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Oxidation of propene and 1-butene by *Methylococcus capsulatus* and *Methylosinus trichosporium*

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SUMMARY

Methane-grown cells of *Methylococcus capsulatus* and *Methylosinus trichosporium* readily oxidized propene and various isomers of butene to their respective epoxides. When examined in a proton NMR spectrum using tris([3-trifluoromethylhydroxymethylene]-*d*-camphorato), europium III derivative as an optically active chemical shift reagent, the products propylene oxide and 1,2-epoxybutane were found to contain equal amounts of both isomers. Methane-grown cells of both bacteria had considerable levels of reducing equivalents to catalyze the epoxidation of gaseous olefins. Cells depleted of reductants catalyzed the oxidation in the presence of low levels of methanol or formaldehyde with a stoichiometry of about 2:1. The rates of epoxidation of propene and 1-butene in a continuous reactor were 2–3-times that of a batch-wise reaction; the epoxidation activity, however, was lost within 3 h. The inactivation was attributed to the reactivity of the accumulated epoxides in the reactor. Propene and 1-butene oxidation by both bacteria were drastically inhibited by the respective products. Thus, the major problem in the application of microorganisms for production of epoxides from gaseous olefins is the rapid separation of the reactive products.

INTRODUCTION

Propylene oxide and epoxides of butene are industrially important monomers. At present, these oxides are made by coproduct oxidation and chlorohydrin processes. Direct epoxidation of propene and 1-butene is an attractive alternative route, but to date, an efficient way of doing this by a chemical process has not been developed. There are several microorganisms that efficiently catalyze the epoxidation of gaseous olefins. These include the methanotrophs [2,5,15,33], *Mycobacterium* [9,10] and other C_3 and C_4 alkane-utilizing bacteria [18,27]. Of all these, methanotrophs are by far the most extensively studied, especially *Methylococcus capsulatus* [2,4,5,15,17,19,25,28,30,32,33] and *Methylosinus trichosporium* [1–3,14,15,17,19,25,30,33]. Oxidation of methane to methanol in *M. capsulatus* and *Mts. trichosporium* is initiated by methane monooxygenase [4,5,15,22]. The latter compound is further oxidized by a series of dehydrogenases to carbon dioxide [2,5,33]. Methane monooxygenase, by virtue of its wide substrate specificity, enables the whole cells to oxidize compounds such as ole-

fins, aromatic hydrocarbons and phenols to non-metabolizable epoxides, phenols and catechols, respectively [3–5,11,14,17,18,32].

We evaluated the application of *M. capsulatus* and *Mts. trichosporium* for the production of olefinic oxides. Propylene oxide and 1,2-epoxybutane produced by both bacteria were achiral. In a continuous reactor, both bacteria produced propylene oxide and 1,2-epoxybutane at impressive rates but the catalysis lasted merely 3 h. The reactive epoxides caused rapid inactivation of the cells. It is concluded that one of the major problems in the application of methanotrophs for direct epoxidation is the rapid separation of highly reactive epoxides.

MATERIALS AND METHODS

Chemicals. All gases were obtained from Scott specialty gases, Troy, MI. Other chemicals used were of highest available purity.

Microorganisms and growth conditions. M. capsulatus N.C.I.B. 11132 and *Mts. trichosporium* NRRL 11202 were used throughout the studies. Both bacteria were routinely grown in 2-l flasks in the presence of 500 ml of Stanier's mineral medium [31] and 250 ml of filter sterilized methane. The gas was introduced by means of a syringe through the silicone septum in the holed screw cap. Incubation conditions were 45°C for *M. capsulatus* and 35°C for *Mts. trichosporium* for 18 h at 200 rpm shaker speed. Large amounts of both bacteria were obtained by growing them in 15-l fermentors in the presence of air and methane. The best ratio of air/methane for maximum yield of cells/l of the medium was determined in a chemostat. Initial flow rate of air/methane was 50:20 ml/min but it was gradually increased to 2000:100 ml/min during the logarithmic phase of growth. At all stages of growth, pH was maintained at 6.8 and cells were kept in dissolved oxygen at 20% or less. Cells were harvested after 40 h at dry weights of 8–10 g/l for *M. capsulatus* and 6 g/l for *Mts. trichosporium*. Harvested cells were washed twice with 20 mM potassium phospate buffer (pH 7.0, KP buffer) and resuspended in the same buffer at 34–38 g/l.

Assays. The oxidation of methane and the oxidation of gaseous olefins were examined in 25-ml baffled flasks fitted with a screw cap and septum. Typically, 0.2–0.5 ml of bacterial cell suspension (7.6 mg of *M. capsulatus* or 19 mg of *Mts. trichosporium*) were taken in 5 ml of KP buffer. Where necessary, appropriate amounts of methanol or formaldehyde were added. The reaction was initiated by the introduction of 5 ml of gaseous substrate (230 μmole) and the flasks were placed in a shaker maintained at appropriate temperature and 200 rpm. Periodically, the reaction was monitored by gas-liquid chromatography (GLC) either by the disappearance of substrate or appearance of product. Tenax GC (60–80 mesh) packed in a 6-f glass column 2 mm i.d. was used as a separation medium. The column was fitted in a Hewlett Packard 5790A instrument and conditioned according to instructions. Methane was detected at −30°C, while olefins and epoxides were detected at 80°C. Helium was used as carrier gas at 40 ml/min. Air and hydrogen were maintained in the detector at 250°C, at flow rates of 240 and 30 ml/min, respectively. The peaks were integrated using a Hewlett-Packard 3270 integrator and the reaction rates were quantitated by construction of standard graphs of substrates and products. The rate of methane oxidation was determined in a Gilson oxygraph by measuring the substrate-dependent oxygen uptake.

Continuous reactor. A jacketed glass column (60 × 3 cm i.d.) was fitted with a ground-glass cover and used as a continuous reactor. The cover carried a sparger along the length of the column and an outlet tube for emerging gas mixture. Typically, the reactor was filled with 350 ml of KP buffer containing 10–12 g cells. A mixture of air and 1-butene was introduced at 300:25 ml/min by precision control flow meters (Forberg Instruments). Frothing of cells was prevented by addition of antifoam SAG-471 (Union Carbide) at 0.001%, v/v. The rate of oxidation of 1-butene was quantitated by analyzing the difference in the concentration of the gas in the inlet and outlet streams. The outlet tube of the reactor was maintained at 120°C to prevent condensation and the pressure inside the reactor was less than 1.5 lb/in^2. Whenever necessary, the outlet

stream was passed over anhydrous Sephadex G-150 and trapped at −70°C to collect 1,2-epoxybutane (BO). Any condensed 1-butene was removed at −20°C from the microbially produced BO and the latter was analyzed for optical purity. The samples of BO produced by both bacteria were taken at 0.1 ml/ml of deuterated chloroform solvent containing trace tetramethyl silane (TMS) for chemical shift reference. Nuclear magnetic resonance spectra were scanned on a Nicolet NT 300 spectrometer in a 5 mm H-1 probe via pulse/FT technique with deuterium field/frequency lock. Pulse width was 2 μs, acquisition time 4.1 s and repeat time 14.1 s. Total number of scans were 32–64. After scanning the NMR spectrum, the optically active shift reagent tris([3-trifluoromethylhydroxymethylene]-*d*-campharato), europium III derivative (Eu-Camph) was added at 0.05 g/ml of solvent.

RESULTS

Oxidation of methane and olefins by methanotrophs

Suspensions of *M. capsulatus* and *Mts. trichosporium* in KP buffer could be stored for 10 days at 24°C and up to 3 weeks at 4°C with little loss of activity. The rate of oxidation of methane by the two methanotrophs is shown in Fig. 1A. Followed by disappearance of substrate, the rates were 40 and 10 mmol/h per g dry weight for *M. capsulatus* and *Mts. trichosporium*, respectively. The comparative values obtained from oxygen uptake studies were, respectively, 22 and 11 mmol oxygen consumed/h per g. The oxidation of propene and 1-butene by the two bacteria is depicted in Fig. 1B. The rates for both olefins were 1.6–2.6 mmol/h per g. The major product of oxidation was identified as propylene oxide and 1,2-epoxybutane, respectively, by comparing the GLC elution profiles with standards and confirming with a GLC mass spectrometer. Although not depicted, *cis* and *trans*-2-butene, isobutene and butadiene were oxidized by both bacteria at rates between 0.5 and 1.0 mmol/h per g. The major products of *cis* and *trans*-2-butene were *cis*-2,3- and *trans*-2,3-epoxybutane, respectively.

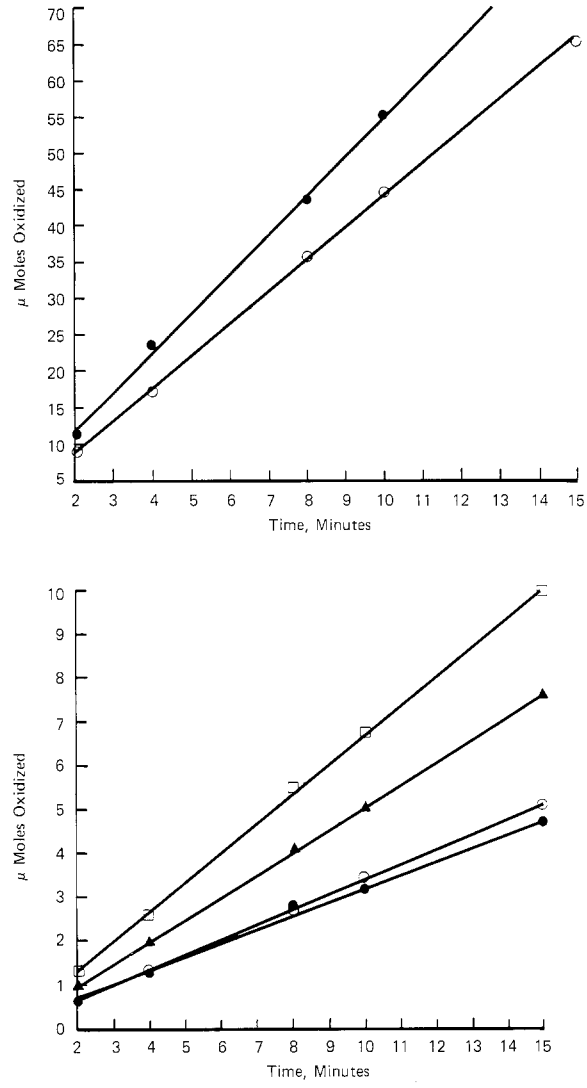

Fig. 1. A, oxidation of Methane by *M. capsulatus* (●) and *Mts. trichosporium* (○). B. Oxidation of propylene (filled symbols) and 1-butene (open symbols) by *M. capsulatus* (●,○) and *Mts. trichosporium* (▲,□). The assay mixture in 5 ml of KP buffer contained 7.6 mg of *M. capsulatus* or 19 mg of *Mts. trichosporium*. The reaction was started by addition of 240 μmol of methane, propene or 1-butene. Other details of assay methods are described in Materials and Methods.

Stoichiometry of olefin oxidation in the presence of methanol and formaldehyde

Both *M. capsulatus* and *Mts. trichosporium* contained considerable amounts of reducing equivalents for oxidation of olefins at the rates shown in

Table 1

Stoichiometry of propene and 1-butene oxidation in the presence of methanol and formaldehyde

The reaction mixture contained 7.6 mg of *M. capsulatus* or 19 mg of *Mts. trichosporium* cells in 5 ml of KP buffer. The cells were expended of all reserve reductants in the presence of 220 μmol of propene over 2 h and equilibrated with air. Amount of propene oxidized during this period was 35 μmol. These cells were designated spent cells and challenged with olefins in the presence of the indicated amounts of methanol or formaldehyde.

Spent cells	Compound added (nmol)	Olefin oxidized (nmol)
M. capsulatus	None	0
	Methanol (500)	Propene (1049)
	Methanol (1000)	1-Butene (2018)
	Formaldehyde (500)	Propene (986)
Mts. trichosporium	None	0
	Methanol (500)	Propene (905)
	Formaldehyde (500)	1-Butene (895)

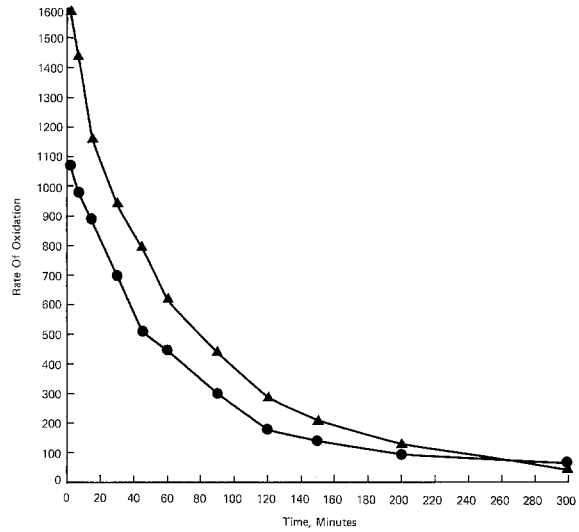

Fig. 2. Kinetics of rate of 1-Butene Oxidation by *M. capsulatus* (●) and *Mts. trichosporium* (▲) in a continuous reactor. The continuous reactor contained 8.64 g of *M. capsulatus* or 12.1 g of *Mts. trichosporium* cells in 400 ml of KP buffer. Rate of oxidation was determined by analyses of 1-butene in 1 ml of inlet and outlet gas streams and is expressed as μmol oxidized/min. For further details on the continuous reactor, see Materials and Methods.

Fig. 1B. Generation of more reductants by addition of methanol or formaldehyde had no effect on the rate of olefin oxidation. Hence, the stoichiometry of methanol or formaldehyde-dependent olefin oxidation was determined using cells expended of reductants in the presence of 220 μmol propene. The spent cells were reequilibrated with air and examined for olefin oxidation. As shown in Table 1, in the presence of low levels (500–1000 nmol) of methanol or formaldehyde, the stoichiometry of olefin oxidation was about 1:2, but the rate was only 0.1–0.2 mmol/h per g. At higher levels of methanol or formaldehyde, the stoichiometry was much lower.

Oxidation of olefin in a continuous reactor

The initial rates of oxidation of 1-butene by *M. capsulatus* and *Mts. trichosporium* in the continuous reactor were 11.1 and 5.3 mmol/h per g, respectively (Fig. 2). However, the epoxidation activity was gradually lost within 3 h (Fig. 2). Addition of methanol or formaldehyde into the reactor caused no increase in the rate of oxidation of 1-butene. During the time course of 1-butene oxidation, there was accumulation of 1,2-epoxybutane in the reactor. When examined for methane- or methanol-dependent oxygen uptake prior to (11–22 mmol/h per g) and after the duration of the experiment in Fig. 2, (0.27–0.59 mmol/h per g) the cells revealed more than 90% loss of activity. Similar results were obtained during the oxidation of propene in the continuous reactor.

Inhibition of 1-butene oxidation by 1,2-epoxybutane

The rate of oxidation of 1-butene by *M. capsulatus* and *Mts. trichosporium* in the presence of various concentrations of 1,2-epoxybutane is shown in Fig. 3. As low as 0.05% v/v of the epoxide reduced the rate of oxidation by 50%. Propylene oxide exerted a similar effect on the rate of propene oxidation by both bacteria. The inhibition caused by the epoxides was not reversed by excess olefins or upon reequilibration of cells with air.

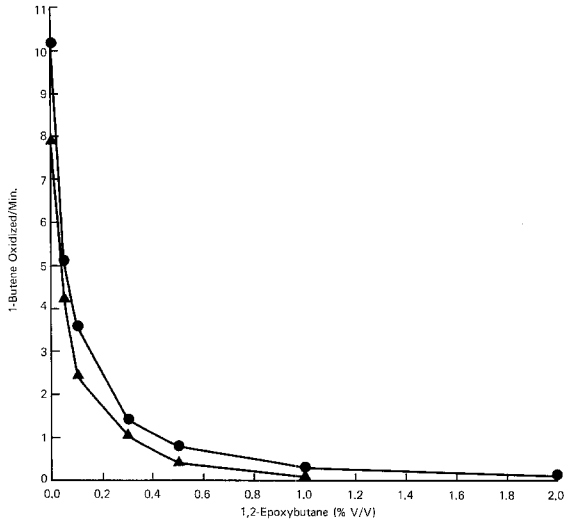

Fig. 3. Inhibition of 1-butene oxidation by 1,2-epoxybutane. The reaction mixture in 5 ml of KP buffer contained the amounts of *M. capsulatus* (●) and *Mts. trichosporium* (▲) indicated in Fig. 1. The cells were incubated for 2 min with various amounts of 1,2-epoxybutane prior to the start of the reaction with 220 μmol 1-butene. Other details of the assay are described in Materials and Methods.

Determination of the optical purity of microbially produced 1,2-epoxybutane

Although the continuous reactor functioned for only 3 h, it was very convenient for trapping 1,2-epoxybutane, the details of which are described in Materials and Methods. The NMR spectrum of the epoxide produced by *M. capsulatus* (S1) and *Mts. trichosporium* (S2) showed peaks of δ = 1.01 (triplet) and 1.58 (multiplet) for the methyl and methylene protons, respectively. The ring protons A, B and C (see Fig. 4) appeared as peaks at δ = 2.90 (multiplet), 2.74 (doublet of doublet) and 2.48 (doublet of doublet), respectively. This spectrum was identical to that of a chemical standard. No impurity was apparent in the sample and only a trace of water was detected. The optical isomers in

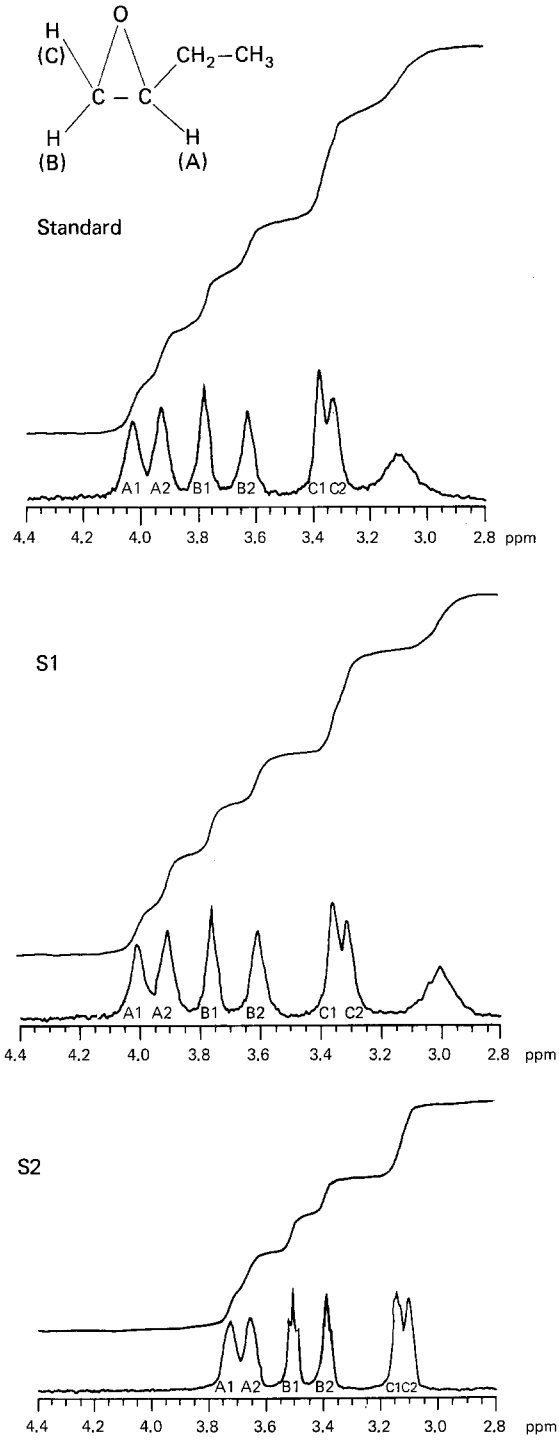

Fig. 4. Chiral shift in ring protons of standard and microbially produced 1,2-epoxybutane in the presence of chiral shift reagent Eu-Camph. NMR spectra were recorded with 0.1 ml of sample and 0.06 g Eu-Camph/ml $CDCl_3$ solvent. The epoxide produced by *M. capsulatus* is labelled S1 and that obtained from *Mts. trichosporium* is designated S2. The splits in ring protons A, B and C in the presence of Eu-Camph are labelled 1 and 2. Details regarding trapping of the epoxide from the continuous reactor and conditions of spectral tracings are described in Materials and Methods.

standard, S1 and S2 were resolved with Eu-Camph which deshielded all epoxybutane protons relative to TMS (Fig. 4). The ring protons labelled A, B and C in Fig. 4 were shifted most, and each proton was further split into two distinct absorptions labelled 1 and 2, where 1 is a greater shift. The magnitudes of induced splitting were B > A > C, with A being the average of B and C. The epoxy ring proton absorptions were also broadened by Eu-Camph as they were shifted. Quantitation was performed by digital integration of the absorptions B1 and B2. In all three samples, standard, S1 and S2 (Fig. 4), the areas B1 and B2 were about equal, indicating the presence of both optical isomers in about equal amounts. The resolution of absorptions A and C were not nearly as well resolved as B and hence not integrated.

DISCUSSION

Direct epoxidation of propene and 1-butene in the presence of air or oxygen is an attractive process. Several microorganisms have been reported to catalyze this reaction in near perfect yields [2,5,9,10,15,20,27,33]. Prominent among these are the methanotrophs which, when grown on methane, produce an NADH-dependent methane monooxygenase [4,5,22,24,25]. The latter, by virtue of its wide substrate specificity, enables the whole cells to oxidize a variety of compounds, including gaseous olefins [3–5,11,14,17,18,32]. A group of less characterized C_2-C_7 alkane-utilizing bacteria also catalyze the epoxidation of olefins with a non-specific monooxygenase [20,26,27]. In contrast, a number of soil bacteria are capable of metabolizing ethylene via ethylene oxide and acetyl-CoA [6–10]. These organisms produce a specific monoxygenase for epoxidation of short-chain olefins [8–10].

Our interest was concentrated on a whole cell microbial epoxidation process and its practical value. Methanotrophs, *M. capsulatus*, and *Mts. trichosporium* were chosen for investigation primarily because (i) they readily accumulate epoxides (ii) they are able to use methanol as in vivo reductant for epoxidation. Also, we were able to optimize the growth of both bacteria in a chemostat by varying the methane-to-air ratio and could obtain high cell yields (g/l medium) in a 15-l fermentor. Finally, methane and olefin oxidation by resting cell suspensions of both bacteria were stable for several days. A similar observation has been recorded by Ribbons and Michalover [29] in a strain of *M. capsulatus*.

The rate of oxidation of methane by both bacteria were 10–12-times that of gaseous olefins, whether assayed by GLC (Fig. 1) or the polarographic method. Amongst the olefins, propene and 1-butene were oxidized 2–4-times the rate of other isomeric butenes. Consistently, *M. capsulatus* showed a 2-fold higher activity with all hydrocarbon substrates than *Mts. trichosporium* in both assay methods. The observed rate of oxidation of methane, propene and 1-butene were either better [1,4,8,9,17–20,23,24,25,27,32] or compared favorably [28] with the values reported for whole cells or cell-free extracts. The major reaction products of various gaseous olefins were identified as the respective epoxides, consistent with the reports of other investigators [4,8–10,13,14,17,20,26].

Methane grown cells of *M. capsulatus* and *Mts. trichosporium* had considerable amounts of reducing equivalents to catalyze the epoxidation of gaseous olefins. This is in accordance with the observations of Hou et al. [18] in several methanotrophs. However, in contrast to Hou et al. [18], Leak and Dalton [21] and Stirling and Dalton [32], addition of methanol, formaldehyde or C_2 to C_4 primary alcohols and their corresponding aldehydes had no effect on the rate of olefin oxidation by resting cell suspensions of both bacteria. Thus, NADH was not rate limiting in the epoxidation reactions. In cells expended of reserve reductants (spent cells) the stoichiometry of methanol or formaldehyde consumption to propene or 1-butene oxidation was 1:2 (Table 1). Both methanol and formaldehyde are potential sources of two molecules of NADH in vivo [2,5,21,33] and hence account for the observed stoichiometry. The rate of olefin oxidation by spent cells was 10–15-fold lower than that of resting cell suspensions. At higher levels of external reductants, both the stoichiometry and rate of olefin oxidation

were much lower. This could be due to the combined effect of inactivation of cells by epoxides and inhibition of oxidation at higher levels of methanol [5].

The oxidation of propene and 1-butene by *M. capsulatus* and *Mts. trichosporium* was examined in a continuous process in order to increase the stability of cells by rapid separation of epoxides. The initial rate of oxidation of 1-butene was 2–4-fold higher than batch-wise assays (Fig. 2), perhaps due to better mass transfer of gaseous substrates aided by reactor configuration. In a continuous process with immobilized *Methylosinus* sp. described by Hou [16], the rate of propylene oxidation was at least 10-fold less. The rate was also much lower for ethylene and propene oxidation by immobilized *Mycobacterium* Py1 in a gas-solid bioreactor [10]. It is conceivable that such a reactor would be mass transfer-limited for gaseous substrates and hence may not achieve the desired rates in a continuous process. Although the rates were higher in our reactor, the activity declined rapidly (Fig. 3) in spite of continuous removal of 1,2-epoxybutane. The steady-state concentration of the product in the reactor was sufficient to inactivate the cells. This is evident from the more than 90% loss in methane and methanol-dependent oxygen uptake in cells after the experiment with the reactor, compared to resting cells. Addition of methanol or formaldehyde failed to revive the activity of the cells. In comparison, propene oxidation by immobilized *Methylosinus* sp. was revived by methanol, albeit to low levels [16]. Likewise, the depleted ethylene-oxidizing activity was restored in immobilized *Mycobacterium* by propionaldehyde [10]. In both of the above cases, the inactivation of cells by epoxides may have been lower due to a lower rate of product turnover in the gas-solid bioreactor [10,16].

The rapid loss of activity in the reactor was due to 1,2-epoxybutane. This is further substantiated by the results presented in Fig. 3. As low an amount as 0.05% v/v of the epoxide reduced the rate of 1-butene oxidation by 50% and the reaction was completely abolished at about 0.25% v/v. The inactivation caused by 1,2-epoxybutane was not reversed by excess substrate or upon reequilibration of cells with air. The effect of propylene oxide on propene oxidation by both cells was the same. The mechanism of inactivation by the epoxide appears to be due to random reactivity with cellular nucleophiles rather than specific inhibition of methane monooxygenase or any other enzyme.

By NMR-spectroscopy in the presence of chiral shift reagent Eu-Camph, 1,2-epoxybutane produced by *M. capsulatus* and *Mts. trichosporium* was found to be racemic. This was not surprising, since the epoxidation is catalyzed by methane monooxygenase, an enzyme with broad specificity towards several structurally unrelated substrates [3–5,22,24,25]. On the other hand, *Mycobacterium* and related strains produced predominantly the *R*-form of 1,2-epoxypropane, 1,2-epoxybutane and (*S*)-1-chloro-2,3-epoxypropane from the respective substrates [13]. These bacteria, unlike the methanotrophs, are capable of utilizing ethylene or propene as sole source of carbon and energy [6–10] and have been shown to elicit a monooxygenase specific for short-chain alkenes [9,10,12,13].

In conclusion, our studies have revealed that methanotrophs do not produce chiral epoxides of propene and 1-butene. However, if a microbial process for production of racemic epoxides is desired, methanotrophs are the organisms of choice. They can be grown in methane to impressive cell yields and the rate of epoxidation is much higher than that in any other bacteria. Methanol can be utilized in stoichiometric amounts as in vivo reductant for epoxidation. The major challenge is the rapid separation of epoxides from the reactor to increase the stability of cells.

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