Bioreactors for Removing Methyl Bromide following Contained Fumigations

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Use of methyl bromide (MeBr) as a quarantine, commodity, or structural fumigant is under scrutiny because its release to the atmosphere contributes to the depletion of stratospheric ozone. A closed-system bioreactor consisting of 0.5 L of a growing culture of a previously described bacterium, strain IMB-1, removed MeBr (>110 μmol L^{-1}) from recirculating air. Strain IMB-1 grew slowly to high cell densities in the bioreactor using MeBr as its sole carbon and energy source. Bacterial oxidation of MeBr produced CO_2 and hydrobromic acid (HBr), which required continuous neutralization with NaOH for the system to operate effectively. Strain IMB-1 was capable of sustained oxidation of large amounts of MeBr (170 mmol in 46 d). In an open-system bioreactor (10-L fermenter), strain IMB-1 oxidized a continuous supply of MeBr (220 μmol L^{-1} in air). Growth was continuous, and 0.5 mol of MeBr was removed from the air supply in 14 d. The specific rate of MeBr oxidation was 7×10^{-16} mol cell^{-1} h^{-1}. Bioreactors such as these can therefore be used to remove large quantities of contaminant MeBr, which opens the possibility of biodegradation as a practical means for its disposal.

Introduction

Approximately 30% of the methyl bromide (MeBr) sold in the United States is used for fumigating structures for pest control or for disinfesting commodities and harvested crops prior to shipment or sale (*1*). In these uses, high levels of MeBr (180–1100 μmol L^{-1}) are directly vented to the atmosphere following several hours to days of exposure to structures or to commodities in closed containers. MeBr has a residence time of greater than 9 months in the troposphere (*2, 3*), long enough for a significant fraction to be transported to the stratosphere (*4*). MeBr participates in reactions in the stratosphere that ultimately result in destruction of ozone (*5*). As a result, the major use of MeBr (preplant field fumigation = 65% of sales) is currently being phased out by international agreement (the Montreal Protocol and its amendments) and as a consequence of the U.S. Clean Air Act (2001). However, no single alternative to use of MeBr as a fumigant has been identified. Recently, there has been renewed interest in the ability of MeBr to kill spores of *Bacillus anthracis* (*6*) (R. Scheffrahn, personal communication). Future regulation of the amount of MeBr released by structural and commodity fumigations is likely. Hence, if MeBr use is to continue in critical applications such as these,

it is imperative to lower the amount released to the atmosphere by collecting the gas following fumigation for eventual recycling or destruction.

Several strategies have been proposed for capturing MeBr from the waste stream of commodity fumigations. Among these, adsorption of MeBr on zeolite or on activated charcoal (*7*) has received considerable attention. Recovery of MeBr adsorbed on zeolite is possible, but no working system for destroying or recycling the captured MeBr has been reported. More than 80% of the added MeBr may be recovered using a single bed or cartridge of activated charcoal adsorber, but emission of 8.9–22 μmol L^{-1} MeBr still results from this practice. Stricter environmental controls in the future will likely require lower emissions, necessitating a second adsorbing bed in series with the first, or further strategies for removing MeBr. Recovery of MeBr adsorbed on activated charcoal has been demonstrated, and the cost is reasonable (*8*). However, recycled MeBr is not currently registered as a fumigant in the United States; hence, there is no market for this product. Catalytic decomposition of MeBr adsorbed on activated charcoal has been shown, but this method operates at elevated temperature and is energy-intensive (*9*). Chemical destruction of MeBr previously adsorbed on activated charcoal using $Na_2S_2O_3$ has also been demonstrated (*10*), although there may be high costs associated with disposal of products ($Na_2S_2O_3$, $NaCH_3S_2O_3$, or Br$^-$) if their concentration in the waste stream exceeds local limits (e.g., 1% solutions in water for California).

Biodegradation offers another solution for removal of MeBr from the waste stream of contained fumigations. Both direct and indirect (e.g., following adsorption on charcoal) methods are considered here. Several newly identified species of α-proteobacteria grow on methyl halides (*11*). Some of these facultative methylotrophic bacteria (including strain IMB-1) are able to oxidize MeBr via the following reaction:

$$CH_3Br + 1.5O_2 \rightarrow CO_2 + H_2O + HBr \qquad (1)$$
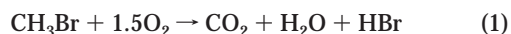
where MeBr is oxidized directly during growth (*12*). Strain IMB-1 (ATCC Accession No. 202107) was isolated from MeBr-fumigated soil and is able to grow on MeBr, methyl chloride (MeCl), and methyl iodide (MeI) as well as methylated amines and non-C_1 compounds such as glucose, acetate, and pyruvate (*13, 14*). Elevated levels of MeBr are toxic to many organisms; however, strain IMB-1 has been shown to oxidize and grow on pulsed additions of moderate concentrations (110–450 μmol L^{-1}) of MeBr. Strain IMB-1 can also oxidize ambient tropospheric concentrations of MeBr ($5 \times 10^{-7} \mu$mol L^{-1}) (*15*). This paper describes the design and operation of two bioreactors that can remove high concentrations of MeBr from the waste stream of contained fumigations. We report on the oxidation of MeBr by strain IMB-1 using pulsed additions of up to 180 μmol L^{-1} MeBr in a recirculating, closed-system bioreactor and during growth on a continuous supply of 220 μmol L^{-1} MeBr in an open-system bioreactor. Limitations arising from exposure of strain IMB-1 to unusually high concentrations of MeBr (2700 μmol L^{-1}) are also considered.

Experimental Section

MeBr gas-phase concentrations are given in μmol L^{-1} air, while liquid-phase concentrations are given in μM and were calculated using Henry's law (see Discussion). Henry's law applies strictly to equilibrium conditions, which likely do not exist during periods of rapid oxidation of MeBr by growing cells. Nonetheless, the equilibrium concentration is a useful

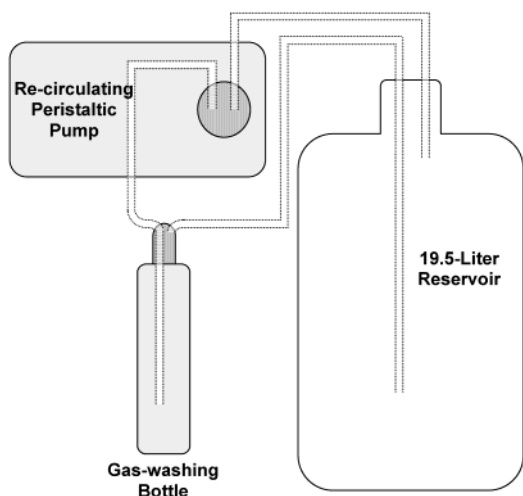* Corresponding author e-mail: lgmiller@usgs.gov; phone: (650)-329-4475; fax: (650)329-4327.

FIGURE 1. Schematic drawing of the closed-system bioreactor (not to scale).

estimate of the maximum liquid concentration in the vicinity of cells. Total amounts of MeBr added or consumed are given in moles of MeBr (MW = 95).

Strain IMB-1 was grown on MeBr in 1-L batch culture in order to obtain an inoculum for the bioreactor. Pulsed additions of MeBr (0.1−1 mmol) were made approximately daily for several weeks. Cells were grown to elevated density (~26 mg L^{-1} dry weight or 1.5×10^8 cells cm^{-3}) in stoppered, 2-L flasks with rotary shaking as described previously (*13, 14*). All cells were grown at 28 °C. The mineral medium used consisted of the following (in g L^{-1}): KH_2PO_4 (6.0), $(NH_4)_2$-SO_4 (2.0), $MgSO_4 \cdot 7H_2O$ (0.125), and $FeSO_4 \cdot 7H_2O$ (0.002) plus 1.0 mL of SL-10 trace minerals (*16*). This recipe represents a 3-fold increase in phosphate concentration over that previously used by our lab to culture methyl halide-oxidizing methylotrophs (*14*) and was substituted in order to provide additional buffering capacity. Cells were washed and resuspended to 0.5 L in fresh media and transferred to the bioreactor. Initial cell density was 3×10^8 cells cm^{-3} (measured) or 10 mg of C L^{-1} (calculated using the cell carbon content of strain IMB-1) (*13*).

The closed-system bioreactor consisted of a suspension of growing culture of strain IMB-1 in a 0.6-L gas-washing bottle connected to a 19.5-L glass reservoir via a recirculating peristaltic pump (Figure 1). Stainless steel tubing (0.64 cm diameter) and PharMed pump tubing (1.0 cm diameter, Cole Parmer, Vernon Hills, IL) were used to make the connections. Samples of the gas phase were collected through septa located at the inlet and outlet of the gas-washing bottle. The gas-washing bottle contained a coarse frit (40−60 μm pore size). The pump had a flow rate of 12.2 L of air h^{-1}, resulting in a system flushing time (τ_f = volume/flow) of 1.7 h. The closed-system bioreactor operated continuously for 46 d. The cell suspension in the gas-washing bottle was maintained at 28 °C using a water bath. Additions of 2.2 mmol of MeBr were made, up to several times a day, by a syringe through the rubber stopper in the top of the glass reservoir. Methane (0.7 mmol) was added at the start as an internal standard. Gases (MeBr and CH_4) were sampled up to 15 times a day both at the inlet and the outlet of the gas-washing bottle. Liquid samples for analysis of dissolved and particulate constituents were collected by syringe through a septum port in the side of the gas-washing bottle. Additions of 2 N NaOH were made through the septum port to maintain the pH of the cell suspension between 6.5 and 7.0.

The open-system bioreactor was a commercial 10-L capacity fermenter (Bioflo 3000, New Brunswick Scientific, Edison, NJ) with a gas supply consisting of 220 μmol L^{-1}

MeBr in air (Matheson Tri-Gas Inc., Newark, CA) introduced at a flow of 6 L h^{-1}, which resulted in a mass flow rate of 1.32 mmol of MeBr h^{-1}. The fermenter initially contained 6 L of cells from a previous culture to which 4 L of sterile, phosphate medium was added. This provided a starting cell density of ~5×10^8 cells cm^{-3}. Culture pH was continuously and automatically adjusted to 7.0 by the addition of 1 N NaOH as called for by the fermenter program (pH stat) when the reading supplied by a pH probe dropped below 7.0. Temperature was maintained at 28 °C, and the cell suspension was agitated with a paddle stirrer (55 rpm). Gas samples were collected downstream of the cells and after an exhaust filter (Sol-Vent DCF, Gellman Sciences, Ann Arbor, MI). Liquid samples were collected by suction using a sampling port on the fermenter.

Bottle experiments were conducted to determine the effects of high concentrations of MeBr upon oxidation of the compound. Strain IMB-1 was grown in 1-L batch culture, as above. The cells were harvested after 3 weeks growth and washed by centrifugation and resuspension in fresh media (final cell density = 3×10^9 cells cm^{-3}). This suspension was diluted with media to obtain cell densities of 3×10^8 and 3×10^7 cells cm^{-3}. Aliquots (5 cm^3) of washed-cell suspensions were dispensed into 13 cm^3 serum bottles and sealed using crimped butyl rubber stoppers (Bellco Glass Inc., Vineland, NJ). MeBr (8.04−55.8 μmol) was added to bottles by a syringe at the beginning of the experiment, and headspace samples were collected and analyzed over time for the determination of the total loss rate of MeBr. Rates of MeBr loss in bottles containing liquid media without cells were defined as chemical loss rates. Bacterial oxidation rates for each bottle were determined using the formula:

$$\text{bacterial oxidation rate} = \text{total loss rate} - \text{chemical loss rate} \quad (2)$$

Selected bottles received 0.5 cm^3 of 1 N HCl at various times in order to lower the pH below 1.5 for the determination of total inorganic carbon as CO_2. Henceforth, these acidified bottles were unavailable for headspace MeBr analysis. Remaining bottles were sampled and analyzed for MeBr concentration over time until fewer than two bottles remained. Rates of CO_2 production in bottles without added MeBr were minor; therefore, rates of CO_2 production due to bacterial respiration were calculated as total CO_2 produced over time.

Bottle experiments were also conducted to compare the rates of MeBr oxidation by cells grown on MeBr with cells grown on glucose and subsequently induced for MeBr oxidation (*14*). Strain IMB-1 was grown in 2-L sealed flasks using pulsed additions of MeBr or with 2 mM glucose as the sole carbon source. There were similar amounts of cells present after several weeks' growth on MeBr or several days' growth on glucose. Before being harvested, glucose-grown cells were exposed overnight to ~50 μM MeBr to induce the enzyme for oxidation of MeBr. Cells were then harvested, centrifuged, and resuspended in media, as above, to final cell densities for MeBr and glucose-grown cells of 1.8 and 1.4 $\times 10^7$ cells cm^{-3}, respectively. MeBr (8.04 μmol) was added by syringe at the start of the experiment to the headspace of 57 cm^3 bottles containing 20 cm^3 cell suspensions. Triplicate headspace samples were collected at various times for analysis of MeBr.

Gas samples were analyzed for MeBr using flame ionization gas chromatography. A separation column consisting of Krytox 143 (60/80 mesh) on a Graphpak support (Alltech Associates, San Jose, CA) was operated at 100 °C with He carrier (25 cm^3 m^{-1}). CO_2 was determined by thermal conductivity gas chromatography following separation of fixed gases using a Porapak S column operated as above.

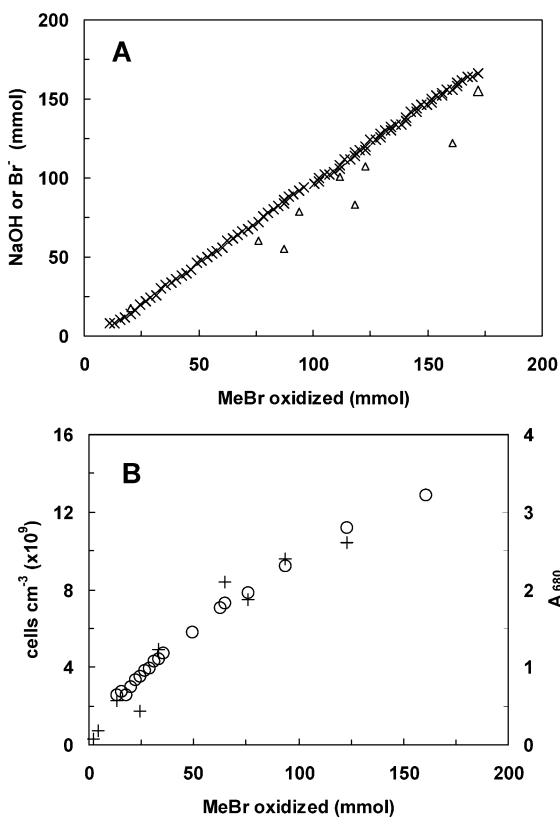**FIGURE 2.** Cumulative oxidation of MeBr, added in pulses to the bioreactor over 45 d showing (A) production of Br$^-$ ($\triangle$) and the cumulative amount of NaOH added ($\times$) to maintain solution pH between 6.5 and 7.2 and (B) growth of strain IMB-1, indicated by acridine orange direct counts (AODC, $+$) and by solution absorbance measured at 680 nm ($\bigcirc$).

Concentrations of Br$^-$ in solution were determined on 0.22-μm filtered (nylon GDX, Whatman Ltd., Maidstone, England) samples using isochratic, background suppressed, ion chromatography (DX 600, Dionex, Sunnyvale, CA). A Dionex separation column (AS9-HC; 0.4 cm diameter, 25.5 cm long) was used with 9 mM CO$_3^{-2}$ mobile phase. Salinity was determined as refractive index of filtered (0.22 μm) samples using a hand-held refractometer (Atago USA Inc., Kirkland, WA). Cell growth was determined by measuring cell density as acridine orange direct counts (AODC) of cells (*17*) and as optical density (OD) of diluted cell suspensions measured as absorbance at 680 nm. Dry weights were determined at five different cell densities to obtain an estimate of cell mass ($\sim$2 $\times$ 10^{-13} g cell^{-1}).

Results

Closed-System Bioreactor. MeBr added in pulses of 2.2 mmol, up to three times a day for 46 d, resulted in the oxidation of 170 mmol of MeBr and production of an equivalent amount of HBr. Solution pH was maintained near neutral by the addition of 1 mol of NaOH for each mole of MeBr oxidized (Figure 2A). Bromide accumulated quantitatively in the spent medium as oxidation of MeBr proceeded. Cell growth, monitored as absorbance and direct counts, was continuous (Figure 2B). Cell density increased from 0.3 to $\sim$12 $\times$ 10^9 cells cm^{-3} in 46 d.

Integrity of the closed-system was demonstrated by constant headspace concentrations of CH$_4$ (internal standard) throughout the experiments (Figure 3). Concentrations of MeBr, both in the reservoir (inlet) and above the cell suspension (outlet), were elevated immediately following injection of the compound, and these levels subsequently

decreased with time. MeBr was lower in the gas exiting the cell suspension than in gas entering the cell suspension. After the first day of operation, there was no detectable MeBr exiting the cell suspension 3 h following each addition, although inlet concentrations at the same time could be >45 μmol L^{-1} MeBr.

The rate of MeBr oxidation increased following the start of the bioreactor. Only 50% of the first addition of 2.2 mmol of MeBr was removed within the first 8 h of operation (Figure 4). The remainder was removed within 24 h. Subsequent additions were removed more rapidly, such that, by the third day of operation, 80% of the added MeBr was consumed within 4 h. Thus, the rate of uptake of MeBr quickly approached the theoretical maximum rate given by the dilution curve in Figure 4. The dilution curve was calculated as an exponential decrease in the concentration of MeBr in the reservoir, using addition of MeBr-free air to the reservoir (i.e., 100% efficient bioreactor) and a system flushing time of 1.7 h.

Open-System Bioreactor. Strain IMB-1 grew immediately upon addition of MeBr to the fermenter. Cell density increased from an initial value of less than 0.5 to 2.5 $\times$ 10^9 cells cm^{-3} after 2 weeks of operation. Removal of 220 μmol L^{-1} MeBr was complete, as evidenced by the absence of detectable MeBr in the exhaust gas. Nearly 0.5 mol of MeBr was oxidized, and an equivalent amount (0.45 mol) of NaOH was consumed during the first 2 weeks of operation of the open-system bioreactor.

Toxic Effects. To determine the upper limit to the concentration of MeBr that could be degraded, above which a toxic effect exists, we conducted bottle experiments using varying MeBr concentrations and amounts of cells. MeBr was removed slowly via chemical reactions from bottles containing liquid media without added cells (Table 1 and Figure 5). In experiments with added cells, bacterial oxidation of MeBr increased with greater cell density. Low levels of MeBr (270 μmol L^{-1}, corresponding to 1.2 mM in solution) were removed within 8 d from bottles containing 3 $\times$ 10^7 cells cm^{-3} and within 35 or 2 h from bottles containing 10- or 100-fold more cells, respectively (Figure 5). Elevated concentrations of MeBr (450 and 2700 μmol L^{-1}, corresponding to 2.1 and 8.2 mM in solution, respectively) were consumed more slowly but generally with the same relationship of increased rate with greater cell numbers (Table 1). Exceptions to this general trend occurred when the levels of MeBr were toxic. Otherwise, the rates of bacterial oxidation of MeBr on a per cell basis were similar (within a factor of 2) at each concentration over the range of cell densities.

In bottles with high cell densities (3 $\times$ 10^9 cells cm^{-3}), low concentrations of MeBr (270 μmol L^{-1}) were removed within 2 h, while intermediate concentrations of MeBr (450 μmol L^{-1}) were removed within 7 h (Figure 6). Oxidation in bottles containing the highest concentration of MeBr (2700 μmol L^{-1}) was rapid during the first 8 h of the experiment but slowed to rates comparable to those displayed by the abiotic controls (i.e., chemical degradation only; data not shown).

Production of CO$_2$ during oxidation of MeBr was inhibited by high MeBr concentrations. Rates of production of CO$_2$ were greatest in bottles with low concentrations of MeBr and decreased as MeBr increased (Table 1). In bottles with low concentrations of added MeBr, rates of CO$_2$ production increased with cell density. At the highest MeBr concentration, CO$_2$ was only produced in bottles containing the greatest number of cells and then only for a brief (8 h) period. Hence, production of CO$_2$ was useful in delineating the duration of enzyme activity. No CO$_2$ was produced in the abiotic controls.

Oxidation by MeBr-Grown versus Glucose-Grown Cells. Cells grown on MeBr were able to oxidize MeBr more rapidly then cells grown on glucose that were induced for MeBr oxidation. Oxidation of 67 μmol L^{-1} MeBr was complete within
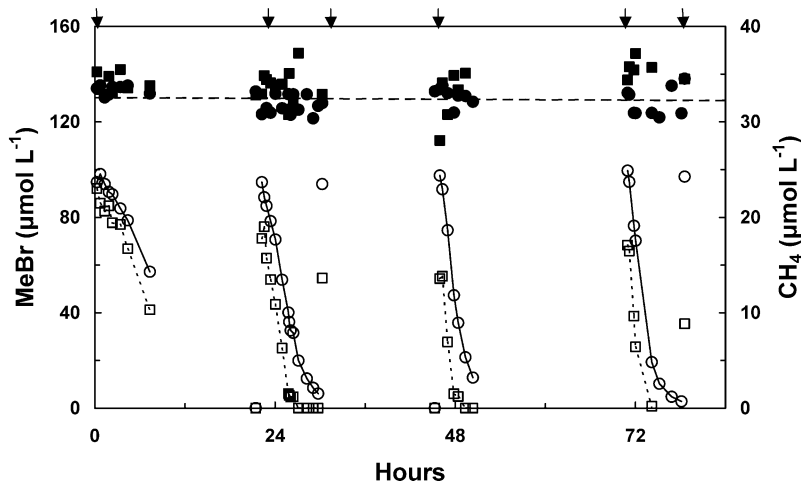
FIGURE 3. Concentration of MeBr (open symbols) and CH_4 (closed symbols) in air entering the gas-washing bottle (circles) and exiting the gas-washing bottle (squares) during the first 4 d of the closed-system bioreactor. Arrows indicate the time of addition of MeBr. Note that CH_4 data are plotted on an expanded scale. The dashed line is a linear regression of the CH_4 concentrations.
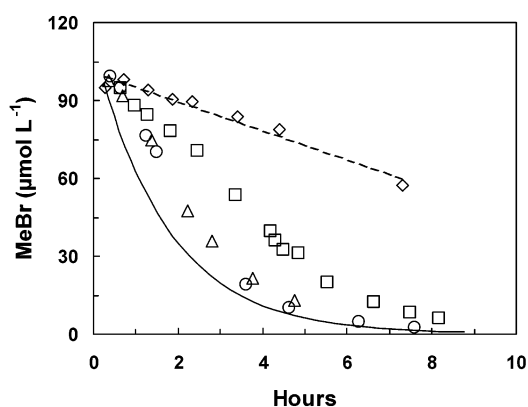

FIGURE 4. Oxidation of sequential additions of MeBr to strain IMB-1 in the bioreactor with the time of each addition set to 0 h; MeBr concentrations measured entering the gas-washing bottle following the first addition ($\diamond$), second addition ($\square$), fourth addition ($\triangle$), and fifth addition ($\bigcirc$) of 2.2 mmol of MeBr. Dashed line is a linear regression of the concentrations following the first edition. Solid line is the dilution curve, calculated from the system flushing time ($\tau_f = 1.7$ h) and represents the theoretical maximum uptake rate of MeBr. The equation describing the line is $C = C_0 e^{-\lambda t}$, where $C_0 = 110$ μmol L^{-1} and $\lambda = 1/\tau_f = 0.58$.

1 h during reaction with cells grown on MeBr whereas the reaction using glucose-grown cells induced for MeBr oxidation required >5 h to remove the same amount of MeBr (Figure 7).

Discussion

We demonstrate here that oxidation of MeBr using microorganisms can provide a practical solution to removing the fumigant from contaminated air. The only caveat is that the concentration of MeBr must be controlled to fall within the range required to sustain high levels of enzyme expression and, hence, oxidation. However, this range is quite broad and may not be a significant concern in actual practice. Growth of strain IMB-1 using MeBr as a substrate was relatively slow and inefficient. For example, during operation of the bioreactors only about 12% of the MeBr was converted to cell carbon while the remaining 88% was oxidized to CO_2. This is a desirable feature of an efficient bioreactor where the major product should be CO_2 rather than cells. The rate of oxidation of MeBr for cells grown by mass culture on MeBr was much faster than for cells grown on glucose and induced with MeBr. We believe this efficiency was achieved by the

TABLE 1. Total Loss of MeBr, Bacterial Oxidation of MeBr, and Production of CO_2 in Bottles Containing Different Numbers of Cells of Strain IMB-1 in 5 cm^3 with Varying Amounts of MeBr Added

amt of MeBr added (μmol)	headspace concn (μmol L^{-1})	MeBr total[a] loss rate (μmol h^{-1})	MeBr oxdn[b] rate per cell $\times 10^{18}$ (mol $cell^{-1}$ h^{-1})	CO_2 prodn rate (μmol h^{-1})
0 cells				
8.04	270	0.01	na[c]	0
14.3	450	0.03	na	0
55.8	2700	0.09	na	0
3×10^7 cells cm^{-3}				
0	0			0
8.04	270	0.04	180	0.01
14.3	450	0.03	0	0
55.8	2700	0.09	0	0
3×10^8 cells cm^{-3}				
0	0			0
8.04	270	0.26	160	0.07
14.3	450	0.11	50	0.07
55.8	2700	0.09	0	0
3×10^9 cells cm^{-3}				
0	0			0.11
8.04	270	3.63	240	1.50
14.3	450	1.54	100	1.35
55.8	2700	1.10	70	0.84[d]

[a] Total loss = bacterial + chemical. [b] Oxidation rate is loss due to bacteria only. [c] na, not applicable. [c] Rate of production of CO_2 during the first 8 h, production ceased after 8 h.

high rate of expression of the enzyme(s) responsible for dehalogenation and subsequent oxidation of MeBr. In addition to providing cells for bioreactors, mass culture of strain IMB-1 on MeBr should provide adequate material to allow for future biochemical studies to be conducted on the pathway of MeBr oxidation (*11*).

Distribution of MeBr between Phases. The disparity in gas and liquid volumes contained in the closed-system bioreactor requires an examination of the how MeBr was distributed between the two phases. The Henry's law constant for MeBr in cell cultures, 0.24 (units of μmol L^{-1} gas/μmol L^{-1} liquid) (*13*) was used with the gas and liquid volumes (21 and 0.5 L, respectively) to calculate the fraction of MeBr contained in the gas phase using the equation in Oremland et al. (*18*). The result of this calculation suggests that 90% of the MeBr resides in the gas phase and 10% of the MeBr resides in the liquid phase. Therefore, changes in the concentration
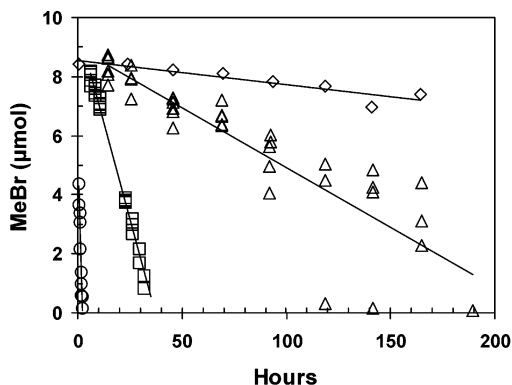
FIGURE 5. Oxidation of 8.04 μmol of added MeBr in bottles containing media without added cells ($\diamond$) and media with the following: 3×10^7 cells cm^{-3} ($\triangle$), 3×10^8 cells cm^{-3} ($\square$), or 3×10^9 cells cm^{-3} ($\circ$). The lines are linear regressions of the data, which include replicate analyses.

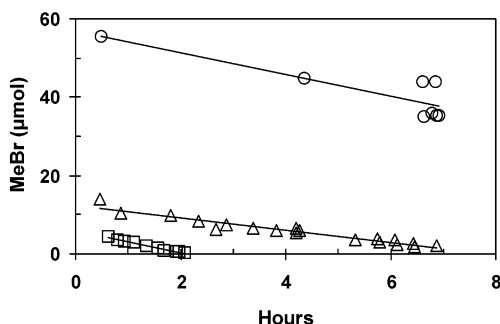**FIGURE 6.** Oxidation of 8.04 ($\square$), 14.3 ($\triangle$), or 55.8 μmol ($\circ$) of MeBr in bottles containing 3×10^9 cells cm^{-3} of strain IMB-1. The lines are linear regressions of individual analyses.
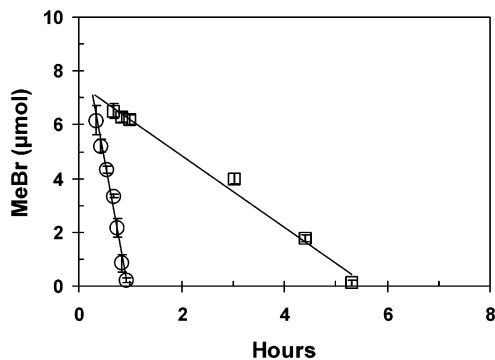

FIGURE 7. Oxidation of MeBr in bottles containing cells of strain IMB-1 grown on MeBr ($\circ$) or glucose ($\square$). Symbols represent the mean of triplicate analyses, and error bars show the standard deviation. The lines are linear regressions of the data.

of MeBr measured in the gas phase are a good approximation of the total changes occurring in the bioreactor. However, a similar calculation applied to the bottle experiments indicates that only about 30% of the added MeBr was present in the gas phase.

Cell Growth in the Bioreactor. The closed-system bioreactor received pulsed additions of 2.2 mmol of MeBr at least once per day (with the exception of weekends) for 46 d. This schedule resulted in the degradation of 170 mmol of MeBr and stoichiometric production of HBr, as determined by both the recovery of Br$^-$ and quantification of the equivalents of NaOH added to maintain the suspension pH at 7.0 (Figure 2A). Titration of the cell suspension with NaOH allowed the oxidation of MeBr to continue beyond the point where the buffering capacity of the media was exceeded. Once this

limitation was relieved, any other constraints to continued operation of the bioreactor could be examined.

Cell growth was relatively slow with an initial doubling time of 20 h and slightly longer doubling times (28 h) 3 weeks later (derived from data used in Figure 2B). No nutrient limitations were observed during growth, and we determined no effects of elevated salinity stemming from the accumulation of NaBr in the medium (up to 26 g kg^{-1}, total dissolved solids). The concentration of O_2 in the gas phase decreased from an initial (atmospheric) value of 21% in the bioreactor to less than 5% after 30 d of operation. During this time, CO_2 in the gas increased from an initial (atmospheric) value of 0.04% to about 10% (data not shown). Addition of 2 L of O_2 to the bioreactor raised the level of O_2 above 11% (v/v) and restored the rate of MeBr oxidation to that previously observed (data not shown). Hence, maintaining sufficient levels of O_2 and keeping the solution pH above 6.5 were both necessary conditions for continuous long-term operation of the closed-system bioreactor. To overcome this O_2 limitation, we carried out subsequent experiments in the open-system bioreactor, which was supplied with MeBr in air (see below).

Oxidation Rates of MeBr. Oxidation of MeBr in the bioreactor was notably rapid. The initial addition of 2.2 mmol of MeBr is removed in less than 1 d (Figure 3). It is unlikely that significant loss of MeBr resulted from abiotic reactions as these processes are relatively slow. For instance, the rate constant for hydrolysis of MeBr at 28 °C is approximately 0.0020 h^{-1} (*19*). The rate coefficient for the initial removal of MeBr in the bioreactor, determined as the measured loss rate divided by the concentration, resulted in a rate coefficient for bacterial oxidation of 0.050 h^{-1} or 25-fold greater than the rate constant for hydrolysis. Hydrolysis, then, could account for only 4% of the total removal of MeBr. This value agrees with the chemical loss (4% of the total loss) measured in bottle experiments conducted at the same starting cell density (3×10^8 cells cm^{-3}) as the closed-system bioreactor (Table 1).

The rate of removal of MeBr calculated from the linear decrease in concentration in the reservoir over the first 8 h was 5 μmol L^{-1} h^{-1} (Figure 4). If 4% of the loss was chemical, then 96% was due to bacterial activity, and IMB-1 in the bioreactor (1.5×10^{11} cells) was able to oxidize MeBr at a rate of 700×10^{-18} mol cell^{-1} h^{-1}. This rate is 500-fold greater than the value reported by Connell Hancock et al. (*13*) for IMB-1 grown on MeBr in bottles. Cells used in that earlier study were grown on pulses of 0.8 mM MeBr, whereas 5-fold higher concentrations of MeBr (4.4 mM) were potentially available to the cells contained in the bioreactor. Cells of strain IMB-1 likely made more methyl halide oxidizing enzyme(s) when exposed to the higher MeBr concentrations, resulting in greater oxidation efficiency. Strain IMB-1 contains a gene cluster with two enzymes (cmuA and cmuC) that have methyltransferase activities similar to methyl halide degrading enzymes found in some other methylotrophs (*20*). These initial enzymes in the oxidation pathway of methylotrophs prepare the methyl group of MeBr for further oxidation and/ or conversion into cell biomass.

Open-System Bioreactor. All of the MeBr in the sparge air (220 μmol L^{-1} or 5000 ppmv) was dissolved and made available to the cells at the low gas flow rate used (6 L h^{-1}). The bioreactor contained 10 L of cell suspension at an initial cell density of 3×10^8 cells cm^{-3} or 3×10^{12} cells. The specific rate of MeBr oxidation, assuming that 4% of the total degradation was due to hydrolysis and 96% was due to bacterial activity, was 0.12 g h^{-1} or 1.3 mmol h^{-1}. Hence, the specific rate of oxidation of MeBr per cell was 430×10^{-18} mol cell^{-1} h^{-1} or 60% of the rate calculated using the change in reservoir concentration in the closed-system bioreactor.

It is possible that the open-system bioreactor was not operating at optimal capacity and that more MeBr could

have been introduced to the cells in the fermenter. However, at higher flow rates (>12 L h^{-1}) breakthrough of about 45 μmol L^{-1} MeBr occurred. Breakthrough was a result of incomplete dissolution of MeBr and not a lack of uptake by cells. Addition of a gas-washing bottle downstream of the fermenter eliminated breakthrough at the higher flow rate (data not shown). The gas-washing bottle contained 0.5 L of cells harvested from the fermenter; hence, the total number of cells was not changed. Only the mechanism of gas dispersion was altered. Consequently, higher flow rates could be applied if the rate of dissolution of MeBr were enhanced, for instance, by reducing bubble size or by increasing the transit time through the fermenter. Thus, elevated concentrations (fumigation levels) of MeBr may be degraded in an open-system bioreactor as long as conditions allowed complete dissolution of the gas.

Toxic Effects. Bacterial oxidation of MeBr followed a general pattern whereby rates of oxidation on a per cell basis decreased with increased MeBr concentrations (Table 1). These results suggest that higher levels of added MeBr partially inhibited oxidation of MeBr by bacteria. Inhibition was further demonstrated by the activity of cells exposed to the highest concentration of MeBr (2700 μmol L^{-1}). Bacteria were able to oxidize this amount of MeBr only in bottles containing the greatest number of cells (3×10^9 cells cm^{-3}) and then only for a brief time. During the first 8 h, the rate of degradation was 30-fold greater than the rate due to chemical processes alone, and there was sustained production of CO_2 (Table 1). After 8 h, there was no further production of CO_2 and degradation of MeBr was by chemical reaction only. Therefore, a toxic effect not attributable to decreased pH or O_2 was observed at the highest MeBr concentration after 8 h of uptake.

Toxic effects were achieved at progressively lower concentrations of MeBr in bottles containing fewer cells. Inhibition was immediately observed at 450 μmol L^{-1} MeBr in bottles with 3×10^8 cells cm^{-3} and at both 270 and 450 μmol L^{-1} MeBr in bottles with 3×10^7 cells cm^{-3}. These results suggest that there is a practical limit to the concentration of MeBr that may be added to a bioreactor above which toxic effects are expected. This concentration limit increases with greater cell density. In bottle experiments with the highest cell density (3×10^9 cells cm^{-3}), strain IMB-1 was inhibited at 2700 μmol L^{-1} MeBr. However, this is above the concentration likely to be realized during commodity fumigations (180–1100 μmol L^{-1} MeBr) (8).

The upper limit to the concentration of MeBr that can be degraded may be even higher in a bioreactor containing more than 3×10^9 cells cm^{-3}. In the absence of inhibition, increased numbers of cells resulted in higher rates of oxidation. Oxidation occurred in all experiments at low concentrations of MeBr (270 μmol L^{-1}; Table 1), and there was a linear relationship ($r^2 = 0.9998$, data not shown) between the number of cells in each reactor and the rate at which MeBr was oxidized. However, a 10-fold increase in cell density resulted in a 15–20-fold increase in the oxidation rate for all but the highest concentrations of MeBr. Thus, the efficiency of degradation increased faster than cell biomass. This suggests that more methyl halide-oxidizing enzyme(s) were expressed as the cells grew on MeBr.

The maximum rate of MeBr oxidation achieved by bacteria in bottles was approximately 240×10^{-18} mol cell^{-1} h^{-1} (Table 1). The rate of oxidation reached in the closed-system bioreactor was 700×10^{-18} mol cell^{-1} h^{-1}, and that in the open-system fermenter was 430×10^{-18} mol cell^{-1} h^{-1}. Using the intermediate open-system rate, a 1000-L (1-m^3) bioreactor containing 5×10^{15} cells would oxidize 1 kg (10.5 mol) of MeBr in 5 h. This is equivalent to the amount of MeBr commonly used in contained fumigations. Thus, provided that cells are dense enough, the concentration of MeBr is

below toxic levels and air is included in the feedstock, the bioreactor can operate efficiently to remove MeBr directly from contained fumigations.

Oxidation of MeBr by Cells Grown on Glucose. The large numbers of cells of strain IMB-1 required for bioremediation can be obtained by growth on substrates other than MeBr, such as glucose (12). Previous studies showed that oxidation by MeBr-grown cells was twice as rapid as oxidation by glucose-grown cells (12–14) and that uptake of micromolar or greater levels of MeBr by glucose-grown cells required a brief induction period (13, 14). By contrast, our study shows that MeBr-grown cells removed MeBr 7-fold more rapidly on a per cell basis than did induced glucose-grown cells. Given the slower rate of oxidation by glucose-grown cells, we do not see any benefit to growing strain IMB-1 on substrates other than methyl halides for use in bioreactors. Also, there is a high risk of contamination associated with growing cells on glucose that is less likely for MeBr.

Example Uses of Bioreactors. It is estimated that fumigation of durable and perishable commodities releases 6.6 (4.8–8.4) and 5.7 (5.4–6.0) Gg MeBr yr^{-1} to the atmosphere, respectively (1). With the phase-out of preplant agricultural use of MeBr underway, recent effort has focused on reducing emissions of MeBr to the atmosphere from post-harvest and quarantine fumigations. The Parties to the Montreal Protocol (21) recognized that upward of 22% of MeBr use was excluded from regulation under quarantine and preshipment exemptions. They recommended that technologies that resulted in MeBr recovery and subsequent recycling or destruction be encouraged in order to reduce emissions of MeBr (Decision XI/13, 2000). Strategies that reduce emissions by recovering MeBr from the waste stream of contained fumigations can thus extend their uses into the future.

Bioreactors can be used alone or in series following a fixed-bed adsorber to obtain the desired remediation depending on conditions (e.g., high concentrations of MeBr) or requirements (e.g., high removal rate for perishables). In a stand-alone operation, a mixture of MeBr and air flows directly into the bioreactor, and the MeBr feed stream concentration can be lowered, if necessary, by dilution with outside air. The high container ventilation rate required by some commodities presents an engineering challenge to reactor design, but it appears that as long as the flow regime allows dissolution of MeBr, then the bacteria are given the opportunity to oxidize MeBr completely.

Bioreactors may be especially useful in destroying MeBr previously adsorbed on activated charcoal or zeolite, where MeBr can be sequestered and later removed by heating and/or flushing with air. This practice of load dampening lends itself to controlled introduction of MeBr into the bioreactor and economical reuse of the activated charcoal (G. Knapp, personal communication). The only additional cost of this strategy beyond those outlined for MeBr desorption from charcoal (7–10) occurs in disposing the spent media from the bioreactor which contains NaBr and viable cells.

The following calculations all use the rate of oxidation determined for the open-system bioreactor (430×10^{-18} mol cell^{-1} h^{-1}) and a cell density of 5×10^9 cells cm^{-3}. The per cell rate times the number of cells gives 2.2 mmol L^{-1} h^{-1}. Therefore, 10 kg (105 mol) of MeBr previously adsorbed from the waste stream of a contained fumigation could be removed by a 1000-L bioreactor in 50 h. However, over 3000 such adsorber/bioreactor systems would be required to completely eliminate the emission of MeBr from commodity fumigations worldwide, thus providing business opportunities for the operators of many such bioreactors.

A structure such as a large greenhouse (2.5×10^3 m^3) would require 300 kg of MeBr to fumigate at a concentration of 25 000 ppmv (1100 μmol L^{-1}), necessitating a very large

bioreactor (50 000 L) to remove all of the MeBr in 1 d. However, practical operation could be achieved by dampening the load of MeBr to the bioreactor using solid adsorber, thus allowing for the use of a smaller volume bioreactor over a longer period. As with commodity and post-harvest agricultural fumigations, structural fumigations may soon come under regulation with regard to their fugitive MeBr emissions.

Another possible use of the bioreactor is in removing MeBr trapped between two layers of plastic applied to the surface of soils during agricultural field fumigations (22). A 1-acre field fumigated with 160 kg of MeBr could be treated thusly, using a 5000-L bioreactor to remove all of the MeBr collected (90% of that applied) in 4 d, providing the inlet concentration remained below toxic levels. Dampening the load of MeBr to the bioreactor using a solid adsorber would allow the use of a smaller volume bioreactor over a longer period or treatment of a larger field.

The previous examples suggest the practicality of using bioreactors to remove MeBr from contaminated air following contained fumigations. Other types of bioreactors employing methylotrophic bacteria, including fixed or fluidized bed, or trickling reactors could also be developed for this purpose. Other strains of bacteria as well as consortia or mixed cultures of microbes should also be investigated for their potential use in bioreactors. One or more of these solutions may be judged desirable on the basis of the economic or public health needs of the users of MeBr and the community at large. In situations where no alternative to the use of MeBr exists, bioreactors may provide the remediation necessary to continue its use in critical applications.

Acknowledgments

Literature Cited

(1) Kurylo, M. J.; Rodriguez, J. M.; Andreae, M. O.; Atlas, E. L.; Blake, D. R.; Butler, J. H.; Lal, S.; Lary, D. J.; Midgley, P. M.; Montzka, S. A.; Novelli, P. C.; Reeves, C. E.; Simmonds, P. G.; Steele, L. P.; Sturges, W. T.; Weiss, R. F.; Yokouchi, Y. In *Scientific Assessment of Ozone Depletion: 1998*; Albritton, G. M., Watson, R. T., Aucamp, P. J., Eds.; World Meteorological Organization: Geneva, Switzerland, 1999; pp 2.1−2.56.

(2) Prather, M. J. *Global. Biogeochem. Cycles* **1997**, *11*, 393−400.

(3) Yvon-Lewis, S. A.; Butler, J. H. *Geophys. Res. Lett.* **1997**, *24*, 1227−1230.

(4) Mellouki, A.; Talukdar, R. K.; Schmoltner, A.-M.; Gierczak, T.; Mills, M. J.; Solomon, S.; Ravishankara, A. R. *Geophys. Res. Lett.* **1992**, *19*, 2059−2062.

(5) World Meteorological Organization (WMO). *Scientific Assessment of Ozone Depletion: 1994*; Report 37; WMO: Geneva, Switzerland, 1995.

(6) Kolb, R. W.; Schneiter, R. *J. Bacteriol.* **1950**, *59*, 401−412.

(7) Leesch, J. G.; Knapp, G. F.; Mackey, B. E. *J. Stored Prod. Res.* **2000**, *36*, 65−74.

(8) Snyder, J. D.; Leesch, J. G. *Ind. Eng. Chem. Res.* **2001**, *40*, 2925−2933.

(9) Gan, J.; Megonnell, N. E.; Yates, S. R. *Atmos. Environ.* **2001**, *35*, 941−947.

(10) Gan, J.; Yates, S. R. *J. Hazard. Mater.* **1998**, *57*, 249−258.

(11) McDonald, I. R.; Warner, K. L.; McAnulla, C.; Woodall, C. A.; Oremland, R. S.; Murrell, J. C. *Environ. Microbiol.* **2002**, *4*, 193−203.

(12) Miller, L. G.; Connell, T. L.; Guidetti, J. R.; Oremland, R. S. *Appl. Environ. Microbiol.* **1997**, *63*, 4346−4354.

(13) Connell Hancock, T. L.; Costello, A. M.; Lidstrom, M. E.; Oremland, R. S. *Appl. Environ. Microbiol.* **1998**, *64*, 2899−2905.

(14) Schaefer, J. K.; Oremland, R. S. *Appl. Environ. Microbiol.* **1999**, *65*, 5035−5041.

(15) Goodwin, K. D.; Varner, R. K.; Crill, P. M.; Oremland, R. S. *Appl. Environ. Microbiol.* **2001**, *67*, 5437−5443.

(16) Widdel, F.; Kohring, G.-W.; Mayer, F. *Arch. Microbiol.* **1983**, *134*, 286−294.

(17) Hobbie, J. E.; Daley, R. J.; Jasper, S. *Appl. Environ. Microbiol.* **1977**, *35*, 1225−1228.

(18) Oremland, R. S.; Miller, L. G.; Strohmaier, F. E. *Environ. Sci. Technol.* **1994**, *28*, 514−520.

(19) Elliott, S.; Rowland, F. S. *Geophys. Res. Lett.* **1993**, *20*, 1043−1046.

(20) Woodall, C. A.; Warner, K. L.; Oremland, R. S.; Murrell, J. C.; McDonald, I. R. *Appl. Environ. Microbiol.* **2001**, *67*, 1959−1963.

(21) UNEP. Methyl Bromide Technical Options Committee, 1998.

(22) Chitwood, D. E.; Deshusses, M. A. *Environ. Sci. Technol.* **2001**, *35*, 636−642.