Bacterial degradation of dichloromethane in cultures and natural environments

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Abstract

Dichloromethane (DCM) is a toxic pollutant showing prolonged persistence in water. DCM biodegradation is usually determined from increases in Cl ions, gas chromatography, or by using radioisotopes. Herein, we present an original and easy spectrophotometric method to estimate DCM concentrations in cultures and environmental samples during DCM biodegradation experiments.

Keywords: Dichloromethane; Biodegradation; Dichloromethane dehalogenase; Enzyme assay

Dichloromethane (DCM) is a highly toxic compound widely used as an industrial solvent. DCM has been in widespread use for several decades, with the principal application being paint removal (U.S. International Trade Commission, 1970–1985). It has been shown to cause lung and liver cancer in rodents (National Toxicology Program, 1986), and a number of harmful effects on humans have been reported (Edwards et al., 1982; Dhillon and Von Burg, 1995). DCM is difficult to remove from contaminated waters (Mackay and Cherry, 1989) and shows high persistence in water (half-life over 700 years) and atmosphere (half-life 79 days). Thus, there is a growing interest in studying biodegradative processes of DCM in natural environments (Edwards et al., 1982; Gälli and Leisinger, 1985; Freedman and Gossett, 1991).

Currently, DCM concentration is determined mainly by capillary gas chromatography (Oldenhuis et al., 1989), and DCM dehalogenase activity has been assessed from the dehalogenation of DCM (Brunner et al., 1980), by enzymatic assays (Leisinger and Kohler-Staub, 1990; Bader and Leisinger, 1994), or by using radioisotopes (Goodwin et al., 1998). Capillary gas chromatography is a sensitive method to estimate a concentration of DCM in a sample; however, it requires relatively costly equipment unavailable to many microbiologists. There are a number of techniques for assessing DCM dehalogenase activity. Continuous monitoring of the acid produced during the dehalogenation of DCM is a simple method.
although fails when using buffered samples (i.e., seawater). Enzymatic assays have also been described allowing estimates of activity by DCM dehalogenases producing formaldehyde (Eq. (1)) by its detection either colorimetrically (Bader and Leisinger, 1994; Doronina et al., 1998) or coupled to the reaction catalyzed by formaldehyde dehydrogenase (Eq. (2)) (Leisinger and Kohler-Staub, 1990). Radioisotopes provide a sensitive alternative to monitor the biodegradation of DCM in cultures and natural samples, but constitute a risk for scientists and the environment.

Herein, we propose an easy, spectrophotometric method to estimate the concentration of DCM during biodegradation experiments. The only required infrastructure is a spectrophotometer, which is a common piece of equipment found in every microbiological laboratory. DCM concentration was estimated from its rate of degradation using a coupled enzymatic assay of two reactions: one catalyzed by DCM dehalogenase in the presence of glutathione (Eq. (1)), and a second reaction catalyzed by formaldehyde dehydrogenase with NAD$^+$ (Eq. (2)).

$$\text{DCM} + \text{H}_2\text{O} \rightarrow \text{formaldehyde} + 2\text{Cl}^- \quad (1)$$

$$2\text{NAD}^+ + \text{formaldehyde} \rightarrow 2\text{NADH}_2 + \text{formate} \quad (2)$$

*Methylobacterium dichloromethanicum* strain DM4 (VKM B-2161) was obtained from the All Russian Collection of Microorganisms of the Russian Academy of Sciences (VKM). Strain DM4 was used as a source of DCM dehalogenase (La Roche and Leisinger, 1991). This is a glutathione-dependent DCM dehalogenase requiring the presence of glutathione to show catalytic activity. The cells were grown in minimal medium (Doronina et al., 1998) which contained (per liter): KH$_2$PO$_4$, 2.0 g; (NH$_4$)$_2$SO$_4$, 2.0 g; MgSO$_4$$\cdot$7H$_2$O, 0.025 g; NaCl, 0.5 g. This medium was supplemented with DCM in three doses to a final concentration of 10 mM (0.85 g/l). Incubations took place in a reciprocal shaker (180 rpm) at 30 °C. The proposed method could also be carried out using recombinant DCM dehalogenase (Gälli and Leisinger, 1988; Vuilleumier and Leisinger, 1996) although, in our hands, the best results were obtained with cell-free extracts from DM4. Thus, cell-free extracts of *Methylobacterium* strain DM4 were used as a source of DCM dehalogenase. DM4 cells in early stationary phase of growth were harvested by centrifugation (6000 × g for 15 min) and passed through a French-Press. This suspension was centrifuged (25,000 × g for 30 min) to remove cell debris. The cell-free extract was used within 2 h since DCM dehalogenase activity decreases during storage (Leisinger and Kohler-Staub, 1990).

Each reaction mix contained K$_2$PO$_4$ (50 mM), reduced glutathione (10 mM), NAD$^+$ (1 mM), formaldehyde dehydrogenase (0.01 units) (Sigma-Aldrich, St. Louis, MO), cell-free extract from *Methylobacterium* DM4 (0.01 units), and an adequate volume of...
sample to be analyzed filtered through 0.2-μm-pore-size nitrocellulose filter (Millipore, Bedford, MA). *Methylobacterium* DM4 DCM dehalogenase units were defined as suggested by Leisinger and Kohler-Staub (1990) for Eq. (2) in this coupled assay and represented the milligram of protein in the cell-free extract required to produce 1 μmol NADH per minute under the specified conditions. Protein concentration in the cell-free extracts was estimated by Bradford’s (1976) method. The reaction mix (500 μl) was incubated in a cuvette at 30 °C in a temperature-controlled Beckman spectrophotometer DU640 (Fullertont, CA). The increase in absorbance at 339 nm due to NADH₂ production was measured over a 5-min period. Controls lacking one of the components of the reaction were carried out. No increase in absorbance was observed when anyone of the components of the reaction mix was missing. Michaelis–Menten kinetic equations, obtained from control reactions using 0.2-μm-filtered deionized water and known DCM concentrations, were used as calibration curves to estimate the actual concentration of DCM in the experimental samples (Fig. 1). Using this method, optimum estimates of DCM concentration were in the range of 0.1 to 10 mM DCM per cuvette. Higher DCM concentrations could be measured by performing appropriate dilutions of the sample. Variability was at most within 15% of average values.

Leisinger and Kohler-Staub (1990) described a coupled enzymatic reaction (Eqs. (1) and (2)) and its use to estimate DCM dehalogenase activity. In this study, we propose the protocol outlined above to estimate DCM concentrations during biodegradation experiments. We used a cell-free extract containing high DCM dehalogenase activity from a well-studied methylotroph (*M. dichloromethanicum*) to dechlorinate the DCM present in a variety of samples. Comparison with a calibration curve following Michaelis–Menten kinetics allowed us to approximate the concentration of DCM in the original samples for concentrations ranging within the non-saturating portion of the reaction curve.

The proposed method was tested with bacterial cultures (Fig. 2), enrichments, and natural samples supplemented with DCM (to be published elsewhere). Fig. 2 shows an example of biodegradation of DCM by a bacterial culture. DCM concentration decreased by about 80% during the growth (2–3 days) of these bacteria.

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References


