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Optimization and maintenance of soluble methane monooxygenase activity in *Methylosinus trichosporium* OB3b

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Abstract

Soluble methane monooxygenase (sMMO) maximization studies were carried out as part of a larger effort directed towards the development and optimization of an aqueous phase, multistage, membrane bioreactor system for treatment of polluted groundwater. A modified version of the naphthalene oxidation assay was utilized to determine the effects of methane:oxygen ratio, nutrient supply, and supplementary carbon sources on maximizing and maintaining sMMO activity in *Methylosinus trichosporium* OB3b. *Methylosinus trichosporium* OB3b attained peak sMMO activity (275-300 nmol of naphthol formed h^{-1} mg of protein^{-1} at 25°C) in early stationary growth phase when grown in nitrate mineral salts (NMS) medium. With the onset of methane limitation however, sMMO activity rapidly declined. It was possible to define a simplified nitrate mineral salts (NMS) medium, containing nitrate, phosphate and a source of iron and magnesium, which allowed reasonably high growth rates (μ_{max} 0.08 h^{-1}) and growth yields (0.4-0.5 g cells/g CH_4) and near maximal activities of sMMO. In long term batch culture incubations sMMO activity reached a stable plateau at approximately 45-50% of the initial peak level and this was maintained over several weeks. The addition of d-biotin, pyridoxine, and vitamin B_{12} (cyanocobalamin) increased the activity level of sMMO in actively growing methanotrophs by 25-75%. The addition of these growth factors to the simplified NMS medium was found to increase the plateau sMMO level in long term batch cultures up to 70% of the original peak activity.

Abbreviations: sMMO – soluble methane monooxygenase, pMMO – particulate methane monooxygenase, NMS – nitrate mineral salts, TCE – trichloroethene, NADH – reduced nicotinamide adenine dinucleotide

Introduction

Soluble methane monooxygenase (sMMO) and particulate methane monooxygenase (pMMO) are two different enzymes which carry out essentially the same function, however they coexist in certain methanotrophic strains. These enzymes have been extensively studied in *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b (Dalton 1992, Murrell 1992) but they are also found in *Methylosinus sporium* strain 5 (Brusseau et al. 1990; Pilkington & Dalton 1991), *Methylocystis* sp. M (Uchiyama et al. 1992), *Methylomonas methanica* 68-1 (Koh et al. 1993), *Methylobacterium* sp. CRL-26 (Patel & Savas 1987), and in various groundwater isolates (Bowman et al. 1993). Though widely distributed sMMO appears to be a strain-specific trait (Koh et al. 1993; Bowman et al. 1993)

while pMMO is universal among all methanotrophs studied so far (Dalton 1992). It has been postulated that methanotrophs producing sMMO may have evolved in habitats which are regularly starved of copper (Prior and Dalton 1985). This cannot be supported empirically, however. sMMO and pMMO synthesis is regulated by copper availability. Copper suppresses the synthesis of sMMO while it promotes pMMO synthesis (Stanley et al. 1983).

Trichloroethene (TCE) has become a major target for developing bioremediation processes using methanotrophs (Hazen 1992; Semprini et al. 1992) owing to its importance as a relatively common, recalcitrant, environmental pollutant (Ensley 1991) as well as its potential carcinogenic threat (Infante and Tsongas 1979). The enzyme sMMO has been shown to co-oxidize trichloroethene (TCE) one- or two-orders of magnitude more efficiently than pMMO (DiSpirito et al. 1992) and other broad specificity monooxygenase and dioxygenase systems (Ensley, 1991). sMMO can insert oxygen into alkanes, haloalkanes, alkenes, ethers, alicyclic, aromatic, and heterocyclic compounds. By comparison pMMO has a significantly narrower substrate range. The mechanistic aspects of sMMO have been recently reviewed by Dalton (1992). Methods are available for specifically quantifying sMMO activity. For instance sMMO can be assayed by its capacity to oxidize cyclohexane to cyclohexanol (Colby et al. 1977) or naphthalene to naphthol (Brusseau et al. 1990; Koh et al. 1993). These substrates cannot be oxidized by pMMO.

Recent interest in developing strategies for removal of chlorinated aliphatic contaminants from groundwater aquifers has lead in a number of directions. A major path has lead to the development of sMMO-based bioremediation systems. In situ systems involving the stimulation of an indigenous methanotrophic microflora to degrade TCE and other similar contaminants have been extensively studied (Hazen, 1992; Semprini et al. 1992). 'Pump and treat' bioreactor(-)based strategies are still in development with relatively few field-scale systems having been implemented. Multi-stage, dispersed growth reactor systems have considerable potential for reaching the field-scale application level (Alvarez-Cohen & McCarty 1991b, McFarland et al. 1992). These systems have the advantage that methanotrophs can be grown in one stage continually maintaining levels of sMMO activity and avoiding the problem of TCE toxicity. Exposure of cells to contaminants takes place in an adjacent stage with subsequent disposal of the cells.

A number of studies have attempted to define cultural conditions needed to obtain sMMO activity in methanotrophs and thus observe heightened TCE degradation. Most studies indicate that copper availability is the most critical factor in obtaining sMMO activity (Oldenhuis et al. 1989; Tsien et al. 1989). Certain studies have focused on obtaining high rates of TCE degradation in resting cell assays. For instance the use of artificial electron donors such as formate enhance sMMO activity (Oldenhuis et al. 1989; Brusseau et al. 1990). Other factors such as the effects of methane competition on TCE degradation (Oldenhuis et al. 1990; Broholm et al. 1992) and TCE toxicity have also been closely studied (Alvarez-Cohen & McCarty, 1991a). Relatively few studies have attempted to determine exactly how sMMO activity can be maximized in methanotrophs during growth and then be maintained over extended periods. Studies by Park et al. (1992) revealed that certain nutrients, including CO_2, iron, nitrate, and phosphate were important in obtaining good growth rates and yields of the methanotroph *Methylosinus trichosporium* OB3b; these studies did not indicate clearly how sMMO activity may be optimized independent of growth rates.

As part of a larger effort involving the development of an innovative bioreactor system for sMMO-based TCE biotreatment, studies involving sMMO optimization in batch culture were undertaken. The factors critical for maximal, long term, and stable sMMO activity in the strain *Methylosinus trichosporium* OB3b was investigated. Factors studied included the supply of reducing equivalents and nutrients, and stimulation of sMMO synthesis with exogenous growth factors. This information is important if sMMO-based bioreactor systems are to be truly optimized for the efficient degradation of TCE and other halogenated aliphatic compounds. (Some of this work has been presented at the 2nd International Symposium on *in situ* and On-Site Bioreclamation, San Diego, USA, April 1993).

Materials and methods

Organisms and culture conditions

The methanotrophs studied here (Table 1) were grown routinely at 25°C in a liquid nitrate-mineral salts medium (NMS) slightly modified from Cornish et al. (1984): 2 mM $NaNO_3$, 2 mM phosphate buffer (pH 6.8), 10 mM $NaHCO_3$, 150 μM $MgSO_4.7H_2O$, 50 μM $FeCl_3.6H_2O$, 50 μM $CaCl_2.2H_2O$, 2 μM $MnSO_4.4H_2O$, 2 μM $ZnSO_4.7H_2O$, 2 μM H_3BO_3, 1 μM K_2SO_4, 1 μM KI, 0.65 μM $CoCl_2.H_2O$, and 0.4 μM $Na_2MoO_4.2H_2O$. The medium was prepared with doubly de-ionized water (resistivity >18 MOhm cm^{-1}) and was essentially copper-free. The pH was adjusted to 6.8 with HCl. All glassware was acid-washed to avoid copper contamination. Cultivation was routinely performed under an atmosphere of 1:4 methane:air unless otherwise specified. Cultures were aerated by shaking at 200 rpm. The culture density was determined spectrophotometrically at 600 nm. Whole-cell protein content was determined by the microbiuret procedure of Munkres and Richard (1965).

Table 1. Naphthalene and TCE transformation kinetic parameters obtained using various methanotrophs.

Strain	Naphthalene		TCE	
	V_{max} (nmol h^{-1} mg protein^{-1})	K_m (μM)	V_{max} (nmol min^{-1} mg protein^{-1})	K_m (μM)
Methylosinus trichosporium OB3b (= ATCC 35070)[a,b]	328 ± 21[c]	40 ± 3	255 ± 62	126 ± 8
Methylosinus trichosporium OB3b (PP358)	305 ± 6	37 ± 5	278 ± 56	138 ± 13
Methylosinus sporium 5(= ATCC 35069)	840 ± 47	96 ± 11	454 ± 84	178 ± 24
Methylococcus capsulatus Bath (ATCC 33009)	47 ± 5	84 ± 6	12 ± 3	249 ± 30
Methylomonas methanica 68-1[a]	551 ± 27	70 ± 4	360 ± 75	225 ± 13
Methylosinus sp. 2CC[d]	383 ± 27	83 ± 5	187 ± 28	200 ± 54
Methylosinus sp. 4CA	249 ± 11	23 ± 2	224 ± 46	89 ± 6
Methylosinus sp. 4CB	217 ± 3	24 ± 5	205 ± 35	96 ± 9
Methylosinus sp. 5CC	521 ± 48	38 ± 4	385 ± 72	120 ± 15
Methylosinus sp. 5CD	678 ± 14	32 ± 4	440 ± 48	136 ± 16
Methylosinus sp. 6CA	674 ± 20	24 ± 4	451 ± 77	99 ± 9
Methylosinus sp. 7CA	280 ± 14	59 ± 4	180 ± 61	153 ± 20
Methylosinus sp. 7CB	546 ± 26	48 ± 4	364 ± 68	117 ± 12
Methylosinus sp. 9BA	231 ± 20	54 ± 9	167 ± 20	177 ± 27
Methylosinus sp. 9CA	53 ± 3	69 ± 3	16 ± 5	310 ± 26
Methylocystis sp. 9BB	77 ± 13	65 ± 12	23 ± 6	246 ± 31
Unidentified group II methanotrophs:				
1C30A	72 ± 3	56 ± 3	29 ± 5	266 ± 22
1C30P2	405 ± 5	88 ± 2	239 ± 20	221 ± 40
1C30L	36 ± 4	72 ± 6	N.D.[b]	N.D.
1C50L1	405 ± 45	53 ± 5	268 ± 49	168 ± 17
2C10P	177 ± 14	89 ± 5	117 ± 37	188 ± 22
3C10P	420 ± 32	82 ± 5	280 ± 30	204 ± 17
3C50	578 ± 38	82 ± 4	347 ± 30	196 ± 23

[a] Data from Koh et al. (1993).

[b] ATCC, American Type Culture Collection, Rockville, Maryland, USA; N.D. = not done.

[c] Parameters were calculated from triplicate analyses.

[d] Methanotrophs were isolated from either groundwater or sediment samples from a trichloroethene and tetrachloroethene-contaminated aquifer at the U.S. Department of Energy Savannah River Laboratories, South Carolina, USA (Bowman et al. 1993).

Determination of sMMO-specific activity

A modification of the naphthalene oxidation assay of Brusseau et al. (1990) was used to quantify sMMO-specific activity. Cell suspensions were diluted to an absorbance of 0.2 (at 600 nm). The diluted cultures were transferred in 1 ml aliquots to 10 ml screw cap test tubes followed by the addition of 1 ml of saturated naphthalene solution (234 µM at 25°C) (Verschueren 1983). The reaction mixtures were then incubated at 200 rpm on a rotary shaker at 25°C for 1 h. Controls included heat-killed cell suspensions and methanotroph cultures grown in the presence of 1 µM $CuSO_4.6H_2O$. Triplicate samples were prepared. After incubation, 100 µl of freshly prepared 0.2% (w/v) tetraotized *o*-dianisidine solution was added to the reaction mixture. Samples were then immediately monitored spectrophotometrically at 525 nm. The intensity of diazo-dye formation is proportional to the naphthol concentration formed by the oxidation of naphthalene by sMMO. Wackett and Gibson (1983) determined the extinction coefficient (ε) of the naphthol diazo dye to be 38000 M^{-1} cm^{-1}. Naphthalene oxidation kinetics were determined by incubating cultures of the various methanotrophs listed in Table 1 with various levels of naphthalene ranging from 7.3 to 195 µM for 1 h and determining naphthalene oxidation specific rate (nmol h^{-1} mg $protein^{-1}$) at each concentration. Kinetic parameters were then determined by fitting the data to rectangular hyperbolic curves ($V = V_{max}S/(K_m + S)$ using the computer program DeltaGraph (DeltaPoint, Monterey, CA). Parameters were found to be in good agreement to values estimated from Lineweaver-Burk plots.

TCE degradation analyses

Cell suspensions were transferred in 1 ml aliquots into screw cap septum vials (14 ml; Pierce, Rockford IL) which were then closed with caps and teflon-lined silicone seals. TCE degradation was initiated by the addition of a saturated TCE aqueous solution (1100 ppm or 8.36 mM at 25°C) (Verschueren, 1983). The vials were then inverted and incubated at 25°C on a rotary shaker at 200 rpm. After incubation (5-15 min) the reaction was terminated by the addition of 2 ml *n*-hexane containing 1 mg/l 1,2-dibromoethane as an internal standard. The undegraded TCE was extracted into the solvent phase by shaking and centrifugation (2000 *g*, 20 min). TCE quantification was performed using a Shimadzu GC 9AM gas chromatogram (Shimadzu Analytical instruments Co., Kyoto, Japan) equipped with a 1: 1 split injector port operated at 220°C, a 60 m x 0.53 mm i.d. R_{TX} volatiles capillary column (Restek Corp., Bellefonte, PA) operated isothermically at 120°C wrth an electron capture detector at 220°C. Nitrogen was used as the carrier gas (flow rate 10 ml/min). The peak areas were integrated with a Shimadzu C-R6A Chromatopac. For determination of TCE degradation kinetics for the various methanotrophs listed in Table 1, the procedure of Oldenhuis et al. (1991) was followed.

Other analytical procedures

Concentrations of methane in cultures were determined by analysing head space samples containing methane by gas chromatography using a Shimadzu GC 9 AM chromatogram equipped with a flame ionization detector and a 15m x 0.53 mm i.d. AT-1 capillary column (Alltech, Deerfield, IL) maintained at 60°C using nitrogen as the carrier gas (1 ml/min). Nitrate, copper, and iron concentrations were monitored following EPA standard methods (Franson 1992). Phosphate was analysed using a inorganic phosphorus kit supplied by Sigma Chemicals Co. (St Louis, MO).

Maximization studies in NMS media

Methane and oxygen availability

Methylosinus trichosporium OB3b was cultivated in 10 ml of NMS medium in 60 ml serum vials (Wheaton Inc., Millville, NJ) in which different initial dissolved methane concentrations were created by adding various amounts of methane with a syringe. The resultant dissolved methane concentrations ranged from 1.6 to 16.5 mg/l as determined by Henry's Law (Atkins 1986). In some experiments,

nitrogen gas was added to the vials to vary the availability of dissolved oxygen (0.4-8.3 mg/l) in the presence of 20% methane. Specific growth rates were determined under these different conditions. sMMO specific activity was determined by the naphthalene oxidation assay.

Effect of nutrients

Various constituents in the NMS medium including nitrate, phosphate, $MgSO_4.7H_2O$, $FeCl_3.6H_2O$, $CaCl_2.2H_2O$, $MnSO_4.4H_2O$, $ZnSO_4.7H_2O$, $CoCl_2.6H_2O$, and $Na_2MoO_4.2H_2O$ were either omitted or added at different concentrations, ranging from 0.01 up to 10 times the original concentration in the regular NMS medium. The effects on *Methylosinus trichosporium* OB3b specific growth rate and sMMO specific activity were assessed.

Effect of exogenous growth factors and carbon substrates

A series of experiments were undertaken to determine if carbon substrates or growth factors had a stimulatory effect on sMMO activity in *Methylosinus trichosporium* OB3b and in five other methanotrophs (Table 2). The carbon substrates were added to the NMS medium to obtain concentrations of 1 or 10 mM. The substrates tested included tricarboxylic acid intermediates and related compounds, including: acetate, DL-lactate, pyruvate, citrate, 2-oxoglutarate, succinate, malate, and, fumarate; serine pathway intermediates: L-serine, glycine, β-hydroxypyruvate, and glyoxylate; D-glucose, various amino acids, and vitamins were also tested. The vitamin solution, a modification of Wolfe's vitamin solution (Balch and Wolfe 1976) consisted of: calcium pantothenate, niacinamide, thiamine-HCI, and riboflavin, all at 5 mg/l; d-biotin, folate, *p*-aminobenzoate, pyridoxal, and L-ascorbate all at 2 mg/l; pyridoxamine and pyridoxine, at 1 mg/l; and vitamin B_{12} at 0.1 mg/l.

Maintenance of sMMO activity

Previous experiments provided information on some of the requirements for obtaining high rates of sMMO activity in *Methylosinus trichosporium* OB3b and other methanotrophs. By utilizing this information, a series of experiments were designed to determine to what extent sMMO can be maintained in long term batch cultures of *Methytosinus trichosporium* OB3b. Maintenance experiments were performed in a simplified nitrate medium consisting of 2 mM $NaNO_3$, 2 mM phosphate buffer (pH 6.8), 50 μM $FeCl_3.6H_2O$, and 50 μM $MgSO_4.7H_2O$. The stability of sMMO activity in *Methylosinus trichosporium* OB3b was observed in long-term batch culture experiments. *Methylosinus trichosporium* OB3b was grown in 2 l flasks in 500 ml of NMS media. A set of flasks received methane (initial dissolved methane concentration was equal to 2 mg/l) at time 0 and on each subsequent day of the experiment. The flasks were also sparged with filter-sterilized air periodically to prevent oxygen limitation. Another set of flasks was treated similarly except that methane was added only at time 0 with no further additions for a period of 20 d. After 20 d addition of methane recommenced, with regular air sparging. A 20 ml sample was taken daily from both sets of flasks and analysed for optical density, sMMO activity, nitrate, phosphate, and iron. Additionally, nitrate, phosphate, iron, and magnesium were added to all of the flasks to the original NMS medium concentration. The recovery

Table 2. The effect of various virtamins on sMMO activity in *Methylosinus trichosporium* OB3b.

Vitamin	sMMO specific-activity (nmol h^{-1} mg $protein^{-1}$)
none added	317 ± 18
complete vitamin solution[a]	398 ± 20
pyridoxine	477 ± 25
d-biotin	472 ± 13
vitamin B_{12}	451 ± 17
folate	348 ± 22
p-aminobenzoate	343 ± 19
pantothenate	339 ± 11
L-ascorbate	325 ± 16
pyridoxal	324 ± 21
pyridoxamine	313 ± 15
thiamine	236 ± 20
niacinamide	228 ± 10
riboflavin	227 ± 16

[a] The concentrations of the vitamins used were a 1:200 dilution of the concentrations in the complete vitamin stock solution (see text).

and maintenance of sMMO specific activity was then monitored for up to 22 d.

Results and discussion

Naphthalene and TCE transformation by methanotrophs

Strain selection and development for a TCE treatment system may be a useful start in an optimization process. As the development of $sMMO^+$ $pMMO^-$ copper tolerant mutants is feasible (Phelps et al. 1992) the problem of suppression of sMMO by copper (Tsien et al. 1989) can be avoided. Similarly the selection of strains exhibiting high rates of TCE transformation and a superior resilience to TCE toxicity is also possible. In this study significant variations were evident in the naphthalene and TCE transformation rates amongst several sMMO-producing methanotrophs examined (Table 1). A number of strains exhibited superior naphthalene or TCE transformation rates when compared to *Methylosinus trichosporium* OB3b and its $sMMO^+$ $pMMO^-$ constitutive mutant PP358 (Phelps et al. 1992). The maximal transformation rates (V_{max}) of naphthalene and TCE by the methanotrophs examined correlated in a linear fashion ($r^2 = 0.91$: $y = 36x + 22$). Overall, the TCE transformation V_{max} was approximately 36 times greater than the corresponding naphthalene oxidation V_{max}. This correlation appears to validate the naphthalene oxidation assay as a way of accurately quantifying sMMO activity. The assay can give an indication of the TCE transformation rate for an sMMO-producing methanotrophs at any given time. The naphthalene oxidation procedure has several advantages in that it does not require gas chromatography, is rapid, and convenient.

Effect of methane and oxygen supply on sMMO specific activity

The specific-sMMO activity and TCE degradation rate in *Methylosinus trichosporium* OB3b was found to increase with increased dissolved methane

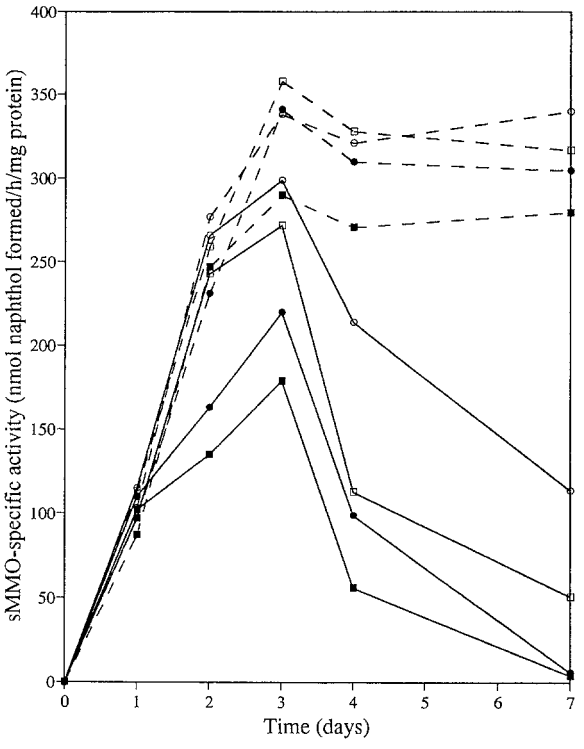

Fig. 1. The effects of dissolved methane concentrations on sMMO specific activity in *Methylosinus trichosporium* OB3b. Specific sMMO activities were determined with resting cells with (---) and without (—) 20 mM sodium formate. The initial dissolved methane concentrations were 1.6 (■), 3.3 (●), 6.6 (□), and 16.5 (○) mg/l.

concentrations. The greatest rates were achieved in the early stationary growth phase after 3 d incubation (Figure 1). Protein content in OB3b cells at this growth stage was found to be 0.70 ± 0.10 mg/mg cells. The initial rate of sMMO activity increase was about the same for the different methane concentration levels. The maximum levels of naphthalene oxidation were obtained with 16.5 mg/l methane at 308 ± 18 nmol h^{-1} mg $protein^{-1}$. After 4 d enzyme activity started to decline with the decline dependent on the initial methane level. By 7 d enzyme activity was virtually absent in cultures grown with 1.6 mg/l or 3.3 mg/l methane with no residual methane detectable. However significant levels of sMMO activity were still present, as well as residual methane (0.5-1.7 mg/l), in the cultures initially supplied with 6.6 mg/l and 16.5 mg/l (Figure 1). To determine if the increase in sMMO activity was due to an increased availability of NADH supply, 20 mM sodium for-

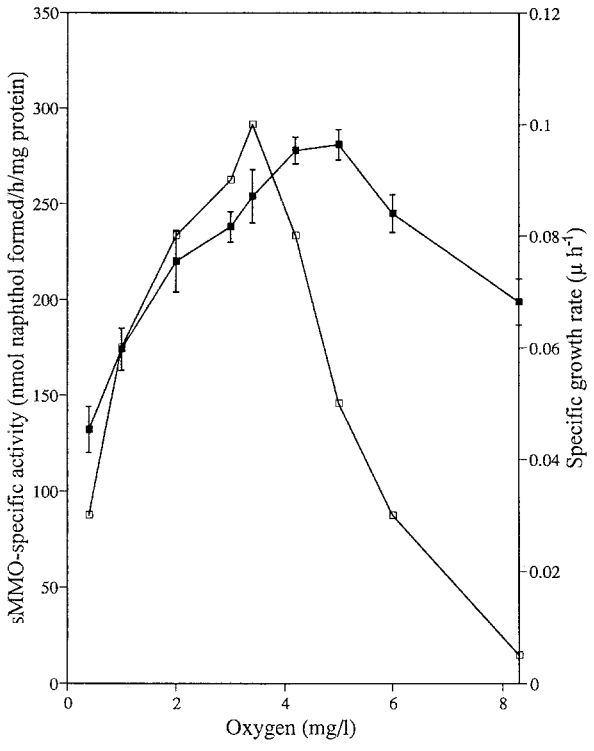

Fig. 2. The effects of oxygen availability on specific growth rate (□) and sMMO specific activity (■) in *Methylosinus trichosporium* OB3b.

mate was included in the sMMO activity assays. It was found that sMMO activities of all the cultures were not appreciably stimulated by the formate until stationary phase was attained and methane became limiting. At this point the cultures with methane initially at 1.6–6.6 mg/l were stimulated to a level comparable to the 16.5 mg/l methane cultures (Figure 1). Thus increased sMMO-specific activity appears to be primarily promoted by the increased availability of reductant supply, i.e. NADH. This is probably due to increased dissimilatory methane oxidation (Rokem & Goldberg 1991). Studies with both *Methylosinus trichosporium* OB3b purified sMMO extracts (Dalton 1992) and whole cells (Alvarez-Cohen & McCarty 1991c) have found that enzyme activity declines rapidly if a hydroxylatable substrate, such as methane, is absent. The reduction in activity is thought to be related to a cellular NADH conservation mechanism mediated by the sMMO B protein preventing complete exhaustion of NADH pools by methane oxidation (Dalton 1992). The degree of reduction in sMMO-activity also seems to be affected by oxygen. Alvarez-Cohen & McCarty (1991c) noticed that low levels of oxygen had a stabilizing effect on sMMO activity when no substrate was present.

In our study oxygen did not seem to be a critical factor for high sMMO expression (Fig. 2); instead oxygen had more bearing on cell growth rates and yields, a fact observed earlier by Park et al. (1991). A modest but significant increase in sMMO-specific activity did occur with ascending dissolved O_2 levels peaking at about 5 mg/l, while at higher O_2 levels a slight decrease in specific sMMO activity was observable. Maintaining high concentrations of dissolved methane (>7 mg/l) could be implemented in two stage dispersed bioreactors. The problems associated with methane reducing TCE degradation rates due to competition (Broholm et al. 1992) can be avoided if TCE degradation takes place in the absence of methane. Oxygen concentrations ideally should be maintained at a level sufficient for high growth rates and sMMO-specific activity. Our study suggests 2–5 mg/l (dissolved O_2) would be sufficient for this.

Effect of nutrients on sMMO activity

It was found the most critical media components for sMMO activity were nitrate, phosphate, and to a lesser extent iron, and magnesium. When completely deprived of a combined nitrogen source and thus actively fixing nitrogen, *Methylosinus trichosporium* OB3b still maintained high growth yields though it grew at a significantly lower growth rate (μ_{max} 0.02 h^{-1}); however, sMMO activities were only 10% of the control cultures (data not shown). When fixing nitrogen a significant proportion of the available NADH is apparently siphoned to the nitrogenase. Significant increases in sMMO activity were observed when 20 mM formate was added to these cultures. With 0-2 mM nitrate present sMMO activity steadily increased (Figure 3). Between nitrate concentrations of 2 and 100 mM there was no significant further increase in sMMO activity, growth rates or yields. Phosphate, added at 2 to 25 mM maintains both sMMO and biomass levels at a simi-

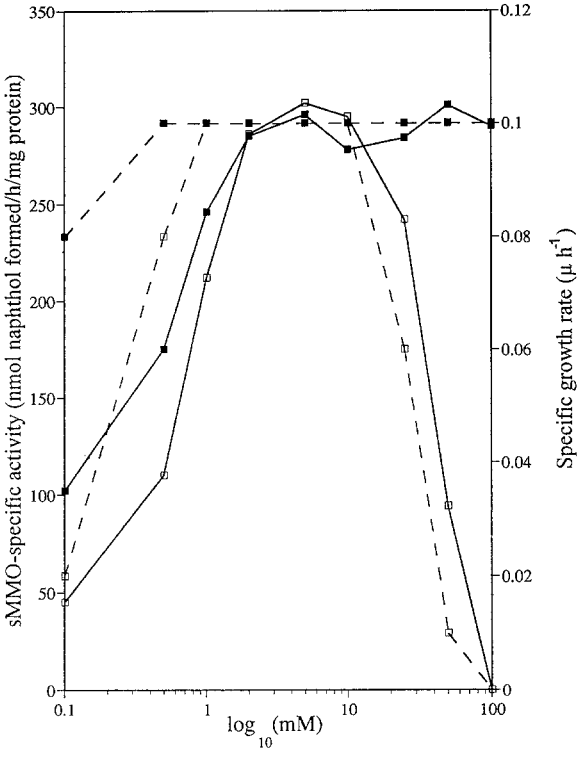

Fig. 3. The effects of nitrate (■) and phosphate (□) levels on sMMO-specific activity (—) and specific growth rates (---).

lar level (Figure 3). Growing *Methylosinus trichosporium* OB3b at phosphate concentrations lower or higher concentrations resulted in a significant decline in growth rate and sMMO activity (Figure 3). High concentrations of phosphate are known to inhibit methanol dehydrogenase activity (Mehta et al. 1987) leading to insufficient formaldehyde production for cell carbon assimilation or for dissimilation to generate NADH.

When iron was provided at less than 10 μM there was a slight reduction in sMMO activity but a more significant decline in growth rate occurred. This result has been previously reported by Park et al. (1991). However in this study it was possible to obtain stable and high sMMO activity at only ≥ 30 μM $FeCl_3.6H_2O$. 50 μM $MgSO_4.7H_2O$ seemed adequate to maintain maximal sMMO activity. When magnesium was not supplied, a moderate decrease in both growth rate and sMMO activity was observed (data not shown). Other trace elements, if absent from the growth medium, did not seem to markedly effect sMMO activity or the growth of *Methylosinus trichosporium* OB3b and presumably are not vital for growth or sMMO synthesis. It was thus possible to define a simplified NMS medium to obtain high sMMO activity. The medium consisted of 2 mM $NaNO_3$, 2 mM phosphate buffer (pH 6.8), 50 μM $FeCl_3.6H_2O$, and 50 μM $MgSO_4.7H_2O$. Reasonable growth rates (0.08 h^{-1}) and yields (0.4-0.5 g cells/g methane) were obtained for OB3b in this medium. Ostensibly contaminated groundwater entering an sMMO-based 'pump and treat' system could be supplemented with nitrate, phosphate, and if they are present in the groundwater only at low concentrations, iron and magnesium.

Effect of supplementary substrates

In this study we found that only modified Wolfe's vitamin solution stimulated sMMO activity in *Methylosinus trichosporium* OB3b (Table 2). The addition of a 1:200 dilution of the Wolfe's vitamin solution gave the greatest increase in sMMO activity compared to unsupplemented controls. The addition of individual vitamins to the medium was performed to find the compounds responsible for the stimulation of sMMO activity. The highest levels of stimulation of sMMO activity occurred when vitamin B_{12} (0.5 μg/l), d-biotin (10 μg/l), or pyridoxine (10 μg/l) were added (Table 2). Only a slight further increase in sMMO-specific activity occurred when these three vitamins were added together. Vitamins seemed to have an oligodynamic effect on sMMO activity. Excessive vitamin concentrations led to a degree of sMMO suppression. This was probably due to the influence of riboflavine, thiamine, and niacinamide which were inhibitory to sMMO activity (Table 2) and to growth rates. Subsequent experiments showed that vitamins do not act as artificial electron donors as is the case for formate and several other compounds (Leak & Dalton 1983). The stimulation of sMMO only occurred when the ultures were actively growing in the presence of vitamin B_{12}, d-biotin, or pyridoxine. No significant increase in protein synthesis, growth rate, or growth yield was observed in the vitamin(-)supplemented cultures. Hypothetically, these compounds could be stimulating the activity of ancillary enzymes which

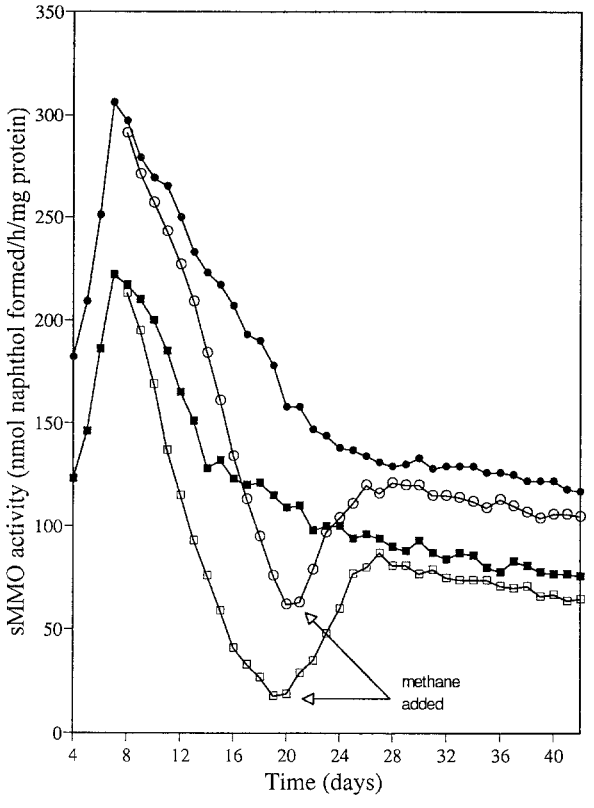

Fig. 4. Maintenance of sMMO specific activity in *Methylosinus trichosporium* OB3b was observed in NMS media supplied with 1:4 methane:air every 24 h (■) and supplied with 1:4 methane:air only once at time 0 (□) Vitamin-ammended (0.5 μg/l vitamin B_{12}, 10 μg/l d-biotin, and 10 μg/l pyridoxine) NMS media was also utilized and with one set receiving methane every 24 h (●) while another set received methane only once at time 0 (○). After 20 d of incubation the addition of 1:4 methane:air to the methane-starved cultures (□, ○) recommenced.

may be indirectly associated with sMMO, alternatively they may broaden the availability of NADH in the cell resulting in the increased sMMO-specific activity.

Maintenance of sMMO activity

Cultures of *Methylosinus trichosporium* OB3b were grown in the simplified NMs medium (see above). The set of cultures that were provided methane only at the time of inoculation experienced methane limitation and a subsequent rapid decline in sMMO activity. No residual methane was detected in these flasks after 13 d incubation. Those cultures given a relatively constant methane supply showed a lower rate of loss of sMMO activity (Figure 4). This decline slowed and eventually plateaued at a level equal to 45-50% of the original peak sMMO activity level (Figure 4). When methane limitation was removed in the methane-starved cultures (after 20 d incubation), sMMO activity was eventually restored back to levels comparable to cultures which were continually maintained under methane (Figure 4). Samples taken from the cultures during the incubation were also tested with the addition of 20 mM sodium formate. No significant stimulation of sMMO activity was found in the cultures maintained with methane, while in those under methane limitation stimulation of sMMO activity was observed. The stimulated sMMO activity was approximately equal to those found for the cultures maintained with excess methane. Since sMMO can be maintained at a reasonably high activity in long-term cultures, bioreactor systems can be adapted to have a higher degree of cell recycling thus reducing the need to continually regrow cells. This approach could be used to improve the economic aspect of such systems; however, TCE toxicity has to be also considered.

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