Journal of Biotechnology 139 (2009) 78–83

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

# Production of soluble methane monooxygenase during growth of *Methylosinus trichosporium* on methanol

Yinghao Yu[a], Juliana A. Ramsay[a], Bruce A. Ramsay[a,b,*]

[a] *Department of Chemical Engineering, Queen's University, Kingston, Ontario, Canada K7L 3N6*
[b] *Polyferm Canada Inc., RR1, Harrowsmith, Ontario, Canada K0H 1V0*

ARTICLE INFO

*Article history:*
Received 20 June 2008
Received in revised form
18 September 2008
Accepted 23 September 2008

*Keywords:*
Methanotrophs
sMMO
Methane
Methanol
Fed-batch
Copper
Allylthiourea

ABSTRACT

Soluble methane monooxygenase (sMMO) can degrade many chlorinated and aromatic pollutants. It is produced by certain methanotrophs such as *Methylosinus trichosporium* when grown on methane under copper limitation but, due to its low aqueous solubility, methane cannot support dense biomass growth. Since it is water soluble, methanol may be a more attractive growth substrate, but it is widely believed that sMMO is not produced on methanol. In this study, when the growth-limiting substrate was switched from methane to methanol, in the presence of the particulate MMO inhibitor, allylthiourea, growth of *M. trichosporium* OB3b continued unabated and sMMO activity was completely retained. When allylthiourea was then removed, sMMO activity was maintained for an additional 24 generations, albeit at a slightly lower level due to the presence of 0.70 μM of $Cu^{2+}$ in the feed medium. While a biomass density of only 2 g $l^{-1}$ could be obtained on methane, 7.4 g $l^{-1}$ was achieved by feeding methanol exponentially, and 29 g $l^{-1}$ was obtained using a modified feeding strategy employing on-line carbon dioxide production measurement. It was concluded that methanol can be employed to produce large amounts of *M. trichosporium* biomass containing sMMO.



## 1. Introduction

Under copper limitation, type II methanotrophs, the type X methanotroph, *Methylococcus capsulatus* Bath, and some type I methanotrophs can produce soluble methane monooxygenase (sMMO) rather than the usual copper-containing particulate type (pMMO) (Hanson and Hanson, 1996; Koh et al., 1993). The soluble form of MMO has a broad substrate range and can degrade many chlorinated and aromatic organic compounds (Colby et al., 1977; Oldenhuis et al., 1989; Tsien et al., 1989). For this reason, methanotrophic bioreactors with high sMMO activities were developed to treat contaminated groundwater (Aziz et al., 1995; Phelps et al., 1990; Pressman et al., 1999), to produce sMMO-containing methanotrophs for bioaugmentation of contaminated sites (Duba et al., 1996; Rockne et al., 1998; Shah et al., 1996) or for industrial biotransformations (Lee et al., 1996). Productivities in such bioreactors are dependant on the amount of sMMO-containing biomass. However, to our knowledge, the highest dry biomass concentration ever attained without biomass recycle by a culture using methane as the sole source of carbon and energy was about 5 g $l^{-1}$ (Asenjo and Suk, 1986) in a baffled shake-flask and about 18 g $l^{-1}$ (with low sMMO activity) in a bioreactor (Shah et al., 1996). Higher biomass concentrations must be obtained to be economically attractive.

The aforementioned limitations in bioreactor productivity were at least partly due to the low aqueous solubility of methane. As a carbon source, methane is first oxidized to methanol by MMO. Many methanotrophs are able to grow on methanol, with no need for methane. Methanol is inexpensive and presents fewer safety concerns than methane. More importantly, methanol is completely miscible in water so there is no mass transfer limitation of growth. Methylotrophs have been produced in high-density culture (Bourque et al., 1995), but none of these produce sMMO. *Methylosinus trichosporium* OB3b (ATCC 35070) is the type II methanotroph most frequently studied for sMMO production. It has been grown to a biomass concentration of 3.2 g $l^{-1}$ in chemostat culture on 1.0% (v/v) methanol (Best and Higgins, 1981). An sMMO constitutive mutant of *M. trichosporium* OB3b (PP358) produced 1.0 g $l^{-1}$ biomass on methanol (Fitch et al., 1996). Both aforementioned cultures retained sMMO activity on methanol. However, many publications have reported, and it is still generally believed, that no sMMO production occurs in type II methanotrophs grown on methanol (Hou et al., 1979; Patel et al., 1980; Sullivan et al., 1998). Based on this belief, significant studies have been conducted on the optimization and scale-up of *M. trichosporium* OB3b fermen-

* Corresponding author at: Polyferm Canada Inc., 4530 CamdenE-Portland Bdry Rd, RR1, Harrowsmith, Ontario, Canada K0H 1V0. Tel.: +1 6135336000x75293; fax: +1 6135336637.
*E-mail address:* bruce.ramsay@polyfermcanada.com (B.A. Ramsay).

0168-1656/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2008.09.005

**Nomenclature**

| | |
|---|---|
| $CCP_t$ | cumulative carbon dioxide production (g) |
| $CPR$ | carbon dioxide production rate (g $h^{-1}$) |
| $S_t$ | total amount of methanol to be fed at time $t$ (g) |
| $t$ | time of fermentation (h) |
| $X_0$ | biomass when exponential feeding begins (g) |
| $X_t$ | desired biomass at time $t$ (g) |
| $Y_{CO_2/S}$ | yield of carbon dioxide from substrate (g $CO_2$ g substrate$^{-1}$) |
| $Y_{X/S}$ | expected yield of biomass from substrate (g biomass g substrate$^{-1}$) |

*Greek symbols*

| | |
|---|---|
| $\mu$ | desired growth rate ($h^{-1}$) |
| $\mu_{CPR}$ | specific growth rate based on carbon dioxide production ($h^{-1}$) |

tations employing methane (Carmen et al., 1996). In this study, we evaluated the efficacy of producing sMMO during growth on methanol and the feasibility of producing dense cultures of *M. trichosporium* OB3b using methanol as the sole source of carbon and energy.

## 2. Material and methods

### 2.1. Organism and growth media

*M. trichosporium* OB3b was maintained on nitrate mineral salts Noble agar plates and incubated in a methane-rich atmosphere (20%, v/v $CH_4$). Two media were used for cultivation. Nitrate mineral salts (NMS) medium consisted of (in g $l^{-1}$) $NaNO_3$, 0.85; $KH_2PO_4$, 0.53; $Na_2HPO_4{\cdot}7H_2O$, 1.62; $MgSO_4{\cdot}7H_2O$, 0.037; $FeSO_4{\cdot}7H_2O$, 0.0112; $CaCl_2{\cdot}2H_2O$, 0.007; and 1 ml $l^{-1}$ of trace element solution containing (in g $l^{-1}$) $ZnSO_4{\cdot}7H_2O$, 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; and KI, 0.083. The pH was adjusted to 6.88 ± 0.10 using 0.2 M $H_2SO_4$. When grown on methanol, a variation of Choi's medium (Bourque et al., 1995) was used. It contained (in g $l^{-1}$): $(NH_4)_2SO_4$, 1.0; $KH_2PO_4$, 1.305; $Na_2HPO_4{\cdot}7H_2O$, 4.02; $MgSO_4{\cdot}7H_2O$, 0.45; $CaCl_2{\cdot}2H_2O$, 0.0033; $FeSO_4{\cdot}7H_2O$, 0.0013; and 1 ml $l^{-1}$ of the trace element solution described above. All media were prepared with deionized water. Stock solutions containing 10 g $l^{-1}$ $CaCl_2{\cdot}2H_2O$ or $FeSO_4{\cdot}7H_2O$ were also prepared.

### 2.2. Inoculum preparation

Erlenmeyer flasks (250 ml) containing 50 ml medium were autoclaved. Colonies of *M. trichosporium* OB3b maintained on noble agar plates were transferred to the medium aseptically. For methanol-grown cultures, 10 g $l^{-1}$ methanol was introduced aseptically. The flasks were covered with foam stoppers and incubated at 30 °C and 200 rpm on a gyro-rotary shaker (Innova 44, New Brunswick Scientific, USA). For methane-grown innocula, the flasks were sealed with rubber stoppers fitted with two 0.2 μM filters. After inoculation, a methane/air mixture (20% $CH_4$, v/v) was introduced to the flasks through the filter for 5 min at a flow rate of 3 l $min^{-1}$ and then incubated at 30 °C and 200 rpm. When growth was observed, this culture was transferred to 2-l Erlenmeyer flasks containing 200 ml NMS medium to prepare inocula for reactor fermentations. Gas feed and incubation conditions were the same as above.

### 2.3. Bioreactors and fermentation conditions

Fed-batch fermentations were performed in a 2-l stirred-tank bioreactor (Multigen F-2000, New Brunswick Scientific, NJ, USA) with 1.25 l Choi's medium and 250 ml of a 72-h inoculum at 30 °C, 500 rpm agitation, and 0.5 l $min^{-1}$ air or an air and oxygen mixture. Headspace $CO_2$ was analyzed by a Guardian infrared $CO_2$ analyzer (Topac Inc. Hingham, MA, USA). pH was controlled at 6.88 by the addition of 14% (w/v) ammonium hydroxide using a Bioengineering AG (Wald, Switzerland) controller. Data acquisition, substrate feeding, and DO control were performed using a data acquisition board (ADR2000, Ontrak Control Systems Inc. Sudbury, ON, Canada), and a Labview™ data acquisition and control program (National Instruments, Vaudreuil-Dorion, PQ, Canada). DO was maintained at 40% or more of air saturation using the Labview™ PID to adjust mass flow controllers and balance the air and oxygen flow rates as described previously (Sun et al., 2006). Substrate feeding was automated using a peristaltic pump (Masterflex® 7520-35, Cole-Parmer International Inc., Chicago, IL, USA) by comparing the predicted substrate feeding and the amount of substrate fed as measured by a balance (P/PI-4002, Denver Instruments, Denver, Colorado).

The study of sMMO activity in different growth media was conducted in a 1.2-l Bioflo IIc reactor (New Brunswick Scientific, NJ, USA) operated as a chemostat at a dilution rate of 0.05 $h^{-1}$. When the culture was grown on methane, headspace methane content was maintained at 22% as described in Yu et al., 2006. Headspace methane content was continuously analyzed by recirculating headspace gas through an infrared $CH_4$ analyzer (Guardian Plus methane monitor, Topac, Inc., Hingham, MA). Temperature was controlled at 30 °C and agitation at 700 rpm.

### 2.4. Substrate feeding strategy

Fed-batch fermentations began in batch mode with 5 g $l^{-1}$ of methanol. At 72 h, exponential substrate feeding was initiated according to Eq. (1):

$$S_t = \frac{X_t}{Y_{X/S}} = \frac{X_0}{Y_{X/S}} \cdot e^{\mu t} \quad (1)$$

where $S_t$ is the total amount of methanol to be fed at time $t$ (g), $X_t$ is the desired biomass at time $t$ (g), $X_0$ is the biomass when exponential feeding began (g), $\mu$ is the desired growth rate ($h^{-1}$), and $Y_{X/S}$ is the expected yield of biomass from substrate (g biomass g substrate$^{-1}$).

The feeding strategy described in Eq. (1) was modified based on carbon dioxide production for better control as shown in Eq. (2):

$$S_t = \frac{CCP_t}{Y_{CO_2/S}} \quad (2)$$

where $CCP_t$ is the cumulative carbon dioxide production (g), and $Y_{CO_2/S}$ is the yield of carbon dioxide from substrate (g $g^{-1}$). This strategy has been shown to be effective in the high-density biomass cultivation of *Pseudomonas putida* (Sun et al., 2006).

### 2.5. Calculation of specific growth rates

Specific growth rates were calculated based on carbon dioxide production rate ($CPR$) as shown in Eq. (3):

$$\mu_{CPR} = \frac{dCPR/dt}{CPR} \quad (3)$$

where $\mu_{CPR}$ is specific growth rate based on carbon dioxide ($h^{-1}$), $CPR$ is carbon dioxide production rate (g $h^{-1}$), and $t$ is time (h).

### 2.6. Analytical techniques

#### 2.6.1. Biomass

The dry weight of biomass per unit volume of fermentation broth was determined by relating the optical density of samples at 600 nm (Spectronic Unicam UV1, Fisher Scientific, Nepean, ON, Canada) to a calibration curve.

#### 2.6.2. Copper

Aqueous samples from the reactor feed or effluent were collected and centrifuged at 8000 × *g* to remove biomass or precipitates. The supernatants were stored in glass tubes pre-washed with 0.2 M sulfuric acid and de-ionized water. Copper content was assayed by inductively coupled plasma-optical emission spectrometer (ICP-OES, Varian, AX-Vista Pro CCD).

#### 2.6.3. Formaldehyde

Samples from the reactor were centrifuged at 12,500 × *g* for 6 min. Formaldehyde concentrations in the supernatants were determined by a colorimetric method (Chrastil and Wilson, 1975).

#### 2.6.4. sMMO activity

sMMO activity was determined in triplicate using the colorimetric method of Brusseau and Tsien (Brusseau et al., 1990; Tsien et al., 1989). Cell cultures were centrifuged and resuspended in NMS medium to a biomass concentration of 0.5 g $l^{-1}$. Concentrated potassium formate was added to a final concentration of 20 mM. The cell suspensions were incubated with naphthalene crystals at 200 rpm, and 30 °C for 1 h. The reaction solutions were then centrifuged and the naphthol content was determined by adding 0.1 ml freshly prepared 1% *o*-dianisidine tetrazotized dye (Sigma–Aldrich) to 1 ml supernatant at the appropriate dilution. A purple color indicates sMMO activity. After mixing, 0.4 ml acetic acid was added to stabilize the color. The absorbance at 530 nm was measured immediately and related to a standard curve (0–7 mg naphthol $l^{-1}$). Specific sMMO activity was calculated as naphthalene oxidation rate per gram of biomass.

#### 2.6.5. Measurement of headspace and aqueous phase methanol concentration

Aqueous samples were withdrawn from the reactor and centrifuged at 12,500 × *g* for 6 min. Methanol concentration in the supernatant was analyzed by a gas chromatograph (Varian 3400, Varian Canada Inc., Mississauga, ON) equipped with a Carbowax®-PEG column and a flame ionization detector (FID). Butanol was used as the internal standard. Injector and detector temperatures were 120 and 250 °C, respectively. Helium was used as the carrier gas at a flow rate of 5 ml $min^{-1}$. The column temperature was held at 60 °C for 1 min, increased to 150 °C at 20 °C $min^{-1}$, and then held for 1 min. Methanol concentration was determined by relating peak area to that of a standard and corrected with the internal standard. For more rapid methanol analysis, 200 μl bioreactor off-gas samples were withdrawn from the reactor exhaust line with a gas-tight syringe and then injected in a Hewlett-Packard 5890 GC with a FID detector and a HP-5 column. Injector temperature, detector temperature, and helium flow rate were the same as the aqueous sample analysis but the oven temperature was maintained at 90 °C. Headspace samples were compared with a methanol standard. Aqueous methanol concentration was then estimated using a Henry's law constant of $1.4 \times 10^2$ M $atm^{-1}$ (Yaws and Yang, 1992).

## 3. Results

### 3.1. sMMO activity

The capacity of *M. trichosporium* OB3b to retain sMMO activity while growing on methanol was investigated. The culture was initially grown in batch mode on NMS medium with methane as the sole source of carbon and energy. Although the medium was formulated to minimize copper content, about 0.70 μM $Cu^{2+}$ was still present in the feed. Therefore, allylthiourea (15 μM) was added to inhibit synthesis of pMMO. Continuous medium flow began at 71 h with a dilution rate of 0.05 $h^{-1}$. Headspace methane was maintained at 22% (v/v). As steady state was approached (judged by methane uptake rate, $CO_2$ production, biomass concentration, and sMMO activity), sMMO activity of about 550 μmol naphthol produced g $biomass^{-1}$ $h^{-1}$ was obtained (Fig. 1), which is comparable to the sMMO activity usually reported for this strain when grown on methane in the absence of copper (Bowman and Sayler, 1994; Koh et al., 1993). After stopping the methane supply and switching to feed containing methanol, the majority of the sMMO activity remained intact. During the brief period when both methane and methanol were present, higher sMMO activity was observed.

After 6 generations of growth on methanol with allylthiourea, the feed was changed to one without allylthiourea. The specific sMMO activity dropped only slightly to 490 μmol naphthol produced g $biomass^{-1}$ $h^{-1}$ (Fig. 2). This level of sMMO activity was maintained for 24 generations. Subsequent addition of 4.5 μM copper to the growth medium caused sMMO activity to diminish to

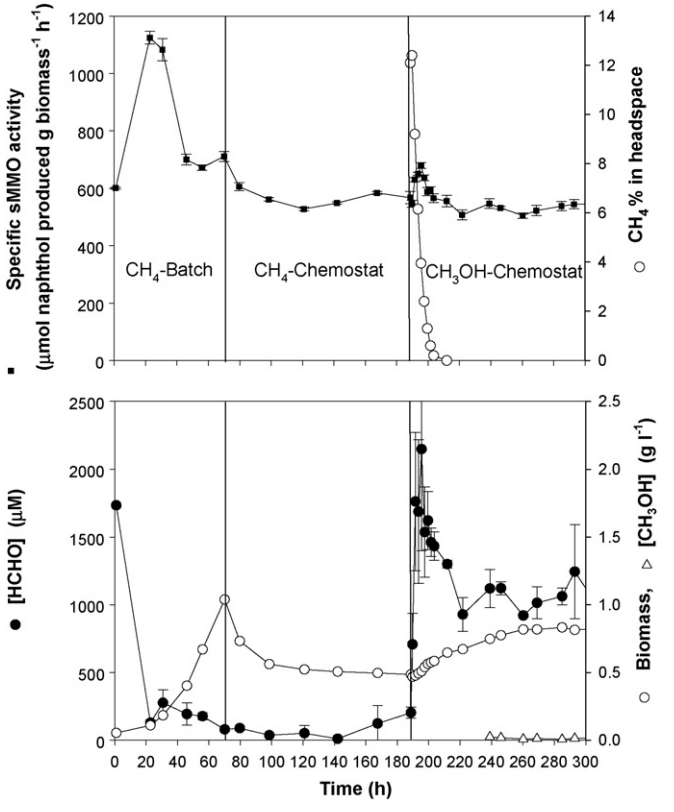


**Fig. 1.** Batch and chemostat growth of *Methylosinus trichosporium* OB3b. The culture was first grown in batch culture on methane with 15 μM of the pMMO inhibitor allylthiourea. Continuous flow of the same medium began at 71 h at a dilution rate of 0.05 $h^{-1}$. After steady state was achieved on methane (190 h), the methane supply was stopped and a medium containing 4 g $l^{-1}$ methanol and 15 μM allylthiourea was continuously fed.

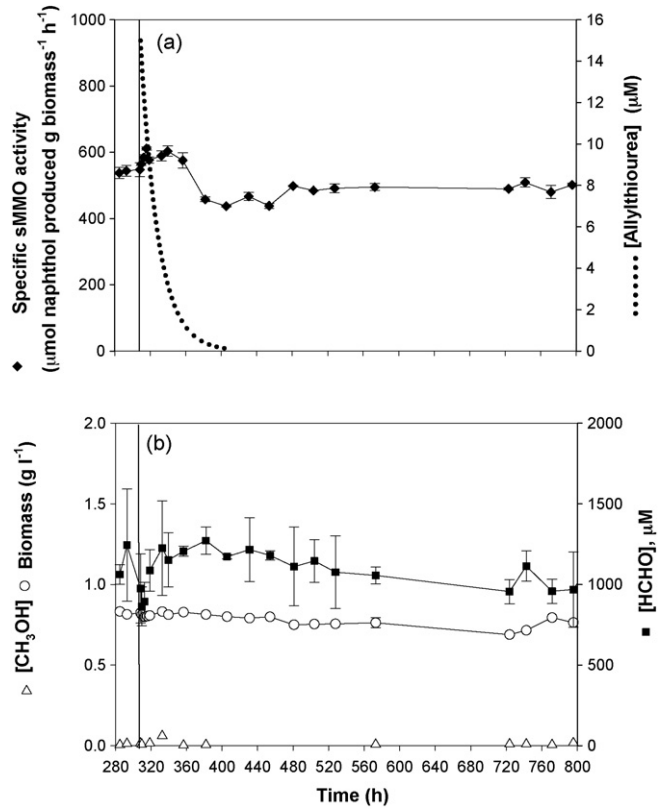


**Fig. 2.** Chemostat culture of *Methylosinus trichosporium* OB3b without allylthiourea. The culture depicted in Fig. 1 was continued, but at 309 h, allylthiourea was omitted from the medium. This resulted in only a small decrease in sMMO activity. The dotted line shows the expected concentration of allylthiourea remaining in reactor due to dilution.

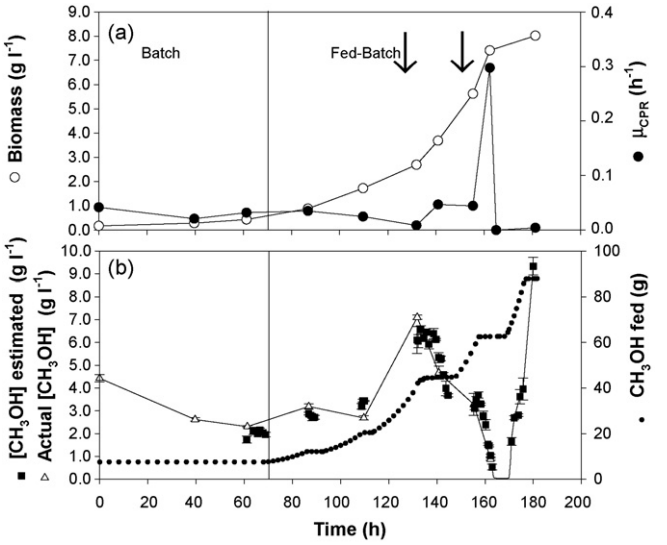


**Fig. 3.** Fed-batch fermentation of *Methylosinus trichosporium* OB3b on methanol using an exponential feeding strategy. Methanol feeding was designed to obtain a $\mu$ of 0.06 h$^{-1}$. Methanol concentration in the aqueous phase was measured in liquid samples (△, actual) or estimated from headspace samples (■, estimated). At 131.8 h (first arrow), 1 ml each of the $CaCl_2{\cdot}2H_2O$, $FeSO_4{\cdot}7H_2O$, and trace element stock solutions was added. At 155.3 h (second arrow), 2 ml of each of these solutions was added.

less than 10 μmol naphthol produced g biomass$^{-1}$ h$^{-1}$ (data not shown).

### 3.2. Fed-batch fermentation using exponential feeding of methanol

Once stability of sMMO in methanol-grown cells was established, a methodology for producing high-density *M. trichosporium* OB3b biomass was investigated. Growth in shake-flasks occurred at up to 40 g l$^{-1}$ of initial methanol concentration but only after a long lag period, so batch cultivation was initiated with 5 g l$^{-1}$ of methanol (Fig. 3). At 72 h, methanol feeding began at a rate designed to produce a $\mu$ of 0.06 h$^{-1}$. However the $\mu_{CPR}$ (specific growth rate calculated based on $CO_2$ production) was only 0.037 h$^{-1}$, this resulted in methanol accumulation in the fermentation broth. At 86.2, 109.4, 135.8, and 157.5 h, methanol feeding was stopped for 6.5, 3.8, 12.2 and 14.0 h, respectively (as seen in the plateaus in the "methanol fed" data in Fig. 3) to allow the concentration to decrease. Over 160 h of fed-batch fermentation, 7.4 g l$^{-1}$ biomass was produced.

The inhibitory effect of methanol was examined at time periods where changes in growth rate and methanol concentration occurred. For example, at 157.5 h, after methanol feeding was stopped, the residual methanol dropped from 3.28 to 0.47 g l$^{-1}$, $\mu_{CPR}$ increased to as high as 0.3 h$^{-1}$ (Fig. 3). The results demonstrated that a methanol concentration above 3 g l$^{-1}$ has a significant inhibitory effect on metabolic activity. The increase in $\mu_{CPR}$ caused the methanol concentration to drop rapidly to growth-limiting values (less than 0.47 g l$^{-1}$). This caused the $\mu_{CPR}$ to decrease to a point where cell growth could not be recovered. These results are typical of several fermentations conducted in a similar manner.

This ineffective feeding strategy was then modified. Instead of using a pre-set $\mu$, CCP was used to control methanol feeding. $Y_{CO_2/S}$ was determined from previous experiments to be 0.73 g g$^{-1}$ and two fermentations with two different methanol concentration set-points were performed to assess the new feeding strategy (Fig. 4). Methanol concentration was better controlled at low biomass concentrations, but high methanol demand (based on CCP) at higher biomass concentrations resulted in over-feeding in both fermentations. A dry weight biomass density of 19 g l$^{-1}$ was obtained in 169 h when the methanol concentration was controlled at about 3 g l$^{-1}$ (Fig. 4a). An even higher biomass density of 29 g l$^{-1}$ was achieved in only 119 h by further lowering the methanol concentration set-point to about 2 g l$^{-1}$ (Fig. 4b). Whether due to deviation of the $CO_2$ analyzer or an actual change of cell yield, the measured $Y_{CO_2/S}$ was not constant throughout the fermentation. Slight adjustments of the yield value were necessary. The $Y_{CO_2/S}$ used in the control equation (Eq. (2)) was gradually increased to 0.87 by the end of both fermentations. For all fed-batch fermentations, mineral salts were supplemented at points indicated by arrows in Figs. 3 and 4.

## 4. Discussion

Since type II methanotrophs such as *M. trichosporium* OB3b grow so slowly, attempts have been made to produce sMMO in recombinant organisms. Although progress is being made in this area (Smith et al., 2002), expression of fully active sMMO in the recombinants is hampered by the complex nature of the enzyme, especially the hydroxylase component. Therefore, we chose to examine sMMO production in a wild type strain growing on a more suitable growth substrate than methane. While a couple of studies have found that type II methanotrophs express sMMO when grown on methanol (Best and Higgins, 1981), most have concluded that they do not (Hou et al., 1979; Patel et al., 1980). In our chemostat experiments, there was no apparent difference in sMMO production whether *M. trichosporium* OB3b was grown on methane or on methanol.

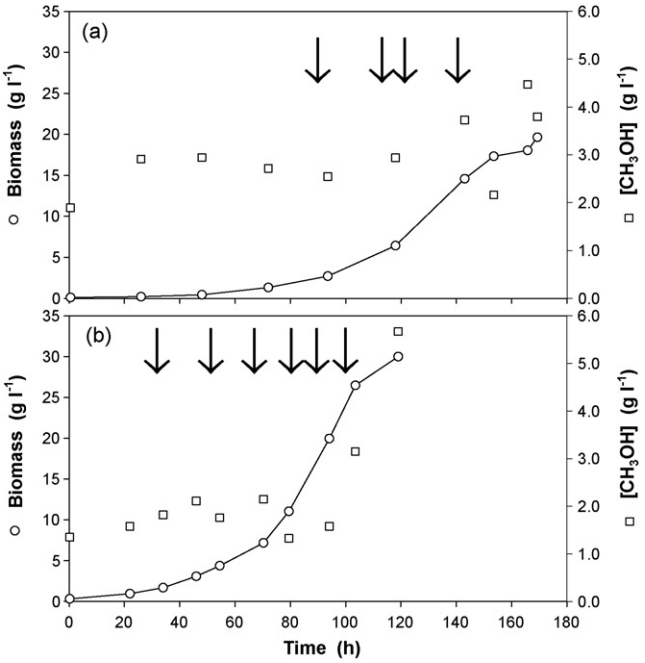


**Fig. 4.** Fed-batch fermentations of *Methylosinus trichosporium* OB3b on methanol using CCP-based feeding strategy. The desired aqueous phase methanol concentration was 3 g l$^{-1}$ (a) and 2 g l$^{-1}$ (b). Methanol concentrations were measured directly from aqueous phase samples. At 93.5, 118, 126, and 144 h, 2 ml each of the $CaCl_2 \cdot 2H_2O$, $FeSO_4 \cdot 7H_2O$ and TES stock solutions were added to fermentation (a). In fermentation (b), 1 ml of each stock solution was added at 34.0 and 54.5 h and 2 ml of each stock solution was added at 70.3, 82.5, 94.1, and 103.5 h. At both 82.5 and 103.5 h, 0.34 g of additional $MgSO_4 \cdot 7H_2O$ was provided.

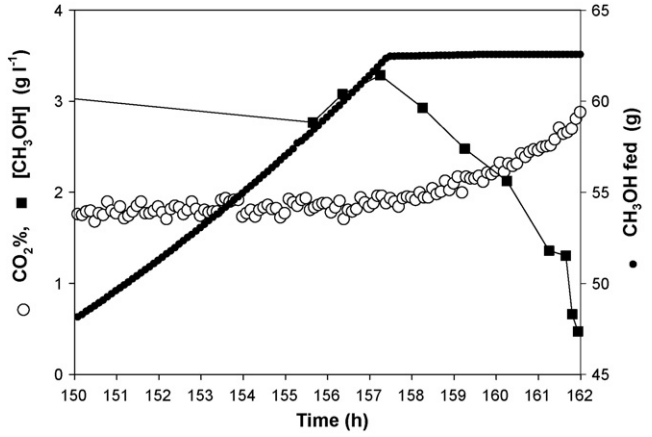


**Fig. 5.** The effect of methanol concentration on carbon dioxide production during a portion of the fed-fermentation depicted in Fig. 3.

However, the microorganism did sometimes lose sMMO activity when stock cultures were maintained on mineral salts agar with methanol. Activity could be recovered by growing it on methane with copper limitation. The reason why MMO activity is maintained while growing on methanol in some experiments but not others, remains to be determined. Never the less, the data from the present study proves conclusively that sMMO activity can be maintained in cultures growing on methanol.

This strain of *M. trichosporium* can tolerate exposure to as much as 40 g l$^{-1}$ methanol, but 3 g l$^{-1}$ was found to significantly inhibit growth. Therefore chemostat or well-controlled fed-batch culture are the most likely methods for the production of sMMO competent *M. trichosporium* from methanol. Control of fed-batch fermentations depends on supplying all of the nutrients at appropriate rates or concentrations. In terms of fermentation control, the key nutrient in this case is methanol. If the methanol level falls too low, growth slows due to limitation in carbon or energy, while, if there is too much methanol, growth will slow because of inhibition. As shown in Fig. 5, before methanol feeding was stopped, the methanol concentration was above 3 g l$^{-1}$ and the $CO_2$ production remained almost constant. Once methanol feeding was stopped at 157.5 h, the $CO_2$ production began to increase as methanol concentration dropped. When entirely deprived of methanol, the cells may enter a physiological state where recovery of rapid growth is severely retarded even after addition of sufficient methanol. This occurred at the end of the fermentation depicted in Fig. 3. We obtained a *M. trichosporium* OB3b biomass concentration of 29 g l$^{-1}$ using a CCP-based strategy to control the methanol concentration. Much higher cell densities should be obtainable if the methanol concentration can be more rigorously controlled.

The specific growth rate on methanol, whether in chemostat or fed-batch, was generally less than 0.1 h$^{-1}$ even when higher feeding rates were imposed. This is similar to growth on methane. Thus, although a much higher biomass density may be obtained from growth on methanol, productivity is still limited by a low growth rate. However, the transient $\mu_{CPR}$ reached as high as 0.3 h$^{-1}$ at about 0.7 g l$^{-1}$ methanol. Although the specific growth rate calculated from carbon dioxide production data may be misleading under transient conditions due to unbalanced growth, these data suggest the intriguing possibility of higher growth rates, but other factors require investigation. For example, $PO_4$ and other ions have been shown to inhibit methanol dehydrogenase activity (Mehta et al., 1989) and previous work (Yu et al., 2006) indicated the presence of a metabolic bottleneck in this organism. However, if these barriers can be overcome, and if sufficient control of the concentration of methanol and other significant medium components during fed-batch fermentation can be maintained, it may be possible to obtain a substantial increase in specific growth rate. Even if the growth rate is not increased, substantially higher biomass concentrations can be obtained on methanol by fed-batch fermentation. This knowledge, combined with the conclusive evidence that sMMO expression can be maintained during growth on methanol should aid in the development of sMMO-based industrial and environmental processes. In future work, we intend to develop an improved process for the control of methanol during fed-batch fermentations and to monitor sMMO production throughout these fermentations. This should result in much higher biomass densities and provide greater insight regarding the effects of methylotrophic fed-batch conditions on sMMO synthesis.

## Acknowledgements

This research was supported by the Natural Science and Engineering Research Council of Canada. Y. Yu was the recipient of an Ontario Graduate Scholarship, McLaughlin Scholarship, and Queen's Graduate Awards.

## References

Asenjo, J.A., Suk, J.S., 1986. Microbial conversion of methane into poly-*b*-hydroxybutyrate (PHB): growth and intracellular product accumulation in a type II methanotroph. J. Ferment. Technol. 64, 271–278.

Aziz, C.E., Fitch, M.W., Linquist, L.K., Pressman, J.G., Georgiou, G., Speitel, G.E., 1995. Methanotrophic biodegradation of trichloroethylene in a hollow fiber membrane bioreactor. Environ. Sci. Technol. 29, 2574–2583.

Best, D.J., Higgins, I.J., 1981. Methane-oxidizing activity and membrane morphology in a methanol-grown obligate methanotroph, *Methylosinus trichosporium* OB3b. J. Gen. Microbiol. 125, 73–84.

Bourque, D., Pomerleau, Y., Groleau, D., 1995. High cell density production of poly-*b*-hydroxybutyrate (PHB) from methanol by *Methylobacterium extorquens*:

production of high-molecular-mass PHB. Appl. Microbiol. Biotechnol. 44, 367–376.
Bowman, J.P., Sayler, G.S., 1994. Optimization and maintenance of soluble methane monooxygenase activity in *Methylosinus trichosporium* OB3b. Biodegradation 5, 1–11.
Brusseau, G.A., Tsien, H.C., Hanson, R.S., Wackett, S.P., 1990. Optimization of trichloroethylene oxidation by methanotrophs and the use of a colorimetric assay to detect soluble methane monooxygenase activity. Biodegradation 1, 19–29.
Carmen, M.L., Jackson, K.J., Knapp, R.B., Knezovich, J.P., Shah, N.N., Taylor, R.T., 1996. Methods for microbial filtration of fluids. US patent 5487834.
Chrastil, J., Wilson, J.T., 1975. Sensitive colorimetric method for formaldehyde. Anal. Biochem. 63, 202–207.
Colby, J., Stirling, D.I., Dalton, H., 1977. Soluble methane monooxygenase of *methylococcus capsulatus* (bath). its ability to oxygenate *n*-alkanes, *n*-alkenes, ethers, and alicyclic, aromatic and heterocyclic compounds. Biochem. J. 165, 395–402.
Duba, A.G., Jackson, K.J., Jovanovich, M.C., Knapp, R.B., Taylor, R.T., 1996. TCE remediation using *in situ*, resting-state bioaugmentation. Environ. Sci. Technol. 30, 1982–1989.
Fitch, M.W., Weissman, D., Phelps, P., Georgiou, G., Speitel, G.E., 1996. Trichloroethylene degradation by *Methylosinus trichosporium* OB3b mutants in a sequencing biofilm reactor. Water Res. 30, 2655–2664.
Hanson, R.S., Hanson, T.E., 1996. Methanotrophic bacteria. Microbiol. Rev. 60, 439–471.
Hou, C.T., Patel, R., Laskin, A.I., Barnabe, N., 1979. Microbial oxidation of gaseous hydrocarbons: epoxidation of C2 to C4 *n*-alkenes by methylotrophic bacteria. Appl. Environ. Microbiol. 38, 127–134.
Koh, S.C., Bowman, J.P., Sayler, G.S., 1993. Soluble methane monooxygenase production and trichloroethylene degradation by a type I methanotroph, *Methylomonas methanica* 68-1. Appl. Environ. Microbiol. 59, 960–967.
Lee, J., Soni, B.K., Kelly, R.L., 1996. Enhancement of biomass production and soluble methane monooxygenase activity in continuous cultures of *Methylosinus trichosporium* OB3b. Biotechnol. Lett. 18, 897–902.
Mehta, P.K., Mishra, S., Ghose, T.K., 1989. Growth kinetics and methanol oxidation in *Methylosinus trichosporium* NCIB 11131. Biotechnol. Appl. Biochem. 11, 328–335.
Oldenhuis, R., Vink, R.L.J.M., Janssen, D.B., Witholt, B., 1989. Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. Appl. Environ. Microbiol. 55, 2819–2826.
Patel, R.N., Hou, C.T., Laskin, A.I., Felix, A., Derelanko, P., 1980. Microbial oxidation of gaseous hydrocarbons: production of secondary alcohols from corresponding *n*-alkanes by methane-utilizing bacteria. Appl. Environ. Microbiol. 39, 720–726.
Phelps, T.J., Niedzielski, J.J., Schram, R.M., Herbes, S.E., White, D.C., 1990. Biodegradation of trichloroethylene in continuous-recycle expanded-bed bioreactors. Appl. Environ. Microbiol. 56, 1702–1709.
Pressman, J.G., Georgiou, G., Speitel, G.E., 1999. Demonstration of efficient trichloroethylene biodegradation in a hollow-fiber membrane bioreactor. Biotechnol. Bioeng. 62, 681–692.
Rockne, K.J., Stensel, D., Herwig, R.P., Stuart, E., 1998. PAH degradation and bioaugmentation by a marine methanotrophic enrichment. Bioremediation J. 1, 209–222.
Shah, N.N., Hanna, M.L., Taylor, R.T., 1996. Batch cultivation of *Methylosinus trichosporium* OB3b V. Characterization of poly-*b*-hydroxybutyrate production under methane-dependent growth conditions. Biotechnol. Bioeng. 49, 161–171.
Smith, T.J., Slade, S.E., Burton, N.P., Murrell, J.C., Dalton, H., 2002. Improved system for protein engineering of the hydroxylase component of soluble methane monooxygenase. Appl. Environ. Microbiol. 68, 5265–5273.
Sullivan, J.P., Dickinson, D., Chase, H.A., 1998. Methanotrophs, *Methylosinus trichosporium* OB3b, sMMO, and their application to bioremediation. Crit. Rev. Microbiol. 24, 335–373.
Sun, Z.Y., Ramsay, J.A., Guay, M., Ramsay, B.A., 2006. Automated feeding strategies for high-cell-density fed-batch cultivation of *Pseudomonas putida* KT2440. Appl. Microbiol. Biotechnol. 71, 423–431.
Tsien, H.C., Brusseau, G.A., Hanson, R.S., Wackett, L.P., 1989. Biodegradation of trichloroethylene by *Methylosinus trichosporium* OB3b. Appl. Environ. Microbiol. 55, 3155–3161.
Yaws, C.L., Yang, H.-C., 1992. Henry's law constant for compound in water. In: Yaws, C.L. (Ed.), Thermodynamic and Physical Property Data. Gulf Publishing, Houston, pp. 181–206.
Yu, Y., Ramsay, J.A., Ramsay, B.A., 2006. On-line estimation of dissolved methane concentration during methanotrophic fermentations. Biotechnol. Bioeng. 95, 788–793.