Batch Cultivation of *Methylosinus trichosporium* OB3b. I: Production of Soluble Methane Monooxygenase

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Methanotrophs have promising applications in bioremediation and in the production of fuel-related chemicals due to their nonspecific enzyme, methane monooxygenase (MMO). The optimal conditions for cell growth and production of the soluble form of MMO (sMMO) were determined from batch cultivations of an obligatory methanotroph, *Methylosinus trichosporium* OB3b, in shake flasks and a 5-L bioreactor. It was confirmed that a copper deficiency is essential for the formation of the cytoplasmic sMMO. Optimum cell growth without added copper was observed at pH 6.0–7.0, temperature of 30–34°C, and phosphate concentration of 10–40 m*M*. In the bioreactor experiments, external CO_2 addition eliminated the long lag period observed in the absence of added $CuSO_4$, i.e., prior to the exponential cell growth phase. When methane was continuously supplied, the profile of the cell growth showed two different phases depending on the availability of nitrate, an initial fast exponential growth phase (specific growth rate, μ = 0.08 h^{-1}) and a later slow growth phase (μ = 0.008 h^{-1}). The cell density at the transition from a fast to a slow growth rate was proportional to the initial medium nitrate concentration in the range 5–20 m*M* and cell yield was estimated to be 7.14 g dry cell wt/g N. Whole-cell sMMO activity remained essentially constant regardless of the growth rate until cell growth stopped. With an initial medium iron concentration below 40 m*M*, an abrupt decrease in sMMO activity was observed. The lowered sMMO activity could be restored by supplying additional iron to the bioreactor culture. Cell yield on iron was estimated to be 1.3×10^3 g dry cell wt/g Fe.

Key words: methanotroph • soluble methane monooxygenase • optimization of culture conditions

INTRODUCTION

Methanotrophs are microorganisms which have the ability to derive both carbon and energy from the metabolism of methane. They are potentially useful in the bioremediation of polluted environments, since they can effectively degrade a number of chlorinated hydrocarbons.[9,17,25,33] Another potentially important application of methanotrophs is the production of methanol[21] and various alkene epoxides.[11,14] Methanol has attractive possible large-scale uses as an alternative cleaner-burning fuel and as a feedstock for the synthesis of the gasoline octane booster, methyl tertiarybutyl ether.[2,24,29] Among the alkene epoxides, propene oxide has an important commercial value and, furthermore, the biocatalyzed conversion of propene to propene oxide is of interest to study as a model gas-solid bioreactor system.[12]

The foregoing potential applications of methanotrophs are attributed to the presence of their rather non-specific enzymes called methane monooxygenases (MMOs). In some methanotrophs such as *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* (Bath), the MMO enzyme is known to be associated with either a membrane-bound (particulate) fraction or as a cytoplasmic (soluble) protein complex, depending on the growth conditions.[8,30,31] While the soluble MMO (sMMO) enzyme system has been purified and studied in some detail during the past five years,[10,19,26] the growth characteristics of microorganisms producing this enzyme have not been investigated and discussed in much detail. Harwood and Pirt examined the growth of *M. capsulatus* (Bath) and provided some basic information which is helpful in assessing the economic feasibility of producing microbial protein from natural gas.[13] They studied the effects of nutrient composition and several operational conditions, but did not address the production of MMO. The effect of oxygen tension on methylotrophic bacteria was examined by Dalton's group[22,23] and Maclennan et al.[20]. In *M. capsulatus* (Bath), Dalton et al. noted that a high oxygen tension above a pO_2 of 0.1 atm inhibited nitrogen assimilation and reduced the growth rate under nitrate- or ammonium-limiting conditions. Maclennan et al. observed that increasing the oxygen tension decreased the cell yield, but it increased CO_2 production during the continuous culturing of *Pseudomonas* AM1 on methanol.

Establishment of the precise culture conditions governing the intracellular location or form of the MMO enzyme has been a conflicting, but important issue. Scott et al.[30] reported that *M. trichosporium* OB3b generated the particulate form of MMO (pMMO) during oxygen-limited, nitrate-excess chemostat culturing. However, Dalton's group reported that the enzyme lo-

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cation in *M. capsulatus* (Bath) and *M. trichosporium* OB3b was determined by copper availability in the culture medium: MMO activity was found in the membrane fraction when adequate copper was added, and in the soluble fraction when copper availability became limited.[31] They also suggested that the conditions described by Scott et al.[30] masked the underlying copper effect. More recently, Higgin's group also demonstrated the role of copper in regulating the intracellular location of MMO in *M. trichosporium* OB3b, but they did not clarify the effects of the nutrients that they previously proposed to be responsible for determining its location and form.[5,8]

This article deals with optimizing the culture conditions for the growth of *M. trichosporium* OB3b and the simultaneous production of sMMO activity. The parameters considered include pH, temperature, and the concentrations of some key culture medium components such as copper, phosphate, nitrate, and iron. The intracellular location of the MMO enzymes was monitored carefully, since defining the culture conditions which would lead to the exclusive formation of sMMO was desired. Experiments were also performed in an attempt to increase the final biomass density.

MATERIALS AND METHODS

Microorganism and Culture Conditions

Methylosinus trichosporium OB3b obtained from Professor R. S. Hanson (Gray Freshwater Biological Institute, University of Minnesota) was used in all experiments. It was maintained on 1.7% agar plates of Higgins nitrate minimal salt medium[6] at 30°C (see Table I). The agar plates were cultured and stored several weeks in a gas-tight jar under a 1:1 (v/v) methane/air gas mixture. For long term storage, glycerol was added at 20% (w/v) to the fully grown shake-flask cultures and the mixture was maintained at −60°C.

The basal culture medium used for both the shake-flask and the bioreactor experiments was the same nitrate minimal salt medium cited above, but it lacked $CuSO_4$. It was prepared from four separate concentrated stock solutions as described in Table I, except that $CuSO_4$ was deleted from solution 3. Cellulose acetate (0.22 μm) membranes, 50-mm-diameter disposable filter unit from Corning Glass Works (Corning NY) or a 142-mm-diameter 316 stainless-steel filter apparatus from Millipore Corp., were used for sterilizing all of the liquid solutions. For the bioreactor experiments which involved the continuous supply of an air/CO_2 gas mixture (see below), the fully constituted culture medium was sparged and saturated with this gas mixture for at least 6 h (along with automatic pH adjustment to 7.0) prior to the inoculations.

Flask experiments were performed at 30°C (unless stated otherwise) in a gyratory incubator (model G24, New Brunswick Scientific Co., New Brunswick, NJ) at a shaking speed of 300 rpm. Shake flasks (300 mL) having a 19-mm (o.d.) sidearm tube (Wheaton Instruments) were employed to read the absorbance directly without removing samples during the cultivation. The liquid working volume was 20 mL. The small screw cap opening on the flask was used for introducing the methane/air mixture. It was sealed with an open-top-closure screw cap and a PTFE-faced red rubber septum (Kimble Glass Inc.). The large opening on the flask was used for adding culture medium and the inoculum. It was plugged with a sponge stopper during autoclaving, but was subsequently sealed tightly during the cultivation with the rubber-lined plastic screw cap that was supplied with the flask. Before starting a new flask incubation, a 1:1 (v/v) methane/air gas mixture was bubbled into the inoculated culture medium for 5 min at a flow rate of 270 mL/min. It was added through a sterile intraveneous placement unit consisting of a needle and a long catheter (Delmed, Inc., New Brunswick, NJ). The methane and air were measured and mixed with a Matheson gas proportioner (model 7300, Matheson Gas Products, Syracuse, NJ) and sterilized through a 0.22-μm disposable filter unit (Corning Glass Works) before the gas mixture passed into the flasks. Generally, during flask culturing the gas phase was either not replenished or else it was replaced one time after 48 h. When

Table I. Composition and concentration of stock solutions for Higgins nitrate minimal salt medium.[a]

Substance	Amount (g/L)
(1) 100× salt solution[b]	
$NaNO_3$	85
K_2SO_4	17
$MgSO_4 \cdot 7H_2O$	3.7
$CaCl_2 \cdot 2H_2O$	0.7
(2) 100× phosphate buffer solution[b]	
KH_2PO_4	53
Na_2HPO_4	86
Adjust solution to pH 7.0	
(3) 500× trace metals solution[b]	
$ZnSO_4 \cdot 7H_2O$	0.287
$MnSO_4 \cdot 7H_2O$	0.223
H_3BO_3	0.062
$NaMoO_4 \cdot 2H_2O$	0.048
$CoCl_2 \cdot 6H_2O$	0.048
KI	0.083
$CuSO_4 \cdot 5H_2O$	0.125
1 mL of 1 m*M* H_2SO_4 per liter of trace metals	
(4) 1000× iron solution[b]	
$FeSO_4 \cdot 7H_2O$	11.2
5 mL of 1 m*M* H_2SO_4 per 100 mL iron	

[a] Culture medium was made up as follows: Appropriate aliquots of concentrated solutions 1–3 were added to the desired volume of double distilled water and the combined diluted mixture was sterilized by passage through a 0.22-μm membrane filter. Separate and freshly prepared sterile iron solution 4 was then added aseptically to this mixture. Higgins standard nitrate minimal medium contains Cu at a final concentration of 1.0 μ*M*.

[b] Each stock solution was stored at 4°C after sterilization, except for the phosphate buffer which was stored at room temperature.

a longer growth period was desired, it was replenished every 24 h.

Fermentor-scale experiments were performed in a 5-L bioreactor (Bioflo II, New Brunswick Scientific Co.) with a continuous flow of gases. The incubation temperature was 30°C and the pH was maintained at 6.8–7.2 through the automatic addition of 2*N* NaOH. Dissolved oxygen (DO) levels were kept above 10% saturation in most cases by controlling the agitation speed (350–700 rpm) and the gas flow rates: 150–500 mL/h for methane and 450–1500 mL/h for air or 10% CO_2-containing air. They were regulated with Matheson mass flowmeters (model 8111) and high accuracy valves (Series 4170).

Analytical Methods

Cell Densities and Nitrate Concentration

Cell densities were determined by measuring the absorbance at 660 nm (A_{660}) in three spectrophotometers, two Spectronic model 21s (Milton-Roy Inc.) and a Gilford model 260 (Gilford Instrument Laboratory, Inc.). Sidearm shake-culture flasks were read in a Spectronic 21 that was equipped with a universal cuvette holder. The cell densities in the bioreactor were monitored continuously by pumping the culture broth into a flow-through cell (glass tube, outside diameter of 10 mm) positioned in a separate Spectronic 21 which was connected to a chart recorder (BD 40, Kipp & Zonen, Holland). The conversion factors from the sidearm flask Spectronic 21 readings and the bioreactor flow-through cell Spectronic 21 readings to the Gilford 260 (10-mm cuvette) readings were 1.5 and 4.0, respectively. One unit of A_{660} in the Spectronic 21 (sidearm flask) corresponded to 0.41 mg dry cell wt/mL; for the Gilford 260 (cuvette) it represented 0.27 mg dry cell wt/mL, based on a linear dry weight curve over an extended range of absorbances. Periodically, serial-dilution platings on 1.7% agar-Higgins medium were performed to validate that the shake-flask and bioreactor A_{660} readings were indeed representitive of cell densities.

Nitrate concentration was determined with an ion-specific nitrate electrode (Orion, model 93-07) and a double-junction reference electrode (Orion, model 90-02) connected to a pH/ion meter (model 701A, Orion Research Instruments). It was possible to measure nitrate concentrations as low as 0.01 m*M* without any pretreatment of the culture medium.

MMO Activity in Intact Washed Cell Suspensions

Microorganisms were harvested by centrifugation at room temperature in an Eppendorf microcentrifuge (model 5413, Brinkmann Instruments, Inc.) at 10^4g for 2 min. The cell pellets were washed once with buffer [25 m*M* 3-*N*-morpholino)-propanesulfonic acid (MOPS), pH 7.0, plus 5 m*M* $MgCl_2$], resuspended in the same buffer to give a cell density of ca. 5.0 mg dry wt/mL, and placed on ice. Enzyme assays were performed immediately after sampling, usually within 30 min.

Total whole-cell MMO (soluble plus particulate) activity was determined routinely by measuring the epoxidation rate of propene.[15] The presence of sMMO in intact cells was assessed by measuring the disappearance rate of chloroform. Chloroform oxidation is a catalytic activity of the soluble, but not the particulate form of *M. trichosporium* OB3b MMO.[32] MMO assay incubations were carried out in 5-mL clear (for propene) or 5-mL amber (for chloroform which is light-sensitive) vials sealed with open-top-closure screw caps and PTFE-faced rubber septa; the PTFE facings of the septa were placed downward toward the gas phase within the vials. The reaction mixtures contained in a total liquid volume of 0.5 mL: 0.1–0.5 mg equivalents of dry cell mass (0.3–1.5 mg of protein in the case of broken cell fractions); 12.5 μmol MOPS buffer, pH 7.0; 2.5 μmol $MgCl_2$; and 10 μmol sodium formate. Propene gas (44.6 μmol) was injected into the headspace of the vial. The alternate sMMO-specific substrate, chloroform (0.5–1.0 μmol), was incorporated into the 0.5 mL liquid volume. Reactions were initiated by placing the vials in a reciprocal water bath shaker (30°C, 180 rpm). Liquid samples of 3 μL (for propene activity) or gas samples of 30 μL (for chloroform activity) were analyzed at 3–5 min intervals over a total time period of 10–30 min with a gas chromatograph fitted with a flame ionization detector (Hach, Carle Chromatography Series 100, Hach Co.). A 6-ft stainless-steel column (outside diameter of 1/8 in.), packed with 0.1% SP1000 on Carbopack C, 80/100 mesh, was used for both the propene and the chloroform substrate assays. The column temperature was 85°C for propene and 130°C for chloroform. Helium was used as the carrier gas at a flow rate of 20 mL/min.

MMO Activity in the Cell-Free Systems

Cells from shake flasks were harvested by centrifugation (10^4g, 10 min, 4°C), washed once in ice-cold 25 m*M* MOPS buffer, pH 7.0, containing 5 m*M* $MgCl_2$, and resuspended in the same buffer to give ca. 25 mg dry cell wt/mL. Bovine pancreas deoxyribonuclease I (Sigma Chemical Co.) was added to give a concentration of 10 μg/mL. Cells in suspension at 4°C were then broken by a single pass through a French pressure cell (SLM Instruments, Inc., IL) at 15,000 lb/in.^{2} and the unbroken cells were removed by centrifugation at 10^4g for 10 in, 4°C. The resulting cell-free extract was further centrifuged two successive times at 3.8×10^4g for 30 min, yielding a supernatant (S38) and an initial particulate (P38 combined sediment) fraction. In order to remove probable contamination from entrapped S38, the initial P38 fraction was washed, recentrifuged one additional time in the same cold buffer, and again resuspended to give the final P38 membrane material.

most constant throughout the cultivation, except for a decreasing trend near the end of the batch operation. It is worth noting that there was no appreciable change in sMMO activity at around 50–60 h, i.e., before and after the large shift in the growth rate. Under these culture conditions the level of sMMO catalysis and, presumably, the rate of methane assimilation are not proportional to the bacterial growth rate. A biphasic growth curve and the nonvarying sMMO activity have not been addressed in the past, but they may explain why the reported values for the methane consumption efficiency (defined as g dry cell wt produced/g methane consumed) of *M. trichosporium* OB3b and several other methanotrophs are not consistent and vary widely.[1,16] Figure 5 also shows that the decrease of the growth rate coincided with the depletion of culture medium nitrate at 50–60 h. This indicated that the transition might be induced by a switch of the nitrogen source from nitrate to the gaseous nitrogen that is carried into the bioreactor as part of the 9:1 air/CO_2 mixture. It has been established that *M. trichosporium* OB3b can fix gaseous nitrogen through a nitrogenase which is inducible under either nitrate- or ammonium-limiting culture conditions.[3,4]

The effect of nitrate on the cell growth and the transition in the growth rate was investigated further: first, by varying its concentration in the bioreactor culture medium, and next, by transferring bioreactor cells growing in two different growth phases into fresh culture media. Figure 6 shows the cell densities both at the transitions (X_t) and at the ends of the batch cultures as a function of the initial medium nitrate concentration. Parameter X_t was determined by extrapolating the straight lines during both growth phases. When the nitrate concentration was varied over the range from 5 to 20 m*M*, X_t increased with increasing nitrate although the final cell densities were practically constant. Cell yield on nitrate was determined from the slope in Figure 6 to be 100 g dry cell wt/mol nitrate or 7.14 g dry cell wt/g N. It is in close agreement with the theoretical value estimated from elemental analyses of various methanotrophs.[1] At a much higher nitrate concentration (40 m*M*), however, the exponential fast growth rate began to decrease well before most of the nitrate was consumed and the proportionality shown in Figure 6 did not extent to this higher nitrate concentration (data not shown). Figure 7 shows the results of the culture transfer experiments. Microorganisms growing in the bioreactor were transferred at two separate times into shake flasks containing fresh media with either no added nitrate or 10 m*M* nitrate. When fast growing early stage bioreactor cells were transferred into the fresh nitrate-containing medium at 20 h, they exhibited the same rapid growth rate. Similarly, when slow growing late-stage bioreactor cells were transferred at 150 h, nitrate quickly restored most of their earlier fast growth rate. But when nitrate-free medium was used, the growth rate was slow regardless of the prior growth rate in the bioreactor. These results, along with those in Figures 5 and 6, demonstrate clearly that nitrate depletion is responsible for the diauxic growth pattern in the batch cultivation of *M. trichosporium* Ob3b in the bioreactor.

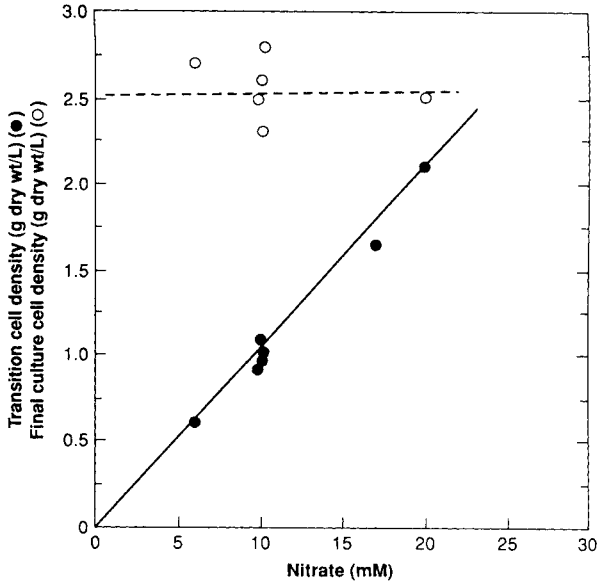

Figure 6. Effect of nitrate concentration on cell density at the transition time (X_t) and at the end of the total growth time in the bioreactor culture.

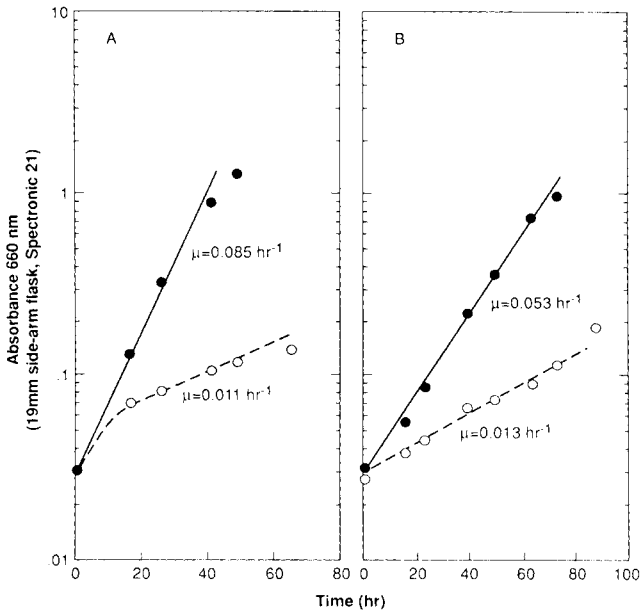

Figure 7. Effect of shake flask nitrate concentration on the growth of prior bioreactor-cultured cells. Inocula cells were transferred from the bioreactor (standard conditions as in Fig. 5) to side-arm shake flasks at 20 hr (A) and at 150 hr (B). They were centrifuged and washed once with nitrate-free medium before inoculation into the flasks. Nitrate concentration was (●) 10 m*M* and (○) 0 m*M*.

Table II. Distribution of MMO activity and protein in broken cell homogenates of *Methylosinus trichosporium* OB3b.[a]

Culture medium	MMO activity (nmol propene oxide/mg protein-min)		Protein (and dry wt) yields/mg dry cell wt	
	Particulate fraction (percent of total)	Soluble fraction (percent of total)	Particulate fraction (dry wt)	Soluble fraction (dry wt)
With 10 μM $CuSO_4$	61.6 ±5.4 (72)	8.8 ±2.3 (28)	0.13 ±0.01 (0.27 ±0.01)	0.41 ±0.01 (0.52 ±0.01)
Without $CuSO_4$	0.6 ±0.3 (2)	36.0 ±3.2 (98)	0.13 ±0.02 (0.24 ±0.04)	0.54 ±0.04 (0.69 ±0.07)

[a] Media contained 40 μM $FeSO_4$ and 10 mM phosphate buffer (pH 7.0); the cellular fractions were prepared from 48-h side-arm shake flask cultures, methane/air (1:1). Data values are the averages of 3–4 independent experiments; ± represent the standard deviations from the mean values.

$3.8 \times 10^4 g$.[27] This explanation is supported by the fact that the S38 fraction from cells grown on 10 μM Cu does not show any chloroform activity in spite of its appreciable propene activity.[32] All of the subsequent experiments described in this paper were carried out in minus-Cu medium.

Effect of Temperature and pH

The effect of temperature on cell growth and sMMO activity was examined (Fig. 2). The specific growth rate increased with increasing temperature up to 34°C and dropped above that. sMMO activity, however, was maximal at 30°C and rapidly decreased with a temperature change in either direction. Chloroform oxidative activities (not shown) were measured and found to be present at a constant ratio of 0.20–0.30 with respect to the propene activity, regardless of the culture temperature. The activation energy for cell growth was estimated to be 2×10^4 cal from a plot of ln μ vs. $1/T$ (Fig. 2, inset). This is slightly higher than the value reported for *Escherichia coli* [$(1.2–1.6) \times 10^4$ cal], but it falls within the broad range that has been determined for most other bacteria [$(1–3) \times 10^4$ cal].[28]

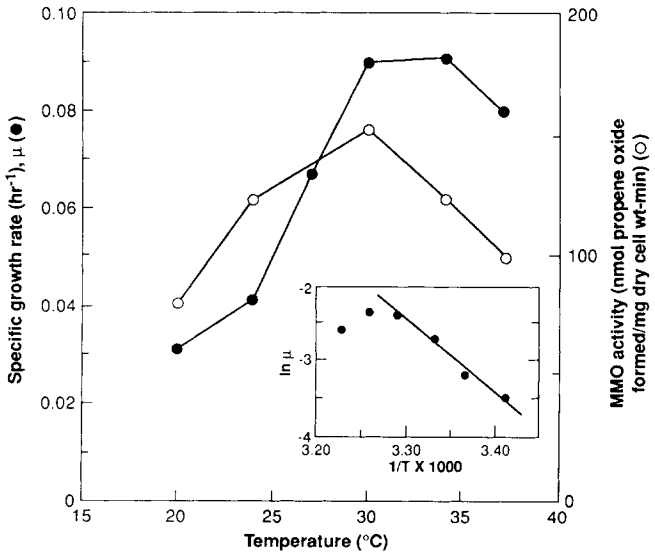

Figure 2. Effect of temperature on the specific growth rate and whole-cell sMMO activity in shake flask cultures (minus-Cu medium). MMO activities were measured at an absorbance of 0.5–0.7 (19-mm sidearm, Spectronic 21). Insert: Arrhenius plot for determination of the activation energy.

The effect of pH was examined in the pH range from 6.0 to 8.5 (Table III). The phosphate concentration was raised from 10 mM (standard level) to 40 mM and held constant to enhance the buffer capacity of the medium. In the pH 6.0–7.0 region, there was essentially no difference in growth rate. But above pH 7.5, an increased lag time was observed and the growth rate decreased with increasing pH. Also, it was found that the culture medium pH significantly shifted towards neutrality during the cultivation, despite buffering with an elevated phosphate concentration. One interesting feature is that the washed whole-cell sMMO activity remained rather constant regardless of significant variations in the culture media pH and the specific growth rates.

Effects of Phosphate Concentration

Figure 3 shows the effects of the culture medium phosphate concentration at pH 7.0 on cell growth and whole-cell MMO activity. Cell growth was inhibited progressively by phosphate concentrations above 40 mM. This is similar to previous results obtained by Harwood and Pirt[13] with *M. capsulatus* (Bath), although the phosphate concentration which is required

Table III. Effect of pH on minus-copper cell growth and whole-cell soluble MMO activity.[a]

pH		Specific growth rate	Lag time	MMO activity (nmol propene oxide/ mg dry cell wt-min)
Initial	Final	(h^{-1})	(h)	
6.0	6.3	0.087	0	132
6.5	6.6	0.087	0	131
7.0	6.9	0.085	0	110
7.5	7.2	0.078	2	108
8.0	7.3	0.071	9	109
8.5	7.5	0.039	15	111

[a] The final pH and sMMO data were obtained with side-arm shake flask cultures (30°C, 300 rpm) at an absorbance of 0.5–0.7, Spectronic 21, and phosphate buffer concentrations of 40 mM in minus $CuSO_4$ media.

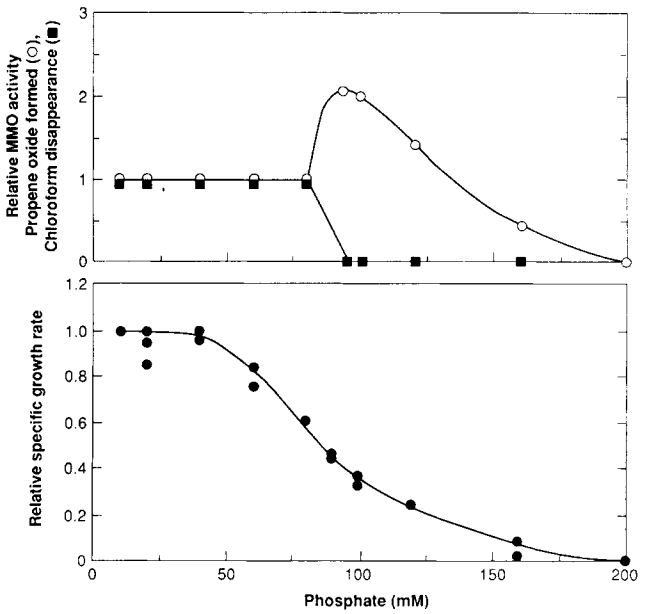

Figure 3. Effect of culture medium phosphate concentration at pH 7.0 on the relative specific growth rate and relative whole-cell MMO activity (shake flask, minus-Cu medium). Relative growth rate and MMO activity were obtained by normalizing each against the corresponding value at 10 m*M* phosphate. MMO activities were measured when the cell cultures reached an absorbance of 0.5–0.7 (19-mm sidearm, Spectronic 21) up to a phosphate concentration of 80 m*M*, and at an absorbance of 0.1–0.3 above that phosphate concentration.

to arrest cell growth completely is more than threefold higher for *M. trichosporium* OB3b. A high phosphate level is known to inhibit whole-cell methanol dehydrogenase (MDH) activity and to result in the accumulation of methanol in the culture medium.[21] But it is not clear why *M. trichosporium* OB3b can tolerate more highly elevated phosphate concentrations than *M. capsulatus* (Bath). Both the propene and the chloroform oxidative MMO activities in Figure 3 are seen to be constant up to 80 m*M* phosphate, irrespective of the decreasing trend in specific growth rate. At approximately 90–100 m*M* phosphate, however, a sharp increase in MMO propene activity was observed, yielding an absolute specific activity of 220 nmol/mg dry cell wt min. This higher propene oxidative activity is due to the production of pMMO, not an elevated production of sMMO, because cells grown on 100 m*M* or higher phosphate do not display any detectable activity with chloroform (Fig. 3).

This observation raised an interesting question concerning the role of phosphate in regulating the form of MMO, namely whether the shift in enzyme location is due to a high phosphate concentration per se or due to Cu contaminants present in the Na/K salts that are used in preparing high phosphate culture medium. Another shake flask experiment combined with culture medium Cu analyses, therefore, was conducted to answer this question. With 100 m*M* phosphate, it was observed that propene oxidative activity was high (ca. 200 nmol/mg dry cell wt min) at a low cell density (A_{660} = 0.28), but it declined to 95 nmol/mg dry cell wt min when the A_{660} reached 0.6. Correspondingly, chloroform oxidative activity was not detected at the low cell density, but it appeared (22 nmol/mg dry cell wt min) at the higher cell density when the propene activity declined. Moreover, inductive coupled plasma-mass spectrometry (ICP–MS) analyses revealed that the Cu concentration was ca. 0.2 μM for 100 m*M* phosphate medium compared to only 0.02 μM for 10 m*M* phosphate medium. Collectively, these results strongly suggest that the production of pMMO in Figure 3 at high phosphate concentrations is due to traces of carry-over Cu contamination in the Na/K salts.

Bioreactor Experiments

Much higher cell densities can be obtained in a bioreactor in which the gaseous substrates are continuously supplied. Our experiments in the bioreactor focussed on the batch culture conditions which yield a high *M. trichosporium* OB3b cell density with an optimal whole-cell sMMO activity.

Effect of CO_2

Figure 4 shows the effect of CO_2 supplementation on cell growth. When CO_2 is either incorporated into the gas stream or supplied as bicarbonate in the culture

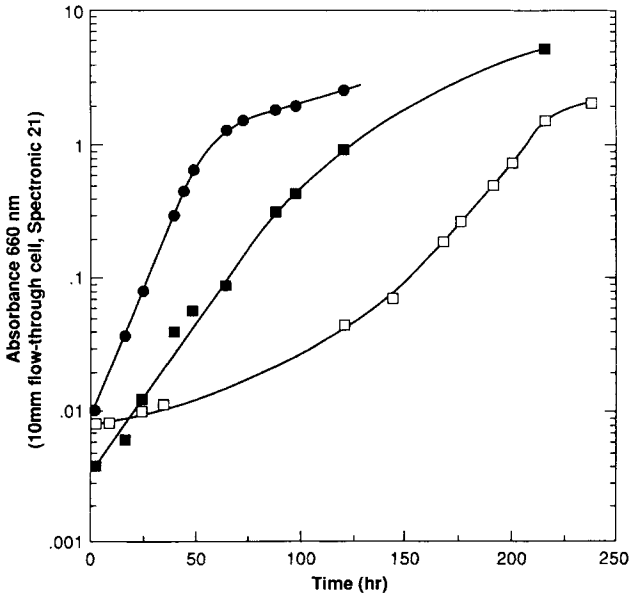

Figure 4. Effect of CO_2 or $NaHCO_3$ on cell growth in a 5-L bioreactor culture (minus-Cu medium). Symbols show (●) CO_2 was added in an air/CO_2 mixture (9:1 volume ratio); (■) $NaHCO_3$ was added to the culture medium at a concentration of 0.1% (w/v); (□) neither CO_2 nor $NaHCO_3$ was added.

medium, the initial lag period was greatly shortened. The maximum specific growth rates during the exponential growth phase, however, remained approximately the same (μ_{max} = 0.065–0.090 h^{-1}). According to the serine pathway by which *M. trichosporium* OB3b is known to utilize formaldehyde for cell mass generation, one molecule of CO_2 is needed per two molecules of formaldehyde assimilated to convert phosphoenol pyruvate to oxaloacetate and to keep the cycle continuously operating.[1] Without an external supplementation, the initial CO_2 level in the culture medium must be too low to support immediate rapid cell growth. This is probably exacerbated in a bioreactor because the continuous sparging with an air/methane gas mixture initially strips off the small amounts of CO_2 produced from methane metabolism. As the cell density slowly increases, the medium CO_2 concentration apparently rises and finally becomes sufficient to support a maximal cell growth rate as indicated by the similar logarithmic slopes in Figure 4. Based on these results, all subsequent experiments in the bioreactor were conducted with 10% CO_2-containing air.

Effect of Nitrate and Diauxic Growth

Figure 5 shows batch profiles of cell growth, nitrate concentration, DO level, and sMMO activity. Exponential cell growth was associated with a large drop in the DO level. The initial specific growth rate (μ) was 0.08 h^{-1} and this fast rate continued until the cell density reached about 1.0 g dry cell wt/L. At 50–60 h, however, a rapid decrease of μ to 0.008 h^{-1} was observed and this slow rate continued for approximately a week before the cell growth completely stopped. The final cell density reached was about 2.5 g dry cell wt/L. The whole-cell sMMO activity was routinely assayed both with and without formate added as an oxidizable agent to regenerate intracellular NADH levels. The former assays of activity were always 4–6 times higher than the latter. In addition, whole-cell sMMO activity was al-

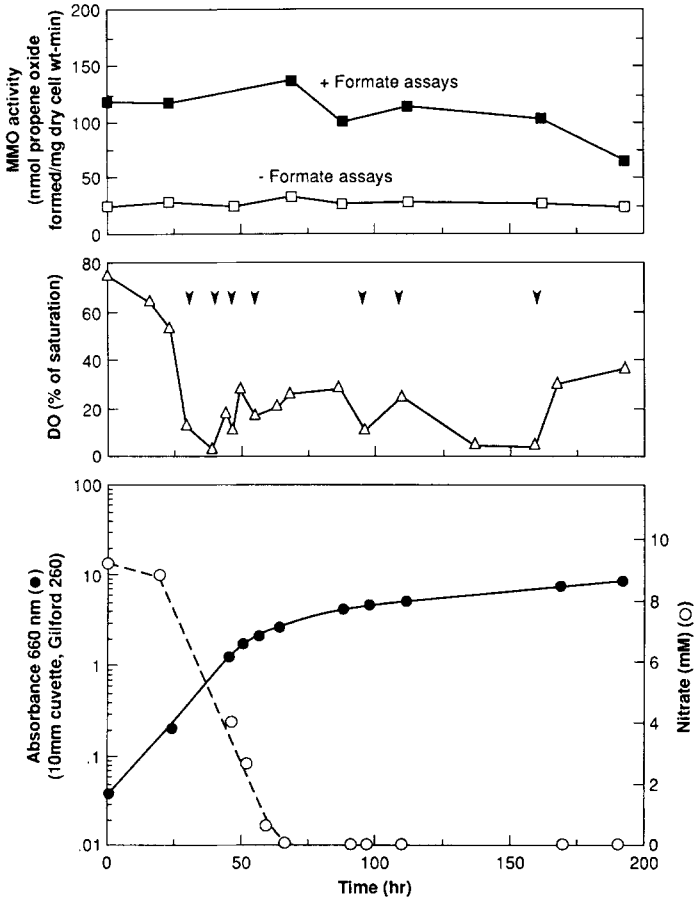

Figure 5. Batch culture profiles of cell growth, nitrate concentration, DO level, and whole-cell sMMO activity in the bioreactor. Cells were grown in Higgins nitrate minimal salts medium in the absence of added $CuSO_4$ and with a continuous supply of methane and 9:1 air/CO_2 gas mixtures (standard conditions for bioreactor culture). Vertical arrows indicate the times at which the agitation speed and gas flow rates were adjusted to maintain DO levels above 10%.

most constant throughout the cultivation, except for a decreasing trend near the end of the batch operation. It is worth noting that there was no appreciable change in sMMO activity at around 50–60 h, i.e., before and after the large shift in the growth rate. Under these culture conditions the level of sMMO catalysis and, presumably, the rate of methane assimilation are not proportional to the bacterial growth rate. A biphasic growth curve and the nonvarying sMMO activity have not been addressed in the past, but they may explain why the reported values for the methane consumption efficiency (defined as g dry cell wt produced/g methane consumed) of *M. trichosporium* OB3b and several other methanotrophs are not consistent and vary widely.[1,16] Figure 5 also shows that the decrease of the growth rate coincided with the depletion of culture medium nitrate at 50–60 h. This indicated that the transition might be induced by a switch of the nitrogen source from nitrate to the gaseous nitrogen that is carried into the bioreactor as part of the 9:1 air/CO_2 mixture. It has been established that *M. trichosporium* OB3b can fix gaseous nitrogen through a nitrogenase which is inducible under either nitrate- or ammonium-limiting culture conditions.[3,4]

The effect of nitrate on the cell growth and the transition in the growth rate was investigated further: first, by varying its concentration in the bioreactor culture medium, and next, by transferring bioreactor cells growing in two different growth phases into fresh culture media. Figure 6 shows the cell densities both at the transitions (X_t) and at the ends of the batch cultures as a function of the initial medium nitrate concentration. Parameter X_t was determined by extrapolating the straight lines during both growth phases. When the nitrate concentration was varied over the range from 5 to 20 mM, X_t increased with increasing nitrate although the final cell densities were practically constant. Cell yield on nitrate was determined from the slope in Figure 6 to be 100 g dry cell wt/mol nitrate or 7.14 g dry cell wt/g N. It is in close agreement with the theoretical value estimated from elemental analyses of various methanotrophs.[1] At a much higher nitrate concentration (40 mM), however, the exponential fast growth rate began to decrease well before most of the nitrate was consumed and the proportionality shown in Figure 6 did not extent to this higher nitrate concentration (data not shown). Figure 7 shows the results of the culture transfer experiments. Microorganisms growing in the bioreactor were transferred at two separate times into shake flasks containing fresh media with either no added nitrate or 10 mM nitrate. When fast growing early stage bioreactor cells were transferred into the fresh nitrate-containing medium at 20 h, they exhibited the same rapid growth rate. Similarly, when slow growing late-stage bioreactor cells were transferred at 150 h, nitrate quickly restored most of their earlier fast growth rate. But when nitrate-free medium was used, the growth rate was slow regardless of the prior growth rate in the bioreactor. These results, along with those in Figures 5 and 6, demonstrate clearly that nitrate depletion is responsible for the diauxic growth pattern in the batch cultivation of *M. trichosporium* Ob3b in the bioreactor.

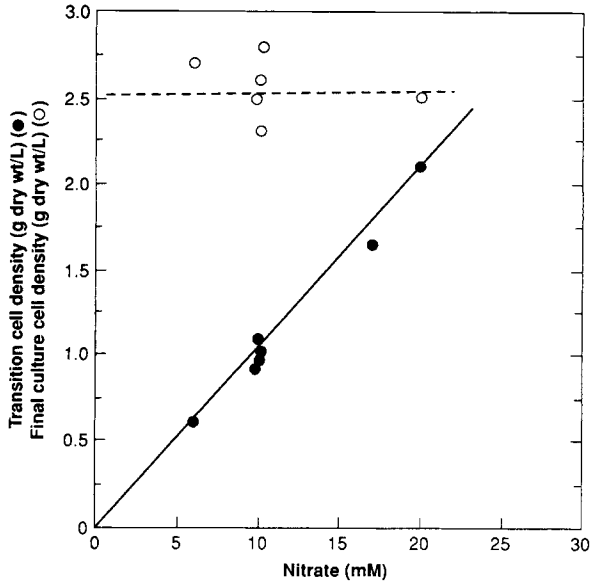

Figure 6. Effect of nitrate concentration on cell density at the transition time (X_t) and at the end of the total growth time in the bioreactor culture.

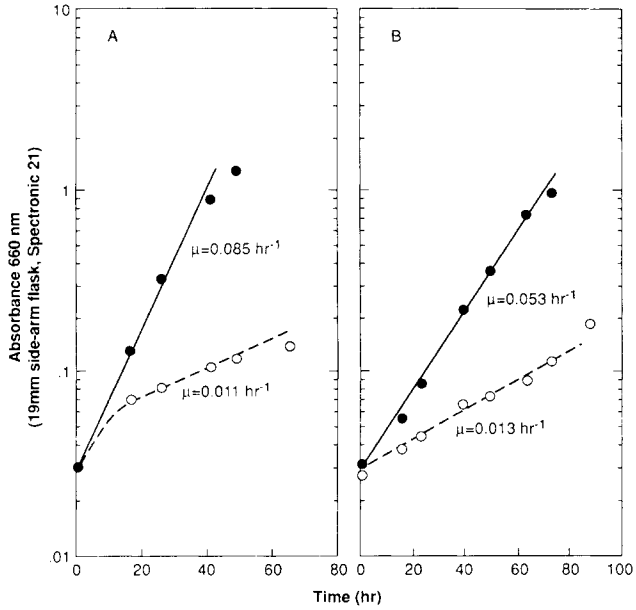

Figure 7. Effect of shake flask nitrate concentration on the growth of prior bioreactor-cultured cells. Inocula cells were transferred from the bioreactor (standard conditions as in Fig. 5) to side-arm shake flasks at 20 hr (A) and at 150 hr (B). They were centrifuged and washed once with nitrate-free medium before inoculation into the flasks. Nitrate concentration was (●) 10 mM and (○) 0 mM.

Effect of Iron

In most batch cultures, microorganisms stop growing at the end of cultivation due to either the depletion of essential nutrients or the accumulation of inhibitory metabolites in the culture medium. Since raising the medium nitrate concentration did not influence the final cell density (Fig. 6) and the metabolites present in the spent bioreactor medium did not inhibit cell growth (data not shown), the maximal cell density attainable with the medium in Table I (lacking $CuSO_4$) seems to be determined by some component(s) other than the C, O, and N sources. In an effort to identify one such limiting component, the effect of the culture medium Fe level was investigated (Table IV). When about half of the usual Higgins medium Fe level (18 μmol/L) was employed, whole-cell propene oxidative sMMO activity decreased sharply from 105 nmol propene oxide formed/mg dry cell wt min to 47 nmol/mg dry cell wt min at around 70 h. The cell density at that time was 1.5 g dry wt/L, which was also about one-half of the final cell density achievable with the standard medium Fe level of 40 μmol/L (Table I). When an additional 50 μmol/L of Fe was supplemented at 90 h (total 68 μmol/L), a normal high level of the sMMO activity was restored within 24 h. Furthermore, cell growth (μ = 0.009 h^{-1}) continued up to 5.4 g dry cell wt/L without any appreciable decline in the whole cell enzymatic activity. This rapid and large Fe effect on the sMMO activity is not too suprising, considering that Fe is an essential cofactor for the sMMO enzyme complex which can represent more than 10% of the total soluble protein in some methanotrophs.[10,19,26] From the dependence of the final cell density on the initial medium Fe level, i.e., up to 40 μmol/L, it was possible to calculate the cell yield on Fe as approximately 1.3×10^3 g dry cell wt/g Fe. This is about five times lower than the value reported for *Klebsiella aerogenes*,[28] and it may be attributable to high Fe requirement for sMMO production by some methanotrophic bacteria. With an initial Fe level of either 40 or 80 μmol/L, no sudden decreases in whole-cell sMMO activity were observed during the bioreactor batch cultivations, although the higher Fe level raised the final cell density from 2.6 to 3.6 g dry wt/L (Table IV). We conclude that an Fe level of 40 μmol/L in the original Higgins nitrate minimal salts culture medium (Table I) is marginal.

Cultivation with Concentrated Medium

In spite of the somewhat increased final cell density with 80 μmol/L Fe (Table IV), it is still not certain that Fe at the standard level of 40 μmol/L is the key limiting medium component that determines the final cell density. Instead of further attempting to identify additional presumptive limiting component(s) in the standard medium, batch cultures were grown in twofold and fourfold concentrated minus-Cu medium in order to increase the final cell density. The results with the twofold concentrated medium are plotted in Figure 8. As anticipated, both the final cell density and X_t were about two times higher than those of the standard medium. The sMMO acitvity remained above 100 nmol of propene oxide formed/mg cell dry wt min until near the end of the cultivation. With fourfold concentrated medium (data not shown), however, there was a serious DO limitation. Initial cell growth was comparable to that of twofold medium, but the DO level decreased to zero when the cell density reached 2.5 g dry cell wt/L and it remained there in spite of our efforts to restore it to 10%. As a result, the growth rate then decreased and some cell lysis occurred causing massive foaming. Minus-Cu culture medium concentrated up to fourfold does not alter the early-stage cell growth and sMMO activity, but the advantages of using it are offset by the oxygen limitations, unless this can be overcome in a given bioreactor.

CONCLUSIONS

In the present article, we studied an obligatory methanotroph, *Methylosinus trichosporium* OB3b, in shake flasks and a 5-L bioreactor in order to optimize the culture conditions for cell growth and the production of

Table IV. Effect of medium iron content on cell growth and soluble MMO activity.

Initally added medium Fe (μmol/L)	Bioreactor batch culture time						Final cell density[a] (>180 h)
	40–50 h		70–80 h		110–120 h		
	Cell density[a]	MMO[b]	Cell density[a]	MMO[b]	Cell density[a]	MMO[b]	
18	0.40–0.78	105 ±5	1.50–1.64	47 ±3	—	—	
+Additional 50 Fe added at 90 h	—	—	—	—	2.55–3.57	126 ±4	5.4
40	0.31–0.64	150 ±10	1.15–1.25	119 ±3	1.58–1.66	120 ±10	2.6
80	0.33–0.64	149 ±7	1.17–1.38	139 ±5	1.94–2.10	160 ±5	3.6

[a] The unit of cell density is g dry cell wt/L culture broth.
[b] The unit of MMO activity is nmol of propene oxide formed/mg dry cell wt-min, determined with formate as a reducing agent.

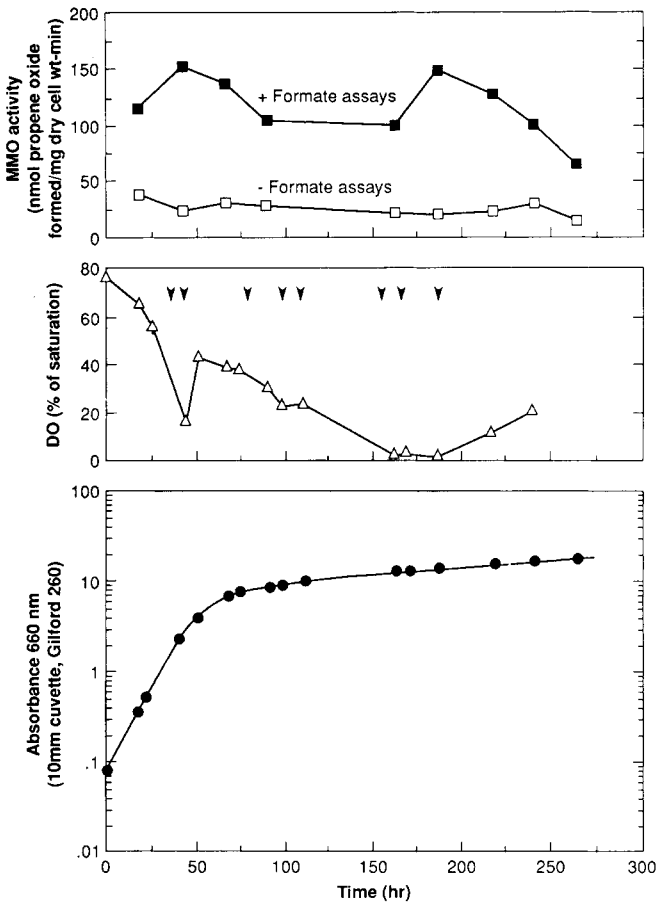

Figure 8. Bioreactor batch culture profiles of cell growth, DO level, and whole-cell sMMO activity with twofold concentrated medium lacking $CuSO_4$. Vertical arrows indicate the times at which the agitation speed and gas flow rates were adjusted in an effort to maintain DO levels above 10%.

sMMO enzyme. Our major findings can be summarized as follows:

1. Exclusively sMMO activity was observed only in cells grown on minus-Cu medium.
2. Cell growth was found to be optimal at pH 6.0–7.0, a temperature of 30–34°C, and a phosphate concentration of 10–40 m*M*.
3. When *M. trichosporium* OB3b was cultivated in a bioreactor with continuous sparging of its gaseous substrates, external CO_2 addition was important to eliminate a long lag period in the cell growth.
4. In bioreactor experiments employing minus-Cu medium and the continuous supply of a methane/air/CO_2 gas mixture, a diauxic growth pattern was observed which was caused by a switch in the nitrogen source from nitrate to gaseous nitrogen. A twofold increase in the culture medium nitrate concentration is recommended to delay the nitrate depletion and hence to shorten the total cultivation time.
5. Sustaining a high whole-cell specific sMMO activity in the bioreactor was dependent on the Fe availability in Higgins culture medium lacking $CuSO_4$. It is recommended that the standard medium Fe level be increased to 80 μmol/L for bioreactor experiments.
6. The final bioreactor cell density was increased either by raising the culture medium Fe level or by using twofold concentrated complete medium. Therefore, the use of medium concentrated possibly up to fourfold is suggested, provided the greater oxygen demand can be satisfied in the bioreactor system to be used.

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References

1. Anthony, C. 1982. The Biochemistry of Methylotrophs. Academic, New York.
2. Boutacoff, D. 1989. EPRI Journal, **Oct./Nov.**: 24.
3. Chen, Y.-P., Yoch, D. C. 1987. J. Bacteriol, **169**(10): 4778.
4. Chen, Y.-P., Yoch, D. C. 1988. J. Gen. Microbiol. **134**: 3123.
5. Cornish, A., McDonald, J., Burrows, K. J., King, T. S., Scott, D., Higgins, I. J. 1985. Biotechnol. Lett. **7**(5): 319.
6. Cornish, A., Nicholls, K. M., Scott, D., Hunter, B. K., Aston, W. J., Higgins, I. J., Sanders, J. K. M. 1984. J. Gen. Microbiol. **130**: 2565.
7. Dalton, H., Prior, S. D., Leak, D. J., Stanley, S. H. 1984. p. 75–82. In: Crawford, R. L., Hanson, R. S. (ed.), Microbial Growth on C1 Compounds, American Society for Microbiology, Washington, D.C.
8. Davis, K. J., Cornish, A., Higgins, I. J. 1987. J. Gen. Microbiol. **133**: 291.
9. Fogel, M. M., Taddeo, A. R., Fogel, S. 1986. Appl. Environ. Microbiol. **51**: 720.
10. Fox, B. G., Froland, W. A., Dege, J. E., Lipscomb, J. D. 1989. J. Biol. Chem., **264**(17): 10023.
11. Habets-Crutzen, A. Q. H., de Bont, J. A. M. 1985. Appl. Microbiol. Biotechnol. **22**: 428.
12. Hamstra, R. S., Murris, M. R., Tramper, J. 1987. Biotechnol. Bioeng. **29**: 884.
13. Harwood, J. H., Pirt, S. J. 1972. J. Appl. Bacteriol. **35**: 597.
14. Hou, C.T. 1984. Appl. Microbial. Biotechnol. **19**: 1.
15. Hou, C.T., Patel, R., Laskin, A. I., Barnabe, N. 1979. Appl. Environ. Microbiol. **38**(1): 127.
16. Leak, D. J., Stanley, S. H., Dalton, H. 1985. Chap. 12. In: Poole, R. K., Dow, C. S. (ed.), Microbial Gas Metabolism, Academic, New York.
17. Little, C. D., Palumbo, A.V., Herbes, S. E., Lidstrom, M. E., Tyndall, R. L., Gilmer, P. J. 1988. Appl. Environ. Microbiol. **54**: 951.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. 1951. J. Biol. Chem. **193**: 265.
19. Lund, J., Woodland, M. P., Dalton, H. 1985. Eur. J. Biochem. **147**: 297.
20. Maclennan, D. G., Ousby, J. C., Vasey, R. B., Cotton, N.T. 1971. J. Gen. Microbiol. **69**: 395.
21. Mehta, P. K., Mishra, S., Ghose, T. K. 1987. J. Gen. Appl. Microbiol. **33**: 221.

22. Murrell, J. C., Dalton, H. 1983. J. Gen. Microbiol. **129**: 3481.
23. Murrell, J. C., Dalton, H. 1983. J. Gen. Microbiol. **129**: 1197.
24. New Fuels Report, **10**(40): October (1989).
25. Oldenhuis, O., Vink, R. M. J. M., Jenssen, D. B., Witholt, B. 1989. Appl. Environ. Microbiol. **55**: 2819.
26. R. N. Patel, 1984. p. 83–90. In: Crawford, R. L., Hanson, R. S. (ed.) Microbial Growth on C1 Compounds, American Society for Microbiology, Washington, DC.
27. Patel, R. N., Hou, C. T., Laskin, A. I., Felix, A., Derelanko, P. 1979. J. Bacteriol. **139**(2): 675.
28. Pirt, S. J. 1975. Principles of Microbe and Cell Cultivation. Wiley, New York.
29. Russel, A. G., Pierre, D. St., Milford, J. B. 1990. Science, **247**: 201.
30. Scott, D., Best, D. J., Higgins, I. J. 1981. Biotechnol. Lett. **3**(11): 641.
31. Stanley, S. H., Prior, S. D., Leak, D. J., Dalton, H. 1983. Biotechnol. Lett. **5**(7): 487.
32. Taylor, R. T., Hanna, M. L., Park, S., Droege, M. W. 1990. Abstracts of the 90th Annual Meeting of the American Society for Microbiology, p. 221.
33. Tsien, H.-C., Brusseau, G. A., Hanson, R. S., Wackett, L. P. 1989. Appl. Environ. Microbiol. **55**: 3155.