mU/mg protein) and lowest in *A. aquaticus* AD27 (4.5 mU/mg protein). *X. autotrophicus* GJ10 showed an almost two-fold higher activity compared to *A. aquaticus* AD27. Three of the South African isolates (DH2, DH5 and UV5) had comparable activity (7.5, 7.23 and 7.13 mU/mg protein, respectively), while isolate DH12 had the highest activity (9.47 mU/mg protein).

Aldehyde dehydrogenase activity

It was found that the aldehyde dehydrogenase activity was extremely low for all isolates tested (Figure 2). The highest aldehyde dehydrogenase activity was observed
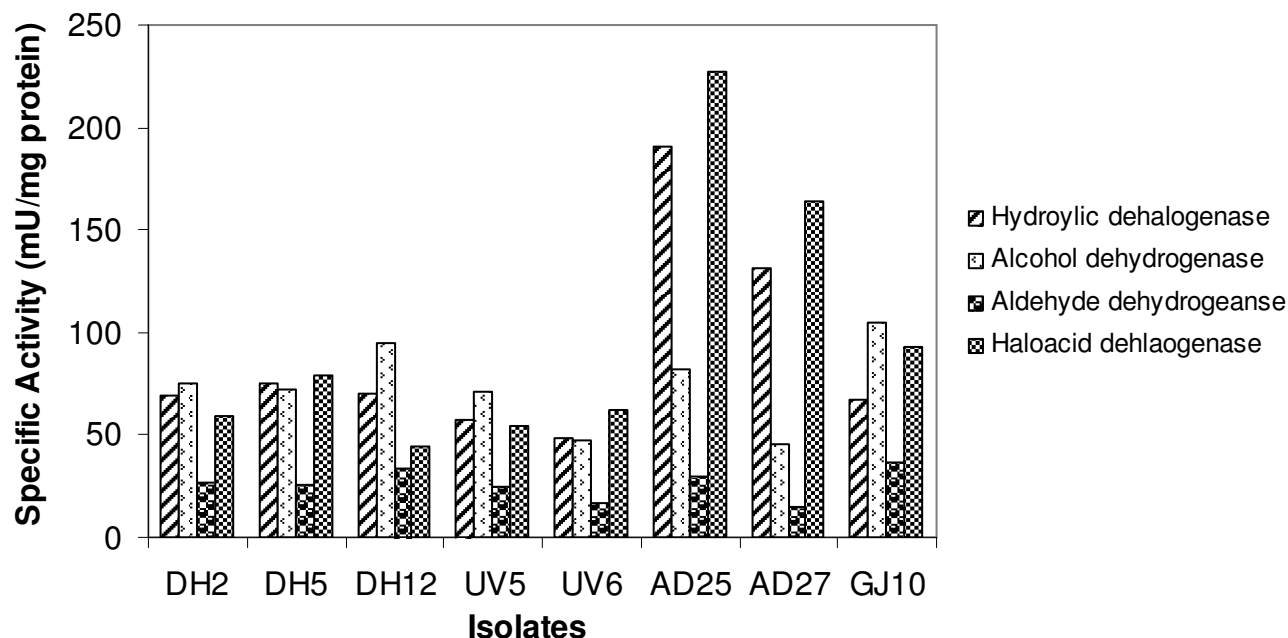
Figure 2. Specific activities of enzymes involved in complete mineralization of DCA.

in *X. autotrophicus* GJ10 (36.6 mU/mg protein) when 1,2-dichloroethane was used as the carbon source during growth and chloroacetaldehyde was the substrate in the assay procedure. One of the South African isolates of *Ancylobacter* (DH12) showed higher activity (33.16 mU/mg) compared with the previously characterized isolates AD25 (29.17 mU/mg) and AD27 (14.5 mU/mg).

Haloacetate dehalogenase activity

The haloacetate dehalogenase and haloalkane dehalogenase specific activities were measured in a similar way; however, in order to measure haloacetate dehalogenase specific activity monochloroacetic acid was used as the substrate. Figure 2 shows the amounts of chloride released over a 20 min time period when monochloroacetic acid was used as the substrate. The rate of the reaction was derived from the regression equation obtained from the graphs drawn for each isolate showing the amount of free halide released over time. The rate of the free halide liberation (μM/min) was then used to determine the haloacetate dehalogenase specific activity of each of the isolates.

A. aquaticus AD25 exhibited the highest haloacid dehalogenase activity (227.3 mU/mg protein) with *X. autotrophicus* GJ10 having an approximately 2.5 fold lower specific activity of 92.9 mU/mg protein (Figure 2). It can be observed that, all of the South African isolates of *Ancylobacter* had significantly lower specific activities toward monochloracetate when compared with *A. aquaticus* AD25 and AD27 (Figure 2). Isolate DH5

showed the highest activity (79.310 mU/mg protein) among all of the South African isolates.

SDS-PAGE analysis

SDS-PAGE analysis of all of the isolates was performed in order to determine whether distinct profiling patterns would be observed in the South African isolates compared to the previously characterized isolates *A. aquaticus* AD25 and AD27 (Figure 3). *A. aquaticus* AD25 exhibited a unique PAGE profile when compared with the other isolates of *Ancylobacter*. It was observed that the profile for *X. autotrophicus* GJ10 is different from the isolates of *Ancylobacter,* although, the intensity of the PAGE profile obtained is low compared with the other isolates which may be due to protein degradation.

Variations in the South African isolates were observed in the 37 kDa region for isolate DH2 (lane 2) where an additional band was observed. Isolates DH5, UV5 and *A. aquaticus* AD27 have a more intense 19 kDa protein band which was not observed in any of the other isolates. Isolate DH12 (lane 4) lacks a well expressed 25 kDa protein which was observed in the other isolates.

DISCUSSION

The key objective of this study was to determine whether the five South African isolates of *A. aquaticus* possessed a similar 1,2-dichloroethane (DCA) degradative route as *X. autotrophicus* GJ10 and two previously characterized
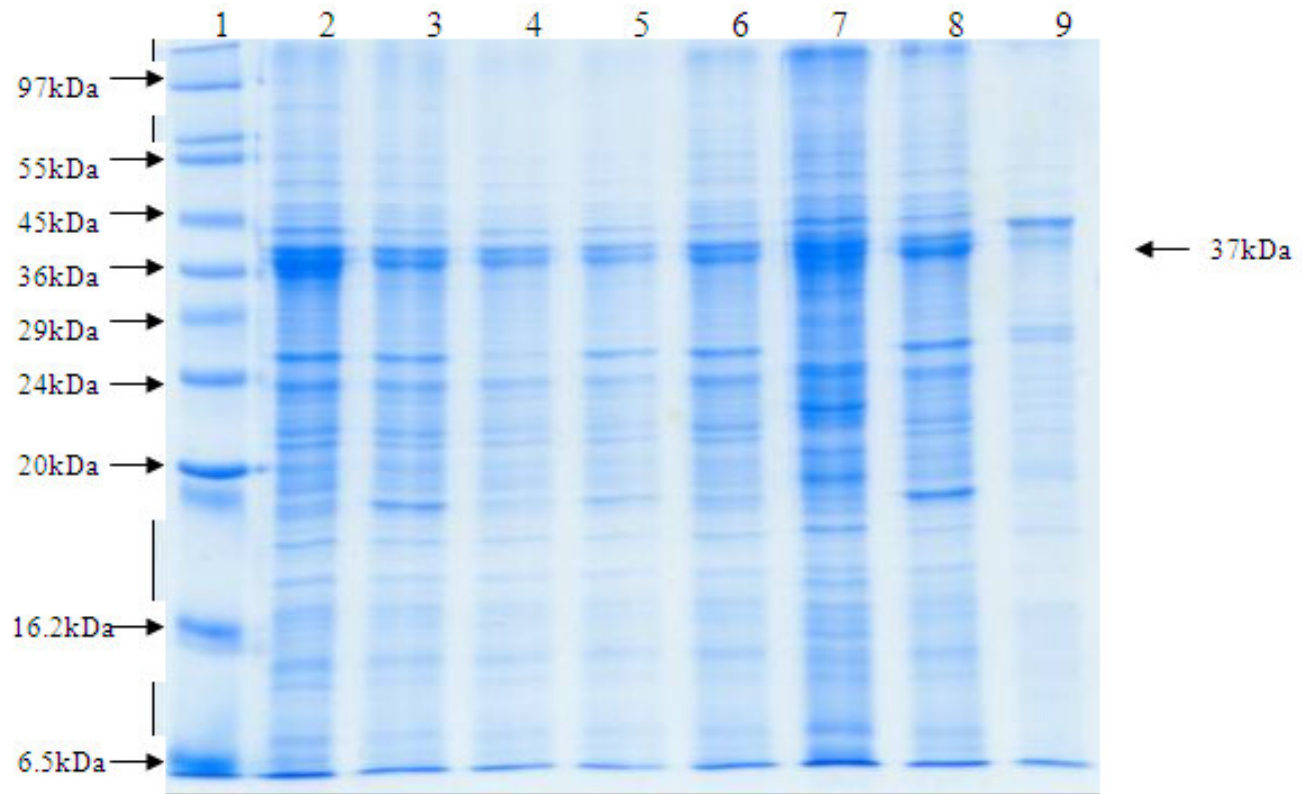
Figure 3. SDS-PAGE analysis of crude cell lysates. Lane 1: Molecular weight marker; lane 2: DH2; lane 3: DH5; lane 4: DH12; lane 5: UV5; lane 6: UV6; lane 7: AD25; lane 8: AD27; lane 9: GJ10.

isolates of *A. aquaticus*, AD25 and AD27. This was achieved using each of the intermediates in the DCA degradative pathway of *X. autotrophicus* GJ10 as substrates in various enzyme assays. The enzyme assays were both a qualitative and quantitative measurement of enzyme activities in crude cell extracts. This allowed for the determination of the exact intermediates in the pathway as well as a comparison to previously characterized DCA degrading microorganisms. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was also carried out in order to compare protein profiles.

It was previously shown that, *X. autotrophicus* GJ10 had a specific activity of 232 mU/mg total proteins when DCA was used as the carbon source during growth and the substrate in the assay procedure (Janssen et al., 1985). This value is significantly higher than the value obtained (Figure 2) in this study (66.979 mU/mg protein). Previous studies have shown that the pH significantly affected hydrolytic dehalogenase activity. At pH 7.5, the activity was shown to be 310 mU/mg protein, whereas at pH 9, the activity had decreased to 210 mU/mg protein (Janssen et al., 1985). Since pH 7.5 was used in this study, it can be assumed that the lower activity was not due to the pH but possible protein degradation.

Initial studies that analysed the dehalogenase activity of

isolates of *A. aquaticus* had shown that isolate AD25 had a specific activity of 4293 mU/mg total protein which is 15 to 20 times higher than that of *X. autotrophicus* GJ10 (232 mU/mg protein), while isolate AD27 had a specific activity of 936 mU/mg protein when 1,2-dichloroethane was used as the carbon source during growth and the substrate in the assay procedure (van den Wijngaard et al., 1992). The highest activity for the South African isolates was observed in isolate DH5 (75.235 mU/mg protein) which is approximately 57-fold lower than *A. aquaticus* AD25 and 12 fold lower than *A. aquaticus* AD27 (Figure 2).

The specific activity of the haloalcohol dehydrogenases from *X. autotrophicus* GJ10 and *A. aquaticus* AD25 towards 2-chloroethanol (Figure 2) was found to be much lower than previously observed (Janssen et al., 1985; van den Wijngaard et al., 1992). In this study, *X. autrophicus* GJ10 showed a specific activity of 10.5 mU/mg protein (Figure 2), while in previous studies the specific activity was shown to be 518 mU/mg protein (Janssen et al., 1985) and 480 mU/mg protein (van den Wijngaard et al., 1992) when DCA was used as the carbon source during growth and 2-chloroethanol was the substrate in the assay procedure. *A. aquaticus* AD25 showed a specific activity of 260 mU/mg protein (van den Wijngaard et al., 1992); however, in this study the specific activity was 8.2

mU/mg protein (Figure 2). In this study, one of the South African isolates (DH12) had higher specific activity (9.47 mU/mg protein) towards 2-chloroethanol (Figure 2) when compared with the previously characterized isolates of *A. aquaticus* AD25 (8.2 mU/mg protein) and AD27 (4.5 mU/mg protein).

When bacterial isolates were grown with DCA as the carbon source and 2-bromoethanol was used as the substrate in the haloalcohol dehydrogenase assay (results not shown), it was found that, *X. autotrophicus* GJ10 showed the highest activity (7.4 mU/mg protein), whereas *A. aquaticus* AD27 showed the lowest activity (2.05 mU/mg protein). Two of the South African isolates of *A. aquaticus* (DH5 and DH12) exhibited higher activities than the previously characterized isolates of *Ancylobacter*, 6.02 and 6.71 mU/mg protein, respectively. These values are much lower than previously observed values for *A. aquaticus* AD25 which had previously been shown to have an activity of 263 mU/mg protein (van den Wijngaard et al., 1992).

Chloroethanol and chloroacetaldehyde are two of the most toxic intermediates in the degradative pathway and have to be rapidly converted or removed from the cell. It has been observed that 2-chloroethanol can be excreted from the cell, while toxic aldehydes generally accumulate within the cell (Janssen et al., 1985). Both the alcohol dehydrogenase and aldehyde dehydrogenase are therefore, key regulatory enzymes required to prevent the accumulation of toxic intermediates. Although, the South African isolates have lower chloroacetaldehyde dehydrogenase activities compared to the previously characterized isolates of *A. aquaticus* AD25 and AD27, isolate DH12 has a higher alcohol dehydrogenase activity compared with both of these isolates. Previous studies have shown specific activity of *X. autotrophicus* GJ10 to be 109 mU/mg protein (Janssen et al., 1985); however, in this study, it was 36.6 mU/mg protein (Figure 2). The isolates of *A. aquaticus* AD25 had a specific activity of 1872 mU/mg total protein, while *A. aquaticus* AD27 had a specific activity of 109 mU/mg protein (van den Wijngaard et al., 1992). The lower activity towards chloroethanol and chloroacetaldehyde may be due to the inhibitory effects of these highly reactive intermediates.

Lastly, the specific activity of the haloacid dehalogenases was found to be extremely low for all the isolates tested (Figure 2). In previous studies, the specific activity of the haloacid dehalogenase from *X. autotrophi*cus GJ10 was shown to be 416 mU/mg protein (Janssen et al., 1985a), while the specific activity of the haloacid dehalogenase from *A. aquaticus* AD25 was approximately 2.5 fold higher at 1035 mU/mg protein (van den Wijngaard et al., 1992). In this study, the highest haloacid dehalogenase activity was observed in *A. aquaticus* AD25 (227.3 mU/mg protein) with *X. autotrophicus* GJ10 having an approximately 2.5 fold lower specific activity of 92.9 mU/mg protein (Figure 2). All of the South African isolates of *Ancylobacter* had

much lower specific activities toward monochloracetate.

Previous studies have shown that, *A. aquaticus* AD25 usually over-expresses the hydrolytic dehalogenase (van den Wijngaard et al., 1992) which could be observed in a slightly more intense 37 kDa band in Figure 3. Based on the hydrolytic dehalogenase enzyme assay, it was also observed that, *A. aquaticus* AD25 has a much higher activity compared with the other isolates of *Ancylobacter* and this may be due to enhanced expression of the enzyme. The determination of whether other proteins involved in the degradation pathway are expressed at high levels is not possible as none of the other enzymes besides the hydrolytic dehalogenase have been previously identified in isolates of *A. aquaticus*. Differences observed in the protein profiles of the different isolates of *Ancylobacter* indicate that, these isolates express proteins differently and may thus, exhibit different degradation potentials.

Lower activities toward some of the compounds may be attributed to poor enzyme stability, over extended periods of time or sub-optimal assay conditions. By performing assays together with known controls, specific activities can be compared even with reduced activities. The lower values for the specific activities of the different enzymes obtained in this study may be due to protein degradation in crude cell lysates during overnight storage at 4°C. Loss of protein expression may also be attributed to repeat sub-culturing in undefined media prior to growth in media containing DCA, as a sole carbon and energy source. This study has been adequately able to demonstrate that the South African isolates of *Ancylobacter* have a similar degradative route to *X. autotrophicus* GJ10 as well as two previously characterized isolates of *Ancylobacter*.

REFERENCES

Bergeron H, Labbe D, Turmeland C and Lau PKC (1998). Cloning, sequence and expression of a linear plasmid-based and a chromosomal homolog of chloroacetaldehyde dehydrogenase-encoding genes in *Xanthobacter autotrophicus* GJ10. Gene. 207: 9-18.
Bergmann JG and Sanik J (1957). Determination of trace amount of chlorine in naptha. Anal. Chem. 29: 241-243.
Bradford M (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.
Fetzner S, Lingens F (1994). Bacterial dehalogenases: biochemistry, genetics and biotechnological applications. Microbiol. Rev. 58: 641-685.
Janssen DB, Oppentocht JE, Poelarends GJ (2001). Microbial dehalogenation. Curr. Opin. Biotechnol. 12: 254-258.
Janssen DB, Keuning S, Withold B (1987). Involvement of a quinoprotein alcohol dehydrogenase and an NAD-dependent aldehyde dehydrogenase in 2-chloroethanol metabolism in *Xanthobacter autotrophicus* GJ10. J. Gen. Microbiol. 133: 85-92.
Janssen DB, Scheper A, Dijkhuizen L , Witholt B (1985). Degradation of halogenated aliphatic compounds by *Xanthobacter autotrophicus* GJ10. Appl. Environ. Microbiol. 49: 673-677.
Jesenska A, Bartos M, Czerneková V, Rychlík I, Pavlík I, Damborski J (2002). Cloning and expression of the haloalkane dehalogenase gene, *dhmA* from *Mycobacterium avium* N85 and preliminary characterization of DhmA. Appl. Environ. Microbiol. 68: 3724-3730.

Keuning S, Janssen DB, Witholt B (1985). Purification and characterization of hydrolytic haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10. J. Bacteriol. 163: 635-639.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

Nagata Y, Nariya T, Ohtomo R, Fukuda M, Yano K, Takagi M (1993). Cloning and sequencing of a dehalogenase gene encoding an enzyme with hydrolase activity involved in the degradation of ¥-hexachlorocyclohexane in *Pseudomonas paucimobilis*. J. Bacteriol. 175: 6403-6410.

Poelarends GJ, Zandstra M, Bosma T, Kulakov LA, Larkin MJ, Marchesi JR, Weightman AJ, Janssen DB (2000). Haloalkane-utilising *Rhodococcus* strains isolated from geographically distinct locations possess a highly conserved gene cluster encoding haloalkane catabolism. J. Bacteriol. 182: 2725-2731.

Poelarends GJ, Wilkens M, Larkin MJ, van Elsas JD, Janssen DB (1998). Degradation of 1,3-dichlorpropene by *Pseudomonas cichorii* 170. Appl. Environ. Microbiol. 64: 2931-2936.

Song JS, Lee DH, Lee K, Kim CK (2004). Genetic organization of the dhlA gene encoding 1,2-dichloroethane dechlorinase from *Xanthobacter flavus* UE15. J. Microbiol. 42:188-193.

Van den Wijngaard AJ, van der Kamp KWHJ, van der Ploeg J, Pries F, Kazemier B , Janssen DB (1992). Degradation of 1,2-dichloroethane by *Ancylobacter aquaticus* and other facultative Methylotrophs. Appl. Environ. Microbiol. 58: 976-983.

Van der Ploeg J, Smidt P, Landa AS, Janssen DB (1994). Identification of chloroacetaldehyde dehydrogenase involved in 1,2-dichloroethane degradation. Appl. Environ. Microbiol. 60: 1599-1605.

Van Hylckama Vlieg JET, Tang L, Spelberg JH, Smilda T, Poelarends GJ, Bosma T, van Merode AEJ, Fraaije MW, Janssen DB (2001). Halohydrin dehalogenases are structurally and mechanistically related to short-chain dehalogenases/reductases. J. Bacteriol. 183: 5058-5066.