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Immobilization of *Methylosinus trichosporium* OB3b for methanol production

Anne Taylor[1] · Paige Molzahn[2] · Tanner Bushnell[2] · Clint Cheney[2] · Monique LaJeunesse[2] · Mohamad Azizian[2] · Lewis Semprini[2]

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Abstract

Due to the natural gas boom in North America, there is renewed interest in the production of other chemical products from methane. We investigated the feasibility of immobilizing the obligate methanotrophic bacterium *Methylosinus trichosporium* OB3b in alginate beads, and selectively inactivating methanol dehydrogenase (MDH) with cyclopropane to produce methanol. In batch cultures and in semi-continuous flow columns, the exposure of alginate-immobilized cells to cyclopropane or cyclopropanol resulted in the loss of the majority of MDH activity (> 80%), allowing methanol to accumulate to significant concentrations while retaining all of *M. trichosporium* OB3b's methane monooxygenase capacity. Thereafter, the efficiency of methanol production fell due to recovery of most of the MDH activity; however, subsequent inhibition periods resulted in renewed methanol production efficiency, and immobilized cells retained methane-oxidizing activity for at least 14 days.

Keywords *Methylosinus trichosporium* OB3b · Alginate immobilization · Cyclopropane · Cyclopropanol · Liquid fuel production

Introduction

The interest in producing commercially marketable products from methane feedstock has surged along with the recent natural gas boom in North America. Moving natural gas from its production source to the site of its consumption, either as a gas or liquefied gas, is difficult and expensive [12], and some effort has been focused on producing liquid fuels such as methanol which are easier to store and transport. The Fischer–Tropsch method is the current technology to produce liquid fuels from gas, and utilizes a series of three reactions that require high temperature (200–300 °C) and pressure (10–60 bar), which have a high overall energy requirement (delta $H = 72$ kJ mol^{-1}) [1]. The resulting liquid fuel can be made into gasoline or can be utilized as the starting point for the production of other compounds such as ethylene or propylene which are precursors in the synthesis of many chemicals. A less energy intensive method to convert methane to methanol is desirable, and bioconversion of methane to methanol has recently received much consideration. Methanotrophs are versatile methane-utilizing bacteria that have the ability to transform a wide range of hydrocarbons, and can be genetically manipulated to produce a variety of products. For example, Strong et al. [26] discuss the potential of producing methanol along with other soluble products such as formaldehyde or organic acids using methanotrophic bacteria, while Kalyuzhnaya et al. [13] suggest the genetic manipulation of methanotroph strains to eliminate the methanol dehydrogenase protein and increase methanol yields. Other workers have shown the feasibility of using methane consuming methanotrophs to produce lipids that could be harvested for biofuel production [6], and [12] advocates the bio activation of methane with genetically modified organisms to produce butanol.

One challenge in utilizing microbes for the production of chemical products is the retention of sufficient biomass to make the process commercially viable. Immobilization

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✉ Anne Taylor
anne.taylor@oregonstate.edu

[1] Department of Crop and Soil Science, Oregon State University, 3017 Agricultural Life Science Building, Corvallis, OR 97331, USA

[2] Department of Chemical Biological and Environmental Engineering, Oregon State University, Corvallis, OR, USA

of the cultures in a gas permeable substrate within a chemostat or a flow through reactor would prevent the loss of biomass as the products are drawn off, and such a reactor might be compact and mobile enough to be utilized at the site at which methane is produced (https://arpa-e.energy.gov/?q=arpa-e-programs/remote). It's been previously reported that immobilization of other cultures at high density for industrial production of alcohols reduced inhibition of ethanol on cells, suppressed growth rates leading to higher yields of product, and increased stress tolerance [15, 32]. Methanotroph cultures may also benefit from immobilization at high densities. In this study, we used a simple method to immobilize the methanotroph *Methylosinus. trichosporium* OB3b in alginate beads to retain biomass in sequencing batch reactors and semi-continuous flow columns with the intent to evaluate the effects of alginate immobilization on methane uptake activities and yields when the culture was manipulated to produce methanol.

Methanotrophs are obligate methane-utilizing bacteria that make methanol in the first step of their metabolic pathway. Most methanotrophs are mesophilic, growing optimally at temperatures between 25 and 30 °C under aerobic conditions at atmospheric pressure [8, 11, 14]. The metabolism of methane is initiated by its oxidation to methanol, a process that is catalyzed by methane monooxygenase (MMO). Reducing equivalents for this energy requiring step are generated further along the metabolic pathway after methanol is oxidized to formaldehyde, and some of the reducing equivalents are shuttled back to MMO [11]. Some of the challenges to utilizing methanotrophs for the production of methanol includes, (i) blocking the oxidation of methanol to formaldehyde by methanol dehydrogenase (MDH) so that methanol can accumulate, (ii) ensuring that enough reducing equivalents are available for the continued activity of MMO and production of methanol, and (iii) retaining methanotroph biomass while decanting the produced liquid fuel for sustained methanol production. If these challenges can be overcome, the production of methanol can be achieved at temperatures < 35 °C and at atmospheric pressure, greatly reducing the energy costs associated with synthetic methanol production.

A possible strategy for meeting the challenges associated with utilizing methanotrophs to produce methanol could be the partial or periodic inhibition of MDH. If this could be achieved methanol would accumulate, some reducing equivalents would cycle back to MMO for continued activity, and no carbon or energy source other than methane would be required for continued operation. In the previous work with the methanotroph *M. trichosporium* OB3b, a variety of compounds including PO_4, $MgCl_2$, NH_4Cl, NaCl, cyclopropane, and cyclopropanol have been used to inhibit the oxidation of methanol to formaldehyde by MDH and allow the accumulation of methanol [5, 10, 16, 22, 23, 27]. We utilized both cyclopropane (gas phase) and cyclopropanol (aqueous phase) treatments for the inhibition of MDH and the production of methanol in *M. trichosporium* OB3b immobilized in alginate beads, evaluating the activity of immobilized *M. trichosporium* OB3b and the potential to manipulate that activity to produce a commercially viable product.

Materials and methods

Culture conditions

M. trichosporium OB3b was grown in nitrate mineral salts (NMS) media without the addition of copper, so that soluble MMO (sMMO) would be expressed [18]. Cultures were grown at 30 °C either in batch culture or in a chemostat with continuous media and methane flow. For batch cultures, 500 ml serum bottles containing 150 ml NMS plus 5% liquid inoculum from a stock culture were sealed with caps with gray butyl septa and shaken at 300 rpm. Methane was added to yield a 20% methane headspace. Continuous growth of OB3b was maintained in a 7-liter New Brunswick BioFlo 110 fermenter. The fermenter, including gas and liquid delivery lines, and 2.7 L of copper-free NMS media minus buffer were sterilized by autoclaving. Upon cooling to room temperature, phosphate buffer and 300 ml inoculum of batch grown culture were added. The fermenter was operated in batch mode with stirring (300 rpm) and continuous methane and airflow for 72 h before switching to chemostat mode with continuous media and gas feed. The media dilution rate was 0.012 h^{-1} and the methane flow rate was 14 ml/min. The air-flow rate was set to maintain the dissolved oxygen concentration between 0.08 and 0.8 mg/L. Sodium carbonate (0.5 M) was automatically added to maintain pH 7. Dissolved oxygen and pH were continuously measured with Mettler–Toledo probes. Cell concentration was estimated by optical density measured on a Beckman–Coulter spectrophotometer at 600 nm. Culture was harvested from the chemostat by closing the gas effluent line and opening the sterilized liquid sample line, allowing pressure in the chemostat to push culture out of the sample line for collection. A naphthalene assay was routinely employed to ensure that sMMO was expressed in the cultures [19]. Cultures were routinely streaked on 1/10 tryptic soy plus glucose plates to check for heterotrophic contamination.

Chemicals

Methane and acetylene were obtained from Industrial Welding (Albany, OR) and Air Products (Allentown, PA), respectively. Cyclopropane was obtained from Matheson Gas Company (Newark, NJ) and cyclopropanol from Manchester Organics (Cheshire, U.K.). Methanol was obtained from

Mallinckrodt Chemicals (Phillipsburg, NJ). Sodium alginate (Alginic acid, sodium salt; Algin) was obtained from Spectrum Chemical MFG. Corp. (Gardena, CA). Propionaldehyde (propanal), 2-propanol (allyl alcohol), and acrylic acid were purchased from Sigma-Aldrich (St. Louis, MO).

Analytical methods

A microbiuret assay was used to determine the protein content of dense cell suspensions [9]. Progress curves of methane disappearance were constructed by analyzing headspace samples (100 µl) by gas chromatography with flame-ionization detection (FID) using a Shimadzu (Kyoto, Japan) GC-14A gas chromatograph (GC) fitted with a Supelco (Bellefonte, PA) 80/100 Carbopack™ C/0.2% Carbowax 1500 column. The FID and injector temperatures were adjusted to 200 °C and the column to 50 °C. Methanol and cyclopropanol accumulation were detected by analyzing aqueous samples (2 µl) with the same GC conditions. The total mass of methane and cyclopropane present was calculated from the concentration in the headspace samples using Henry's Law (H_{cc} = 31.25 and 3.5 for methane and cyclopropane, respectively) and volumes of the gas and liquid phases. Methanol and cyclopropanol are miscible in water and were not considered to partition significantly between the aqueous and gas phases.

Gas chromatography–mass spectrophotometry (GC/MS) was utilized to identify cyclopropanol. Details of the analysis are provided in the Supplemental Materials, and are briefly presented here. Cyclopropanol was purged from the aqueous phase sample with a Tekmar–Dohrmann 3100 sample concentrator equipped with a Tekmar–Dohrmann Aqua 70 liquid auto sampler. The sample concentrator had the following conditions: preheat 15 min to 80 °C, purge 20 min, followed by a dry purge time of 10 min. The sorbent trap was heated at 225 °C for 10 min and back flushed with helium to desorb the trapped cyclopropanol onto a Hewlett–Packard (HP) 6890 GC/5973 MS, which was interfaced with and with Restek capillary column-Rtx-VMS (30 m, 250 µm ID, and 1.4 µm thickness). The resulting peaks were analyzed with an HP Chem Station data system. The cyclopropanol eluting from the GC column was identified by comparing the measured mass spectra and retention time to reference spectra and the retention time for a cyclopropanol standard.

M. trichosporium OB3b methane uptake activity in suspension and alginate beads

Cultures of *M. trichosporium* OB3b (here after referred to as OB3b) were grown as described above to OD_{A600} ~ 0.6, and harvested and concentrated by centrifugation. A dense suspension of the culture was made with fresh NMS media and aliquots added to 4 ml of NMS media in 70 ml serum vials to obtain specific cell protein concentrations of 0.1–1.1 mg. The vials were capped with gray butyl stoppers and crimped with aluminum caps.

For assays of immobilized culture, the dense cell suspension in fresh NMS media was thoroughly mixed with a sterile sodium alginate solution to yield the desired cell protein concentrations and a final alginate concentration of 2%. The alginate/culture mix (5 ml) was extruded at a constant rate from a 20 ml plastic syringe through 16G, 18G, or 23G needles to form beads, which were dropped into a 100 mM $CaCl_2$ solution to externally crosslink the alginate. The alginate beads were exposed to $CaCl_2$ for up to 5 min, after which they were rinsed 3× with dH_2O before being suspended in 4 ml of NMS in 70 ml serum vials and capped with gray butyl stoppers and crimped with aluminum caps. Methane was added (100 µM in the aqueous phase, C_{aq}) and the vials shaken on an orbital shaker at maximum speed (300 rpm) at 20 °C, and methane consumption followed. Rates were normalized per mg protein. Controls included vials with media, media plus alginate beads but without OB3b, or media plus alginate beads with boiled OB3b to evaluate methane loss from the experimental system. Over the time course of our experiments, no methane was lost in control treatments that did not contain active OB3b culture.

Inhibition of methanol dehydrogenase

Cultures of OB3b were grown, harvested, and immobilized in alginate as described above for use in (i) batch reactors, (ii) sequencing batch reactors, and (iii) semi-continuous packed columns.

1. For the batch reactors, 5 ml of alginate beads (~ 0.5 mg protein) were suspended in 4 ml of NMS in 70 ml serum vials and capped with gray butyl stoppers and crimped with aluminum caps. Cyclopropane was added to vials to achieve specific aqueous concentrations and vials were shaken on an orbital shaker at 300 rpm at 20 °C for 90 min. The GCMS spectra and GC retention time of the single-oxidized product of cyclopropane was compared with purchased standards of cyclopropanol, propionaldehyde (propanal), 2-propanol (allyl alcohol), and acrylic acid to identity that the oxidized product of cyclopropane was cyclopropanol. The results of the GC–MS analysis are presented in Figure S2 in the Supplemental Information.

   Beads were rinsed 3× with dH_2O to remove cyclopropanol, before the addition of fresh media and 100 µM C_{aq} methane. The consumption of methane and accumulation of methanol were monitored over time. In other batch experiments with alginate-immobilized OB3b, uptake of 200 µM methanol was compared before and after 2, 6, or 18 h cyclopropane treatments.

2. In the sequencing batch reactor (SBR), 5 ml of OB3b containing alginate beads (~ 0.5 mg protein) were suspended in 4 ml of NMS in 70 ml serum vials and capped with gray butyl stoppers and crimped with aluminum caps. Cyclopropane was added to the vials (100 µM C_{aq}) and shaken at 20 °C for 2, 6 or 18 h. Beads were rinsed 3× with dH_2O, before the addition of fresh media and 100 µM C_{aq} methane. Half of the treatments also included 20 mM formate as an energy source, while the others had no carbon or energy source other than methane. The consumption of methane and accumulation of methanol were monitored over time; methanol sorption into alginate beads was accounted for in determining methanol accumulation and efficiency (Supplemental material Table 1 and Figure 1). At 24 h intervals, the alginate beads were rinsed before suspension in fresh media plus methane.
3. For immobilized OB3b in a packed column, cultures of OB3b were chemostat grown, harvested, and immobilized in alginate as described above (30 ml total of OB3b immobilized alginate beads) and packed into a column with dimensions of 2.5 cm ID and 13 cm packing height, with filters on the inlet and outlet. Three column trials were performed with variations in the conditions (Supplemental Material Table 2). Total cell protein in the column trials was 1.0 g, 400, and 350 mg for Trial 1, 2, and 3, respectively. Inhibition of MDH was achieved with 100 µM cyclopropane in Trial 2, and with 500 µM cyclopropanol in Trial 1 and Trial 3. Cyclopropanol for these tests was biotically produced by OB3b using the procedure described above. For the inhibition periods, the column was incubated under batch conditions for 18 h with cyclopropane or cyclopropanol in media, then quickly flushed with ~ 10 pore volumes of NMS, before media flow containing saturating methane (calculated to be ~ 200 µM) and air (8 mg/L) was begun at a rate of 1–10 ml/min, corresponding with hydraulic residence times in the column of 3–30 min. Methane consumption and methanol accumulation were monitored by gas chromatography of the liquid samples in the column influent and effluent solutions until methanol was no longer detectable. In this semi-continuous flow system, methanol retardation due to alginate beads sorption was kept to a minimum and no correction was needed to evaluate methane-to-methanol efficiency. To see if methanol production could be reinstated once MDH recovery had occurred, the columns were incubated under batch conditions for 18 h with cyclopropane or cyclopropanol, and flushed with NMS, before media flow containing dissolved methane and dissolved air or oxygen was resumed.

Statistical analysis

ANOVA analysis using the Holm–Sidak method was used for pair-wise comparisons in SigmaPlot (Systat Software, San Jose, CA) of methane consumption or methanol accumulation between treatments.

Results

OB3b activity immobilized in alginate beads

Methane consumption by OB3b was compared in suspension and in alginate beads made with three standard needle gauges (16G, 18G, and 23G) resulting in beads with diameters of 4.2 ± 0.4, 3.2 ± 0.3, and 2.9 ± 0.0 mm. In all treatments, the total protein mass was kept at ~ 0.5 mg per replicate, and methane was added to the headspace to achieve 100 µM in the aqueous phase (C_{aq}), which was near the half-saturation constant (K_s) of OB3b. Rates of methane consumption in all treatments were sustained over 24 h (Fig. 1a), and were not significantly different in suspension in comparison with alginate beads ($p > 0.05$), suggesting that the presence of alginate under these conditions did not interfere with methane diffusion to the cells and subsequent methane uptake. Rates of methane consumption ranged from 129 to 157 nmol/min/mg protein, and were lower than maximum reported rates (k_{max} ~ 700 nmol/min/mg protein [17, 20]). This was likely because the initial methane concentration was less than rate-saturating, and because the temperature for all experiments was at 20 °C rather 30 °C, where the reported maximum rates were measured. The low protein content of the beads in suspension resulted in methane uptake over a period of ~ 24 h; thus, mass transfer limitations were not observed with transport into the alginate beads. The similar rates of methane utilization with beads of different size are consistent with negligible mass transfer limitations with the long time course exposures. In ensuing experiments, 23G needles were used to make beads.

Effect of biomass concentration on methane consumption in alginate beads

Rates of methane consumption by OB3b in suspension and immobilized in alginate beads were compared at three different protein concentrations to evaluate the effects of biomass concentration on methane consumption (Fig. 1b). Methane was consumed most quickly in the treatments with the highest concentration of OB3b biomass. The rates in suspension vs. alginate beads were not significantly different ($p > 0.1$), confirming that consumption was not limited in alginate beads compared with that in suspension. However, when the rates of methane consumption were normalized for cell

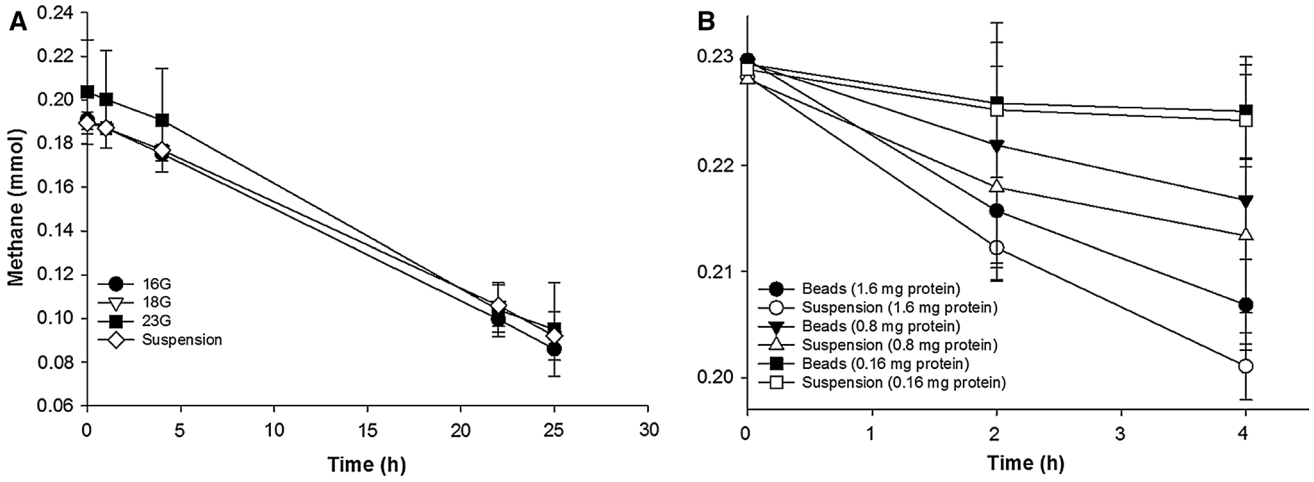

Fig. 1 Methane consumption by OB3b in batch incubations. **a** Comparison of cells immobilized in alginate beads made with 16G, 18G, and 23G needles to yield beads of different sizes, with culture in suspension; and **b** comparison of cells in alginate beads vs. suspension at different total concentrations of protein. Error bars represent the standard deviation of the average of three replicate measurements

Table 1 Comparison of the effects of protein concentration on the rates of methane consumption by OB3b in suspension and in alginate beads over 4 h in batch incubations

Protein (mg)	Suspension nmol/min/mg protein	Beads
0.16	123 ± 25^{a}	114 ± 17^{a}
0.80	76 ± 10^{b}	68 ± 7^{b}
1.60	70 ± 3^{b}	59 ± 12^{b}

Rates are the average of three replicate measurements ± the standard deviation. Values that have lower case letters in common are not significantly different ($p < 0.05$)

protein, the treatments with higher protein concentrations had the lowest rates of methane consumption (Table 1). This suggested that as biomass concentration and uptake of methane increased, limitations for transfer of methane or oxygen from the headspace to the aqueous phase were manifest despite shaking at 300 rpm. For ensuing batch experiments, protein concentrations were held at ~ 0.5 mg/replicate treatment to minimize mass transfer limitations while maintaining a rate of methane consumption that could be measured accurately over a short time period.

Effect of cyclopropane treatment on methanol accumulation by OB3b immobilized in alginate beads

Previously, it had been shown that methanol accumulated in OB3b cultures pre-treated with cyclopropane before the introduction of methane [22]. The oxidation of cyclopropane resulted in the production of cyclopropanol which irreversibly inactivated MDH allowing methanol to accumulate [23]. We evaluated the effect of cyclopropane pre-treatment on methanol accumulation by OB3b immobilized in alginate beads.

Alginate-immobilized OB3b were exposed to 100, 200, and 400 µM C_{aq} concentrations of cyclopropane for 90 min and the supernatant was evaluated for cyclopropanol accumulation. Using gas chromatography and GC/MS, cyclopropanol accumulation was identified only in treatments with live culture immobilized in alginate, and not in alginate beads made with killed cells (data not shown). The GC/MS analysis of the cyclopropanol formed via the cometabolism of cyclopropane is shown in Figure S2 of the Supplemental Materials. Both the retention time elution of cyclopropanol and the GC–MS mass spectrum of cyclopropanol matched those of the purchased standard. The results indicate that a very pure form of cyclopropanol was biosynthesised via the cometabolic transformation of cyclopropane by OB3b.

The alginate-immobilized OB3b were then rinsed, and resuspended in fresh media with 100 µM C_{aq} methane. After exposure to all three cyclopropane concentrations, methanol accumulated for up to 18 h, then accumulation ceased. There was an insignificant trend for methanol to accumulate to a greater degree in the treatments with 100 µM C_{aq} cyclopropane (3.7 ± 0.5 mM methanol) than in those with 200 or 400 µM cyclopropane (3.0 ± 0.3 and 2.5 ± 0.4 mM methanol, respectively) ($p > 0.05$).

Methanol is a substrate for MMO with an apparent K_m of 0.95 mM [4]. In batch incubations, the accumulation of methanol rose to levels at which it could successfully compete with methane for the active site of MMO causing methanol accumulation to stop; therefore, incubations were performed in sequencing batch reactor (SBR) mode in an effort to minimize any negative effects of methanol

accumulation. Cultures of OB3b immobilized in alginate beads were exposed to 100 μM C_{aq} cyclopropane for 2, 6, or 18 h to evaluate the effect of exposure time. After the cyclopropane incubation, the beads were rinsed before resuspension in NMS with methane. To determine if methanol yields could be improved by adding an electron donor, the methane incubations were done in the presence and absence of 20 mM formate [16, 28]. The consumption of methane and accumulation of methanol (Fig. 2) were monitored over several days until methanol no longer accumulated. At 24 h intervals, the alginate beads were rinsed before suspension in fresh NMS plus methane. There was no significant difference in methane consumption or methanol accumulation between plus and minus formate treatments ($p > 0.18$) for any exposure time, and formate was not included in future experiments.

Methane consumption was not significantly different between the three treatments ($p > 0.1$) indicating that longer exposures to cyclopropane did not negatively affect MMO and methane uptake, and there was a non-significant trend for methane consumption to increase over time ($p > 0.1$). In all exposure length treatments, methanol accumulated to the highest degree during the first day after cyclopropane exposure, where the efficiency of methanol accumulation (methanol accumulated/methane consumed) ranged from 34 to 47% (Table 2), suggesting that MDH had either never been fully inhibited, or that MDH activity partially recovered within the 24 h SBR cycle. Methanol accumulated to a greater degree during the first day of the 6 h cyclopropane treatment than in the other treatments ($p < 0.01$), but afterwards, there was not a significant difference in methanol accumulation between the three treatments ($p > 0.1$). After the first day, accumulation of methanol decreased signaling a recovery of MDH activity; the 2 h treatments did not accumulate methanol after the second day, and the 6 and 18 h treatments accumulated some methanol on the third day after cyclopropanol treatment.

To determine if any of the cyclopropane treatments completely inhibited MDH, the rates of methanol consumption by alginate-immobilized OB3b were measured in a separate

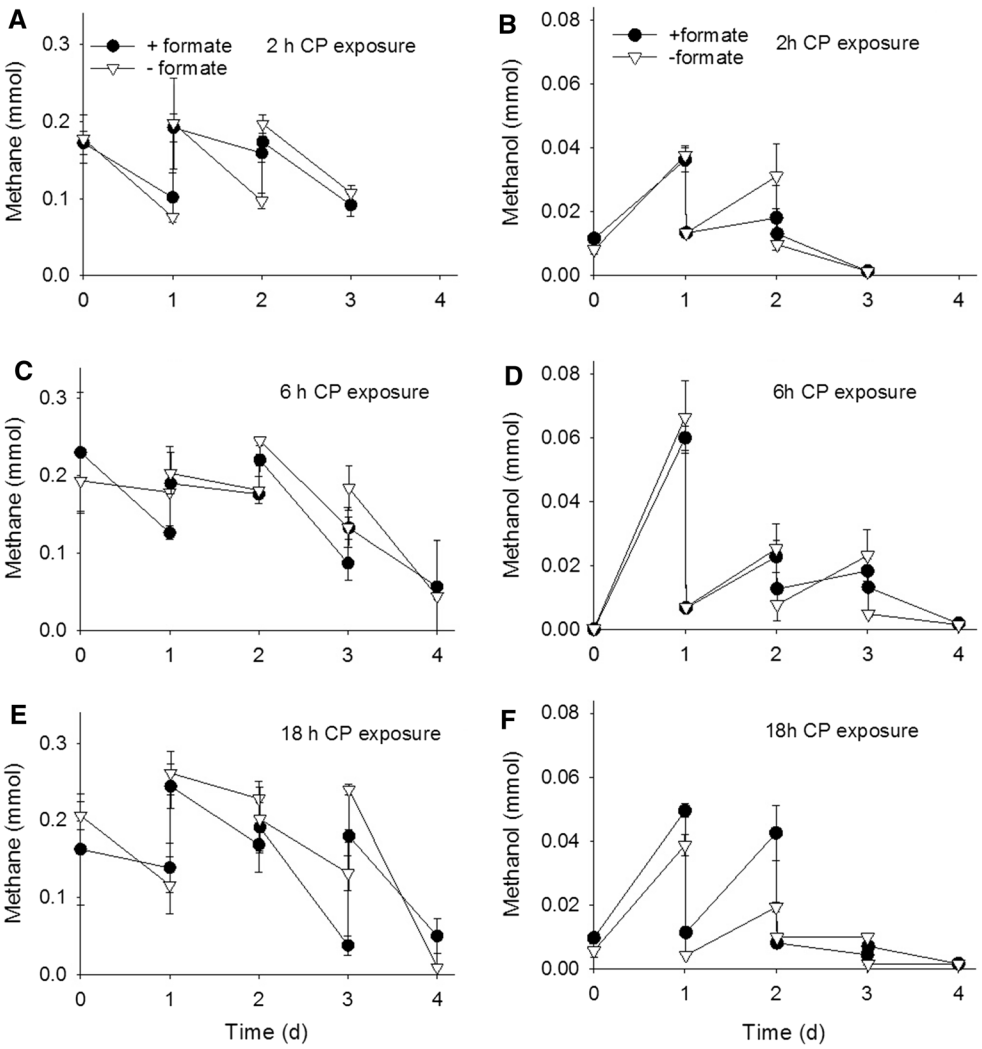

Fig. 2 Methane consumption (**a**, **c,** and **e**) and methanol accumulation (**b**, **d,** and **f**) in sequencing batch reactors (SBR) containing OB3b immobilized in alginate beads. Prior to methanol accumulation, beads were exposed to cyclopropane (CP) for 2, 6, or 18 h. Alginate beads in the SBR were rinsed to remove accumulated methanol daily before resuspension in fresh media with methane. Error bars represent the standard deviation of the average of three replicates

Table 2 Effects of cyclopropane (CP) exposure time on methane consumption and methanol (MeOH) accumulation during the first day of operation in SBR containing OB3b immobilized in alginate beads

Length of CP exposure (h)	Methane consumption + formate (μmol)	Methanol accumulation + formate (μmol)	Methanol efficiency + formate (%)	Methane consumption − formate (μmol)	Methanol accumulation − formate (μmol)	Methanol efficiency − formate (%)
2	96 ± 2Aa	29 ± 5 Aa	35 ± 8Aab	84 ± 16Aab	34 ± 5 Aa	41 ± 6 Aa
6	136 ± 64Aa	60 ± 4Ab	43 ± 3 Aa	15 ± 32 Aa	66 ± 12 Ab	47 ± 8 Aa
18	109 ± 16Aa	40 ± 1Aa	34 ± 0Ab	90 ± 29 Ab	33 ± 1Ba	37 ± 1Ba

Methanol efficiency is defined as methanol accumulated/methane consumed. Mass balances for methanol included the estimates of methanol sorbed into alginate beads based on partitioning studies presented in Table S1. Results are the average of three replicates ± the standard deviation. Upper case letters denote a significant difference ($p < 0.05$) between ± formate treatments. Lower case letters indicate significant differences ($p < 0.05$) between the different CP exposure lengths. Treatments with the same letters are not significantly different

experiment before and after 2, 6, and 18 h of 100 μM cyclopropane exposure. Methanol uptake was not completely inhibited after 2 or 6 h of cyclopropane treatment and MDH retained 53 ± 15 and 55 ± 05% of its initial capacity. However, 18 h of cyclopropane treatment had a significantly greater effect on MDH ($p < 0.05$) resulting in the loss of the majority of MDH activity (80 ± 07%).

Inhibition of MDH in column studies and the production of methanol

In the SBR, methanol accumulated to concentrations that might be oxidized by MMO which lead to the evaluation of the production of methanol in packed columns. In a short-term test column packed with OB3b in alginate beads, the flow rate (10 ml/min) was adjusted so that nearly all (87 ± 12%) of methane in solution was consumed and then the flow was halted, whereupon 500 μM C_{aq} cyclopropanol was added (Fig. 3). After 18 h of cyclopropanol exposure in batch mode, flow of media saturated with methane and oxygen was resumed and methane and methanol monitored. The percentage of methane consumed after cyclopropanol inhibition (73 ± 10%) was not significantly different from before the inhibition step ($p > 0.1$). In this column with a constant flow of media and methane, the efficiency of methanol production was 86 ± 27% of the methane consumed.

This success led to the execution of multiple columns maintained for longer times under semi-continuous or continuous flow conditions. This approach conveniently allowed the evaluation of multiple inhibition periods of alginate-immobilized OB3b, and the long-term viability of OB3b in alginate beads. Several trials were performed with multiple periods of inhibition followed by methanol production and then full or partial recovery of MDH activity (see Supplemental Materials Table 2 for specific conditions in each column). The first long-term column of OB3b immobilized in alginate beads was inhibited with 500 μM cyclopropanol for 18 h in batch mode before flow began on day 1 (Fig. 4). After about 1 h of continuous flow the concentration of methanol produced reached the concentration of methane utilized indicating stoichiometric conversion of methane to methanol (methanol production efficiency was 82 ± 33%). After 5 h of operation, the methanol concentration began to decrease indicating the beginning of

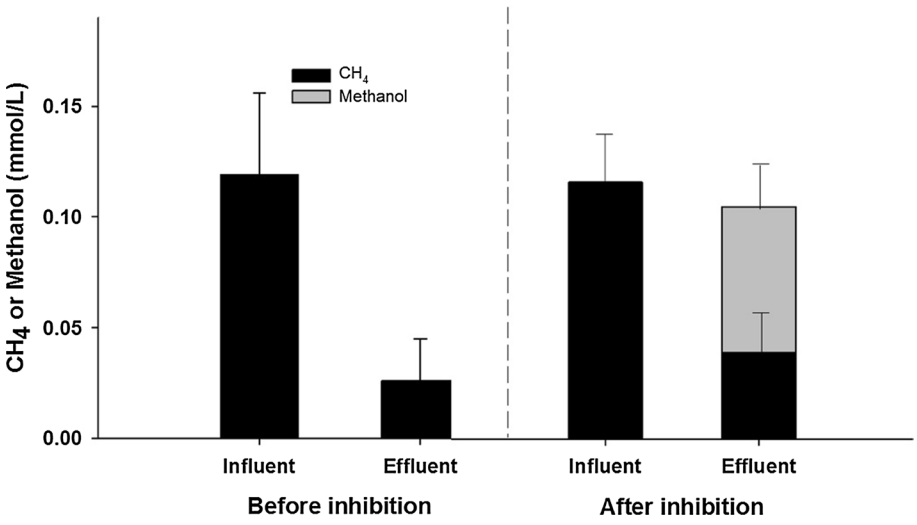

Fig. 3 Methane consumption in a in semi-continuous flow column packed with OB3b in alginate beads before and after inhibition with 500 μM cyclopropanol, and methanol production after cyclopropanol inhibition. The bars are the average of the concentrations measured during steady state resulting before and after cyclopropanol inhibition (three measurements taken 15 min apart), and the error bars represent the standard deviation of the mean

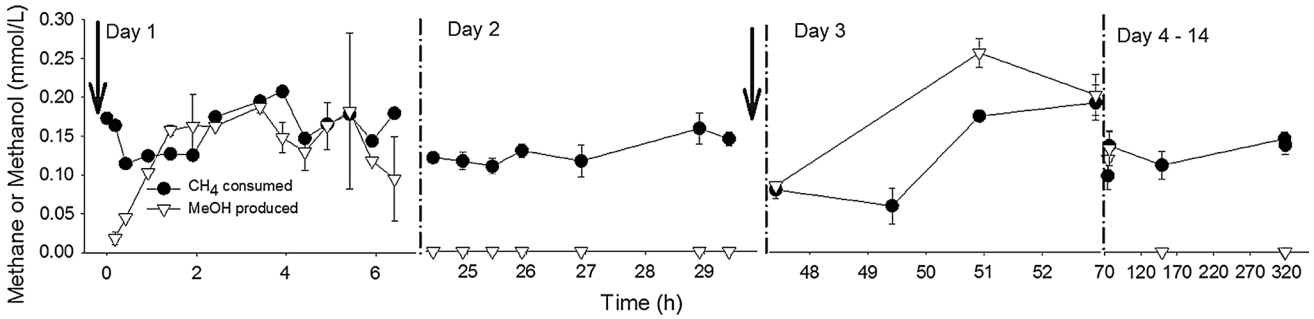

Fig. 4 Consumption of methane (CH_4) and production of methanol (MeOH) in a column packed with OB3b immobilized in alginate beads after exposure to cyclopropanol. Days of active operation are shown. The arrows indicate when the column was exposed to 500 μM cyclopropanol in batch mode for 18 h. Error bars represent the standard error of three replicate measurements

recovery of MDH activity. The stoichiometric conversion of methane to methanol continued to decrease up to 6.5 h whereupon flow was ceased for 18 h. When flow was resumed on day 2, methanol was not detected; however, methane utilization remained high, indicating that MDH was no longer inhibited in the immobilized OB3b cells. At the end of day 2, the column was again exposed to cyclopropanol in batch mode for 18 h. This resulted in MDH activity being effectively blocked during operation on day 3, with methanol produced with high efficiency until the flow was ceased at 53 h (methanol production efficiency was ~ 100%). Flow was resumed briefly on days 4, 6, and 14 to check for activity. On day 4 methanol was detected, indicating that MDH was still inactivated; but on days 6 and 14, methane was consumed, but no methanol detected. This column experiment demonstrated that multiple inhibitions and periods of methanol production by alginate-immobilized OB3b were possible and that OB3b immobilized in alginate could retain activity for up to 14 days.

Two other columns packed with alginate-immobilized OB3b were inhibited in batch mode for 18 h with cyclopropane (Column 2), or cyclopropanol (Column 3, Fig. 5). Consistent with Column 1, there was conversion of methane to methanol on day 1 after inhibition with either cyclopropane or cyclopropanol (efficiency averaged 82 ± 06%). This result indicated that MMO activity was able to convert cyclopropane to cyclopropanol in situ and achieve methanol production efficiencies similar to those achieved by direct exposure to cyclopropanol. In both Columns 2 and 3, methane consumption continued on day 2, but the efficiency of methanol production fell to an average of 38 ± 18% due to recovery of most of the MDH activity. After the second inhibition of 18 h in batch mode with cyclopropane or cyclopropanol, the efficiency of conversion of methane to methanol on day 3 of Columns 2 and 3 averaged 86 ± 08%, demonstrating that multiple inhibitions and periods of methanol production by alginate-immobilized OB3b were possible. Similar to Column 1, significant methanol production efficiency was retained in Column 2 into day 4 (Fig. 5).

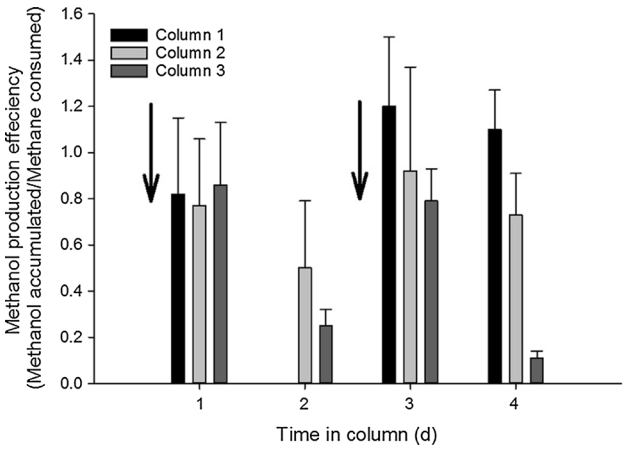

Fig. 5 Methanol production efficiency in semi-continuous flow columns packed with OB3b immobilized in alginate beads (see Supplemental Table 2). The arrows indicate when the columns were exposed to 500 μM cyclopropanol (Columns 1 and 3) or 100 μM cyclopropane (Column 2) in batch mode for 18 h. Methane consumption and methanol accumulation were recorded over time. The bars represent the average methanol production efficiency over the course of each day with indicated standard deviation

Discussion

In this study, we evaluated the methane uptake rates and the potential of methanol production by the selective inhibition of MDH in *Methylosinus trichosporium* OB3b immobilized in alginate beads. Although methane-oxidizing bacteria in general, and OB3b in particular are well studied, there is limited information in the literature with methane-oxidizing cultures immobilized or attached to surfaces to retain biomass in a bioreactor. Most of the existing work centered around the degradation of chlorinated solvents. For example,

Fitch et al. [7] grew OB3b biofilms on diatomaceous earth or glass beads in sequencing biofilm reactors with alternate phases of trichloroethylene (TCE) degradation and growth on methane. Oxidation of TCE resulted in loss of activity that was not recovered during the growth phase, and the authors came to the conclusion that OB3b recovery did not occur, because it did not attach well to surfaces. Chu and Alvarez-Cohen [3] obtained better results with mixed cultures containing OB3b in unsaturated biofilm columns packed with diatomaceous earth, glass beads, or silicalite pellets. Sustained TCE degradation was achieved for several days when passed through the column in the gaseous phase. Unfortunately, this approach would not work during the production of methanol from methane since liquid flow is needed for the removal of methanol, as accumulated methanol has the potential to become a substrate for MMO [4].

Significant concentrations of methanol (up to 3.7 mM) were produced by inhibiting methanol dehydrogenase (MDH) of alginate-immobilized OB3b with cyclopropane or cyclopropanol in batch reactor and column studies. Previous work using methanotroph cultures immobilized within alginate for engineered systems was done some years ago with *Methylocystis* strain M for the degradation of TCE [24, 29, 30]. In kinetic studies, V_{max} of TCE degradation with alginate-immobilized *Methylocystis* strain M was no different than in free cell suspensions [29], indicating that TCE and oxygen transport via diffusion was not impacted by immobilization in alginate. Consistent with this result, we found that OB3b maintained high rates of methane uptake when immobilized in alginate. Scaling up for the production of methanol with higher concentrations of cells to promote faster rates of methane consumption and methanol production presents a practical challenge since we observed effects of mass transfer limitation on methane consumption as cell protein concentrations increased from 0.032 to 0.32 mg protein/ml of alginate beads in batch reactors (0.16–1.6 mg total protein) shaken at 300 rpm. A similar observation had been made, although at much higher protein concentrations, in regards to TCE degradation by *Methylocystis* strain M, where mass transfer limitations were observed when cell protein concentrations ranged from 6 to 42 mg protein/ml of alginate beads [29]. However, mass transfer limitations were not due to alginate immobilization, because the same effect was observed in suspended cultures, pointing to the insolubility of oxygen in the aqueous phase of the two compartment liquid/gas reactors that were used [29]. Mass transfer limitations in batch and column studies might be overcome by maintaining the reactions under greater than atmospheric pressure. Modest increases in pressure (up to 1.4 atm) increased solubility of methane and oxygen, and the methanotroph *Methylomonas albus* (BG-8) methane uptake rates were increased 1.6-fold in batch culture suspensions [25], while the maximum methane utilization rates by mixed methanotroph cultures (including OB3b) in a biofilm reactor under elevated oxygen tension were almost ninefold higher than in biofilm reactors under ambient pressure [2].

During our study, we observed that OB3b activity in alginate beads in semi-continuous columns was sustained for at least 14 days; however, there is evidence that methanotrophs can sustain activity even longer in alginate beads. *Methylocystis* strain M immobilized in alginate beads maintained methane consumption for 24 days in a fluidized bed reactor [24]. Despite the toxicity of TCE, its degradation was maintained in this system for 10 days when operation for TCE degradation and reactivation with methane occurred on alternate days. In contrast, we did not observe negative effects of longer or multiple incubations of cyclopropane/cyclopropanol on MMO activity. Cyclopropane is not thought to be toxic to OB3b, since it demonstrated little effect on MMO activity, and its oxidation product cyclopropanol is thought to be a specific inactivator of MDH and probably not generally toxic to OB3b [21, 27]. Incubations with cyclopropanol or cyclopropane were equally effective at inhibiting MDH activity, and cyclopropane has the advantage of being more readily available for purchase. We found that a very pure form of cyclopropanol could be biosynthesized by the cometabolic transformation of cyclopropane by OB3b; thus, inhibiting MDH activity. The production of cyclopropanol also illustrates how OB3b can be used to biosynthesize high-valued products via cometabolic transformations.

In most cases, during this study, complete inhibition of MDH was not observed (maximum loss of MDH activity was 80%), but this may work in favor of maintaining sustained methanol production. In the semi-continuous flow columns exposed to cyclopropane or cyclopropanol, there was near stoichiometric conversion of methane to methanol (methanol production efficiency was 82 ± 33%) on the first day of operation. This was followed by a decrease in efficiency of methanol production to 38 ± 18% due to recovery of most of the MDH activity. A second inhibition with cyclopropane resulted in renewed methanol production efficiency (86 ± 08%) in the column, demonstrating that multiple inhibitions and periods of methanol production by alginate-immobilized *M. trichosporium* OB3b were possible. The partial or periodic inhibition of MDH allows some reducing equivalents to return to enable continued methane oxidation activity by MMO. Longer studies will be required to determine if periodic recovery of MDH activity would allow for the resynthesis of enzymes required to maintain activity over time, or if there is the potential for growth of OB3b while immobilized in alginate beads. Growth of a wide range of bacteria within alginate bead pore space or along the periphery of the alginate beads has previously been observed, however, at slower rates than in suspension [31]. In OB3b cultures with cyclopropane or cyclopropanol inhibited MDH, most of the methanol will not be further

metabolized to produce energy for cellular biosynthesis, and growth in this system may be constrained. As we have shown in this study, the retention of methanotrophic biomass in alginate and the selective inhibition of MDH lead to substantial concentrations of methanol to be produced. This method could be further developed with immobilization of genetically engineered cultures for the conversion of gas to other chemical products.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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